T.C.
BOLU ABANT İZZET BAYSAL UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF BIOLOGY

DEVELOPMENT OF TRANSGENIC SUGAR BEET (*BETA VULGARIS* L.) PLANTS VIA CHLOROPLAST TRANSFORMATION TECHNOLOGY

DOCTOR OF PHILOSOPHY, BIOLOGY

MEHMET ÖRGEÇ

THESIS SUPERVISOR
Prof. Dr. Ekrem GÜREL
SECOND THESIS SUPERVISOR
Assist. Prof. Dr. Muhammad SAMEEULLAH

BOLU, 6/16/2025

ACCEPTANCE AND APPROVAL PAGE

The thesis entitled "**DEVELOPMENT OF TRANSGENIC SUGAR BEET (*BETA VULGARIS* L.) PLANTS VIA CHLOROPLAST TRANSFORMATION TECHNOLOGY"** prepared and submitted by **Mehmet ÖRGEÇ** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in Department of **BIOLOGY** with unanimity vote is hereby approved. 6/16/2025

Jury Members	**Signature**
Supervisor	
Prof. Dr. Ekrem GÜREL Bolu Abant İzzet Baysal University Member	.....
Prof. Dr. Bahtiyar Buhara YÜCESAN Bolu Abant İzzet Baysal University Member	.....
Prof. Dr. Fatma PEHLIVAN KARAKAŞ Bolu Abant İzzet Baysal University Member	.....
Prof. Dr. Mehmet Cengiz BALOĞLU Kastamonu University Member	.....
Prof. Dr. İlker BÜYÜK Ankara University	.....

Institute of Graduate Studies Approval

Prof. Dr. İbrahim KÜRTÜL

Director of Institute of Graduate Studies

ETHICAL DECLARATION

In this thesis study, which I have prepared in accordance with Bolu Abant İzzet Baysal University, Institute of Graduate Studies' Thesis Writing Guide and Template;

- I have obtained the data, information and documents presented in the thesis within the framework of academic and ethical rules,
- I have presented all information, documents, evaluations, and results in accordance with the rules of scientific ethics and morality,
- I have cited and referenced all the works I have benefited from in the thesis study,
- I have not made any changes to the data used,
- I declare that the work I have presented in this thesis is original, and in any other case, I declare that I accept all loss of rights that may arise against me.

According to the similarity report obtained by applying the filters determined by the directorate of the institute in the Turnitin program related to the thesis, the similarity index of the thesis does not exceed 30%.

.....

MEHMET ÖRGEÇ

ABSTRACT

DEVELOPMENT OF TRANSGENIC SUGAR BEET (*BETA VULGARIS* L.) PLANTS VIA CHLOROPLAST TRANSFORMATION TECHNOLOGY
PHD THESIS
MEHMET ÖRGEÇ
BOLU ABANT IZZET BAYSAL ÜNIVERSITESI
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF BIOLOGY
(SUPERVISOR: PROF. DR. EKREM GÜREL)
(CO-SUPERVISOR: ASSIST. PROF. DR. MUHAMMAD SAMEEULLAH)
BOLU, 16 JUNE 2025
XVIII + 173 Pages

Chloroplast transformation is a powerful tool in plant biotechnology, offering major advantages for genetic engineering through features like genetic stability, high levels of gene expression, and maternal inheritance. In this study, our objective was to develop a transplastomic sugar beet plant by utilizing the pExpPN-phaA-SB vector, which was engineered to include the *phaA*, *adaA*, and *ampR* genes, along with the *trnA* and *trnI* sequences serving as homologous recombination sites.

A total of 66 independent transformation experiments were conducted using the biolistic bombardment method to insert the pExpPN-phaA-SB vector. Putative transplastomic explants were initially subjected to selection on MS medium with 0.5 mg/L BAP, 0.1 mg/L NAA, and 50 mg/L spectinomycin. The induced calli were then transferred to MS medium containing 1 mg/L BAP, and 0.5 mg/L ABA for further development. Regenerated plants were planted in MS medium with 0.25 mg/L KIN medium.

Long-term antibiotic treatments were found to negatively impact both callus formation and regeneration efficiency. Additionally, it was observed that MS medium with 0.25 mg/L KIN effectively reduced the incidence of vitrification, a common issue in sugar beet tissue culture studies. Following the biolistic bombardment of 5846 explants, regeneration of 516 putative transplastomic plants was successfully achieved. Genomic DNA was isolated from these plants, and PCR analysis confirmed the partial integration of the pExpPN-phaA-SB vector, resulting in the generation of 10 transplastomic plants. These results provide a significant reference for chloroplast transformation in difficult-to-transform species such as sugar beet. Moreover, the data generated from this project are invaluable, offering key insights and experience that will be instrumental for future genome editing initiatives in sugar beet and related crops.

KEYWORDS: Sugar beet, Chloroplast Transformation, Biolistic Bombardment, Vitrification

ÖZET

KLOROPLAST TRANSFORMASYON TEKNOLOJİSİ İLE TRANSGENİK ŞEKER PANCARI (*BETA VULGARIS* L.) BİTKİLERİNİN GELİŞTİRİLMESİ
DOKTORA TEZİ
MEHMET ÖRGEÇ
BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ
BİYOLOJİ ANA BİLİM DALI
(TEZ DANIŞMANI: PROF. DR. EKREM GÜREL)
(İKİNCİ DANIŞMAN: ÖĞR. GÖR. DR. MUHAMMAD SAMEEULLAH)
BOLU, 16 HAZİRAN 2025
XVIII + 173 Sayfa

Kloroplast transformasyon bitki biyoteknolojisinde güçlü bir araçtır ve genetik mühendislik için genetik stabilite, yüksek düzeyde gen ekspresyonu ve maternal miras gibi özelliklerle önemli avantajlar sunar. Bu çalışmada, amacımız pExpPN-phaA-SB vektörünü kullanarak transplastomik şeker pancarı bitkisi geliştirmekti. Bu vektör, *phaA*, *adaA* ve *ampR* genlerini içerecek şekilde tasarlanmış ve *trnA* ve *trnI* dizileri homolog rekombinasyon bölgeleri olarak kullanılmıştır.

Atmış altı bağımsız transformasyon deneyi, pExpPN-phaA-SB vektörünün eklenmesi için biyolistik bombardıman yöntemi kullanılarak gerçekleştirilmiştir. Potansiyel transplastomik eksplantlar, başlangıçta 0,5 mg/L BAP, 0,1 mg/L NAA ve 50 mg/L spektinomisin ile içeren MS besin ortamı üzerinde seçilime tabi tutulmuştur. İndüklenen kalluslar daha sonra gelişim için 1 mg/L BAP ve 0,5 mg/L ABA içeren MS ortamına aktarılmıştır. Rejenerasyon sonucu elde edilen bitkiler, 0,25 mg/L KIN içeren MS ortamına ekilmiştir.

Uzun süreli antibiyotik uygulamalarının hem kallus oluşumu hem de rejenerasyon verimliliği üzerinde olumsuz bir etkisi olduğu belirlenmiştir. Ayrıca, 0,25 mg/L KIN içeren MS ortamının, şeker pancarı doku kültürü çalışmalarında sıklıkla karşılaşılan camlaşma sorununu azaltmada etkili olduğu gözlemlenmiştir. 5846 eksplantın biyolistik bombardımanı sonrası, 516 potansiyel transplastomik bitki başarılı bir şekilde rejenerasyonu sağlanmıştır. Bu bitkilerden genomik DNA izole edilerek yapılan PCR analizleri, pExpPN-phaA-SB vektörünün kısmi entegrasyonunu doğrulamış ve 10 transplastomik bitki elde edilmiştir. Bu sonuçlar, şeker pancarı gibi zorlu türlerde kloroplast transformasyonu için önemli bir referans noktası sağlamaktadır. Ayrıca, bu projeden elde edilen veriler, şeker pancarı bitkilerinde ve gelecekteki projelerde kullanılabilecek gelecek nesil genom düzenleme çalışmalarında önemli bilgiler ve deneyimler sunmaktadır.

ANAHTAR KELİMELER: Şeker pancarı, Kloroplast Transformasyonu, Biyolistik Bombardıman, Camlaşma

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF PHOTOS

Page

LIST OF ABBREVIATIONS AND SYMBOLS

BAP : 6-benzyloaminopurine
KIN : Kinetin
NAA : Napthalenacetic acid
MS : Murashige and Skoog
ABA : Abscisic acid
°C : Centigrade degrees
Gr : Gram
L : Liter
FAO : The Food and Agriculture Organization of the United Nations
DNA : Deoxyribonucleic acid
RNA : Ribonucleic acid
RF : Fertility
ORFs : Open reading frames
CMS : Cytoplasmic male sterility
GOI : Gene of interest
SM : Selectable marker gene
cDNA : Complementary DNA
GFP : Green fluorescent protein
NaCIO : Sodium hypochlorite
Kg : Kilogram
HCl : Hydrogen chloride
NaOH : Sodium hydroxide
LB : Luria-Bertani medium
CTAB : Cetrimonium bromide
EDTA : Ethylenediaminetetraacetic acid
Tric-HCI : Tris-hcl hydrochloride
PCR : Polymerase chain reaction
OD : Optical density
Bp : Base pair
UV : Ultraviolet radiation
NCBI : National Center for Biotechnology Information
IPTG : Isopropyl β-D-1-thiogalactopyranoside

ACKNOWLEDGEMENT

I wish to extend my heartfelt gratitude to my supervisor, Prof. Dr. Ekrem GÜREL, for his invaluable guidance, constructive criticism, encouragement, and mentorship throughout my research. His expertise has profoundly influenced not only the current document but also my growth as an individual.

I would like to express my sincere thanks to Prof. Dr. Mehmet Cengiz BALOĞLU Prof. Dr. Bahtiyar Buhara YÜCESAN, Prof. Dr. Fatma PEHLİVAN KARAKAŞ, and Prof. Dr. İlker BÜYÜK for their valuable suggestions and for serving on my thesis committee.

I am deeply grateful to Prof. Dr. Songül GÜREL for her unwavering patience, thoughtful suggestions, and insightful feedback. Her encouragement and guidance played a pivotal role in helping me remain committed to my objectives.

I am deeply grateful to Dr. Muhammad SAMEEULLAH for his support and guidance.

I am deeply thankful to Dr. Özge KAYA, Doc. Dr. Salih Tunç KAYA, Dr. Ömer Can Ünüvar and Dr. Dipul Biswas for their experimental support.

I want to convey my deepest thanks to my father Mustafa Murat ÖRGEÇ, my mother Gülfer ÖRGEÇ and my sister Gülsün ÖRGEÇ for their supports and love. My heartfelt thanks go to my beloved wife Aysu PELİT ÖRGEÇ, whose endless love, patience, and unwavering belief in me gave me the strength to keep going. This journey would have meant little without her by my side.

During my doctoral studies, I was supported by the TÜBİTAK Scientist Support Programs Directorate (BİDEB), 2211-A National PhD Scholarship Program. I would like to express my sincere gratitude to TÜBİTAK-BİDEB for providing this valuable scholarship opportunity.

I gratefully acknowledge the scholarship support of the Council of Higher Education through the (YÖK) 100/2000 Doctoral Scholarship Program.

I would like to thank the Turkish Sugar Institute for their help and support.

This study was supported by the Technological Research Council of Türkiye (TUBİTAK), Grant No: 120O596

1. INTRODUCTION

Humans were first used agriculture as far back as 11000 BC. Since then, people have used agricultural products both in their own diet as a main source and in animal husbandry. Globally, people obtain about 90% of their energy and 80% of protein from plant-based sources.

Over the past fifty years, the rapid growth of the world population, the adverse effects of climate change (Basso et al., 2020), and wars in various regions of the world have posed some of the greatest challenges to agricultural production. The Food and Agriculture Organization of the United Nations (FAO) has made feeding the world's population by 2050 a primary priority because of these difficulties (Clarke & Daniell, 2011). Growing attention has been placed on the production of plants that are resistant to both stress factors (biotic and abiotic), show enhanced yield and quality, and can adapt to a variety of ecological environments.

Conventional plant breeding techniques have substantially advanced agriculture by incorporating basic features into appropriate plant species. Classical plant breeding is generally a challenging process, with disadvantages such as being labor-intensive, having a long processing time, limited genetic diversity, and involving random gene changes (Koya & Daniell, 2005; Basso et al., 2020).

Over the last twenty years, plant biotechnology, especially genetic engineering, has been crucial in the progression of contemporary agriculture. Plant biotechnology, particularly plant genetic engineering (Hou et al., 2003), has significantly impacted modern agriculture. Unlike classical plant breeding, genetic engineering takes less time and facilitates the transfer of important and beneficial genes between species. The plant biotechnology, whether used independently or in conjunction with classical breeding methods, plays a vital role in producing plants that are durable, highly productive, and able to grow in different environmental conditions.

Currently, plant genetic engineering has been employed to develop plant types with distinct traits. The genomes of many plant species, such as wheat, corn, and tobacco, have been manipulated with these methods. Biolistic bombardment and *Agrobacterium tumefaciens* are widely used plant genetic engineering methods for integrating heterologous deoxyribonucleic acid (DNA) into plants (Altpeter et al., 2016).

1.1 Sugar Beet (*Beta vulgaris* ssp. *vulgaris* L.)

Plants convert sunlight into a form that is useful for both people and animals. The most important of these converted forms is sugar. While the plants utilize sugar for their own metabolic processes, certain plants, such as sugar beets and sugar cane, both use for metabolic process and accumulate sugar for storage purposes (Pathak et al., 2014). For thousands of years, sugar has been an essential part of human diets. It is an important part of foods and beverages, especially with its high energy-giving, food sweetening, and preservative properties. The rapid growth of the world population has resulted in a significant increase in the need and demand for sugar over time (Draycott, 2007). Sugar is a valuable agricultural product due to its ease of storage, transportation, and trading.

The first beets were domesticated nearly 2500 years ago. It was originally eaten as vegetables. Throughout the years, beets were selected as swollen parts by humans (Lange et al., 1999). It is believed that the development of root types and enlarged roots originated in the Near East, particularly in regions like Iraq, Iran, and Türkiye, before spreading into Europe (Galewski & McGrath, 2020). It has been used as an important source of sugar, especially in Europe and other temperate regions of the world last 250 years (Bojović et al., 2024; Ćirić et al., 2024). Sugar beet and sugar cane (*Saccharum officinarum* L.) are the two main sources of table sugar (Tayyab et al., 2023a). In sugar beet, sucrose accumulates in the taproot (Figure 1.1), whereas in sugar cane, it accumulates in the stem (Mitchell Mcgrath & Panella, 2019). Over 20% of the global sugar demand is supplied by sugar beet, while the remainder comes from sugar cane (Ćirić et al., 2024).

Sugar beet will remain a strategic crop in many countries as long as sugar continues to play an important role in both human nutrition and industrial production. Sugar beet can also be used as biofuel, livestock, cellulose for papermaking, and biodegradable plastics. Additionally, due to their high nutritional value, sugar beet leaves are also utilized as raw materials for advanced industrial processing (Mukherjee, 2023; Bojović et al., 2024; Ćirić et al., 2024).

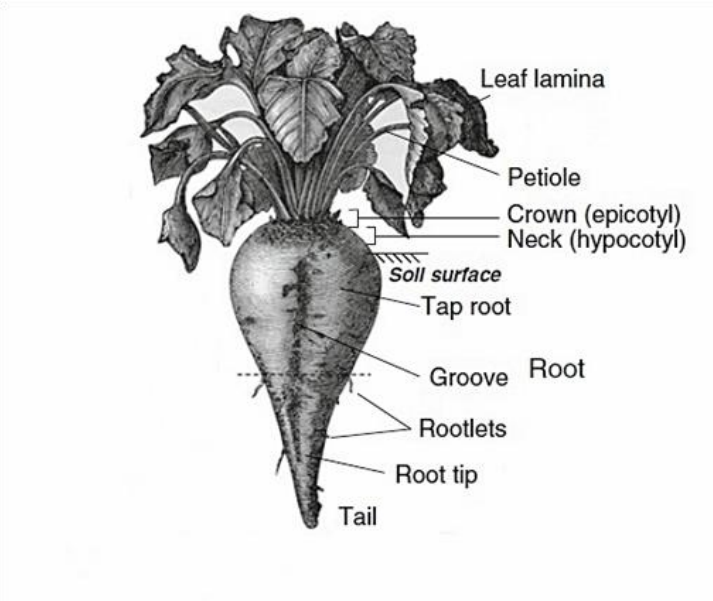


Figure 1. 1. Main parts of sugar beet (*Beta vulgaris* ssp. *vulgaris* L.) modified from (Biancardi et al., 2010a)

1.1.1 General Description and Taxonomy of the Sugar Beet

A detailed description of the beet was first made by Andrea Cesalpino in his book De *Plantis*, 1538. The first binomial name for *Beta vulgaris* was given by Linnaeus in the first edition of Species Plantarum. In the second edition of Species Plantarum, the species were categorized into wild and cultivated plants. Wild beets were classified as *Beta maritima*, while cultivated varieties were categorized under *Beta vulgaris* (Lange et al., 1999). Nevertheless, many changes have been made in the taxonomic grouping of sugar beet from past to present (Sandell et al., 2022).

The sugar beet is a eudicot plant species that belongs to the order *Caryophyllales*, within the family *Amaranthaceae* and the subfamily *Betoideae* (Table 1.1). Scientists think that *caryophyllales* are branched off from the other two eudicot clades around 100 million years ago. Evidence indicates that *Beta* species diverged from their close relatives approximately six million years ago (Mcgrath & Panella, 2019). The Beta genus has four sections, which are called Beta, Nanae, *Procumbentes*, and *Corollinae*.

Table 1.1. Scientific classification of *Beta vulgaris* ssp. *vulgaris* L.

Scientific Category	Classification
Kingdom	*Plantea*
Phylum	*Magnoliophyta*
Class	*Angiospermae*
Category	Basal core eudicots
Order	*Caryophyllales*
Family	*Amaranthaceae*
Subfamily	*Chenopodioideae*
Genus	*Beta*
Species	*Beta vulgaris*

Beta vulgaris (L.) subsp. *maritima* (L.) Arcang, also called sea beet, is accepted as the ancestor of the plants called *B. vulgaris* (L.) *subsp*. *Vulgaris* (Goldman & Janick, 2021; McGrath et al., 2023). *Beta* consists of beets in the *vulgaris* subspecies (Subrahmanyeswari & Gantait, 2022). *B. vulgaris* ssp. *vulgaris* includes all beet species that are domesticated for their leaves or roots. Today, all four varieties of cultivated beet crops sugar beet, fodder beet, leaf beet, and garden beet are encompassed by vulgaris (Moore, 2016). Cross-fertilization has no barriers, and all these cultivated beet and wild varieties can cross each other (Bartsch, 2010; McGrath et al., 2023).

Beta maritima was domesticated in the Middle East, and people used their leaf as a food source (Moore, 2016). Sea beet is found in the Middle East, India, Mediterranean coast, Iran, UK, North Africa, and Azerbaijan. Wild sea beet has two varieties as biennial and annual. Cultivated forms of sea beet are likely focused on biennial forms. Roots of sea beet are unswollen and usually forked (Goldman & Janick, 2021). Unlike sugar beets, sea beets do not require vernalization to flower (Kaffka & Grantz, 2014).

Sugar beet is a long-day herbaceous plant that grows as a biennial. A phase of vegetative growth, cold-induced vernalization, the development of the upright, prolonged flowering stem, and seed production comprise the sugar beet's full life cycle. During its first year, it forms a rosette of leaves and develops a white, conical root where sugar is produced and stored (Subrahmanyeswari & Gantait, 2022). In the second year, it produces seeds (Bojović et al., 2024). Sugar beet requires vernalization process for seed production (Brar et al., 2015). Flowering and seed

development and stem elongates start soon after vernalization. The perianth hardens and turns woody, encasing the seed inside the ovary. Clusters of two to seven flowers are common, and when adjacent flowers in the same cluster come together, a hard, wrinkled particle with a diameter of three to five millimeters is created (Elliott & Weston, 1993).

Sugar beet has taproot and most of the sugar is stored in this swollen taproot. It has a hypocotyl neck with long and strong-petiole leaves (Moore, 2016). It has many leaves, and the leaf shape can vary, such as ovate to cordate shape. Their color can be purple-green, and they can grow up to ~ 35 cm long. Leaves arise from an underground stem, forming a rosette (Subrahmanyeswari & Gantait, 2022). Sugar beet has a C3 photosynthetic system (Zicari et al., 2019). It can grow in a variety of air and soil conditions and is also resistant to salt and frost (Pathak et al., 2014). Sugar beet grows faster than sugar cane. Therefore, it produces more products per unit time. In comparison to sugar cane, it also needs less water (Brar et al., 2015; Stevanato et al., 2019).

Sugar beet has 2n=2x=18 chromosomes and its genome size is approximately 714–758 mb (Wang et al., 2023). On the other hand, for breeding purposes, sugar beet triploid and tetraploid types have also been produced (Zicari et al., 2019). Chromosomes are morphologically similar during mitotic metaphase, and ~60% or more of the genome is highly repetitive DNA sequences. (McGrath et al., 2023). The number of protein-coding genes in sugar beet is estimated to be 27,421 (Subrahmanyeswari & Gantait, 2022). Scientists reported that *B. vulgaris* has between 714 and 758 million base pairs for each haploid genome, and this can show variation through sub-species (Arumuganathan & Earle, 1991).

1.1.2 History of Sugar Beet Cultivation

People have utilized beet for medicinal purposes and as a leafy pot herb at the late Mesolithic. They first started to use beet's enlarged taproot as a vegetable in the Middle Ages (McGrath et al., 2023). Ancient civilizations of Greek, Roman, and Egyptian used beets in their cuisine and drugs, with most recipes pointing out the use of the leaves rather than the root (Goldman & Janick, 2021). There is no clear information about the origin of the enlarged taproot. The modern sugar beets originated from fodder beets, which were grown in Silesia, Germany, at mid-eighteenth (Subrahmanyeswari & Gantait, 2022).

The first breakthrough in the modern industry of sugar beet was the discovery by Andreas Sigismund Marggraf. In 1747, he proved that sugar cane crystals (sucrose) were the same as the crystals that resulted from a crude extraction from pulverized *B. vulgaris* root (Kaffka & Grantz, 2014). His research on red and white beet revealed that the sugar content was 1.6% of the fresh weight (Winner, 1993). Marggraf's student Franz Carl Achard, known as *'father of the beet sugar industry'*, searched the beet crops for more details. He pioneered industrial sugar production from beet root. After studying different forms of *Beta*, his results suggested that roots with white skin, white flesh, and conical shape had the most sugar content (Francis, 2007).

Franz Carl Achard constructed the world's first beet sugar factory in Silesia in 1801. They obtained 4% sucrose of fresh weight. Archard shared his main work about growing and processing beet for sugar production in 1809. He described selecting, cultivating, and processing beet for sugar production and utilizing the by-products. Moreover, he figured out that growth conditions, type of soil, and cultivation process could affect sugar yield and quality. After Achard's report, his innovative ideas about the potential for sugar production spread rapidly across borders. (Francis, 2007).

1.1.3 Production and Distribution of Sugar Beet

Sugar beet is a second-important plant for sucrose production worldwide (Aşkın & Küçüköner, 2020). Sugar beets have a wide distribution area due to their ability to grow in various agro-ecological regions. It can grow between latitudes 30° and 60° north, as well as between 25° and 35° south (Bojović et al., 2024). The development of new sugar beet varieties that grow in warmer climates results in the cultivation of sugar beets in tropical and subtropical regions such as India (Tayyab et al., 2023).

The largest cultivated areas are in Europe and Asia (Bojović et al., 2024) (Figure 1.2). 41 different countries produce sugar beet, and the top five countries are Russia, France, United States of America, Germany, and Türkiye (Subrahmanyeswari & Gantait, 2022). The world's total sugar beet production was around 261 million tons in 2022 (FAOSTAT).

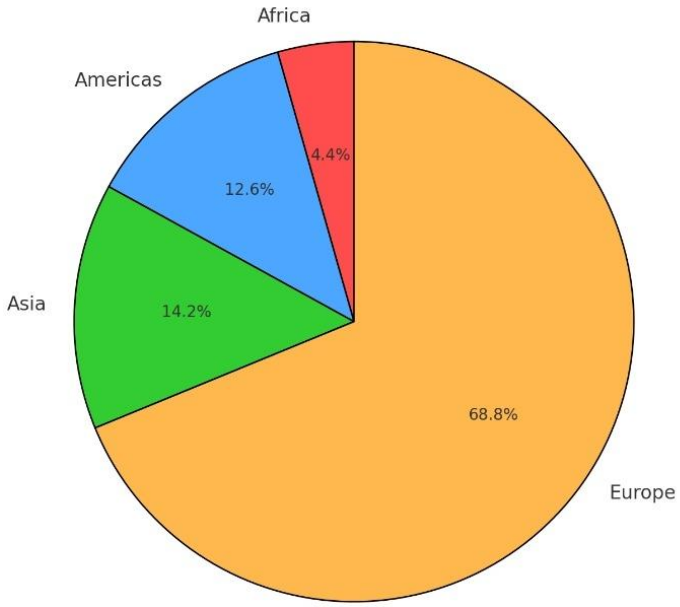


Figure 1. 2. Production share of Sugar beet by region between 2002-2022 (Taken from FAOSTAT)

Türkiye is the world's 5th largest and Europe's 4th largest sugar beet producer. Sugar beet production increased by 22.1% in 2024 compared to the previous year, reaching 23 million tons in Türkiye (TUİK, 2024). Sugar beet production in Türkiye has varied between approximately 12 and 23 million tons in the last 22 years (Figure 1.3). Türkiye has given importance to the production and development of sugar beet varieties since 1932. During this period, various sugar beet varieties have been developed and registered. When considered the current profile of the research, outputs raised from collaborative research by the Turkish Sugar Institute, Bolu Abant İzzet Baysal University, and Kastamonu University, two *Rhizomania*-resistant sugar beet varieties have been officially registered in 2024. These two new sugar beet varieties were named Türkşeker-2023 and Türkşeker-2053.

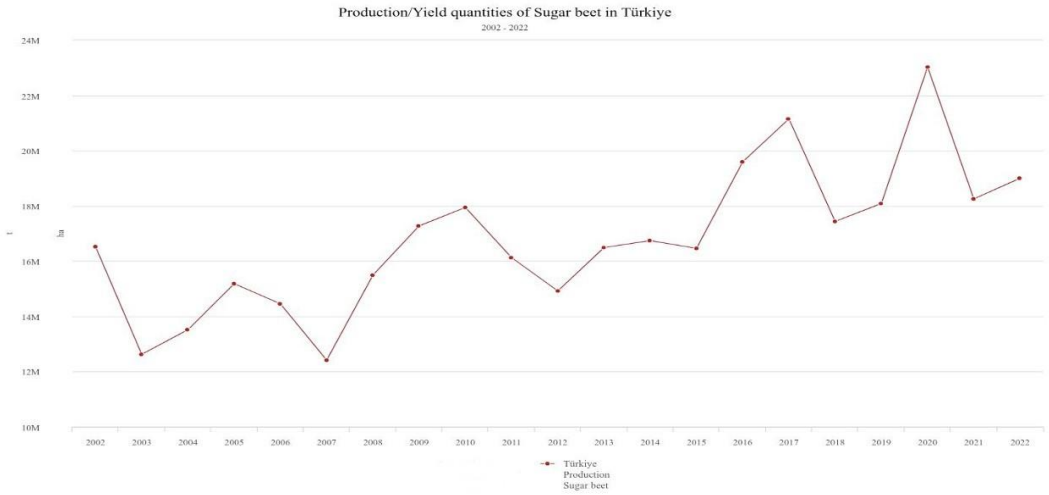


Figure 1. 3. Production (tons) share of Sugar beet in Türkiye between 2002-2022 (Taken from FAOSTAT)

1.1.4 Biochemical Composition of Sugar Beet

Normally, a sugar beet weighs between 0.5 and 1.5 kg. Their roots are composed of approximately 75% water, 20% sugar, and 5% pulp (Subrahmanyeswari & Gantait, 2022). Cellulose, hemicellulose, lignin, and pectin are all present in the pulp, and it is insoluble in water. Moreover, it contains a high amount of Mg, Mn, and Fe (Ćirić et al., 2024).

The sucrose content can vary depending on environmental conditions and cultivar. In mid-18th, sucrose content was approximately 6% in sugar beet. Breeding methods have increased the amount of sucrose content over time (Mitchell Mcgrath & Panella, 2019). The average sucrose content in sugar beets is between 15 and 21% of their fresh weight (Subrahmanyeswari & Gantait, 2022; McGrath et al., 2023). While 98% of the sugar in the roots consists of sucrose, glucose and fructose are present in very small amounts (Gruska et al., 2022). Sugar beet roots also include saponins, free amino acids, soluble saccharides, proteins, betaine, inorganic/organic ions and, nitrogen-free acids. Sugar beets are known as the only source of betalains. They produce two betalain pigments known as red betanin and yellow vulgaxanthin I (Subrahmanyeswari & Gantait, 2022). The protein content of sugar beet roots is from 2% and 10% according to literature (Zicari et al., 2019). Fresh weight of sugar beet leaf contains 1.20-1.75 mg/g chlorophyll and 3.6-7.76 mg/g carotenoid contents (Brar et al., 2015).

1.1.5 Usages of Sugar Beet

Sugar beets are mainly used for sucrose production. Furthermore, the sugar beet plant is used in many different areas. For example, they can be utilized, such as class II preservatives, bioplastics, pectins, cellulose for paper making, biodegradable plastics, etc (Mukherjee & Gantait, 2023). Betalain pigments, which are produced by sugar beets, are used as natural food colorants (Subrahmanyeswari & Gantait, 2022). Pulps from sugar beets have been successfully utilized as a precursor molecule to create other biochemicals, to bio-adsorb contaminants from wastewater, and to reinforce polymer composites (Rana et al., 2022).

Sugar beet can also be used as a biofuel for energy production. Ethanol as the biofuel can be produced from sugar beet. Sucrose can be directly fermented into ethanol using conventional and industrial-scale methods and thus can be used as a biofuel (Finkenstadt, 2014). Ethanol production in sugar beet is much easier compared to other plants (Mujtaba et al., 2020).

Beets, together with their many byproducts, can also be used as fodder sources. Thus, it can be used as an alternative product by countries that have difficulties with animal feed resources. Sugar beet, when combined with proteins, is a suitable feed for many farm animals. (Ćirić et al., 2024). The tops, leaves, and roots can be used as animal feed.

1.2 Cytoplasmic Male Sterility in Sugar Beet

The German botanist Joseph Gottlieb Köllreuter figured out the plant male sterility in 1763 (Chen & Liu, 2014). The term "male-sterility" describes a flowering plant's incapacity to generate functioning pollen grain (Swamy et al., 2017). Early in the 20th century, cytoplasm that cause male-sterility were identified. Bateson and Gairdner (1927) showed in flax that male sterility was inherited from female plants, while genes from both parents influenced its expression. In 1927, Chittenden and Pellow identified that male-sterility in flax results from an interaction among cytoplasm and nucleus. According to them, male sterility results in being induced by a homozygous recessive nuclear genes combined with sterility-inducing cytoplasm (Havey, 2007).

Male sterility has been identified around 610 plant species (Chen & Liu, 2014). Male-sterility provide good chance to decrease production cost of hybrid seeds. Three different male sterility structures are seen in plants: genetic male

sterility (GMS), cytoplasmic male sterility (CMS), and cytoplasmic genetic male sterility (CGMS). GMS is regulated by one or more nuclear genes and follows Mendelian inheritance patterns. On the other hand, CMS is determined by the interaction of nuclear genes and sterile cytoplasm (S), and it does not adhere to Mendelian inheritance (Swamy et al., 2017).

CMS has been identified around 150 plant species for more than a century. It is a maternally inherited feature that prevents the production of functional pollen. Incompatibility between cytoplasm and nuclear genes causes the CMS phenotype. It is one of the important tools for hybrid seed production in self-pollinating species (Sofi et al., 2007). Plants with S cytoplasm are sterile and can only produce seeds when there is foreign pollination. Due to the dominant effect of sterile cytoplasm, the cytoplasm of the hybrid seeds also shows sterile properties (Karaağaç & Balkaya, 2009). Cytoplasm is maternally inherited, leading to complete sterility in the female parent. It serves as the optimal system for hybrid seed production if sterility is stable, and restorer genes are accessible (Swamy et al., 2017). CMS is inhibited by a nuclear gene known as restorer of fertility (*Rf*). A dominant *Rf* allele typically inhibits CMS expression. Therefore, the seed parents utilized in hybrid seed production must be homozygous recessive. To produce seeds or fruit, it is essential that the F_1 hybrid plant undergoes fertility restoration to ensure effective pollination (Arakawa et al., 2020).

CMS is a maternally inherited characteristic frequently linked to typical open reading frames (ORFs) present in mitochondrial genomes. In the last 20 years, mitochondrial genes linked to male sterility have been identified in sources of cytoplasmic male sterility in beets. One of these genes is called *attp6* (36 kDa) and exhibits a strong association with the mitochondrial membrane. The other gene source called *orf129* (12 kDa) and its translation product was found in matrix and inner mitochondrial membrane (Mikami et al., 2011).

CMS phenotypes include various reproductive abnormalities. These anomalies can be listed as follows; stamens can be transformed into petals, which serve a non-reproductive function, or into female carpels. It arises from the breakdown of stamens or particular stamen sections, including the anther and tapetal cells, which line the anther and facilitate pollen formation. In certain instances, CMS appears not within the pollen-producing structures but in the developing pollen itself; the pollen either does not complete its development or, if

it does, fails to function effectively (Chase, 2007). The mitochondria are likewise impacted by cytoplasmic male sterility. It has been noted that mitochondria are substantially smaller in CMS plant microspores and tapetum. A lot of adenosine triphosphate (ATP) is needed for the steps of cell division and differentiation. CMS plants' tapetum's smaller mitochondria hinders adequate energy generation, which has an adverse effect on pollen formation. (Sadzık, 2024).

The key benefit of CMS is that the maternal lines, characterized by their 100% male-sterile plants, negate the necessity for artificial emasculation. This results in high labor and other expense costs while also guaranteeing seed purity. As a result, these systems have been extensively utilized for the exploitation of crop heterosis (Lin et al., 2014).

CMS in sugar beet plant was first found and studied by Owen (1945). According to Owen's findings, sugar beet sterility is contingent on the interaction of at least two recessive genes with an S cytoplasm. He suggested that the 100% male-sterile sugar beet plant carries the (S)xxzz genotype, while other eight genotype combinations showed varying degrees of pollen sterility. CMS plants need to be pollinated by O-type plants that have the same genes as the male-sterile plants but contain N cytoplasm ((N)xxzz) so that offspring can be obtained from the male-sterile plants. Identifying the O-type of sugar beet plant is a very difficult and time-consuming process. Only 3-5% of the population exhibits O-type of plant (Bosemark, 2007; Biancardi et al., 2010). The sugar beet CMS is linked to a distinct 39 kDa mitochondrial protein, which is encoded by an open reading frame of unknown origin, designated *preSatp6*. The translation product of *preSatp6* is present in all analyzed organs (Arakawa et al., 2020). Every single CMS line requires a corresponding O-type for reproduction. A minimum of six to eight backcrosses is required to generate comparable genotypes that primarily differ in their N- and S-cytoplasms (Figure 1.4). The commercialization of hybrids was made feasible in 1955 following the identification of genetic-cytoplasmic male sterility systems (Biancardi et al., 2010). Plants with the CMS and O-type trait can be selected from the population using pollen tests and/or molecular markers.

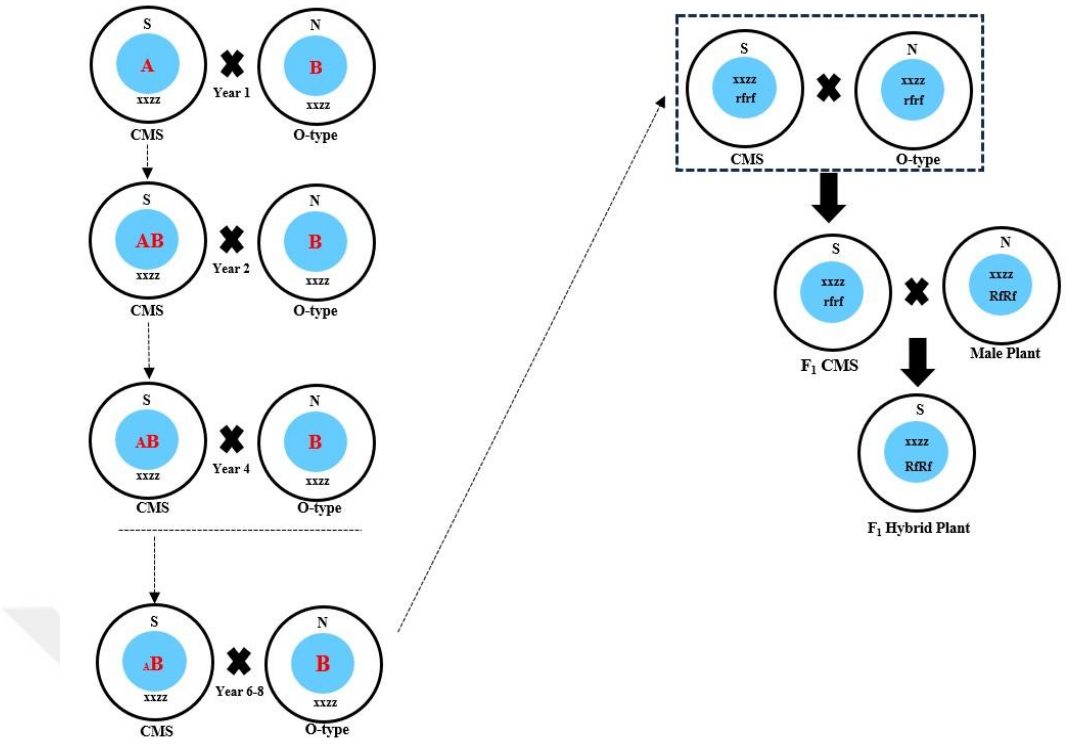


Figure 1. 4. Schematic representation of hybrid seed production using Cytoplasmic Male Sterility (CMS) and fertility restoration systems

1.3 Plant Tissue Culture in Plant Genetic Engineering

Growing cells, tissues, and organs in a synthetic medium under aseptic conditions is known as plant tissue culture. This technique relies on the principle of totipotency (Gosal & Kang, 2012). Totipotency refers to the genetic potential of a cell to develop into a multicellular organism. (Loyola-Vargas & Ochoa-Alejo, 2018). The concept of totipotency and the capacity for plants to generate from single cells was discovered by Haberlandt in 1902 (Sehgal & Khan, 2020). The other important step in the tissue culture began with the discovery and application of auxins and other plant growth regulators. Following research on nucleic acids resulted in the identification of the first cytokinin, kinetin (Yancheva & Kondakova, 2018). With the discovery of cytokines, researchers figured out that the balance of hormones (auxins vs cytokinins) in the plant growth medium leads to the production of different tissues in the plant. Normally, a high ratio of cytokinins to auxins promotes shoot formation, while a high ratio of auxins to cytokinins favors root formation. A balanced ratio promotes callus formation. A callus is a mass of undifferentiated cell tissue characterized by its disorganized structure. Normally, it occurs in plants as a response to injuries or pathogen invasion. Callus can arise from

a single differentiated cell. With many callus cells exhibiting totipotency, enabling them to regenerate the entire plant body (Chandimali et al., 2024).

The formation of plant organs, either directly from an explant or from a callus, is known as plant organogenesis. There are two stages of organogenesis which are called dedifferentiation and redifferentiation. Dedifferentiation leads to callus formation from explants accompanied by increased cell division. Redifferentiation is caused by the formation of primordia from a cluster of callus cells.

Direct organogenesis is defined as the formation of shoots, roots, and buds from an explant without an intermediary callus phase, achieved through the sole application of cytokinins or in combination with auxins, ultimately leading to the regeneration of plantlets (Subrahmanyeswari & Gantait, 2022). Indirect organogenesis refers to the process by which a plant organ develops from callus tissue, ultimately resulting in the formation of a complete plant.

Protocols for tissue culture are significantly influenced by the type of explant, genotype, and the medium utilized during various phases of cell proliferation, embryo or organ formation, and regeneration (Sehgal & Khan, 2020). Tissue culture exhibits significant species dependence and genotype specificity. Certain plants, including tobacco (*Nicotiana tabacum*), *Arabidopsis thaliana*, and rice, exhibit high capacity for *in vitro* regeneration. In contrast, other species, such as soybean (*Glycine max*), wheat (*Triticum aestivum*), and maize (*Zea mays*), present greater challenges in regeneration phase (Long et al., 2022). The physical conditions of a growth medium influence plant tissue response (Gurel et al., 2008). For all these reasons, determining tissue culture protocols suitable for each plant genotype is of enormous importance. The development of plant tissue culture significantly improved control on morphogenesis through the manipulation of various levels and combinations of plant hormones. This capability enabled the regeneration of almost all plants, facilitating the use of *in vitro* systems to investigate essential aspects of cell differentiation and development, as well as expanding the applications of tissue culture in areas such as plant genetic engineering (Loyola-Vargas & Ochoa-Alejo, 2018).

Over a century since the first paper, tissue culture remains a vital technique for the generation of transgenics and for the industrial micropropagation of plant species. Plant tissue culture is a significant strategy in agriculture, horticulture, and

biotechnology, providing effective methods for the propagation, preservation, and improvement of plant varieties. Tissue culture facilitates large-scale plant propagation and plays a significant role in plant growth and physiology, somatic embryogenesis, artificial seeds, and pathology studies.

Plant tissue strategies serve as effective auxiliary tools in plant cultivating and genetic transformation applications (Loyola-Vargas & Ochoa-Alejo, 2018). Understanding the regulatory network and genetic mechanisms that control plant regeneration in tissue culture enhances genetic transformation efficiency (Long et al., 2022). The integration of plant tissue culture with genetic transformation has emerged as a significant tool for advancing studies in molecular biology in the past decade (Purwantoro et al., 2022). Tissue culture is crucial in the selection of genetically modified plants or explants (callus, petiole, etc.) that are derived from methods such as agrobacterium or biolistic within the selection medium, as well as in the regeneration of the chosen plants. Changes resulting from the genetic transformation of cells make plant tissues and individual plant bodies easily identifiable during development in an *in vitro* culture medium. Genetic transformation devoid regeneration, and regeneration absent genetic transformation possesses limited utility (Sharma et al., 2005). Over the last two decades, the integration of recombinant DNA technology, gene transfer strategies, and the use of tissue culture has facilitated the effective transformation and production of transgenic crops across various plant species (Gosal & Kang, 2012).

1.4 Plant Genetic Transformation

The production of higher-quality plants is a common objective in plant breeding applications. These programs may be established through three components: variability, selection, and genetic recombination. These aspects can be addressed concurrently through either classical or modern breeding methods. Moreover, integrating classical and modern breeding techniques is very important for the best outcomes in plant breeding programs (Purwantoro et al., 2022). Classical plant breeding, alongside enhanced agricultural practices, has significantly advanced crop yields over the last five decades and is expected to yield further advantages in the future. There are significant pressures for enhancements in crop quality and quantity due to increases in population, social demands, environmental stresses, and ecological considerations. Classical plant breeding

technology faces limitations stemming from a restricted gene pool and the species barriers that constrain gene transfer among organisms. Plant biotechnology advances the enhancement of crops through precise methodologies, enabling the transfer of individual genes with known functions into current crop varieties (Sharma et al., 2005). By transferring helpful genes, plant biotechnology has a lot of potential to improve crop quality and the ability of plants to handle both biotic and abiotic stresses (Gürel et al., 2019).

Plant genetic transformation represents a new strategy and a significant technological development in modern breeding, offering advanced methods for crop enhancement and making it easier to obtain basic information about plant biology. Plant genetic transformation methods are now frequently used over several plant species, both annual and perennial crops, as well as dicotyledonous and monocotyledonous plants. Genetic transformation in plants through genetic engineering targets to develop new varieties, which are disease and pest resistance, enhanced protein production in plant cells, drought tolerance, increased pharmaceutical compounds, and improved specific traits, while also analyzing the functional genes of plants (Purwantoro et al., 2022). Plant genetic engineering offers methods to incorporate single elements into intricate pathways and to control their expression in a spatial and temporal manner (Rivera et al., 2012).

The synthesis of recombinant DNA molecules occurred in the early 1970s through the application of restriction enzymes, which function as biochemical scissors on the genome (Rivera et al., 2012). Genetic engineering refers to the method of modifying an organism's genetic composition through recombinant DNA technology. This process utilizes laboratory instruments to insert, modify, or remove nucleotides that encompass one or more target genes. Genetic engineering facilitates the transfer or introduction of novel and beneficial traits into plants. In contrast to traditional breeding methods, which involve selecting and incorporating specific traits into various cultivars, genetic engineering facilitates the direct transfer of one or several genes of interest between closely or distantly related organisms to achieve the desired agronomic characteristics. Not all genetic engineering methods require the incorporation of DNA from external organisms. Plants can be altered through the removal or deactivation of their own genetic material (Sehgal & Khan, 2020). Plant genetic transformation involves two related phases: the insertion of DNA into plant cells, often referred to as transient

transformation, where transgenes have not yet integrated into the genome, followed by the integration of the GOI into the plant genome, known as stable transformation (Altpeter et al., 2016).

Following the preliminary reports by three research teams regarding the genetic transformation of plants utilizing the *Agrobacterium tumefaciens* tumor-inducing plasmid at the Winter Symposium in 1983, genetically transformed crops are currently cultivated in 41 countries (Anjanappa & Gruissem, 2021). The earliest successful regeneration of a transgenic plant from transformed plant cells that express a bacterial gene encoding neomycin phosphotransferase occurred in 1983 (Barton et al., 1983). During the 1980s, genetically stable transformed plants such as maize, grape, petunia, cassava, rice, Brassica napus, wheat, tobacco, tomato, millets, and chrysanthemum were successfully developed. The tomato was the first genetically engineered crop approved by the FDA for food consumption and distribution in the U.S. market in 1994 (Rivera et al., 2012).

Plant genetic transformation has produced a large number of genetically modified plants containing DNA regions that cannot be transferred by any existing breeding method (Rommens, 2004). The transgene may originate from different plant species, non-plant sources or bacteria. Screening and identification of genes is potentially important for improved traits in plants is crucial for plant genetic transformation (Saeed & Shahzad, 2015).

In order to develop transgenic plants, all of the following stages must be correct and effective (Sharma et al., 2005);

- The setting up of dependable *in vitro* regeneration systems,
- Development of gene constructs and transformation into appropriate vectors,
- Effective transformation methodologies for the incorporation of genes into agricultural plants,
- Regeneration and propagation of transgenic plants,
- Characterization of putative transgenic plants at the molecular and genetic levels for stable and effective gene expression,
- Assessment of transgenic plants for their efficacy in managing biotic and abiotic pressures.

Plant genetic transformation methods are categorized into two groups: indirect genetic transformation and direct genetic transformation (Figure 1.5). Bacterial plasmids are used as carriers for indirect genetic transformation, like Agrobacterium-mediated gene transfer into target cells. In contrast, direct genetic transformation utilizes external forces to introduce genes of interest through the cells, including methods such as particle bombardment/gene gun, electroporation, etc. (Su et al., 2023).

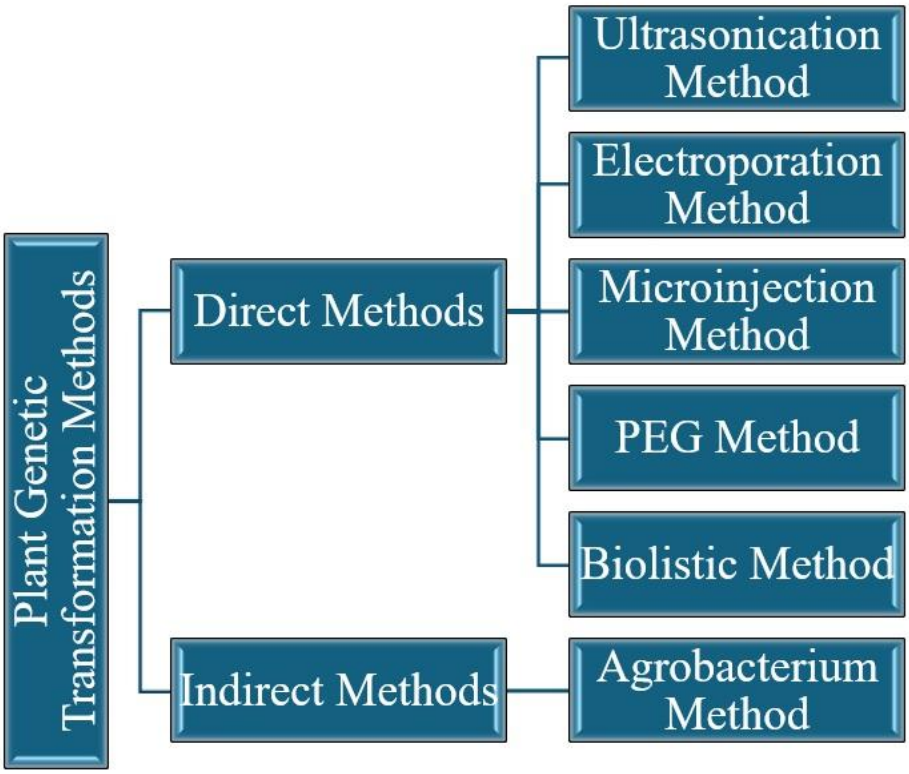


Figure 1. 5. Plant genetic transformation methods

Indirect transformation methods involve the delivery of plasmids—independent circular DNA molecules that exist in bacteria, distinct from the bacterial chromosome—into the target cell using bacteria that can transfer genes to plant species. *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* are the most utilized microorganisms, both of which are native to soil environments. The size of a plasmid used for genetic transformation can range from 5 to 12 kilobase pairs in *Agrobacterium* (Rivera et al., 2012). Agrobacterium species, *A. tumefaciens* and *A. rhizogenes*, possess plasmids that trigger tumor (Ti) or hairy root (Ri) formation. Modification of the plasmid allows for the transfer and integration of a segment of the T-DNA from the Ti/Ri plasmid into the plant genome, facilitating

the co-integration of the target gene with the T-DNA. Plant genetic transformation via *Agrobacterium*-mediated offers high efficiency, easy procedures, and genetic stability. This method is applicable for the transformation of most dicot species and a few monocot species (Su et al., 2023).

The use of *Agrobacterium* for gene transfer presents certain disadvantages. Multiple parameters influence plant regeneration and transformation, resulting in a slow process. The introduction of unnecessary common vectors that induce unknown genetic expressions in plants may adversely impact growth and development. The requirement for acquiring bacteria-free plants post-transformation represents a notable disadvantage (Rivera et al., 2012).

1.4.1 Biolistic: Particle Bombardment

Direct transfer methods for plant transformation utilize physical or chemical methods exclusively to introduce DNA into the target cells (Altpeter et al., 2005). Particle bombardment, (or gene gun) method, is a physical technique utilized for the direct introduction of foreign DNA into the plant genome. This method allows for plant transformation to occur on a variety of targets, including cells, callus, immature embryos, organs, or mammalian cells (Su et al., 2023). The particle bombardment machine was developed at Cornell University in 1987 by Sanford and his colleagues for the genetic transformation of cereals and monocotyledons, though it is applicable to various plant species. The initial stable transformation employing particle bombardment has been discovered in soybean. Immature embryonic cells successfully generated stably transformed callus tissue. The technique successfully achieved the first genetic engineering of chloroplasts and mitochondria, respectively (Ozyigit & Yucebilgili Kurtoglu, 2020).

This method involves coating the target gene onto the surface of gold or tungsten particles (0.6 – 1.0 μm) to create a DNA-coated microcarrier. The DNA-coated microcarrier is moved through the gas acceleration tube by high-pressure helium pulses and a stream of pressurized helium gas (Su et al., 2023). The microcarriers are uniformly distributed on the macrocarrier, which is composed of circular plastic film. This complete assembly is maintained within the primary vacuum chamber located beneath the rupture disk in the apparatus. A wire mesh, referred to as a stopping screen, is positioned beneath the macrocarrier to retain it while permitting microcarriers to pass through. Also, the target cells are situated

below the entire assembly (Saeed & Shahzad, 2015). The particles acquire adequate speed to penetrate recipient cells rapidly, while the target gene, coated externally, remains within the cell and is ultimately integrated into the plant's chromosome, resulting in the transformed plant (Figure 1.6). Various types of rupture discs are currently available, designed to burst at pressures from 450 to 2200 psi.

Biolistics is a widely recognized direct method for the genetic modification of plants, applicable to various species, subcellular organelles, bacteria, fungi, and animal cells. Its advantages include a brief processing time and ease of introducing multiple genes or chimeric DNA. Additionally, it is independent of the cell's electrophysiological properties, such as electrical potential and the structural components of the cellular membrane. Nonetheless, the transformation factors require optimization for each biological target utilized (Rivera et al., 2012).

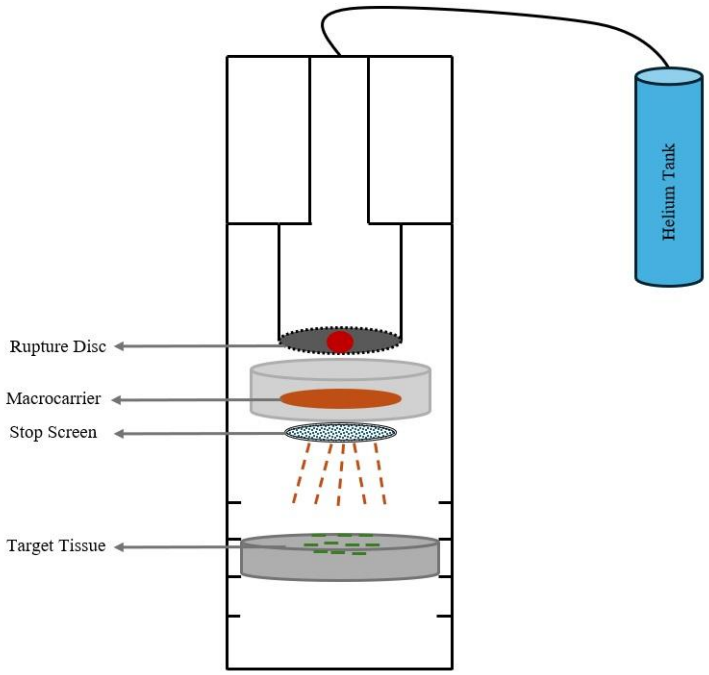


Figure 1. 6. Diagrammatic representation of particle bombardment machine (PDS-1000/He)

As previously mentioned, plant genetic transformation occurs in two stages: The DNA is initially transferred into the recipient cell, followed by its integration into the host genome. The transformation efficiency with this method is influenced by various parameters, including temperature, cell quantity, regenerative capacity, the number of DNA-coated particles, the DNA coverage on each particle, and

pressure. The success of a particle bombardment experiment relies on the optimization of several key variables. These factors can be categorized into physical, environmental, and biological variables (Ozyigit & Yucebilgili Kurtoglu, 2020).

Physical parameters encompass the composition and size of microprojectiles, the conformation and quantity of DNA, as well as machine settings (Kikkert et al., 2005). The selection of particle structures with desired physical properties is a critical factor influencing the efficacy of DNA transformation. Dense metal particles are chosen due to their ability to provide the necessary kinetic energy for cell penetration. Chemically inert metals are favored to prevent cell toxicity. Metal substances including gold, tungsten, palladium, platinum, and rhodium are utilized in DNA coating. Gold and tungsten are extensively utilized in coating processes (Ozyigit & Yucebilgili Kurtoglu, 2020). Although both meet most requirements, they still exhibit disadvantages in certain applications. Studies indicate that tungsten use is associated with DNA damage and inhibited cell growth, suggesting that gold is preferable for achieving higher transformation efficiency. Tungsten, in contrast, is recognized for its enhanced ability to bind DNA (Mazuś et al., 2000; Twyman & Christou, 2004). Additional chemicals include spermidine, protamine, and calcium chloride, which are utilized for DNA coating. Their concentration and preparation methods are also highly important. The concentration of DNA influences the shot load and requires careful optimization. An elevated DNA load enhances the occurrence of transient expression; however, it may also result in harm to tissues. Instrument parameters such as helium pressure, vacuum level, and sample position within the chamber should be adjusted based on the study's objectives and the specific plant genotype (Rasco-Gaunt et al., 1999).

Environmental factors, including moisture, temperature, and light intensity, may influence the rate of successful transformation. Elevated humidity can influence the dissociation of micro/microcarriers, leading to the aggregation of microparticles and a subsequent decrease in the delivery rate (Ozyigit & Yucebilgili Kurtoglu, 2020).

Biological factors are essential for the success of transformation experiments. The factors include the gene construct, explant type, physiological status of the target, and post-bombardment culture conditions. The DNA intended

for transfer typically consists of an expression cassette cloned within a bacterial cloning plasmid. The cloning vector backbone generally comprises a bacterial origin of replication and a selectable marker, facilitating the cloning of the expression construct in *Escherichia coli*. There are a few main parts that make up the expression cassette: a promoter, an open reading frame, a multilinker sequence with specific restriction sites for the gene(s), and a terminator that works in plant cells. The expression cassette alone is sufficient for the expression of the transferred gene, independent of the vector backbone (Fu et al., 2000). A promoter sequence, located upstream of the gene of interest, regulates both the place and degree of gene expression in transformed cells. *35S*, *adh1*, or *actin 1* can be some examples of the promoter sequences (McElroy et al., 1990; Petrillo et al., 2008). A selectable marker gene sequence is an essential component of the gene construction employed to identify transformed cells following the bombardment. Genes conferring resistance to antibiotics or herbicides, such as *nptII*, *bar*, *adaA*, or *ESPS*, are frequently employed to select viable transformed cells (Petrillo et al., 2008). Reporter genes, including *GUS* and *GFP*, serve as indicators of transformation with a specific gene or its expression within target cells (Ozyigit & Yucebilgili Kurtoglu, 2020). The selection of target tissue may influence both transient and stable transformation frequencies. The efficiency of transformation is primarily influenced by the ability to regenerate and totipotency of the cells. Meristematic and embryogenic cells represent the most common explant types utilized for stable transformation.

Particle bombardment refers to the process of directing high-energy particles towards a target material for stable genetic transformation not only nuclear but also mitochondria, and chloroplast organelles (Sharma et al., 2005). This method is acknowledged as the first to accomplish organelle transformation, such as chloroplasts and mitochondria, as well as nuclear transformation in recalcitrant crop species, primarily monocots like rice, wheat, and maize (Ozyigit & Yucebilgili Kurtoglu, 2020). A key benefit of particle bombardment is its independence from the biological constraints of any specific group of microorganisms. Furthermore, it is not contingent upon any specific cell type, provided that the DNA can be integrated into the cell without causing cell death. The generation of transgenic plants from transformed cells relies solely on the capacity of these cells to demonstrate totipotency within the utilized culture conditions. In this context, particle bombardment outperforms other techniques for transformation as it allows

for the use of both individual cells and organized tissues as targets transformation (Altpeter et al., 2005). Furthermore, co-transformation using multiple plasmids enables the concurrent transformation of several genes with a single selectable marker. Furthermore, it is essential for numerous agronomic traits that are governed by multiple genes. This method gained appeal among plant scientists for producing plants with enhanced desirable traits at a specific period (Zorrilla-López et al., 2013).

This method is also limited to transferring DNA smaller than 10 kb, as larger DNA sizes are prone to breakage during bombardment or exhibit weak adherence to metal particles, leading to disorganized DNA integration events (Su et al., 2023). In comparison to Agrobacterium-mediated transformation, it presents certain disadvantages, including a reduced transformation rate and increased costs (Keshavareddy et al., 2018).

1.5 Plastid Transformation

Plastids are organelles found in the cells of plants, algae, and certain protists that are crucial for survival (Maliga, 2004). It is now well acknowledged that plastids emerged approximately 1.5 billion years ago when a cyanobacterial cell was absorbed by a heterotrophic eukaryote. Throughout the years, the host and the arriving bacterial cell established a symbiotic relationship (Bock, 2015). The morphology and function of plant plastids differ across various plant tissues; for instance, green chloroplasts are found in photosynthetically active cells, while red, orange, and yellow chromoplasts are located in fruits and flowers, and colorless ones are found in storage organs (Scotti et al., 2012). Additional specialized plastid types encompass gerontoplasts, which are associated with senescent leaves and play a crucial role in resource allocation; oleoplasts, responsible for oil storage; and etioplasts, present in the final stage of proplastid development within photosynthetic tissues under dark conditions (van Wijk & Baginsky, 2011). Each plastid type originates from a small, unpigmented organelle known as proplastid, which is found in meristematic cells (Scotti et al., 2012). Plant cells include DNA in three different cellular sections: the nucleus, plastids, and mitochondria. Plastids contain their own genetic materials and transcriptional - translational machinery. The plastid genome, also referred to as plastome or ptDNA, is a strongly polyploid, circular double-stranded DNA ranging from 120 kb to 180 kb in size, encoding

approximately 120 genes (Maliga, 2004). ptDNA typically exhibits a tetrapartite genome structure, comprising a large single copy region (*LSC*) and a small single copy region (*SSC*), which are divided by two inverted repeat parts (*IRs*) that differ solely in their relative orientation. Estimates indicate that, varying by species, tissue, phase of development, and growth conditions, a plant cell may contain as many as 10.000 identical copies of ptDNA (Scotti et al., 2012). The DNA sequence of chloroplasts is significantly smaller than that of free-living cyanobacteria; however, the remaining genomic sequences exhibit notable similarities. Chloroplast genomes in land plants generally possess around 120 unique genes, in contrast to cyanobacteria, which contain over 1500 genes. A significant number of absent genes are found within the host's nuclear genome (Martin et al., 2002). Despite the relatively small size of ptDNA in comparison to higher plant nuclear genomes, chloroplast DNA generally constitutes approximately 10-20% of the total DNA in cells content. The underlying reason is that a diploid plant cell contains thousands of copies of ptDNA (Bock, 2001).

In almost all of angiosperm plants, plastid genes exhibit maternal inheritance (Verma & Daniell, 2007). Even if transgenic plastids may exist in pollen, plastid DNA is removed from the male germ line at various stages of sperm cell development, contingent upon the plant species. This reduces the chance of transgene outcrossing to related weeds or crops (Hagemann, 2007).

Plastids show an endosymbiotic origin, as previously noted. In comparison to their cyanobacterial ancestors, they have a limited number of genes categorized into three primary groups: genes for protein synthesis (including rRNAs, RNA polymerase subunits, tRNAs, and ribosomal proteins), genes associated with photosynthesis, and additional genes (which encompasses those with various functions, conserved sequences, and other open reading frames) (Scotti et al., 2012).

Plastid gene editing represents a significant advancement in agricultural development initiatives, as it allows for the effective manipulation of plastid genomes to achieve desirable quality traits. The process of developing transplastomic plants involves two primary steps. The first step involves the transfer and integration of a target gene into the ptDNA via plasmid DNA. In the second stage, transformed cells are selected using selective media that contains antibiotics, leading to the restoration of fully developed plants (Filipenko et al., 2011). Plastid

transformation is generally achieved through particle bombardment of plant tissue using a transformation vector or through polyethylene glycol-mediated transformation of protoplasts. A novel strategy for plastid transformation utilizing nanoparticles has recently been introduced. This method facilitates the delivery of DNA to chloroplasts via single-walled carbon nanotubes, eliminating the requirement for supplementary instruments or protoplast isolation (Hanson et al., 2013; Yu et al., 2020).

1.5.1 Chloroplast Transformation

Chloroplasts function as metabolic process centers that convert solar energy into carbohydrates via photosynthesis while also releasing oxygen (Daniell et al., 2016). In addition to its well-established role in photosynthesis, chloroplasts participate in essential plant cellular processes, including amino acid synthesis, secondary metabolite production, fatty acid synthesis, starch, pigments, vitamins synthesis, and nitrogen metabolism (Kumar & Ling, 2021; Rascón-Cruz et al., 2021). The chloroplast genome encodes approximately 120 genes within a size range of 150 to 220 kb, characterized by a quadripartite structure. A notable feature in higher plants is the duplication of a substantial region, approximately 25 kb, in an inverted orientation (IRs) (Kusnetsov, 2018), which contributes to genomic stability (Rascón-Cruz et al., 2021; Yue et al., 2008). Chloroplasts contain approximately 3000 proteins that regulate their metabolism, predominantly encoded by the cell nucleus, with a limited number encoded by the chloroplast itself (Leister, 2003).

Plastid transformation involves the introduction of foreign DNA into chloroplasts or non-green plastids. Chloroplast transformation involves modification of the genome or introduction of a new foreign gene into the chloroplast (Kumar & Ling, 2021). Upon achieving stable genetic transformation, all plastid types within the plant will possess an identical transgenic plastome (Day & Goldschmidt-Clermont, 2011). As a result of endosymbiosis, plastids received both membranes forming the envelope from gram-negative cyanobacteria. Therefore, for chloroplast transformation, DNA must pass through at least three membranes (plasma membrane and envelope) along with the cell wall (Gould et al., 2008).

Chloroplast transformation is not the same as transgene integration by the nuclear transformation because chloroplast transformation method depends on two homologous recombination events between the targeting regions of the transformation vector and the wild type ptDNA. Thus, chloroplast transformation provides integration of the gene of interest into a predictable, predetermined location (Scotti et al., 2012). The design of vectors for plastid transformation necessitates the inclusion of flanking sections of homology corresponding to the intended integration site within the plastid genome (Figure 1.7) (Bock, 2015).

Transformation vectors originate from *E. coli* plasmids and, in addition to the two plastid sequences necessary for recombination (left and right flanking sequences), the expression cassette consists of the selectable marker gene (SM) and the gene of interest (GOI) (Figure 1.7) (Scotti et al., 2012).

Chloroplast transformation consists of three stages: in the beginning foreign DNA is introduced into the cells of an explant. The foreign DNA is integrated into the plastome via homologous recombination at a specific site. Third, candidate transformants undergo repeated screening on selection medium until the wild type plastome is fully eliminated. Positive explants are subsequently regenerated into stable transplastomic plants (Yu et al., 2020).

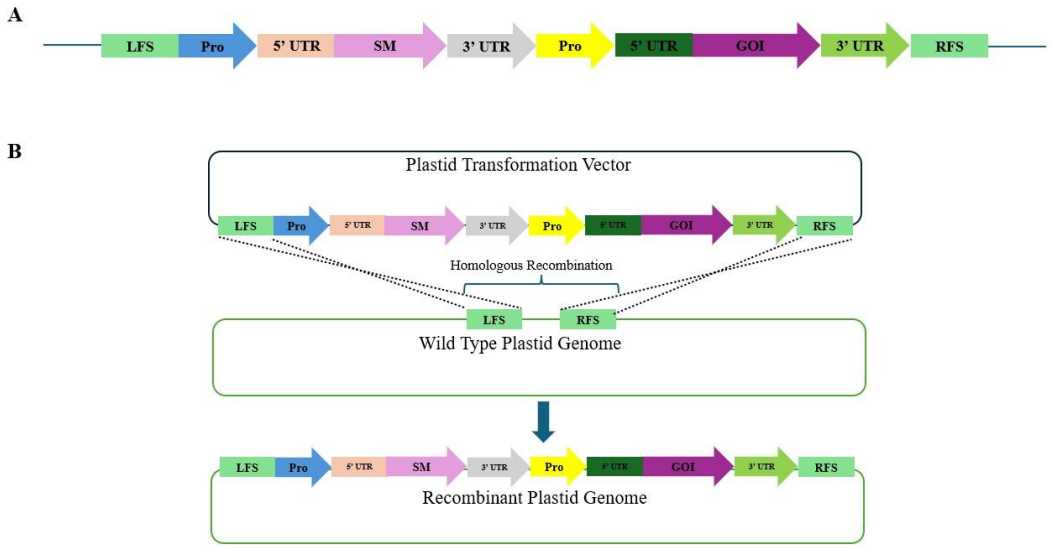


Figure 1. 7. Vector design. **A:** Chloroplast expression vector cassette. **B:** Homologous recombination between plastid transformation vector and wild-type plastid genome

The chloroplast transformation technique presents several significant advantages, such as elevated transgene expression levels, the capability for multi-gene engineering within a single transformation experiment, prevention of transgene escapes through maternal inheritance, absence of gene silencing vector sequences, mitigation of position effects due to site-specific transgene integration (Kumar et al., 2004). The chloroplast genome can host numerous transgene copies, potentially reaching up to 10.000 transgene copies for each cell, due to the presence of 10–100 genomes per chloroplast and 10–100 chloroplasts per cell (Koya & Daniell, 2005). Because of this feature of chloroplast transformation is the capacity to accumulate significant quantities of foreign protein, reaching up to 46% of total leaf protein, when the homoplastomic stage is reached (Verma & Daniell, 2007).

Chlamydomonas reinhardtii, a green eukaryotic alga, has served as a first significant organism for chloroplast genetics and molecular biology, facilitating advancements in stable chloroplast transformation. The *Chlamydomonas* cell contains a single chloroplast (Day & Goldschmidt-Clermont, 2011) and the stable chloroplast transformation was first succeeded in Chlamydomonas (Boynton et al., 1988). In 1989, Pal Maliga and colleagues achieved the first successful chloroplast transformation in a higher plant (Bock, 2001). They presented stable transformation of tobacco (*Nicotiana tabacum*) plastids using a chloroplast 16S ribosomal RNA

gene modified with point mutations that provide resistance to spectinomycin and streptomycin. They achieved this by biolistic bombardment of sterile tobacco leaves, followed by selection for spectinomycin-resistant cell lines (Svab et al., 1990). Chloroplast transformation through organogenesis has been successfully achieved in several crops, including lettuce (Lelivelt et al., 2005), cabbage (Liu et al., 2007), oilseed rape (Cheng et al., 2010), poplar (Okumura et al., 2006), sugar beet (De Marchis et al., 2009), potato (Valkov et al., 2011), tomato (Ruf et al., 2001). Nonetheless, the transformations of *Chlamydomonas* and tobacco have become sufficiently routine to serve as a model system for chloroplast transformation (Kong et al., 2017).

The chloroplast transformation process entails the design of the DNA construct, which includes selectable markers, and the subsequent introduction of this construct into the chloroplast. The design of vectors and selectable markers is crucial for inserting the desired gene into the vector construct, particularly in chloroplast transformation, and for identifying successfully transplastomic plants.

1.5.2 Vector Construct

As we mentioned before, transformation vectors originate from E. coli plasmids. Basically, the transformation vector has two plastid sequences, the left and right flanking sequences, that allow recombination. It also has the selectable marker gene (SM) and the expression cassettes for the gene of interest (GOI). Cassettes typically comprise a 5'-regulatory region that contains a robust promoter (*Pro*) and a 3'-regulatory region, that has a terminator sequence corresponding to the mRNA 3'-untranslated region (*3'-UTR*) (Figure 1.7) (Scotti et al., 2012).

Flanking sequences refer to the DNA sequences from the ptDNA that exhibit homology to the target integration site. Their role is to promote site-specific recombination and determine the insertion location of the transgene. The flanking sequence should be unique to the targeted ptDNA; these sequences are about 1 kb in length and are positioned on both sides of the expression cassette (Venkatesh & Park, 2012). Currently, several plastid sequences serve as homolog recombination sites, with three being predominantly utilized in plastid transformation vectors: the *trnV-3'/rps12* and *trnI*/*trnA* regions found in the *IRs*, and the *trnfM*/*trnG* region located in the *LSC* (Zoubenko et al., 1994; Daniell et al., 1998; Ruf et al., 2001). The most frequently used of integration region from this three is between the *trnI-*

trnA genes, within the *rrn* operon. This site is found in the inverted repeat regions of the ptDNA (Verma & Daniell, 2007).

Determination and selection of the GOI region is important for transformation experiments. A GOI is frequently present in multiple copies within the genome; therefore, selection among them should be based on criteria including expression level, gene structure, and the presence of conserved domains (Basso et al., 2020). The GC content of the gene of interest can enhance its expression level. Additionally, both codon usage and GC content are factors that contribute to increased mRNA stability and protein accumulation (Sidorenko et al., 2017). A high GC content in the *5′-UTR* may impede ribosome loading and interfere with translation (Kawaguchi & Bailey-Serres, 2005).

Promoters are DNA sequences typically ranging from 300 to 1500 nucleotides in length, situated upstream of the *5′-UTR* of a gene. It encompasses various regulatory elements that play a crucial role in the regulation of transcription initiation. The effective transcription of GOI requires the transcription initiation complex to associate with other proteins, such as activators or repressors, to initiate the transcription of DNA by RNA polymerase. *psbA*, *rrn*, and *rbcL* are the most used promoter sequences in plastid transformation. The *psbA* promoter was initially utilized more than three decades ago in Chlamydomonas and continues to be the optimal site for target gene insertion, as the *psbA* gene product is the most abundantly translated plastid protein (Yu et al., 2020). Transcriptional terminator region are conserved sequences consisting of cis-regulatory elements located downstream of the protein-coding region (*mRNA 5′-* or *3′-UTR*), which are identified by the transcriptional machinery as signals to finish transcription, thereby causing the dissociation of this machinery from the DNA (Basso et al., 2020).

1.5.3 Selectable Markers

The difficulty of genetic transformation involves inserting the foreign DNA into the plastome and subsequently selecting the transplastomic cell for its regeneration. The key role of the selectable marker is to facilitate the detection of transformants, allowing for the differentiation between transformed and non-transformed chloroplasts (Kumar & Ling, 2021). The selection process involves the incorporation of selective agents into the *in vitro* culture medium, followed by multiple subculture phases and the application of hormones. Selection begins

following a coculture period under conditions of darkness or low light, which may be incrementally intensified to enhance selection and minimize tissue oxidation (Basso et al., 2017). The achieving of the homoplastomic status is estimated to require approximately 20 cell divisions and a few rounds of subculture on explants to selection medium (Figure 1.8). Greening, rapid proliferation, and shoot regeneration are employed to identify transplastomic plants on selective media (Maliga, 2004). An efficient selection marker is essential for successful plastid transformation. Selection pressure under the *in vitro* conditions provides wild type plastid sorting during repeated cell divisions to achieve the regeneration of homoplasmic transplastomic shoots (Venkatesh & Park, 2012). The regeneration of homoplastomic cells produces genetically stable plants (Lutz et al., 2007).

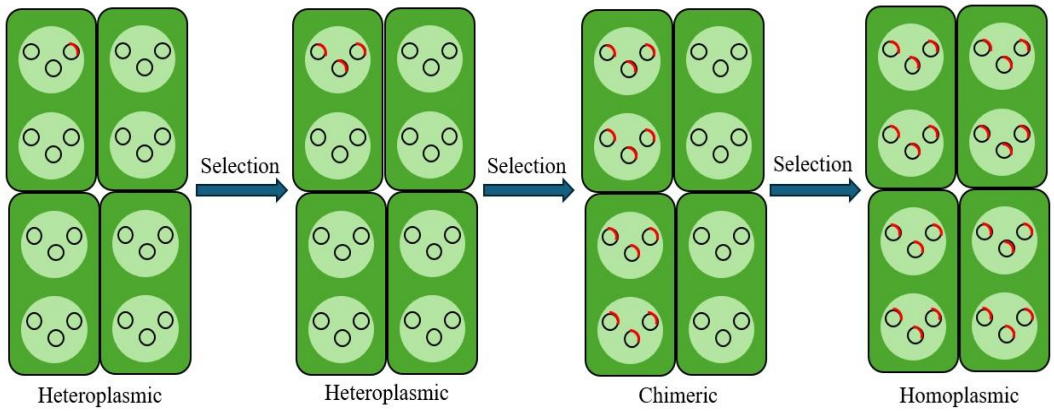


Figure 1. 8. Progression of chloroplast cells from a heteroplasmic to a homoplasmic state following a transformation experiment

Choosing the most suitable marker gene for the specific plant species is a preliminary step prior to the design and synthesis of the transformation vector (Basso et al., 2020). However, the performance of each selection marker can vary depending on the genotype of the plants. Enhancement of selection efficiency can be achieved through the utilization of robust species-specific promoters. Plastid marker genes are classified into two categories: positive selective markers, which enable the direct selection of transplastomic cells. The other marker is called negative selective markers, which impart a phenotype when present in a majority of ptDNA copies however are ineffective for the recovery of transplastomic cells when present in only a limited number of ptDNA copies (Lutz et al., 2007).

Positive markers provide resistance to cells against spectinomycin, streptomycin, and kanamycin. These markers block the production of proteins on ribosomes of prokaryotic-type plastids (Maliga, 2004). Resistance to spectinomycin and streptomycin in plastids is mediated by the Streptomycin 3'-Adenylyltransferase (*aadA*) gene (Goldschmidt-clermont, 1991), while resistance to kanamycin results from the Neomycin Phosphotransferase (*noe*) gene (Carrer et al., 1993) or Aminoglycoside 3'-Phosphotransferase (*aphA-6*) gene (Huang et al., 2002). Greening, rapid proliferation, and shoot regeneration are employed to identify transplastomic plants under the antibiotic selection (Maliga, 2004).

At the beginning, scientists used the plastid *16S rRNA* (*rrn16*) gene for selection of the transplastomic cells. *rrn16* have a point mutation that inhibits the binding of spectinomycin or streptomycin to the *16S rRNA* (Staub & Maliga, 1992). The first study of stable transformation and expression of the *aadA* gene occurred in the ptDNA of Chlamydomonas (Goldschmidt-clermont, 1991). The *aadA* gene originates from the intestinal bacterium *Escherichia coli* (Bock, 2015). The *aadA* gene produces aminoglycoside 3'adenylyltransferase, an enzyme that inactivates spectinomycin and streptomycin through adenylation, thus stopping their binding to chloroplast ribosomes (Verma & Daniell, 2007). The *aadA* gene subsequently served as a selectable marker in tobacco, resulting in a transformation rate that was 100-fold greater than that of the mutant *16S rRNA* genes (Svab & Maliga, 1993). The *aadA* is the primary and widely utilized antibiotic resistance marker specific to chloroplasts.

Alongside positive markers that aid in the selection of transplastomic cells, several negative marker genes have been identified in plastids. These exhibit conditional lethality and are expected to be valuable in genetic screenings for regulators of plastid gene expression (Bock, 2015).

In addition to antibiotic resistance-based selectable markers, reporter genes facilitate the monitoring of gene expression without providing a selective advantage or disadvantage to putative transplastomic cells. Beta-glucuronidase (*GUS*/*uidA*) and green fluorescent protein (*GFP*) are two markers commonly utilized as reporter genes in transformation studies (Maliga, 2004). The enzymatic activity of *GUS* in chloroplasts is quantified through fluorogenic assays and can be imagined via histochemical dyeing (Staub & Maliga, 1993). *GFP* serves as a visual marker, facilitating the direct visualization of the fluorescent gene product within living

cells (Reed et al., 2001). Other examples of reporter genes used include luciferase (*LUC*), yellow fluorescent protein (*YFP*), and red fluorescent protein (*RFP*).

1.5.4 Applications of Plastid Transformation

Plastid transformation is especially advantageous for utilizing plants as bio factories, owing to the high expression levels of recombinant proteins achievable through this method (Scotti et al., 2012). Consequently, numerous proteins of pharmaceutical interest and various recombinant enzymes have been expressed in transplastomic plants in recent years. Examples include CTP-proinsulin (Boyhan & Daniell, 2011), insulin-like growth factor (Daniell et al., 2009), or aprotinin etc. (Tissot et al., 2008).

Researchers have genetically modified various genes in chloroplasts to impart desirable agronomic traits. Simultaneous expression of protease inhibitors and chitinase has been utilized to create plants resistant to biotic and abiotic stresses, especially in tobacco. Traits of agronomic importance, such as herbicide resistance, insect resistance, and tolerance to drought and salinity, have been effectively introduced into plants via plastid transformation (Adem et al., 2017). The earliest usage of transplastomic methods in pest control involved the expression of the Bacillus thuringiensis (*Bt*) *cry1A(c)* gene within the tobacco plastome. Transplastomic tobacco plants exhibited significant accumulation of the *Bt* insecticidal protein, constituting 3%–5% of total soluble protein, and demonstrated elevated resistance to herbivorous insects (McBride et al., 1995). Daniell et al. documented the creation of glyphosate-resistant transplastomic tobacco plants through the expression of the wildtype petunia *EPSPS* gene, which conferred resistance to approximately tenfold the lethal dose. Furthermore, several genes have been effectively expressed in plastid genome to impart herbicide resistance. The bacterial bar gene was expressed in tobacco to confer resistance to phosphinothricin, achieving a level of resistance up to 50 mg L^{-1} (Kang et al., 2003).

The chloroplast genome has been effectively modified to generate significant biomaterials. Polyhydroxyalkanoates (PHAs) represent a significant category of biodegradable polyester biopolymers, serving as a viable alternative to petroleum-derived plastics (Dobrogojski et al., 2018). Polyhydroxybutyrate (PHB) is most extensively studied PHA (Yu et al., 2020). Multiple systems have been tested for PHB production, encompassing microbial cells and diverse plant tissues.

The highest PHB accumulation recorded so far was achieved in tobacco plants by chloroplast transformation method, reaching 18.8% of dry weight (Bohmert-Tatarev et al., 2011).

1.6 Aim and Scope of the Study

Current research aims to produce a genetically modified sugar beet plant with chloroplast-targeted modifications using biolistic bombardment. In order to provide transplastomic sugar beet lines we utilized the pExpPN-phaA-SB vector, which contains the *ampR*, *adaA* and *phaA* genes. The purpose of using these genes within the vector is, respectively, the amplification of the pExpPN-phaA-SB vector in *E. coli*, providing an advantage during the selection stage of potential transplastomic plants after biolistic bombardment, and obtaining CMS sugar beet plants.

2. MATERIALS and METHOD

2.1 Growing Plants for Chloroplast Transformation Applications

2.1.1 Selection, Sterilization and Germination of Plant Material

In the first step of sterilization of SG-DH seeds, the seeds were sterilized with 70% ethanol for 5 minutes and then washed three times with sterile distilled water. Following washing, seeds were treated with 20% (v/v) sodium hypochlorite (NaCIO) and 0.1% (v/v) Tween-20, and sterilization was conducted on a magnetic stirrer for 40 minutes. Subsequently, the seeds were washed at least five times with sterile distilled water (Photo 2.1). After these procedures, sterile seeds were kept in warm water in a sterile cabinet overnight. After 24 hours, seeds were planted in germination medium for germination. Germination medium consisted of 4.4 g/L MS (Murashige & Skoog, 1962), 3% sucrose (Merck) and 0.8% (w/v) agar (Duchefa). The pH value of the germination medium was adjusted to 5.8 using 1 N HCl and 1 N NaOH. The prepared medium was then sterilized using an autoclave at 121°C, 1.06 kg cm^{-2} pressure for 15 min. Every three weeks, seeds were subcultured to fresh media. Germinated shoots were transferred to magenta containers to continue their development. Seeds planted in germination medium were placed in a plant growth room with 16 hours of light and 8 hours of darkness at 25 °C. SG-12, SG-13, SG-6M and ELK345 genotypes were supplied as germinated from the Turkish Sugar Institute. These supplied plants were directly planted in the petiole development medium.

Photo 2. 1. Sterilization stage of seeds. **A:** Selection of large and healthy seeds. **B:** Sterilization of seeds

2.1.2 Planting Germinated Plants into Shoot and Petiole Development Medium

To obtain a sufficient number of petioles and leaf explants to be used in biolistic transformation, the roots of germinated and growing plants were cut and removed (Photo 2.2) and planted in 4.4 g/L MS, 3% sucrose, 0.4% phytagel, and 0.25 mg/L BAP (6-Benzylaminopurine) (Duchefa, Netherlands) medium. The pH value of the growth medium was adjusted to 5.8 using 1 N HCl and 1 N NaOH. The prepared medium was then sterilized using an autoclave (NC 40M NUVE, Turkey) at 121°C and 1.06 kg cm^{-2} pressure for 15 minutes. Plant growth hormones were sterilized using the filter sterilization method and added to the medium in a sterile cabinet after the autoclave stage. The plants planted in the medium were placed in a plant growth room with 16 h light and 8 h dark conditions at 25 °C. The plants were subcultured at the end of every three weeks. During subculture, aging leaves were cut to encourage young leaf formation, and the plants were grown in this medium for approximately 1.5-2 months. With this method, the number of young leaves and petioles increased, and their regeneration was achieved. At the end of this period, petiole explants (approximately 0.5 cm long) taken from young and healthy plants were used for biolistic transformation and callus formation (De Marchis et al., 2009). Leaf tissues were also cut from these plants to be used in biolistic bombardment.

Photo 2. 2. Cutting the plants and planting them in a shoot and petiole development medium

2.1.3 Culturing Petiole Explants in Callus Medium

Petioles taken from plants planted in petiole growth medium were cultured in callus medium for callus formation. The callus medium was composed of 4.4 g/L MS, 3% sucrose, 0.4% phytagel (Caisson-labs) supplemented with 0.5 mg/L BAP + 0.1 mg/L NAA (1-alpha-Naphthaleneacetic acid) (Duchefa, Netherlands). The pH value of the medium was adjusted to 5.8 using 1 N HCl and 1 N NaOH. The medium was sterilized using an autoclave at 121°C and 1.06 kg cm^{-2} pressure for 15 minutes. Plant growth hormones BAP and NAA underwent sterilization via the filter sterilization method and were subsequently introduced to the medium within a sterile cabinet following the autoclave process. After the petiole explants were placed in the callus medium, the petri dishes were covered with aluminum foil to prevent light from passing through and placed in a box. The box was kept in a plant growth chamber at 25 ℃. Petiole explants were transferred to fresh medium every 3 weeks and kept under these conditions until they formed callus (De Marchis et al., 2009). The calli obtained were then used for biolistic transformation.

2.1.4 Preparation Of Petiole, Leaf and Callus Explants for Biolistic Transformation

Petiole (approximately 0.5 cm), leaf, and callus explants taken from plants grown in petiole medium were used for biolistic transformation. Before explants were shot with the PDS1000He biolistic gun (BioRad Laboratories, Hercules, CA, USA), the explants were planted in a medium with 4.4 g/L MS, 3% sucrose, 0.4% phytagel, 0.5 mg/L BAP, and 0.1 mg/L NAA. The pH value of the medium was adjusted to 5.8 using 1 N HCl and 1 N NaOH. The prepared medium was then sterilized by autoclaving at 121°C, 1.06 kg cm^{-2} pressure for 15 min. BAP and NAA were sterilized using the filter sterilization method and added to the medium in a sterile cabinet after the autoclave stage. The planting locations of the plants in the petri dish were determined by marking them on the petri dish with the help of the PDS1000He device. The maximum number of explants was placed in the marked locations (Photo 2.3). After the biolistic shots for the transformation of the final expression vector pExpPN-phaA-SB designed for sugar beet chloroplast transformation, the petri dishes were wrapped with aluminum foil to prevent light exposure and placed in a cardboard box. The box was kept in a plant growth

chamber at 25 ℃ for 48 hours. At the end of this period, the bombarded explants were planted in the selection medium.

In order to balance the osmotic pressure in target explants, explants were planted in MS medium containing 0.2 M mannitol (Bioshop, Canada), 0.2 M sorbitol (Merck, Germany), and 0.5 mg/L BAP, 0.1 mg/L NAA for 4 hours before and 4 hours after bombardment. At the end of the period, plants were planted in MS medium containing 0.5 mg/L BAP and 0.1 mg/L NAA and kept in the dark for 2 days. Explants were kept in the dark for 2 days and then planted in selection medium. We followed this method between the 53rd and 66th bombardments.

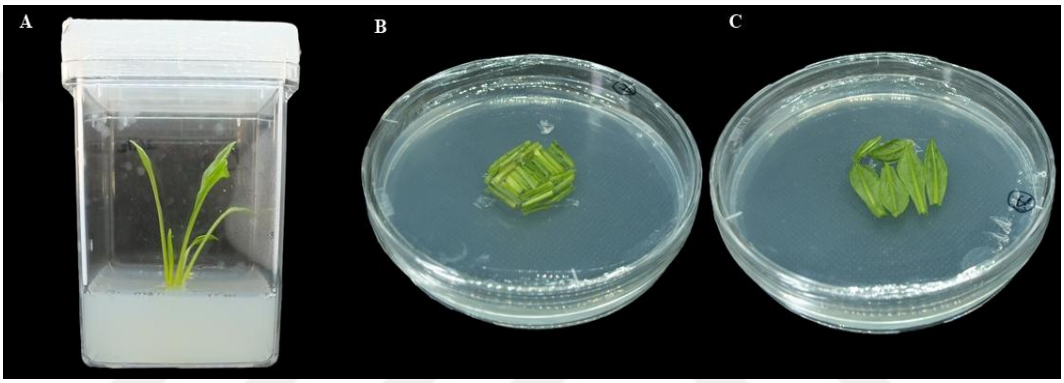


Photo 2. 3. Examples of explants used in biolistic bombardment. **A:** Sugar beet plants grow in petiole culture before cutting. **B-C:** Preparation of petiole and leaf explants before bombardment

2.2 The Properties of the pExpPN-PhaA-SB Vector

The pExpPN-PhaA-SB vector was designed as a sugar beet-specific chloroplast vector in the TÜBİTAK project number 120O596. As mentioned before, the pExpPN-PhaA-SB vector contains 3 different gene regions (*ampR*, *adaA* and *phaA*) and uses *trnA* and *trnI* regions as homologous recombination sites. The expression cassette is under the control of the *5'-psbA* promoter and the *3'-rbcL* (*Chlamydomonas*) terminator and consists of the spectinomycin gene *aadA* for the selection of transplastomic plants. The *phaA* gene is controlled by the *PrrnPEP+NEP* promoter and the *3' tobacco rbcL* terminator (Figure 2.1).

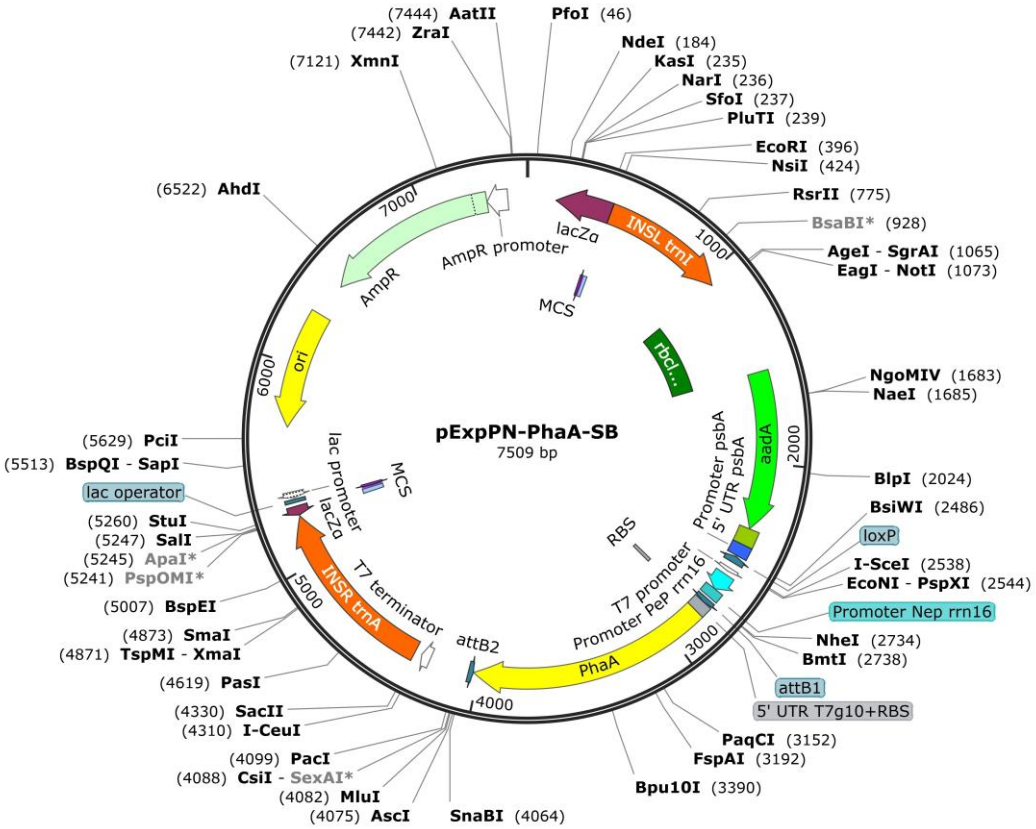


Figure 2. 1. Structure of the pExpPN-PhaA-SB vector

2.3 Amplification of the pExpPN-PhaA-SB Vector for Use in Biolistic Bombardment

The targeting vector pExpPN-PhaA-SB was propagated using competent *E.coli* (Dh5alpha) cells. Firstly, the pExpPN-PhaA-SB vector was transferred into *E. coli* cells using the 'Heat-shock' method. According to this method, 1 µl of vector was placed into competent *E. coli* cells in Eppendorf that had been removed from -20 °C. Then, it was slowly and carefully mixed clockwise with a pipette and placed in a container containing ice for 30 minutes. At the end of this period, it was kept at 42 °C for 30 seconds and then immediately kept on ice again for 3 minutes. Following that, 250 µl of S.O.C (Invitrogen) medium was added and shaken at 180 rpm for 1 hour at 37°C. At the end of the period, 20 µl and 50 µl were taken from S.O.C medium and seeded in Luria-Bertani (LB) medium containing 100 mg/L ampicillin (Duchefa, Netherlands) antibiotic and incubated in the oven at 37 °C overnight to form single cell colonies. At the end of the period, single cell colonies were formed (Photo 2.4).

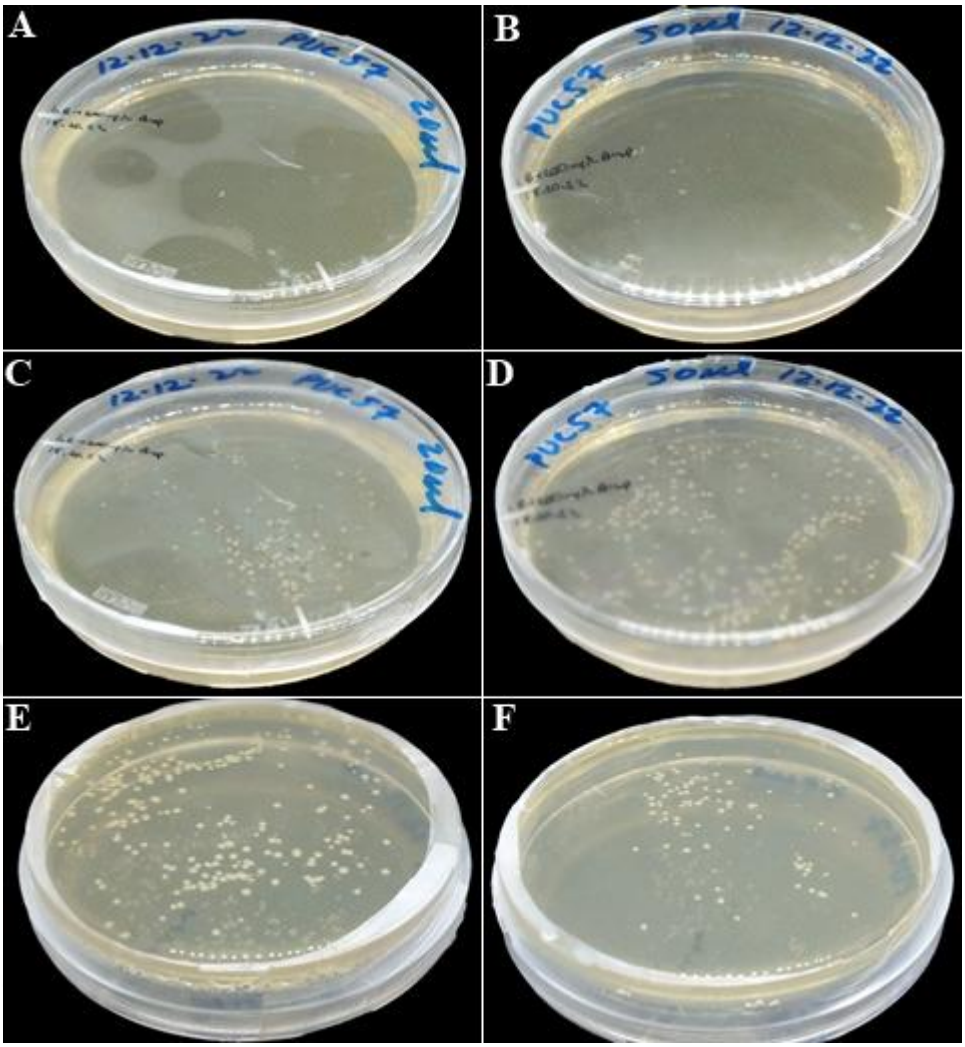


Photo 2. 4. Propagation of pExpPN-PhaA-SB vector in competent *E. coli* cells. **A-B:** First day of culturing *E. coli* cells containing the pExpPN-PhaA-SB vector in LB medium containing 100 mg/L ampicillin. **C-F:** Single cell colonies containing the pExpPN-PhaA-SB vector were grown in LB medium containing 100 mg/L ampicillin after incubation at 37 °C overnight

Cells taken from a single cell colony in solid LB medium kept at 37°C overnight were transferred to a medium containing 3 ml of liquid LB and 3 µl of ampicillin. After the transferred tubes were shaken at 180 rpm and 37°C for 1 night (Photo 2.5), plasmid isolation was performed using geneJET Plasmid Miniprep KIT (Thermo Scientific) following the manufacturer's protocol. Plasmid isolated samples were verified both by electrophoresis on 1% agarose (invitrogen) gel and by PCR. In addition, the purity and DNA concentration of the plasmid were examined with micro-drop.

The LB medium used for bacterial cultivation was prepared as follows; 10 g/L peptone (Fluka/Biochemika), 5 g/L yeast extract (Merck, Germany) and 15 g/L NaCl (Merck/Germany) were added and adjusted to pH 7.0. Then, 15 g/L agar (Oxoid, England) was added to the medium and sterilized using an autoclave at 121°C, 1.06 kg cm^{-2} pressure for 15 minutes. After the autoclave was completed, the LB was left outside to cool for a while. An ampicillin antibiotic was sterilized using the filter sterilization method and then added to the medium in a sterile cabinet. Cells were cultured in solid and liquid LB medium in a sterile cabinet (MiproLab, Msafe1200).

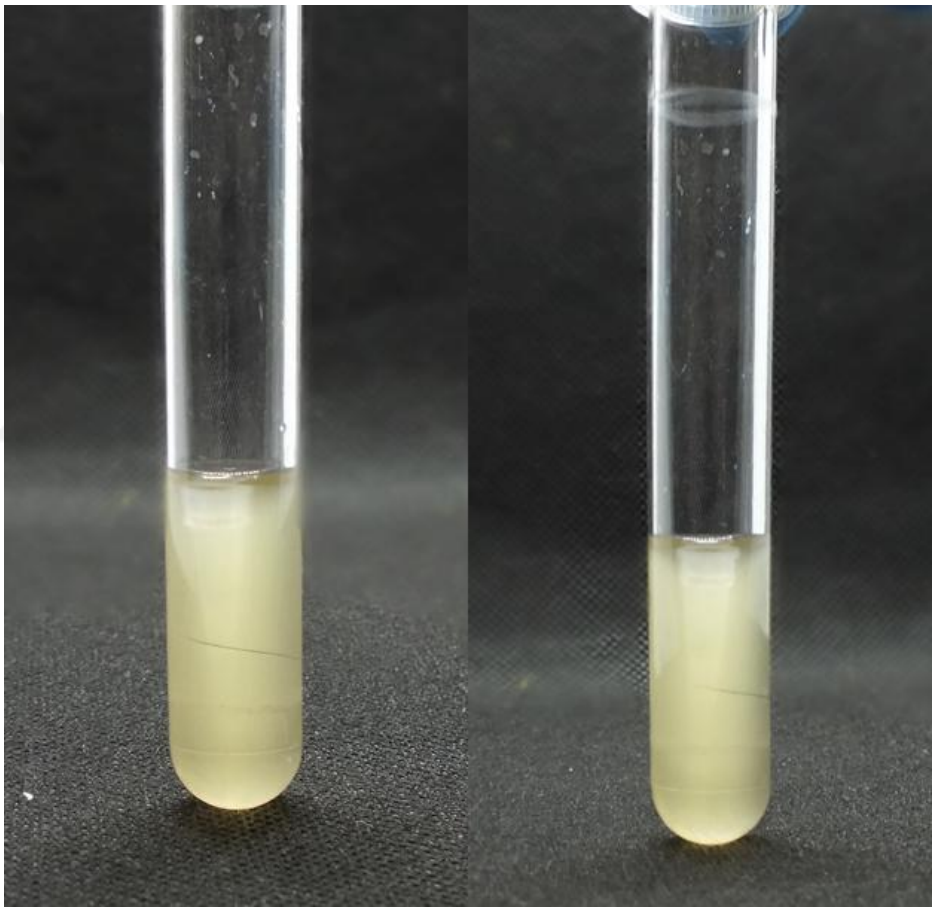


Photo 2. 5. *E. coli* cells containing the pExpPN-PhaA-SB vector were grown overnight in liquid LB medium supplemented with ampicillin

2.4 Coating of the pExpPN-phaA-SB Vector with Gold Particles and Biolistic Bombardment

Two different methods were used to prepare the gold particle stock and coat the pExpPN-phaA-SB vector with gold particles. All steps of the two methods were performed in a sterile cabin (Photo 2.6).

The first of these methods (Verma et al., 2008);

The procedure started with the preparation of gold particles (0.6 µm) (Bio-Rad, USA) stock. First, 50 mg of gold particles were weighed and placed in a 1.5 ml eppendorf tube, then 1 ml of pure ethanol (IsoLab) was added and mixed with vortex for two minutes. Following that, it was centrifuged at 10000 g for 3 minutes. After centrifugation, the supernatant was carefully removed, and 1 ml of 70% v/v ethanol was added and mixed using a vortex for 1 minute. At the end of the period, it was kept at room temperature for 15 minutes and the centrifuge tube was gently mixed by hand every three minutes. At the end of the 15 minutes, it was centrifuged at 5000 g for two minutes and the supernatant was carefully removed. After this step, the washing phase started. 1 ml of sterile distilled water was added and vortexed, then it was left at room temperature for one minute. Then, it was centrifuged at 5000 g for two minutes and the supernatant was removed. The washing phase was repeated four times. At the end of the fourth wash, 1 ml of previously sterilized 50% vol/vol glycerol was added to the gold particles and stored at -20°C. The gold stock was renewed every six months. Glycerol was prepared before the experiment and sterilized using an autoclave.

The previously prepared gold stock was used to coat the pExpPN-PhaA-SB vector with gold particles. While the gold stock was vortexed, 50 µl of gold particles were taken and placed in a 1.5 ml centrifuge tube. While the centrifuge tube was vortexed, 5 µl of pExpPN-PhaA-SB vector (1 µg/µl), 20 µl of 0.1 M spermidine and 50 µl of 2.5 M $CaCl_2$ were added, and vortexing was continued for 20 minutes at 4°C. Afterwards, it was centrifuged at 10000 g for one minute, and the supernatant was carefully removed. The pellet was first washed with 70% v/v ethanol and then with pure ethanol. Finally, 50 µl of pure ethanol was added to the gold-coated pExpPN-phaA-SB vector and kept on ice to be used in biolistic bombardment as soon as possible. 10 µl of gold particle-coated pExpPN-PhaA-SB vector was used in each bombardment set.

The second method is (Brant et al. 2021);

In the beginning, 60 mg of gold particles (0.6 µm) were weighed and placed in a 2 ml centrifuge tube, then 2 ml of 70% v/v ethanol was added. nuclease-free water was used when preparing 70% v/v ethanol. It was mixed with vortex for three to five minutes and then left at room temperature for 15 minutes. Then, it was centrifuged at full speed for five seconds with a small table-top centrifuge. After

centrifugation, the supernatant was carefully removed. After this step, the washing phase started. At this stage, 2 ml of sterile nuclease-free water was added and centrifuged for five seconds with a table-top centrifuge and the supernatant was removed. The washing phase was repeated three times. In the third washing phase, 2 ml of nuclease-free water was added and mixed with vortex for 1 minute and left at room temperature for 1 minute. At the end of the washing process, 1mL of 50% v/v pre-sterilized glycerol was added to the gold particles and stored at -20℃. The prepared gold particles were used for 6 months.

The previously prepared gold stock was used to coat the pExpPN-PhaA-SB vector with gold particles. First, the gold stock was vortexed for one minute and at the end of the period, 30 µl of gold particles were taken and placed in a 1.5 ml centrifuge tube and immediately 30 µl of vector was added. The solution was vortexed for one minute. At the end of the period, first 20 µl of 0.1 M spermidine and then 50 µl of 2.5 M $CaCl_2$ were added and vortexed for another one minute at half speed. Afterwards, centrifugation was performed for one second with a table-top centrifuge set at 14000 g and the supernatant was carefully removed. To wash the pellet, 200 µl of 70% v/v ethanol was added without disturbing the pellet and the washing step was performed again with centrifugation for one second. Finally, the gold-coated pExpPN-phaA-SB vector was kept in 100 µl of 100% ethanol and stored on ice to be used in biolistic bombardment as soon as possible. 5 µl of solution was used in each bombardment. To adjust the DNA amount to 30 µl, plasmid DNA was first measured with a micro-drop (Thermo Scientific/ Multiskan SkyHigh) device. Then, its concentration was adjusted to 30 µl with nuclease-free water.

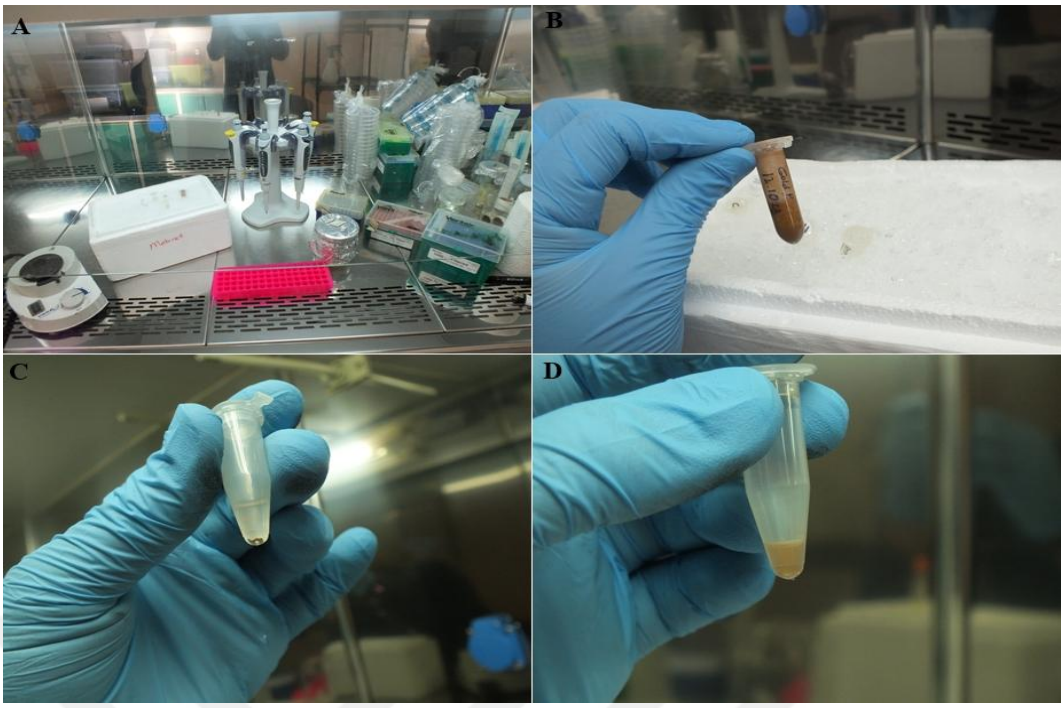


Photo 2. 6. Coating of the pExpPN-PhaA-SB vector with gold particles. **A:** Sterile cabinet where experiments were performed. **B:** Previously prepared gold stock. **C-D:** Coating of the pExpPN-PhaA-SB vector with gold particles

Biolistic bombardment of the pExpPN-phaA-SB vector coated with gold particles was performed with the PDS1000He (BioRad Laboratories, Hercules, CA, USA) device (Photo 2.7A). Bombardment was performed at a pressure of 1100 psi and 1350 psi to obtain stable transformants. The petiole, leaf and callus explants were placed in the petiole under 28 Hg vacuum and placed 6 cm away from the stop screen (Photo 2.7D) (Verma et al., 2008; De Marchis et al., 2009; Cheng et al., 2010).

Photo 2. 7. Biolistic bombardment experiments. **A:** Biolistic bombardment device. **B-C:** Placing the pExpPN-phaA-SB vector on the macro carrier. **D:** Application of biolistic bombardment to previously prepared explants. **E-F:** Rupture disc and macro carrier after bombardment

2.5 Determination of Antibiotic Selection Dose

After biolistic bombardment, antibiotic selection method was applied under *in vitro* conditions to identify putative transplastomic plants. The expression cassette of sugar beet specific pExpPN-phaA-SB vector contains *aadA* gene which provides resistance and advantage to spectinomycin and streptomycin antibiotics for selection of transplastomic plants under the control of *5'-psbA* promoter and *3'-rbcl* (*Chlamydonas*) terminator. To determine the antibiotic dose, petiole and leaf explants were cultured on 4.4 g/L MS, 3% sucrose, 0.4% agar and 0.5 mg/L BAP and 0.1 mg/L NAA media containing seven different doses of spectinomycin. These doses were 0, 25, 50, 75, 100, 150 and 200 mg/L spectinomycin. The sample size of petioles for each dose was 30 and for leaves was 15. To determine the best antibiotic dose, both statistical and plant developmental status in the medium were taken into consideration.

2.6 Selection of Transformants and Shoot Regeneration

After particle bombardment, petiole, leaf and callus explants were incubated in the dark for 48 hours. Following that transferred to media containing 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 50 mg/L spectinomycin, 0.5 mg/L BAP, 0.1 mg/L NAA for the selection of transplastomic plants. The pH value of the medium was

adjusted to 5.8 using 1 N HCl and 1 N NaOH and the prepared medium was sterilized using an autoclave at 121°C and 1.06 kg cm^{-2} pressure for 15 minutes. Spectinomycin, BAP and NAA were sterilized via the filter sterilization method and added to the medium after the autoclave step. Explants were incubated under light/dark conditions for 16/8 hours. The explants were carefully planted in the medium to maximize contact between their tissue surfaces and the selection medium. The antibiotic selection period was changed in parallel with the results obtained during the experiments. At the end of the selection period, if the explants did not form callus and/or shoots, they were transferred to media containing 4.4 g/L MS, 3% sucrose, 0.4% phytagel supplemented with 0.5 mg/L BAP, 0.1 mg/L NAA.

Callus-forming explants were transferred to media containing 4.4 g/L MS, 3% sucrose, 0.4% phytagel, 1.0 mg/L BA and 0.5 mg/L ABA (abscisic acid) (Duchefa, Netherlands) hormones to induce regeneration.

Shoot-forming explants in the regeneration medium were cultured in 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 0.25 mg/L BAP medium. Due to the vitrification problem experienced in this medium, the medium was later changed to 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 0.25 mg/L KIN (Kinetin) (Sigma).

In order to balance the osmotic pressure in the target explants for successful and stable transfer of the pExpPN-phaA-SB vector to sugar beet, the explants were cultured in MS media containing 0.2 M mannitol (Bioshop, Canada), 0.2 M sorbitol (Merck, Germany), 0.5 mg/L BAP, and 0.1 mg/L NAA 4 hours before and 4 hours after bombardment. After osmotic treatment, plants were planted in MS medium containing 0.5 mg/L BAP and 0.1 mg/L NAA and kept in the dark for 2 days. Then, they were planted in MS medium containing 0.5 mg/L BAP, 0.1 mg/L NAA, and 50 mg/L spectinomycin for 1 month and kept in 16 h light and 8 h dark. This method was followed in experiments between and including the 53rd and 66th bombardments.

2.7 PCR Validation of Chloroplast Integration of the pExpPN-phaA-SB Vector

To verify the integration of the pExpPN-phaA-SB vector, genomic DNA (gDNA) was first isolated from healthy and sufficiently growing plants. DNA isolation was performed using the manual method as explained in detail below

(Lefort et al., 1988). Petiole and leaf parts of the plant were taken to be used in gDNA isolation (Photo 2.8).

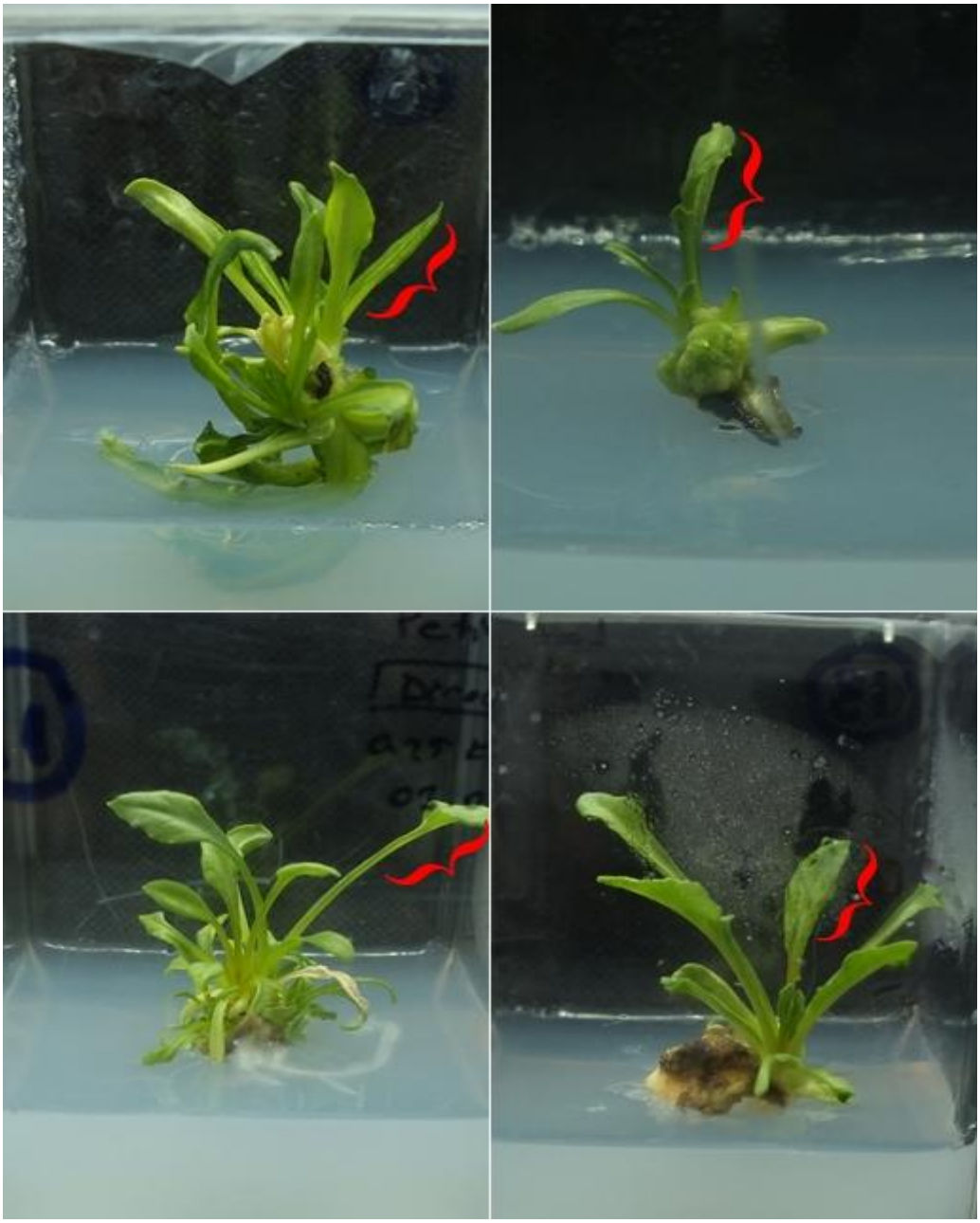


Photo 2. 8. Tissue sections taken from plants for use in gDNA isolation

2.7.1 Manual gDNA Isolation Procedure

1. Tissue samples were taken from putative transplastomic plants, then covered with aluminum foil and labeled. These samples were stored at -86˚C for use in DNA isolation.
2. For DNA isolation, samples were placed in a mortar and crushed with liquid nitrogen to form powder.

3. 100 mg of the crushed samples were placed in 2 ml Eppendorf tubes.
4. Samples were placed in Eppendorf tubes and then 1 ml of DNA extraction solution and 10 µl of 2-Mercaptoethanol were added.
5. The samples were kept in a water bath set at 65 ˚C for 60 minutes and turned upside down every 15 minutes. At the end of this period, they were cooled to room conditions.
6. 1 ml of chloroform (Sigma, Germany)/isoamyl alcohol (24:1) was added and rotated on ice for 30 minutes.
7. At the end of the 30 minutes, the samples were centrifuged at 14000 rpm for 20 minutes.
8. After centrifugation, the supernatant was transferred to a new eppendorf tube, and 1 ml of chloroform was added, then centrifuged at 12,000 rpm for 10 minutes. The supernatant was transferred to new eppendorf tubes.
9. Afterwards, 1 ml of isopropyl alcohol (Merk, Germany) was added to the eppendorf tubes, they were inverted 5-6 times and kept in the refrigerator at -20˚C for 1 hour.
10. At the end of the period, the samples were centrifuged for 20 minutes at 14000 rpm. Following that the supernatant was discarded.
11. 1 ml of 70% ethanol was added to the pellet and centrifuged at 14000 rpm for 10 minutes. This step was repeated twice.
12. After the washing step, ethanol was removed from the eppendorf tubes, and the pellet (gDNA) was left to dry.
13. After drying, 30-50 µl of nuclease-free water was added to each gDNA sample.
14. Isolated gDNAs were stored at -20 ˚C.

Preparation of DNA Extraction Solution

To prepare the DNA extraction solution, firstly 2x 100 ml CTAB solution was prepared. For this purpose, 2 gr of CTAB was weighed, 25 ml of sterile pure water was added and mixed at 150 ˚C until it was thoroughly dissolved. Then, 10 ml of 1 M Tris-HCl (Bioshop, Canada) (pH 8.0), 4 ml of 0.5 M EDTA (Thermo, USD) (pH 8.0) and 28 ml of 5 M NaCl were added and mixed. The prepared 2x CTAB solution was poured on top of the other mixtures, and the total volume was completed to 100 ml. The solution was mixed until its color became clear.

To prepare 1 M TrisCI, 1.21 grams of Tris was weighed, and 8 ml of sterile distilled water was added. The pH value of the mixture was adjusted to 8.0. The mixture was then made up to 10 ml with sterile distilled water. To make 0.5 M EDTA, 0.584 grams of EDTA were weighed and dissolved with 3 ml of sterile pure water. The pH value of the mixture was adjusted to 8.0. The mixture was then completed to 4 ml with sterile pure water. To prepare 5 M NACI, 8.182 grams of NaCI were weighed and dissolved with 20 ml of sterile pure water. The mixture was then completed to 28 ml with sterile pure water.

2.7.2 Confirmation of Isolated gDNA by Gel Electrophoresis

gDNAs isolated from putative transplastomic plants were run on electrophoresis using a 2% agarose gel to verify DNA isolation. For 2% agarose gel, 2 gr of agarose (invitrogen) were weighed and dissolved in 100 ml of 1X TAE solution. 5 µl of RedSafe (iNtRON Biotech) was used for gel staining.

2.7.3 Determination of Purity and Concentration of Isolated gDNA Samples

Micro-drop (Thermo Scientific/ Multiskan SkyHigh) device was used to determine the purity and concentration of the isolated gDNA. The purity and concentration of the isolated gDNAs were measured according to nuclease free water. After the measurements, all gDNA samples were adjusted to 100 nanograms (ng).

2.7.4 Polymerase Chain Reaction (PCR) Experiments

gDNA samples were used to verify the plastid genome integration of the pExpPN-phaA-SB vector. To test the integration of the pExpPN-PhaA-SB vector into sugar beet, 11 primers (forward and reverse) were designed and used (Table 2.1).

Table 2. 1. Nucleotide sequences of forward and reverse primers designed to test integration of the pExpPN-PhaA-SB vector.

Primer Name	**Forward and Reverse Primer sequences (5'-3')**	**Product Length (bp)**
adaA Full	Forward: TATTTGCCAACTACCTTAGTGATCTCGC Reverse: ATGGCTCGTGAAGCGGTTATCG	795
adaA Partial	Forward: GTACCAAATGCGGGACAACG Reverse: TTCTCCGCGCTGTAGAAGTC	341
phaA Full	Forward: ATGAAAGATGTTGTTATTGTGGC Reverse: TCAATCTCTCTCAACAGCCAAT	1179
phaA Partial	Forward: TGGAAGTGGTCTTAGGGCTG Reverse: TCAGCGAAAACAACTGGCTC	381
trnI Full	Forward: CCAAGCATTCTTCTTACGGC Reverse: CGATGGGCTATTAGCTCAGTG	626
trnI Partial	Forward: CGGGAAGGGAGGATTAGGGA Reverse: CAAGGCACATTAGCATGGCG	419
trnA Full	Forward: GGCTCGGGGGGATATAGCTC Reverse: TGGAGATAAGCGGACTCGAACC	908
trnA Partial	Forward: GTCGTTGCGATTACGGGTTG Reverse: CTTCTCCGACCCTTACTGCC	518
ampR Full	Forward: TTACCAATGCTTAATCAGTGAGGC Reverse: CACCCAGAAACGCTGGTGAA	792
ampR Partial	Forward: AATAAACCAGCCAGCCGGAA Reverse: CTTGATCGTTGGGAACCGGA	210
Optimized *PhaA* gene	Forward: CGAGATGGCGCTCGATGAC Reverse: GCATCAACTGGACCAAGTCCC	1449

These primers were designed to cover a portion of the pExpPN-PhaA-SB vector, the entire nucleotide sequences of all elements (*adaA*, *phaA*, *ampR*, *trnI*, or *trnA*) present on the vector, and specific nucleotide sequences of these elements (Figure 2.2 -2.3).

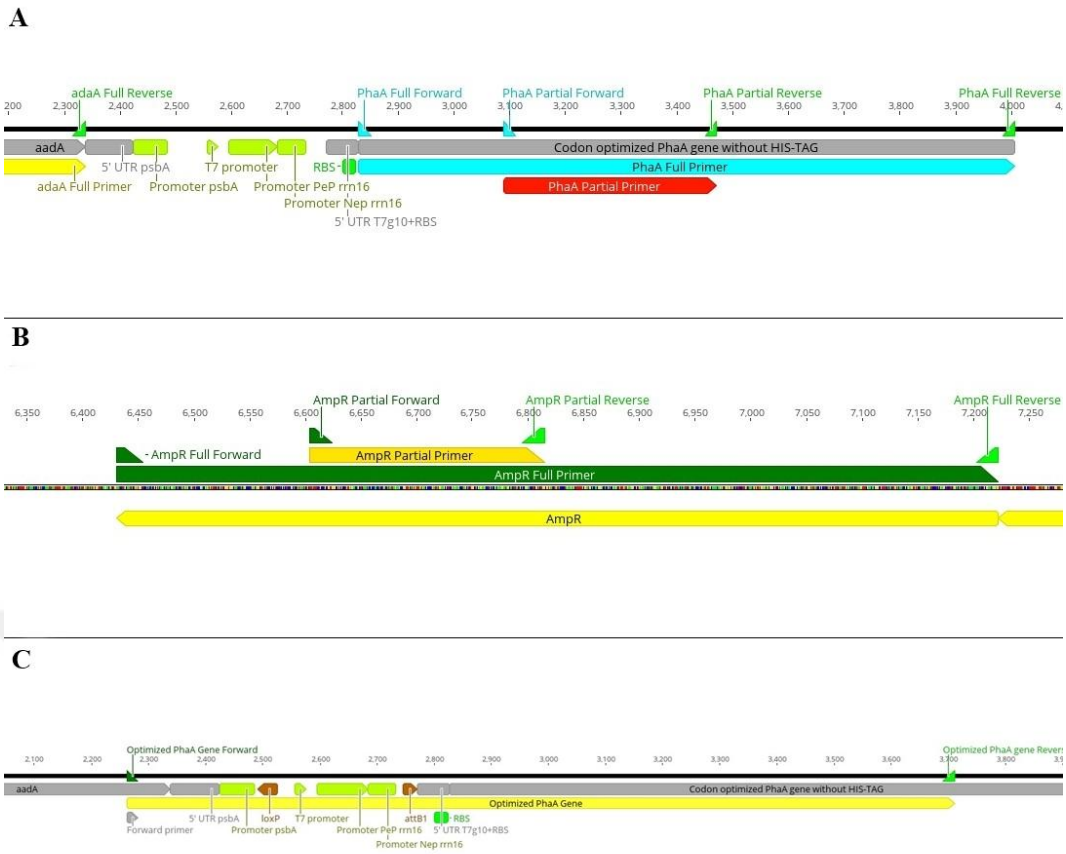


Figure 2. 2. Regions to which primer sequences were ligated in the pExpPN-PhaA-SB vector. **A:** *phaA* full and *phaA* partial. **B:** *ampR* full and *ampR* partial. **C:** Optimized *PhaA* gene.

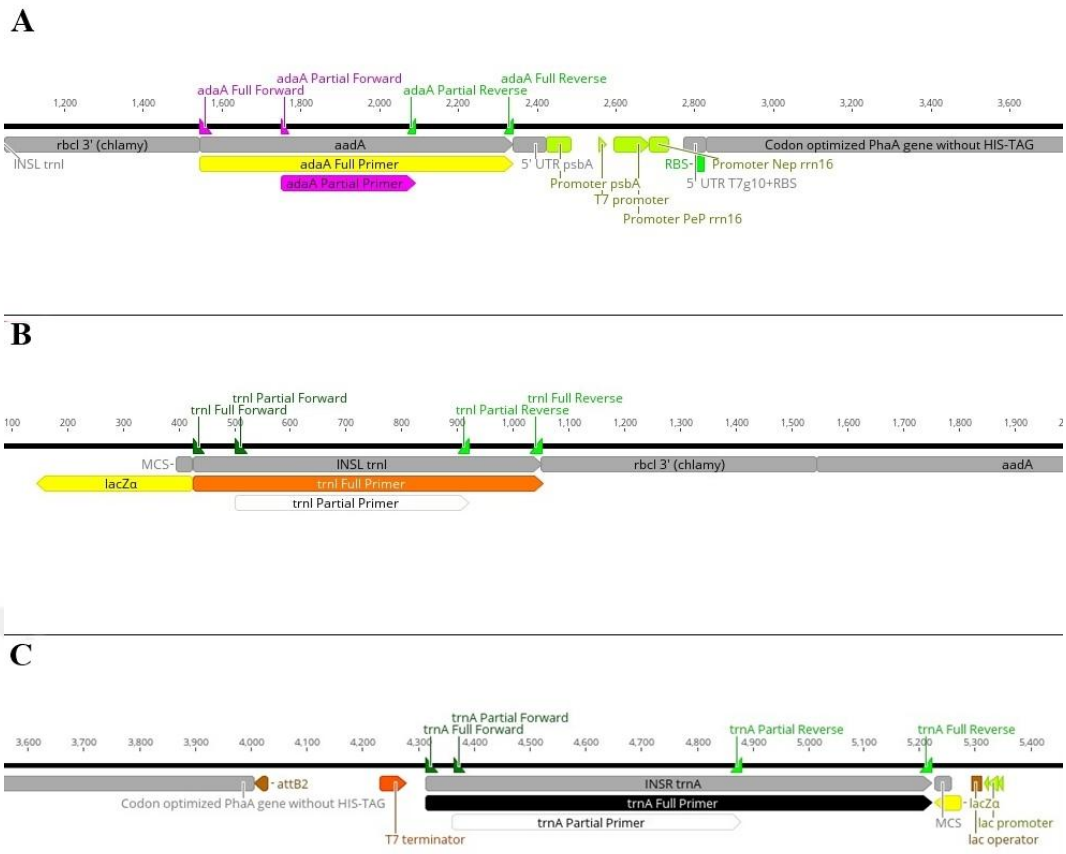


Figure 2. 3. Regions to which primer sequences were ligated in the pExpPN-PhaA-SB vector. **A:** *adaA* full and *adaA* partial. **B:** *trnI* full and *trnI* partial. **C:** *trnA* full and *trnA* partial

To optimize the PCR protocol, a gradient study was performed to determine the suitable annealing temperature of our primers using the pExpPN-PhaA-SB vector. PCR experiments were performed at different temperatures to determine the best annealing temperatures for successful screening (Photo2.9-2.10-2.11). Annealing temperatures as a result of the PCR experiments are given in Table 2.2.

Table 2. 2. Annealing temperatures and band lengths of the designed primers.

Primer Name	Annealing Temperatures ˚C	Product Length (bp)
adaA Full	52 ˚C	795
adaA Partial	50 ˚C	341
phaA Full	54 ˚C	1179
phaA Partial	50 ˚C	381
trnI Full	54 ˚C	626
trnI Partial	55 ˚C	419
trnA Full	57 ˚C	908
trnA Partial	55 ˚C	518
ampR Full	54 ˚C	792
ampR Partial	56 ˚C	210
Optimized *PhaA* gene	53 ˚C	1449

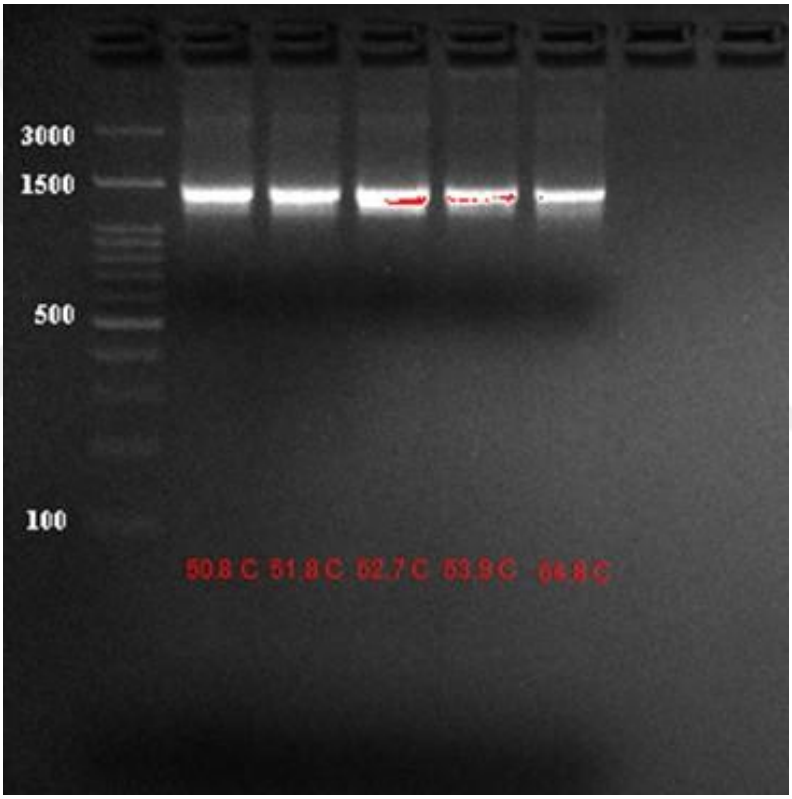


Photo 2. 9. Agarose gel (2%) electrophoresis image of gradient PCR results of the optimized *PhaA* gene primer (1449 bp)

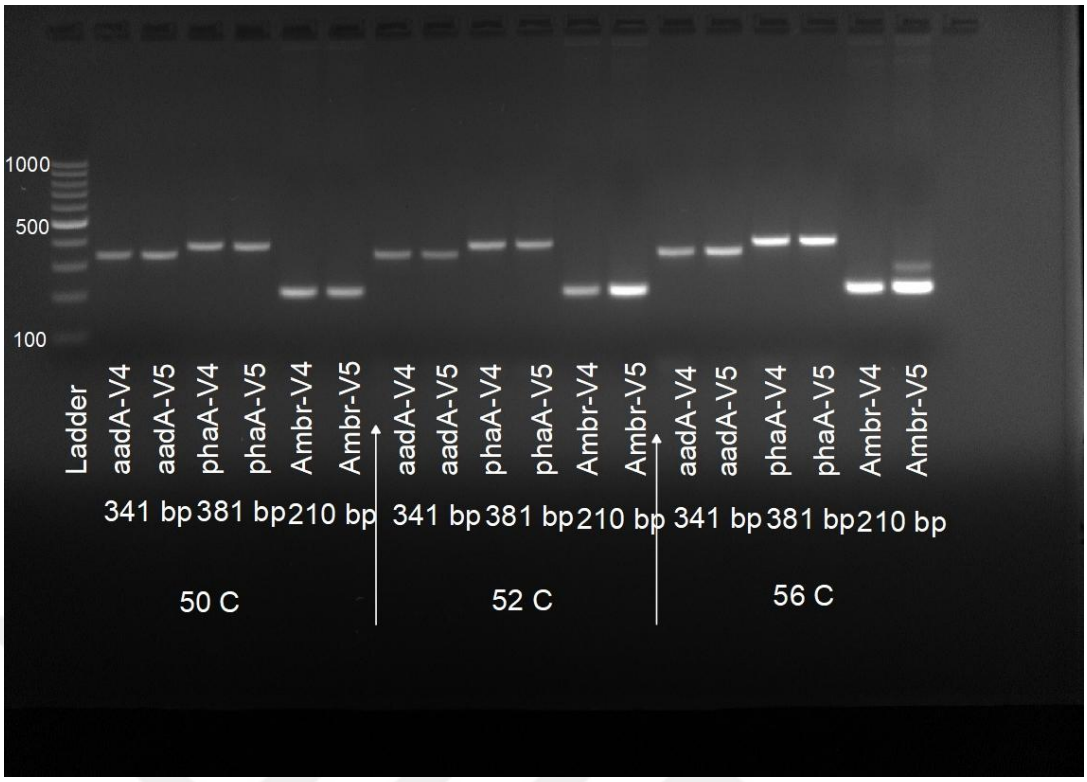


Photo 2. 10. Agarose gel (2%) electrophoresis analysis of the results of the PCR reaction performed at different temperatures with the pExpPN-PhaA-SB vector for PCR optimization of the *phaA* partial (381 bp), *adaA* partial (341 bp) and *ampR* partial (210 bp) primers

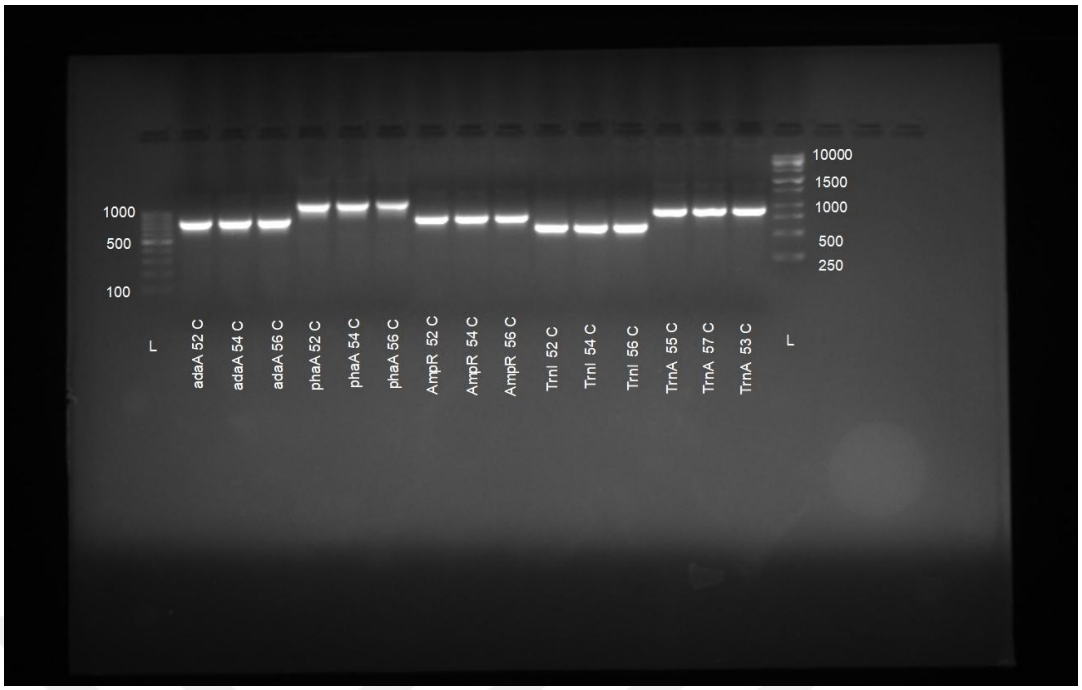


Photo 2. 11. Agarose gel (2%) electrophoresis image of the results of PCR reactions performed at different temperatures with the pExpPN-PhaA-SB vector for PCR optimization of *phaA* full (1179 bp), *adaA* full (795 bp), *ampR* full (792 bp), *trnA* full (908 bp) and *trnI* full (792 bp) primers

After optimization, the temperature, time and cycle numbers determined for the PCR reaction are as follows;

- Initial Denaturation: 3 minutes at 95 ˚C
- Denaturation: 1 minute at 95 ˚C
- Annealing: temperature adjusted according to the primer (Table 2.2), 1 minute
- Primer Extesion: 2 minutes at 72 ˚C
- Ending Temperature: 10 minutes at 72 ˚C
- PCR reaction cycle is 35.

The master mix solution determined for the PCR reaction is as follows (the described solution preparation is only for one PCR reaction, Table 2.3);

Table 2. 3. PCR master mix solutions.

PCR Master Mix	**Amount (µl)**
10 mM dnTPmix	0,5
10 mM Forward primer	1
10 mM Reverse primer	1
10x Taq Buffer (invitrogen)	1
50 mM $MgCl_2$ (invitrogen)	1
gDNA Sample	1
Taq DNA polymerase	0,2
Nuclease-free water	14,3
Total	**20**

In PCR reactions, pExpPN-PhaA-SB vector was used as positive control and NFW was used as negative control. After PCR reactions, PCR products were analyzed by electrophoresis with 2% agarose gel. In each gel image, the first well was 100 bp DNA ladder (GeneDirex- Cat. No. DM003-R500), the second well was positive control and the third well was negative control.

2.7.5 Isolation of Samples with Positive Bands in PCR from Agarose Gel

In PCR screenings performed with putative transplastomic plants, some samples have positive results. From these positive bands, especially samples that gave single and the brightest bands, were selected, and PCR studies were performed again. In these studies, the PCR reaction mix was set to 40 µl and the amount of DNA used was set to 150 ng (Table 2.4). These positive bands were cut from the gel under UV light and isolated with the help of the Kit (FavorPrep Gel/PCR Purification). Isolation was done according to the manufacturer's protocol.

The first isolated samples were sent to SentebioLab firm and bioinformatics analyses were performed. Forward and reverse Sanger sequencing was performed on the obtained PCR products. Bioinformatics analyses of the sequence results received from SentebioLab were performed with Geneious Prime, Snapgene Viewer, NCBI/BLAST and Clustal Omega. First, forward and reverse reads coming from the sequence company were aligned using Clustal Omega. Following the first step, a blast analysis was performed using NCBI. In the last step, the obtained sequences were aligned with the pExpPN-phaA-SB vector using the Geneious prime program.

The samples isolated from the second experiment were sent to Intergen firm for sequence analysis and bioinformatics analysis was performed. Spike-in De novo sequencing was performed on the PCR products. The sequence results from Intergen were tested with the pExpPN-phaA-SB vector using the IGV genome program. Then a BLAST analysis was performed using NCBI.

Table 2. 4. 40 µl of PCR master mix solutions.

PCR Master Mix	**Amount (µl)**
10 mM dnTPmix	1
10 mM Forward primer	2
10 mM Reverse primer	2
10x Taq Buffer (invitrogen, including 50 mM $MgCI_2$)	4
gDNA Sample	3
Taq DNA polymerase	0,4
Nuclease-free water	27,6
Total	**40**

2.8 Expression Analysis of pExpPN-phaA-SB Vector by qRT-PCR

2.8.1 Total RNA Isolation from Plants

Total RNA was isolated from 10 putative transplastomic plants specified in Table 3.4 and the wild type of SG-DH plant as a control plant. For total RNA isolation, callus, petiole and leaf tissue were taken from the plant and crushed with liquid nitrogen. Then, total RNA isolation was performed using the FavorPrepTM Plant Total RNA Mini Kit, following the manufacturer's protocol. RNase-Free DNas Set (Qiagen) kit was used for DNase treatment. After total RNA isolation, samples were run on 1% agarose gel to confirm that the RNA isolation was problem-free, and the average RNA amount was determined by measuring RNA concentration twice with the help of nanodorp (Thermo 2000).

2.8.2 Complementary DNA (cDNA) Synthesis

Complementary DNA (cDNA) synthesis was performed using total RNA of plant samples with iScript™ cDNA Synthesis Kit (Biorad). For each cDNA sample,

- ❖ 4 µl 5x iScript Reaction Mix
- ❖ 1 µl iScript Reverse Transcriptase
- ❖ 15 µl RNA

was used. When adjusting the RNA concentration, the amount of nuclease water was adjusted according to the cDNA reaction, so nuclease-free water was not added to the cDNA Mix solution again. The PCR protocol for cDNA synthesis was set as follows,

- ❖ Priming: 5 minutes at 25 ˚C
- ❖ Reverse transcription: 20 minutes at 46 ˚C
- ❖ RT inactivation: 1 minute at 95˚C

After the cDNA reaction, the samples were stored at -20 ˚C

2.8.3 Expression Analysis of pExpPN-phaA-SB Vector by qRT-PCR

For qRT-PCR analyses, plants that have partially pExpPN-phaA-SB vector were selected according to sequence results (Table 3.4). The absolute qRT-PCR method was preferred to determine the presence and amount of expression of *adaA* and *ampR* genes partially contained in transplastomic plants. For absolute qRT-PCR analyses, the pExpPN-phaA-SB vector was used as a standard. The other primers used were *ampR* and *adaA*, the primer sequences and optimized annealing temperatures are shown in Table 2.5. For qRT-PCR, we set the reaction cycle to 40.

Before starting qRT-PCR experiments, the concentration of the pExpPN-phaA-SB vector was measured with nanodorp. As a result of the concentration measurement, the concentration of the vector was found to be 64 ng/µl. In order to create a standard curve, 4 serial dilutions of the vector were made at a ratio of 1/5. The expression levels of the putative transplastomic plants were calculated according to the created standard and the standard error was used as the error bar.

Table 2. 5. Sequences and basic properties of primers used in qRT-PCR.

Primer Name	Forward and Reverse Primer sequences (5'-3')	Annealing Temperatures ˚C
adaA	Forward: GTACCAAATGCGGGACAACG Reverse: TTCTCCGCGCTGTAGAAGTC	54 °C
ampR	Forward: AATAAACCAGCCAGCCGGAA Reverse: CTTGATCGTTGGGAACCGGA	54 °C

Real time PCR system (Biorad, CFX96) and SYBR Green qPCR Master Mix (Universal) (MCE, America) kit were used in the expression analyses of the pExpPN-phaA-SB vector. Reaction mixtures are shown in Table 2.6.

Table 2. 6. Real-time-PCR reaction components.

Components	Amount
Super mix	5 µl
10 µM Forward Primer	0,2 µl
10 µM Reverse Primer	0,2 µl
cDNA	1 µl
Nuclease free water	3,6 µl

2.9 Western Blot Analysis

2.9.1 Expression of Beta-ketothiolase Enzyme in *E. coli* Cells

E. coli (Dh5alpha) cells containing the pExpPN-phaA-SB vector were cultured on LB Agar plates containing ampicillin. After 16 hours of incubation at 37 °C, one of the single colonies formed was selected, and the *E. coli* cells containing the gene were passage into 5 ml of LB containing 5 µl of ampicillin. Incubation was carried out for 16 hours at 37 °C in a shaking incubator at 180 rpm. 300 µl was taken from this bacterial culture and passage into 30 ml LB liquid medium containing 30 µl ampicillin. It was incubated at 37 °C, 180 rpm, until the optical density (OD) absorbance reached 0.6 in the measurement made with a UV-visible spectrophotometer at 600 nm. When OD_{600} reached 0.6, IPTG (Isopropyl β-D-1-thiogalactopyranoside) induction was performed at 0.5 mM for 30 ml and the falcons were placed in an incubator at 180 rpm at 37 °C. When the OD_{600} of the culture reached a value between 1.5 and 3.0 in the measurement made at 600 nm,

the cells were centrifuged at 6000 rpm at 4 °C for 15 minutes and pellet was stored at -80 °C.

2.9.2 Total Protein Isolation from *E. coli* Cells

E. coli pellets stored at -80 °C were taken and protein isolation was performed on ice. 50 mM Tris HCl was used as lysis buffer. 150 µl lysis buffer was added to 15 ml bacterial culture and protein isolation was performed on ice with the help of a sonicator. Sonicator was applied for 5 seconds and allowed to cool for 20 seconds and applied 3 times.

2.9.3 Total Protein Isolation from Sugar Beet Plant

Leaf samples were collected from sugar beet plants that were found to be partially positive in the sequencing result for protein extraction. The collected plant tissues were stored at -80 °C. On the day of protein studies, the leaves were ground into powder with liquid nitrogen. Afterwards, 5 ml of lysis buffer was added while the samples were on ice. The lysis buffer includes 2 mM EDTA, 2% PVP, and 50 mM potassium phosphate (pH 7.8). Samples with 5 ml of lysis buffer were sonicated for 10 seconds, 20 seconds, and 20 seconds, respectively. There was 1 minute between each sonication. Then, centrifugation was applied at 15000 rpm and 4 °C for 30 minutes. At the end of this period, the pellets were stored at -80 °C and the supernatants were centrifuged again at 15000 rpm and 4 °C for 20 minutes. Then, the supernatants were stored at -80 °C to be used in protein determination and western blot experiments. All experiments were performed with 3 sets of experiments and 2 replications in each set, and protein determinations were performed by the Bradford method.

2.9.4 Western-Blot Analysis of Isolated Proteins

After total protein samples taken at -80°C were thawed on ice, the protein content of the protein samples was evaluated using the Bradford method (Bradford, 1976). Protein amounts of the samples were determined using the Bradford method then the protein amount of each sample was calculated to be used in the western-blot experiment. An equal volume (30 µl) of soluble protein from each sample was mixed with 10 µl of sample loading buffer solution (4x). Then, the proteins were denatured by keeping them at 95°C for 5 minutes and they were ready to be loaded onto the SDS gel. Then, 20 µL of soluble fraction was loaded onto 15%

polyacrylamide SDS-PAGE gel. Protein-loaded gels were run at a constant voltage of 100 V for 90 min. The method given by Harlow and Lane in 1988 was followed to transfer the proteins separated on the gel to PVDF (Millipore Immobilon 0.45 μm) membranes. The transfer was carried out using Mini Trans Blot Cell (Bio-Rad) at a constant current of 0.34 A for 1 hour. All equipment was kept at 4 °C during the transfer period. After the transfer, the PVDF membrane on which the protein was transferred was blocked with a mixture of skim milk powder (BioShop, Canada) in tris-buffered saline (TBS) at pH 7.4 for 2 hours. Beta-ketothiolase was used for the primary antibody. The blotted membranes were then probed with the relevant anti-polyclonal antibodies (primary antibodies prepared at a dilution of 1:500 in TBST solution containing 5% skim milk) for 1 hour. Following this step, the western blot was washed 3 times for 5 min with TBST buffer (a combination of TBS buffer with Tween-20). Then, the membranes probed with primary antibodies were placed in the secondary antibody (Goat anti-Rabbit IgG(H+L) Secondary antibody, HRP) solution prepared by diluting 1:5000 in TBST containing 5% skim milk powder for 16 hours. At the end of the process, the probed membranes were washed with TBST 3 times for 5 min. Then, the membrane was stained using Clarity™ and Clarity Max™ Western ECL Substrates (BioRad, Hercules, CA, USA) for 5 min and the results were observed using a hi-chemiluminescence detection system ChemiDoc MP (Biorad). After imaging, membranes were washed again with TBST for 5 min and PonceuS staining was performed for 5 min to confirm protein transfer.

3. RESULTS

3.1 Selection, Sterilization and Germination of Plant Material

The main plant source engaged in transplastomic sugar beet plants was obtained from the seed of the doubled haploid breeding line SG-DH. To reduce and test the effect of genotype on transformation, four different sugar beet genotypes, SG-12, SG-13, SG-6M and ELK345, were employed in biolistic bombardment experiments. These four genotypes were obtained from the Turkish Sugar Institute under *in vitro* conditions and then the plants were cultured in petiole development medium.

No problem was encountered during the sterilization stage of the seeds. After the sterilization, the seeds were planted in a germination medium consisting of 4.4 g/L MS, 3% sucrose and 0.8% agar. Ten seeds were planted in each petri dish (Photo 3.1). They were subcultured every 3 weeks and the developing seeds were transferred to Magenda boxes to continue their development. On average, it took 20-25 days for SG-DH seeds to start germinating. This can be explained by the fact that sugar beet seed coats are very hard.

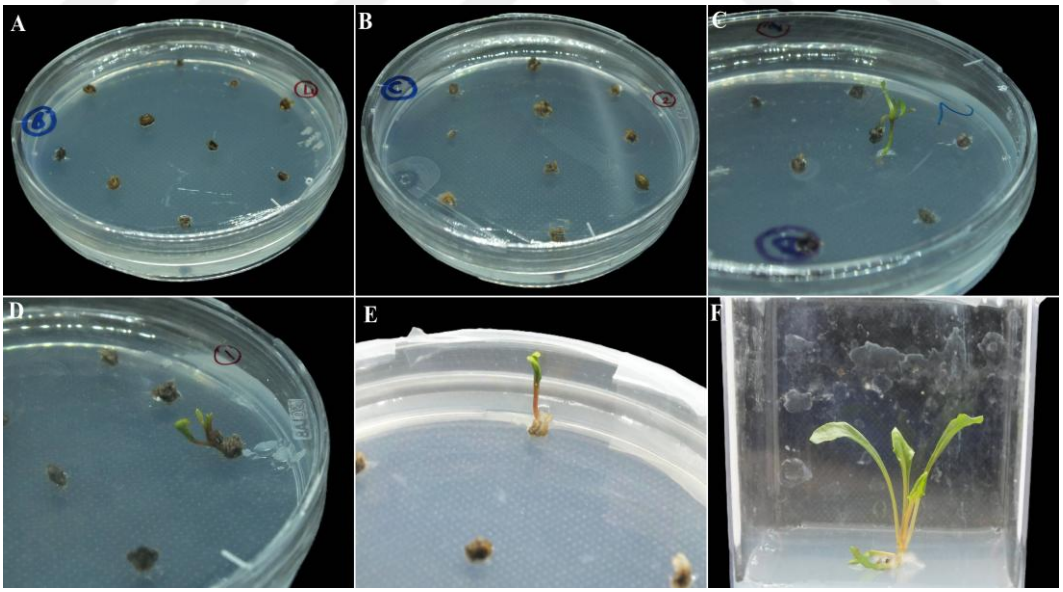


Photo 3. 1. Germination stages of SG-DH seeds. **A-B:** First day of sowing seeds in germination medium. **C-E**: Average 25-30th days of seeds in germination medium. **F:** 40-day-old SG-DH plants subcultured in germination medium

To analyze the success of SG-DH seeds in the germination environment, a germination experiment was carried out with 3 repetitions, each with 150 seeds. As

a result of this experiment, the germination rate was 56% in the first repetition, 48% in the second repetition and 44% in the third repetition. The average of these three germination data was 49.3% (Figure 3.1).

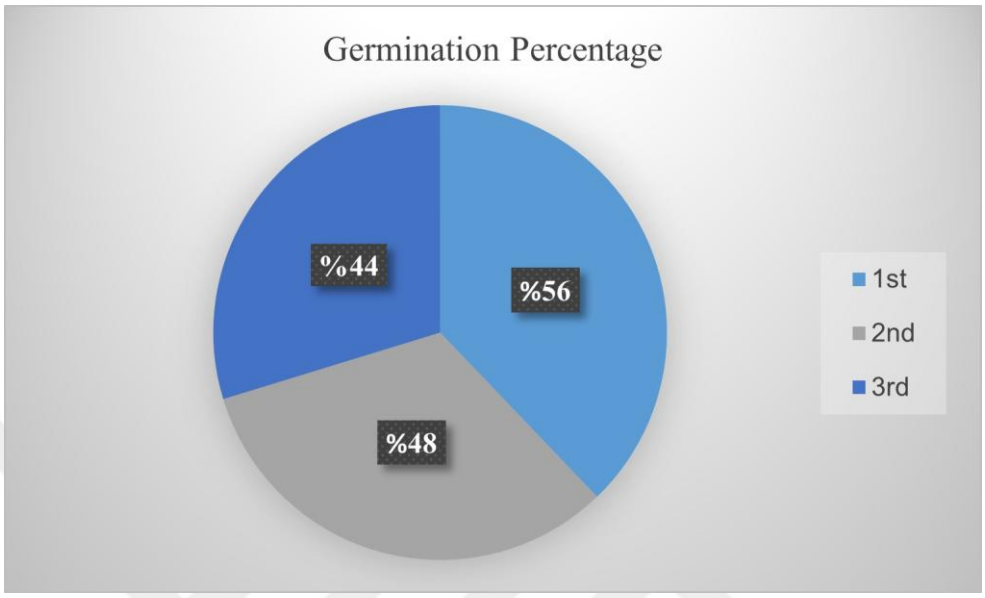


Figure 3. 1. Germination percentages of seeds of the SG-DH genotype

3.2 Planting Sugar Beet Plants in Shoot and Petiole Development Medium

Germinated healthy SG-DH seeds and SG-12, SG-13, SG-6M and ELK345 plants were planted in 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 0.25 mg/L BAP medium by cutting the roots to increase the number of leaf petioles (Photo 2.2). Plants were subcultured every 3 weeks. During subculture, senescent leaves and petioles were cut. Plants grew in this medium for approximately 1.5-2 months (Photo 3.2). At the end of this period, explants taken from young and healthy leaves (approximately 0.5 cm in length) were used for biolistic transformation and callus formation (De Marchis et al., 2009). No problems were observed in the development and formation of petioles planted in the regeneration medium to increase the number of young and healthy petioles.

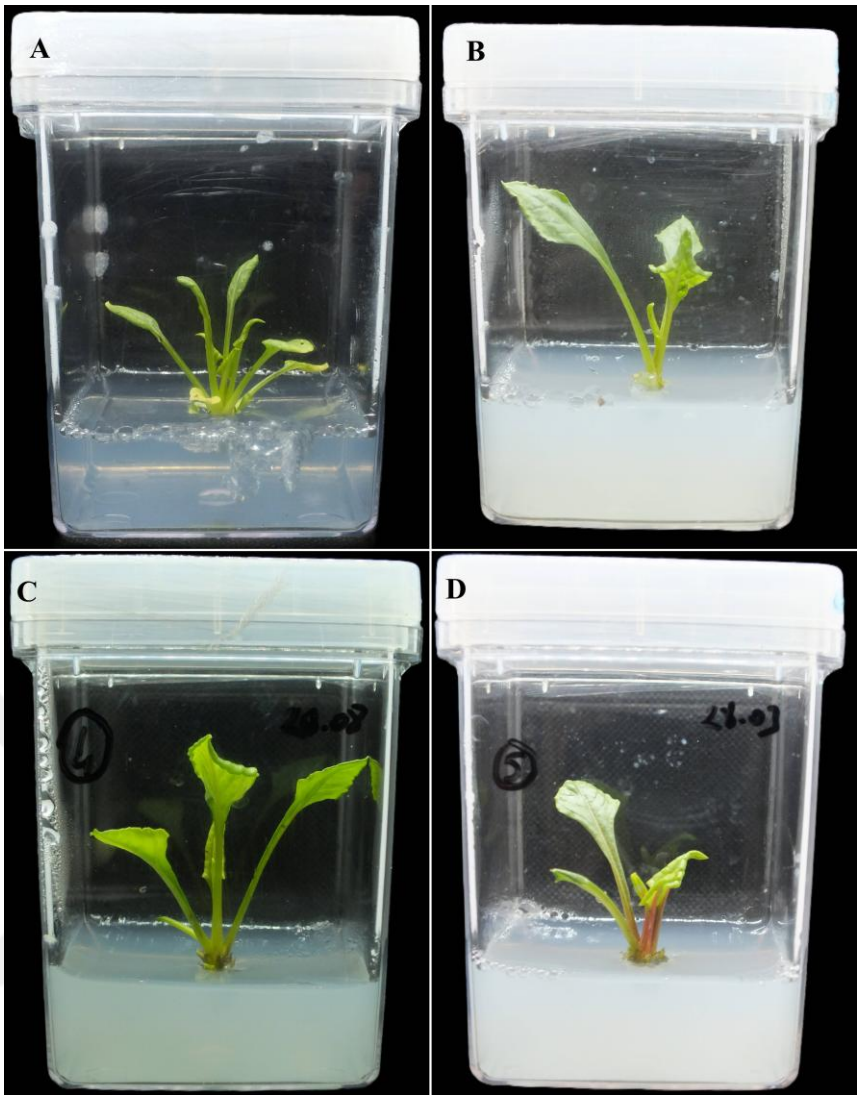


Photo 3. 2. Growth of sugar beet genotypes in regeneration medium. **A:** SG-DH genotype. **B:** SG-12 genotype. **C:** SG-13 genotype **D:** SG-6M genotype

3.3 Culturing Leaf Petiole Explants in Callus Medium

Petiole explants cut from plants grown in regeneration medium were transferred to callus medium for callus induction. Sugar beet has a recalcitrant structure against tissue culture and callus formation is slow in this plant. In addition, as previously known, tissue culture has been shown to be directly related to plant growth medium, growth conditions, explant type and especially genotype (Örgeç et al., 2021). Plant growth in tissue culture is a time-consuming and difficult process. This process may differ in plants with different genotypes.

As mentioned in De Marchis et al. 2009 study, leaf petiole explants formed callus even though they were blackened (Photo 3.3). The callus formed here was used as an explant in biolistic bombardment.

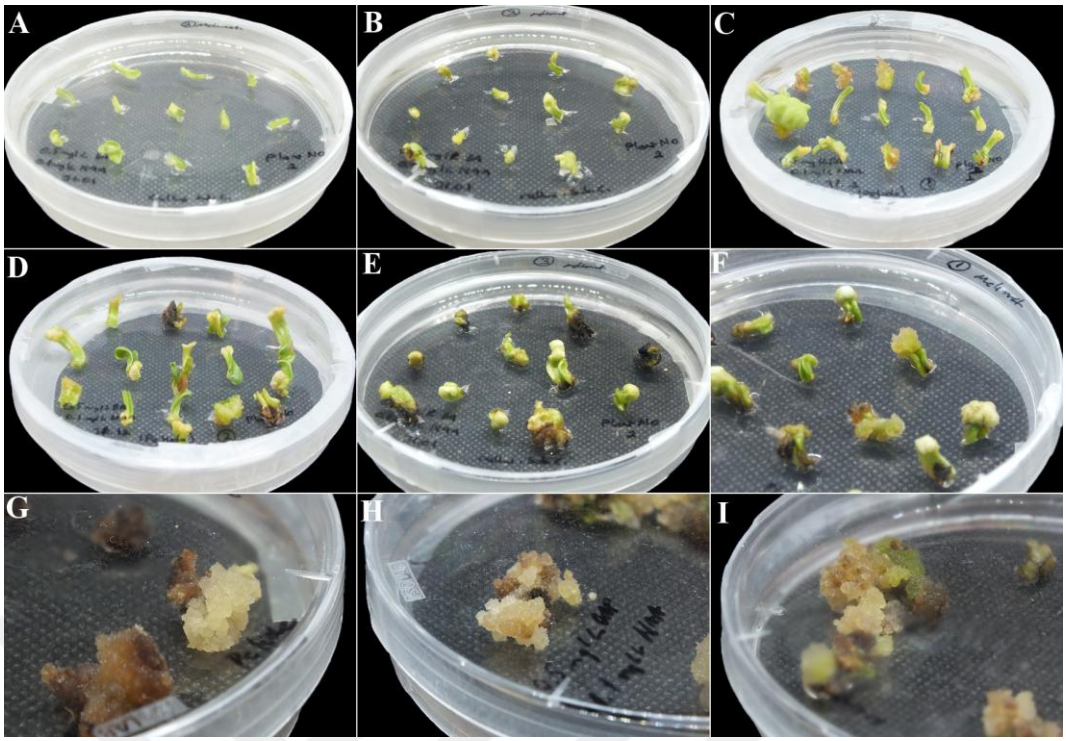


Photo 3. 3. Callus formation stages of petiole explants planted on callus medium. **A-B:** 7th day **C-D:** 1-month-old **E-F:** 2-month-old callus medium and **G-I:** 4-month-old petiole explants grown on callus medium

3.4 Determination of Antibiotic Selection Conditions

Antibiotic selection method was applied under *in vitro* conditions to identify putative transplastomic plants after biolistic bombardment.

To determine the optimum antibiotic dose, petiole and leaf explants were grown on media containing six different spectinomycin doses (0, 25, 50, 75, 100, 150 and 200 mg/L spectinomycin) 4.4 g/L MS, 3% sucrose, 0.4% agar with 0.5 mg/L BAP and 0.1 mg/L NAA. For each spectinomycin dose tested, sample number was 30 for petiole explant and 15 for leaf explant.

The observational results showed that in the 25 mg/L spectinomycin medium, a spot like white color was observed on the explants (Photo 3.4). In the 50 mg/L spectinomycin medium, in addition to the white spots, tinny blackenings were also observed at the tips of some leaf petioles. In the 75 mg/L spectinomycin medium, the size of the white and black spot increased. In the selection medium containing 100 mg/L and 150 mg/L spectinomycin, development came to a halt especially in leaf explants, and blackening covering almost the entire tissue was observed. In the 200 mg/L spectinomycin medium, it was observed that all leaf

explants planted turned white, petiole explants turned black, and development stopped (Photo 3.4).

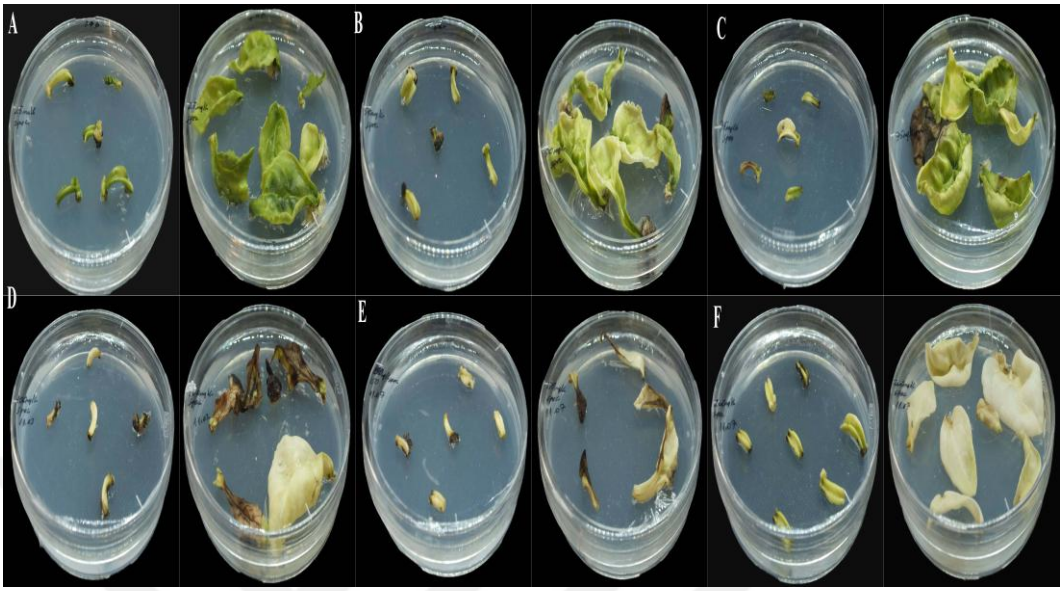


Photo 3. 4. 70th day of SG-DH plants in antibiotic selection with different doses. **A:** Petiole and leaf explants in 25 mg/L **B:** in 50 mg/L **C:** in 75 mg/L. **D:** in 100 mg/L. **E:** in 150 mg/L **F:** in 200 mg/L spectinomycin selection

Spectinomycin doses of 0, 25, 50, 75, 100, 150 and 200 mg/L were applied to leaf and petiole explants obtained from SG-DH sugar beet genotype as mentioned above. The data obtained were analyzed with Fisher Exact test GraphPad Prism 10.4.1 (trial version) application to find the survival rates (Figure 3.2). As a result of the statistical analysis, no significant difference was found between the control and 20 mg/L spectinomycin doses in both explant types. The first dose that gave a significant difference was 50 mg/L spectinomycin. Increasing the antibiotic dosage resulted in a decrease in the vitality of the explants. This shows that high antibiotic doses in sugar beet create a toxic effect and reduce viability. Since it was thought that the use of high antibiotic doses would negatively affect the growth of the plants *in vitro*, it was decided to use 50 mg/L spectinomycin for antibiotic selection.

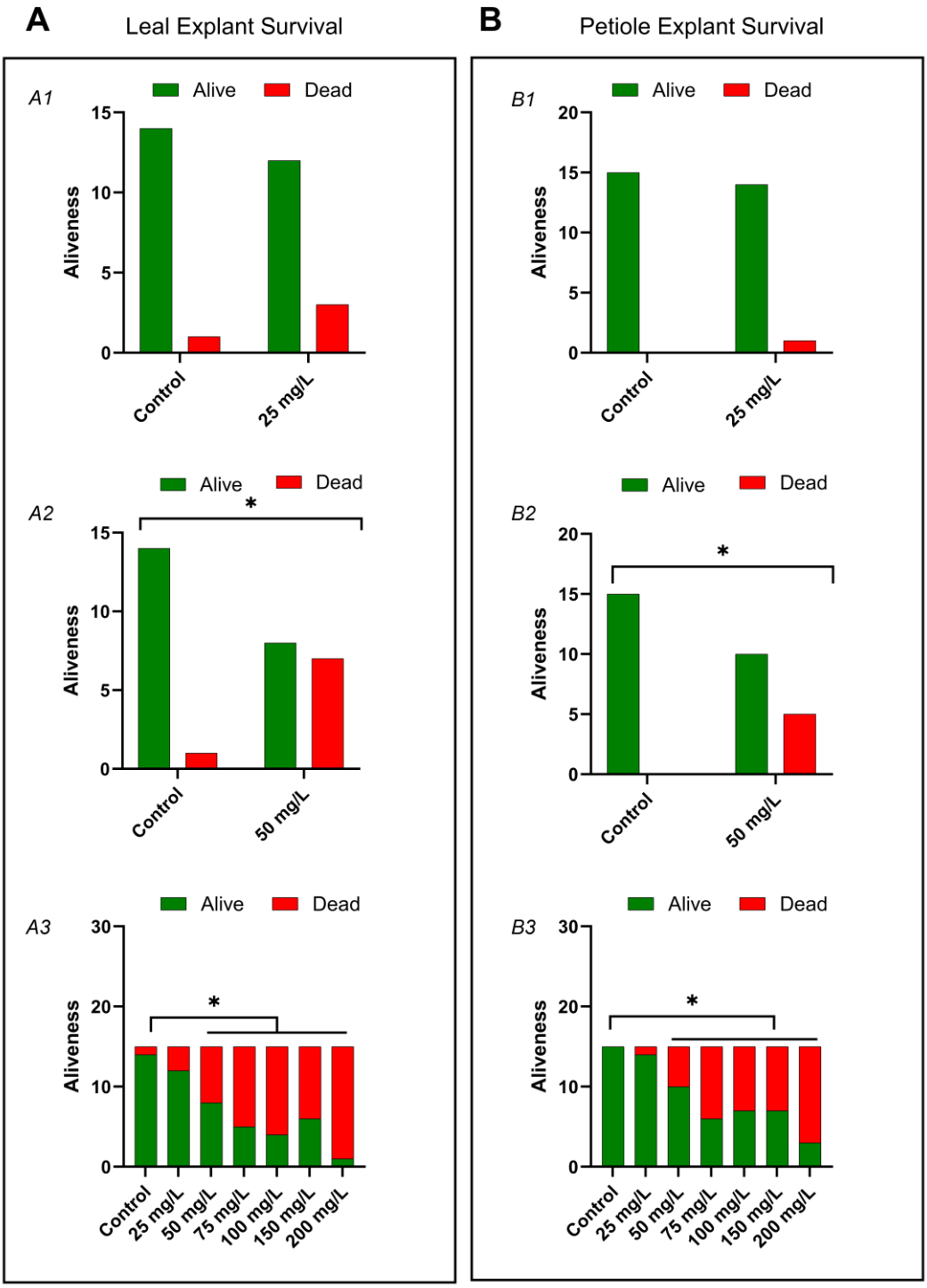


Figure 3. 2. Determination of spectinomycin antibiotic dose across aliveness of the tissues. **A:** Analysis of leaf explants and **B:** petiole explants excised from SG-DH sugar beet genotype ($P<0.05$)

3.5 Biolistic Bombardment of the pExpPN-phaA-SB Vector, Selection of Transformants and Shoot Regeneration

To ensure the integration of the pExpPN-phaA-SB vector into sugar beet chloroplast, the main explant source, SG-DH genotype, as well as SG-12, SG-13, SG-6M and ELK345 genotypes, were used as explant sources.

A total of **66** independent bombardment experiments were conducted and bombardment experiments were carried out on a total of **5846** explant sources including leaves, callus and petioles. Table 3.1 shows the total number of bombardments, the type and number of explants, and the callus, direct and indirect shoot formation numbers. Table 3.2 shows the bombardment number of sugar beet genotypes, and the total number of explants used. Under *in vitro* conditions, **2167** calli were formed and **376** direct shoot regeneration and **155** indirect shoot regeneration resulted in a total of **531** plants. Unfortunately, some of these shoots could not form full plants due to vitrification problems. Molecular verification analyses were performed on **516** explants, including some that remained at the callus stage, to understand the integration of the pExpPN-phaA-SB vector into sugar beet chloroplast.

Table 3. 1. Total number of biolistic bombardments for integration of the pExpPN-phaA-SB vector into sugar beet chloroplast.

Bombardment No	Explant Number	Callus Formation	Direct Shoot Regeneration	Indirect Shoot Regeneration
1	85 Petiole	9	2	4
	15 Callus	0	0	0
2	130 Petiole	108	40	32
	15 Callus	0	0	0
3	83 Petiole	51	21	5
4	131 Petiole	87	15	24
5	145 Petiole	94	9	7
6	26 Petiole	38	4	0
	26 Leaf	144	0	1
7	56 Petiole	74	1	5
	30 Leaf	97	0	8
8	152 Petiole	93	13	1
9	143 Petiole	120	20	10
10	145 Petiole	102	10	6
11	126 Petiole	17	10	6
12	116 Petiole	68	36	3
13	23 Petiole	9	14	3
	14 Leaf	3	7	1

Table 3. 2. (continued). Total number of biolistic bombardments for integration of the pExpPN-phaA-SB vector into sugar beet chloroplast.

Bombardment No	Explant Number	Callus Formation	Direct Shoot Regeneration	Indirect Shoot Regeneration
14	8 Petiole	0	6	0
	31 Leaf	0	3	0
15	46 Petiole	16	13	1
	26 Leaf	59	2	0
16	67 Petiole	5	8	0
	27 Leaf	9	6	0
17	42 Petiole	12	3	2
	27 Leaf	2	1	0
18	34 Petiole	0	1	0
	18 Leaf	0	0	0
19	70 Petiole	1	0	0
	27 Leaf	0	0	0
20	81 Petiole	3	0	0
	35 Leaf	1	0	0
21	63 Petiole	3	0	0
	23 Leaf	0	0	0
22	50 Petiole	7	0	0
	33 Leaf	2	0	0
23	91 Petiole	0	4	0
	28 Leaf	4	0	0
24	59 Petiole	13	2	0
	25 Leaf	9	0	0
25	17 Petiole	2	0	0
	2 Leaf	1	1	0
26	57 Petiole	10	2	1
	14 Leaf	7	1	0
27	45 Petiole	1	0	0
	16 Leaf	2	0	0
28	63 Petiole	6	0	0
29	112 Petiole	15	0	0
	9 Leaf	2	0	0
30	121 Petiole	47	3	0
	6 Leaf	17	0	0
31	87 Petiole	13	0	0
32	66 Petiole	0	3	0
33	84 Petiole	32	0	1
	6 Leaf	10	0	0
34	57 Petiole	15	2	1
	10 Leaf	2	0	0
35	75 Petiole	60	3	2
	6 Leaf	6	0	0
36	92 Petiole	40	2	3
	10 Leaf	16	0	0
37	80 Petiole	25	3	1
	9 Leaf	7	1	0

Table 3. 3. (continued). Total number of biolistic bombardments for integration of the pExpPN-phaA-SB vector into sugar beet chloroplast.

Bombardment No	Explant Number	Callus Formation	Direct Shoot Regeneration	Indirect Shoot Regeneration
38	79 Petiole	14	2	0
	6 Leaf	0	0	0
39	75 Petiole	13	4	1
	3 Leaf	0	0	0
40	75 Petiole	9	2	1
	3 Leaf	1	0	0
41	99 Petiole	6	2	3
	8 Leaf	12	1	0
42	42 Petiole	5	1	0
43	91 Petiole	35	8	3
	7 Leaf	5	1	0
44	71 Petiole	18	2	1
	6 Leaf	9	0	0
45	68 Petiole	11	13	0
	12 Leaf	6	1	0
46	96 Petiole	21	4	2
	13 Leaf	4	0	0
47	77 Petiole	20	2	1
	13 Leaf	4	1	0
48	105 Petiole	32	6	1
	8 Leaf	4	1	0
49	83 Petiole	33	2	0
	18 Leaf	3	0	0
50	83 Petiole	8	3	1
	6 Leaf	2	1	0
51	83 Petiole	17	3	2
	8 Leaf	3	0	0
52	68 Petiole	12	2	3
	10 Leaf	2	1	0
53	97 Petiole	21	7	3
	10 Leaf	2	1	0
54	57 Petiole	12	2	1
	10 Leaf	2	1	0
55	69 Petiole	24	2	1
	6 Leaf	4	0	0
56	35 Petiole	10	3	1
	7 Leaf	2	0	0
57	54 Petiole	11	2	0
58	74Tam Bitki	13	5	2
	12 Leaf	4	1	0
59	68 Petiole	11	2	2
	20 Leaf	2	3	0
60	42 Petiole	7	1	1
	11 Leaf	3	0	0

Table 3. 4. (continued). Total number of biolistic bombardments for integration of the pExpPN-phaA-SB vector into sugar beet chloroplast.

Bombardment No	Explant Number	Callus Formation	Direct Shoot Regeneration	Indirect Shoot Regeneration
61	91 Petiole	17	3	1
	8 Leaf	1	0	0
62	60 Petiole	18	5	2
63	92 Petiole	19	3	0
64	74 Petiole	14	3	1
	8 Leaf	3	0	1
65	101 Petiole	42	3	1
	9 Leaf	4	1	0
66	68 Petiole	14	3	1
	10 Leaf	2	1	0
Total	**5846**	**2167**	**376**	**155**

Table 3. 5. Genotype distribution of biolistic bombardment experiments.

Sugar Beet Genotype	Bombardment No	Total Number of Explants
SG-DH	1-2-3-4-5-6-7-8-9-10-11-12-15-17-19-20-21-22-23-30-33-36-37-39-41-42-43-44-46-48-50-51-52-53-54-56-57-58-59-60-62-63-64-66	4186
SG-12	13-14-16-18-32	288
SG-13	24-27-28-31-35-38-47-49-55	727
SG-6M	26-34-45-65	328
ELK345	25-29-40-61	317

The duration of the plants remaining in the selection medium after the biolistic bombardment was optimized several times during the experiments according to the responses of the explants. Accordingly;

First, after the bombardment process, leaf, petiole and callus explants were incubated in the dark for 48 hours and then cultured in media containing 4.4 g/L MS, 3% sucrose, 0.4% phytagel supplemented with 50 mg/L spectinomycin, 0.5 mg/L BAP and 0.1 mg/L NAA for the selection of putative transplastomic plants. Petri dishes were kept in this medium for 2 months and incubated under 16/8 hour light/dark conditions (Photo 3.5). When planting the explants in the medium, they were planted in a way that the tissue surfaces would have maximum contact with the medium. During the subculture stage, the leaf and petiole explants that grew in length were cut again and planted. At the end of 2 months, transplastomic plants were planted in medium containing 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 12 mg/L spectinomycin, 0.5 mg/L BAP, 0.1 mg/L NAA (Photo 3.7). 12 mg/L is the lowest antibiotic concentration that causes whitening of sensitive cells, as described in the article by De Marchis et al. 2009. In both antibiotic concentrations, plants

were subcultured after every 3 weeks. Keeping the plants under 50 mg/L spectinomycin for 2 months caused chlorosis in the explants. For this reason, at the end of 2 months in 50 mg/L spectinomycin medium, the spectinomycin concentration in the medium was reduced to 12 mg/L (Photo 3.7). De Marchis et al. 2011 reported that explants formed callus even if they turned dark brown. For this reason, explants subjected to biolistic transformation were replanted in each subculture even if they turned dark brown. It was observed that some petiole plants formed callus within 1 month after the bombardment experiment (Photo 3.6). These callus-forming plants and callus explants were planted in MS media containing 1.0 mg/L BA and 0.5 mg/L ABA hormones to ensure plant regeneration.

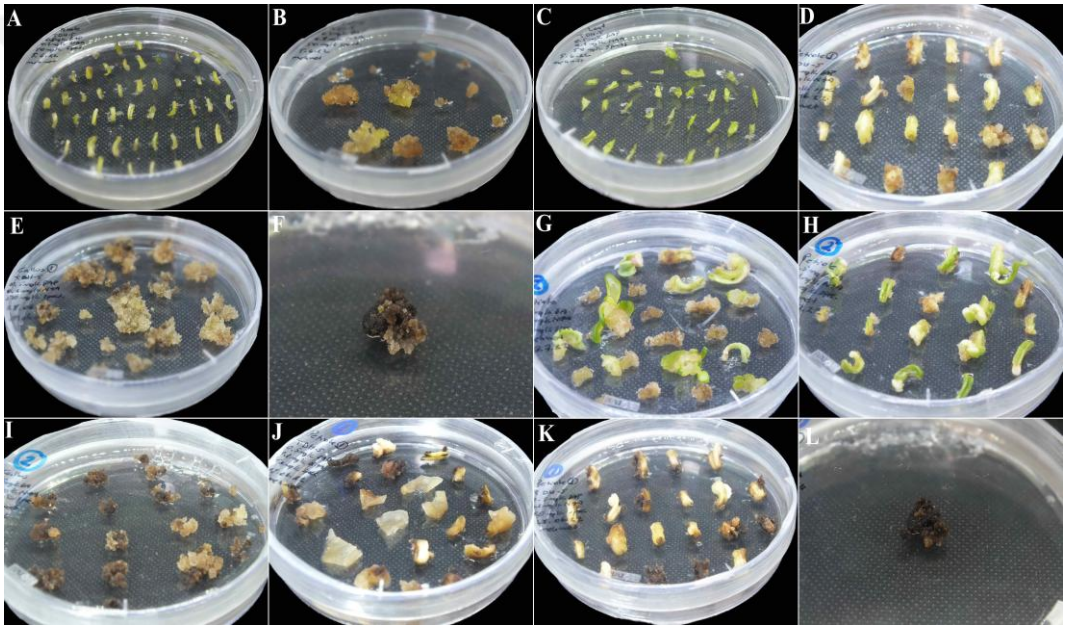


Photo 3. 5. Selection of putative transplastomic leaf, petiole and callus explants on antibiotic medium. **A-C:** First day of explants on 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 50 mg/L spectinomycin, 0.5 mg/L BAP, 0.1 mg/L NAA medium. **D-I:** Biolistic transformed plants on medium during their first month. **J-L:** The second month

Photo 3. 6. Putative transplastomic petiole explants forming callus in 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 50 mg/L spectinomycin, 0.5 mg/L BAP, 0.1 mg/L NAA medium. Picture taken 25 days after the bombardment experiment

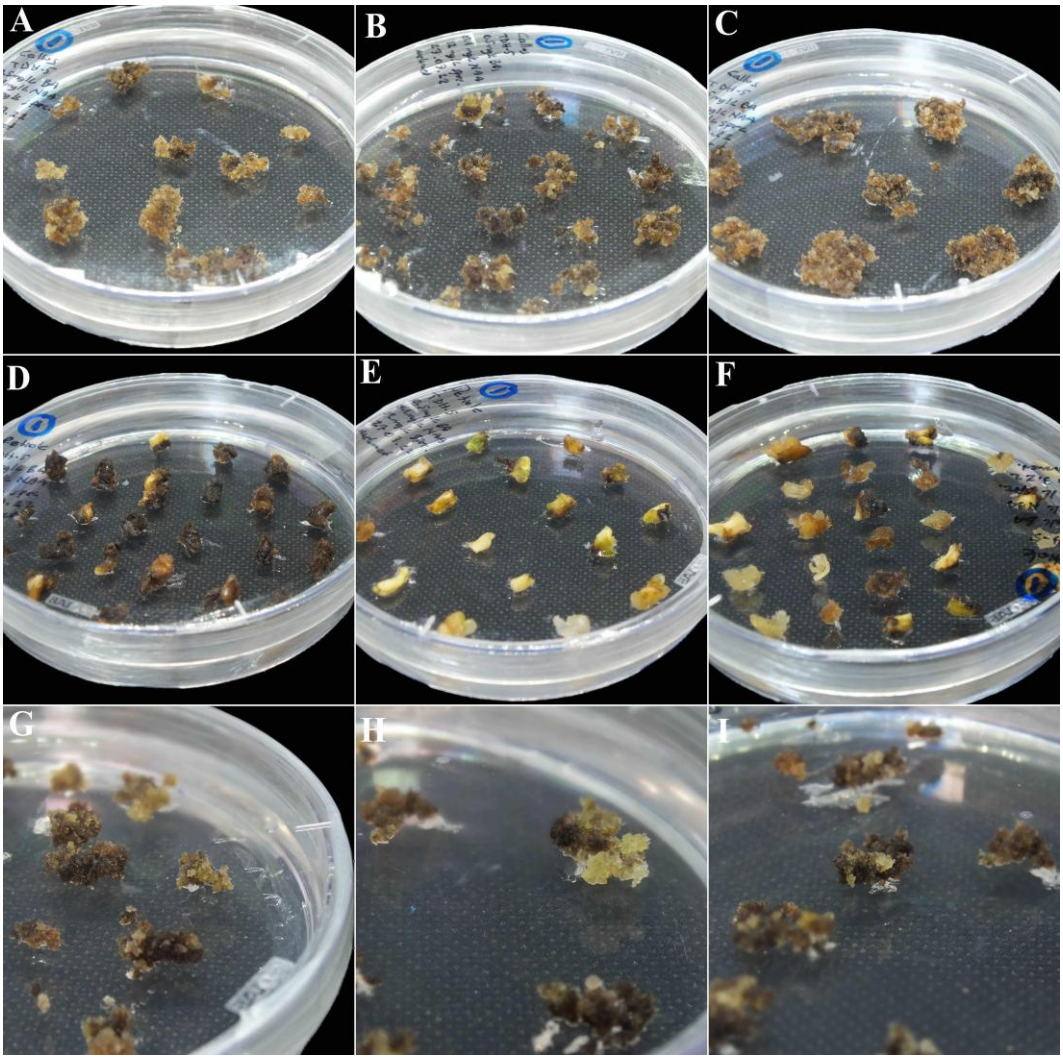


Photo 3. 7. Growth of transplastomic petiole and callus explants in 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 12 mg/L spectinomycin, 0.5 mg/L BAP, 0.1 mg/L NAA medium. **A-F:** First day in 12 mg/L and **G-I:** 10th day in 12 mg/L spectinomycin medium

The explants in the first biolistic experiments were cultured in 50 mg/L spectinomycin medium for two months and then in 12 mg/L spectinomycin for 16 days. At the end of this period, the plants were transferred to antibiotic-free media containing 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 0.5 mg/L BAP, 0.1 mg/L NAA and incubated in the plant growth chamber at 25 ℃ with 16 h light and 8 h dark conditions. Due to the darkening and loss of vitality of the explants, the explants were transferred to antibiotic-free media (Photo 3.8). As seen in Table 3.1, callus formation (7 pieces) or direct (2 pieces) / indirect shoot (4 pieces) regeneration was achieved in the explants in the first bombardment, albeit in low

amounts. As a result of the regeneration studies, full plants could not be obtained from the explants used in the first bombardment.

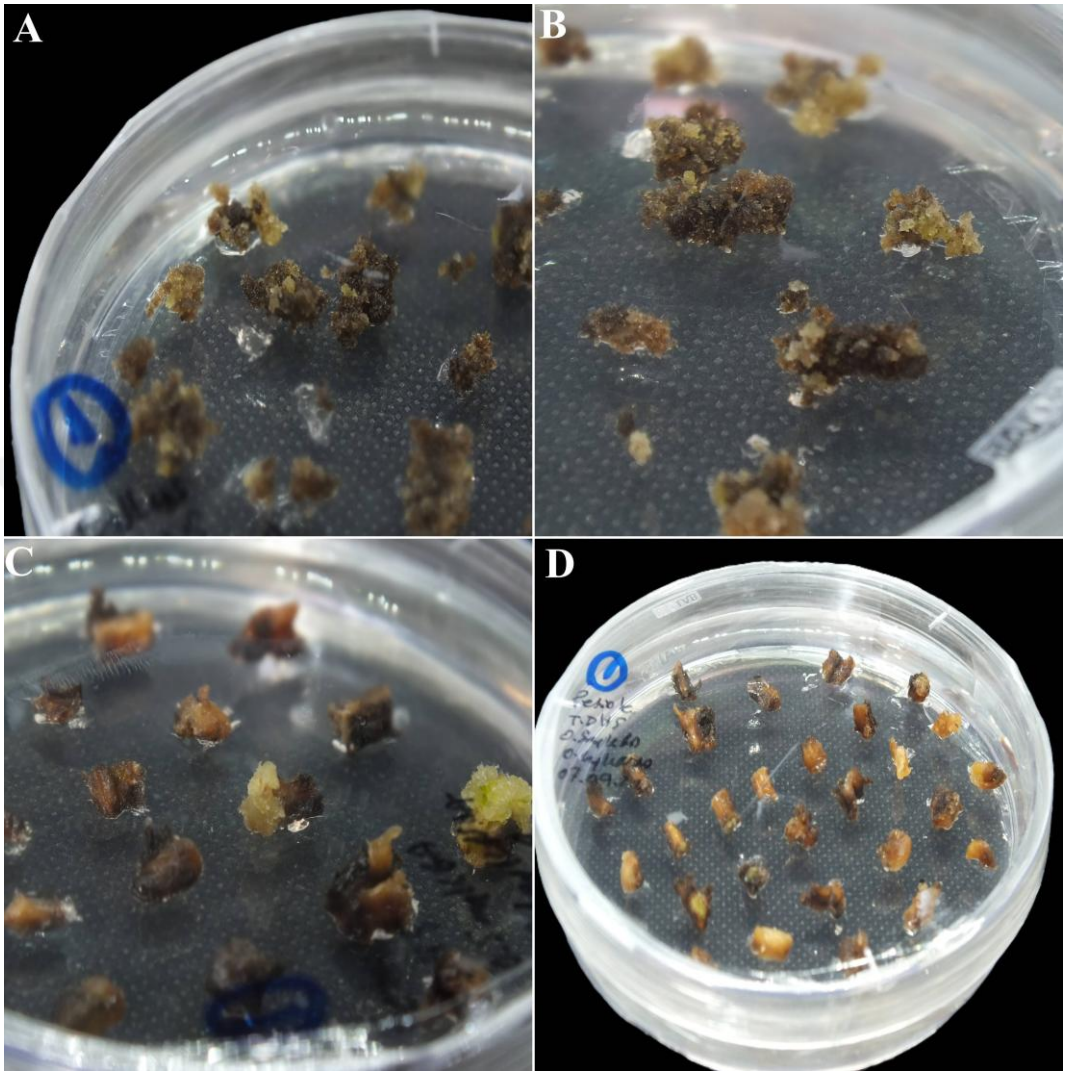


Photo 3. 8. Development of callus and petiole explants in media containing 0.5 mg/L BAP, 0.1 mg/L NAA. **A-B:** Callus explants. **C-D:** Petiole explants

Petiole and callus explants grown in antibiotic-free medium showed shoot and callus formation 10 days after being cultured. These callus and shoots were first transferred to the medium containing 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 1.0 mg/L BAP, 0.5 mg/L ABA and incubated in the plant growth room with 16 hours light and 8 hours dark conditions at 25 ℃ (Figure 3.9).

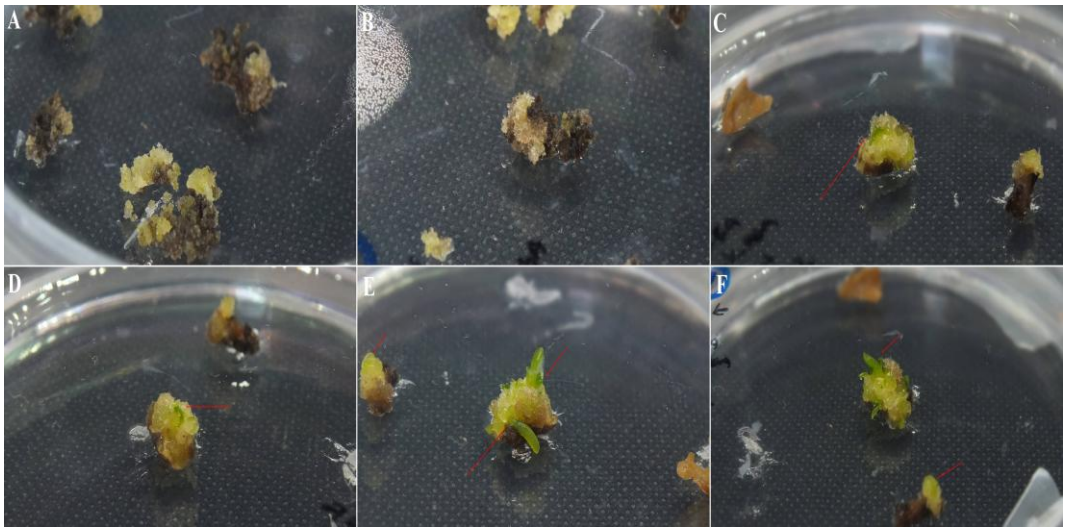


Photo 3. 9. Culturing callus and petiole explants in media containing 1.0 mg/L BAP, 0.5 mg/L ABA. **A-D:** Callus and petiole explants on day 1 in media. **E-F:** Petiole explants on day 6 in the media

In the shoots obtained from the leaf petiole shown in Photo 3.9, vitrification was observed in the medium containing 1 mg/L BAP and 0.5 mg/L ABA. The vitrified shoots were transferred to the medium containing no hormones (4.4 g/L MS, 3% sucrose, 0.4% phytagel). However, on later, due to the yellowing of the shoots (Figure 3.10), 0.25 mg/L BAP was added to the medium and they were cultured at 25 ℃ under 16 hours of light and 8 hours of darkness. The shoots on which vitrification was observed lost their vitality.

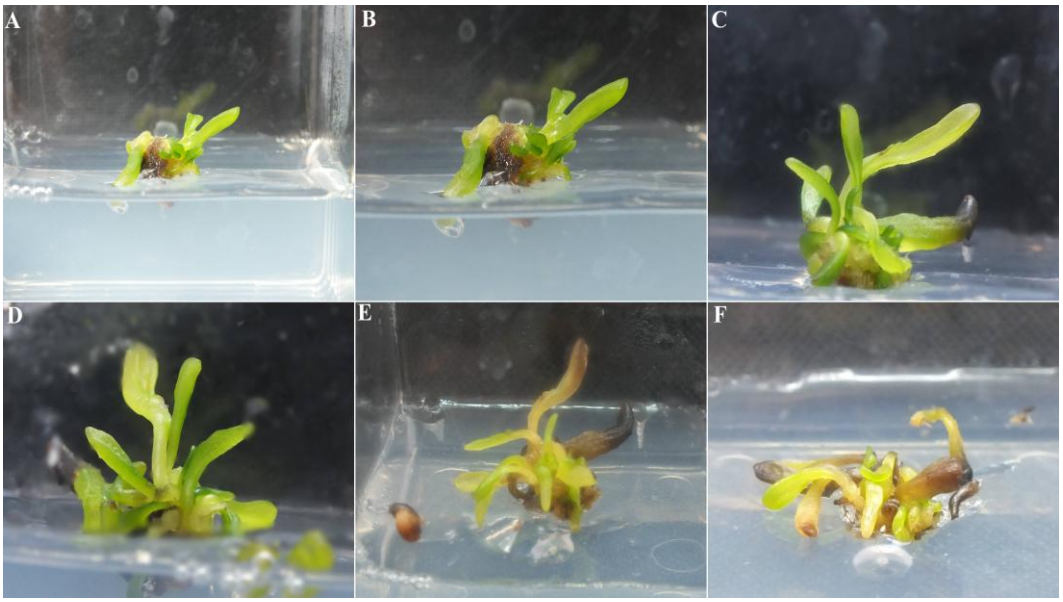


Photo 3. 10. Growth of vitrified shoots obtained from bombarded leaf petiole explants on MS medium (without hormone) and medium containing 0.25 mg/L BAP. **A-B:** 1st day **C-D:** 15th day and **E:** 1st month of shoots on MS (without hormone) medium. **F:** 7th day of shoots on MS medium containing 0.25 mg/L BAP

In the first and second biolistic bombardment experiments, although some of the calli used as explants formed green dots, shoot and root regeneration could not be achieved. During this process, callus explants were also cultured in media containing 0.25 mg/L KIN and no regeneration was achieved from these explants (Photo 3.11).

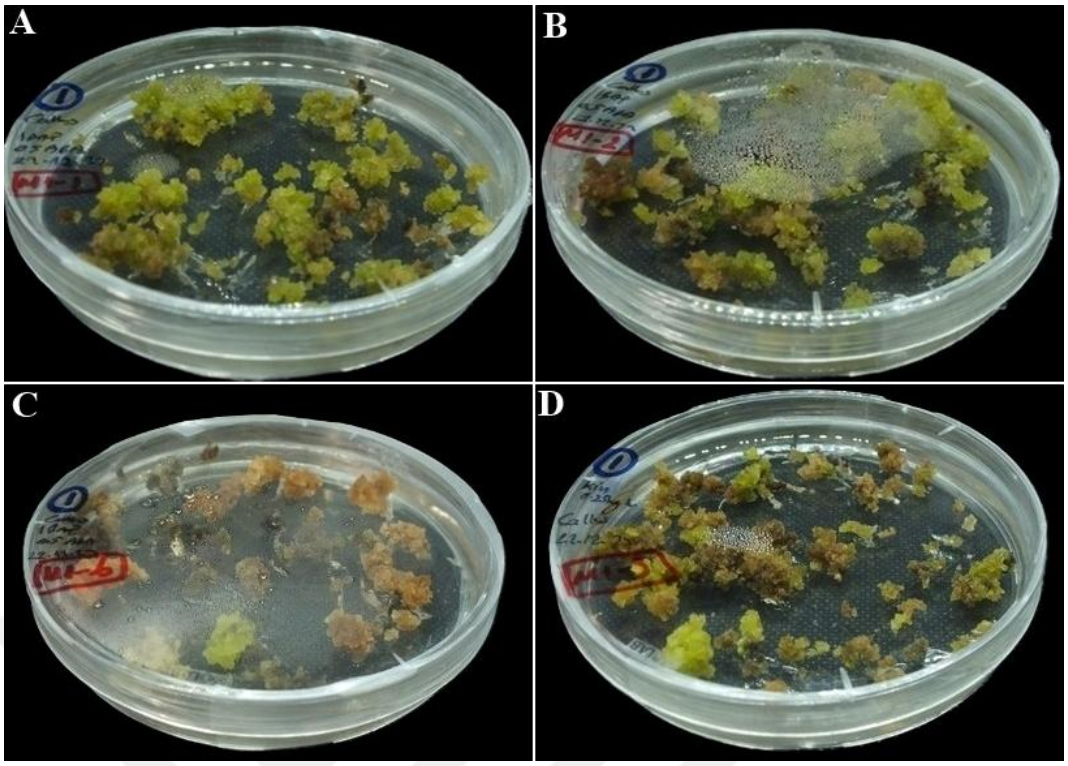


Photo 3. 11. Bombarded callus explants were first cultured in medium containing 1 mg/L BAP and 0.5 mg/L ABA and then grown in medium containing 0.25 mg/L KIN. **A-C:** Development of callus explants in medium containing 1 mg/L BAP and 0.5 mg/L ABA (4^{th} month). **D:** Development of callus explants in medium containing 0.25 mg/L KIN (2^{nd} month)

In the first bombardment experiment, it was observed that the explants lost their viability along with the growth retardation or cessation under antibiotic selection. Therefore, the explants used in the second bombardment experiment were grown in media containing 50 mg/L spectinomycin and 0.5 mg/L BAP and 0.1 mg/L NAA for one month and at the end of this period they were transferred to same media without antibiotics. The explants were incubated at 25 ℃ for 16 hours of light and 8 hours of dark conditions. It was observed that the blackened petiole explants formed callus after being cultured in media without antibiotics (Photo 3.12).

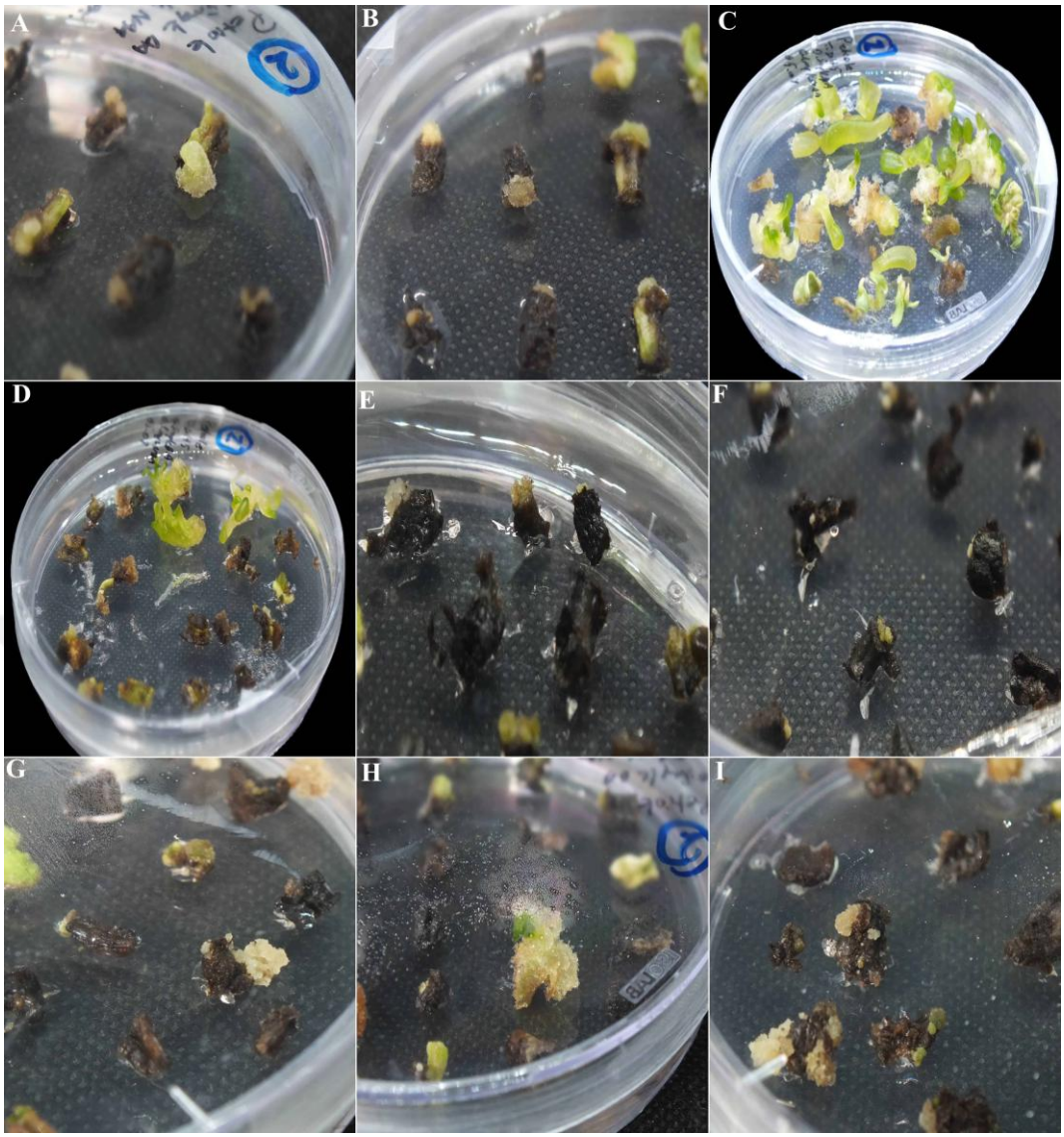


Photo 3. 12. Growth of petiole explants on media containing and not containing antibiotics. **A-B:** 1st month of petiole explants in medium containing 50 mg/L spectinomycin and 0.5 mg/L BAP and 0.1 mg/L NAA. **C-D:** 10th day **E-F:** 1st month and **G-I:** 55th day of petiole explants in medium containing 0.5 mg/L BAP and 0.1 mg/L NAA

Calli formed from petiole explants used in the second bombardment experiment were first cultured in a medium containing 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 0 mg/L BAP and 0.5 mg/L ABA for regeneration (Photo 3.13). Calli formed from petiole regenerated in the same medium and formed shoots. However, when the formed shoots were kept on the same medium for a long time, vitrification was observed in the shoots.

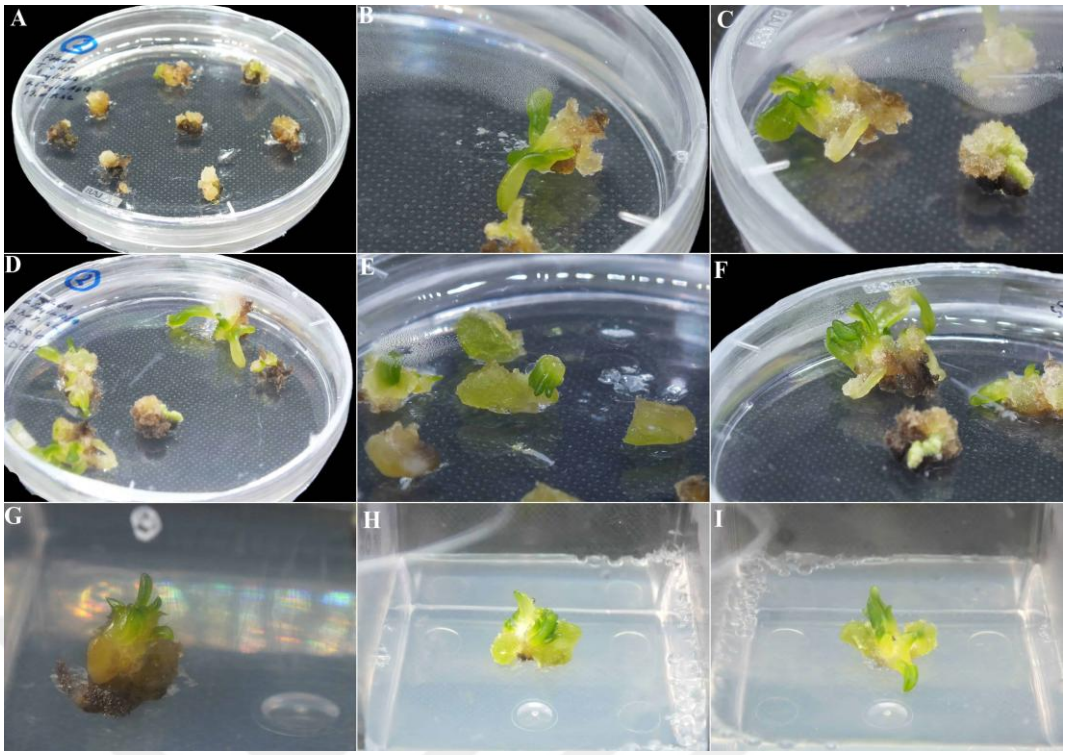


Photo 3. 13. Growth of bombarded petiole explants in medium containing 4.4 g/L MS, 3% sucrose, 0.4% phytagel with 1 mg/L BAP and 0.5 mg/L ABA. **A-C:** First day **D-F**: 7th day and **G-I:** 15th day of petiole explants in medium

Direct and indirect shoots formed from leaf petiole explants were cultured in hormone-free medium containing 4.4 g/L MS, 3% sucrose, 0.4% phytagel since they started to vitrify in their own regeneration medium (Photo 3.14). Vitrification was seen more and more intensely in plant samples that showed indirect shoot regeneration. In addition, shoot regeneration was not observed even though green spots were formed in compact calluses.

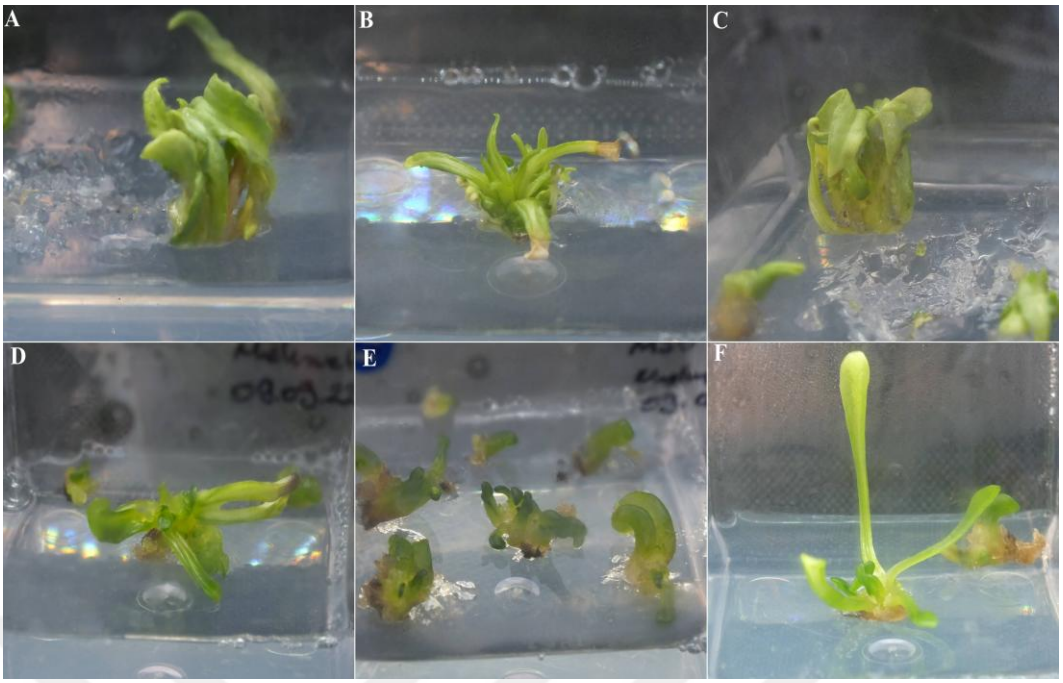


Photo 3. 14. Day 1 of the obtained shoots in 4.4 g/L MS, 3% sucrose, 0.4% phytagel medium (without hormone). **A-C:** Directly regenerated shoots. **D-F:** Indirectly regenerated shoots

Since no improvement was observed in the vitrification problem and slowdown in growth in plants grown in hormone-free media, these plants were transferred to media containing 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 0.25 mg/L KIN (Photo 3.15). It was observed that 0.25 mg/L KIN hormone concentration had a positive effect on the vitrification problem in plants compared to other media.

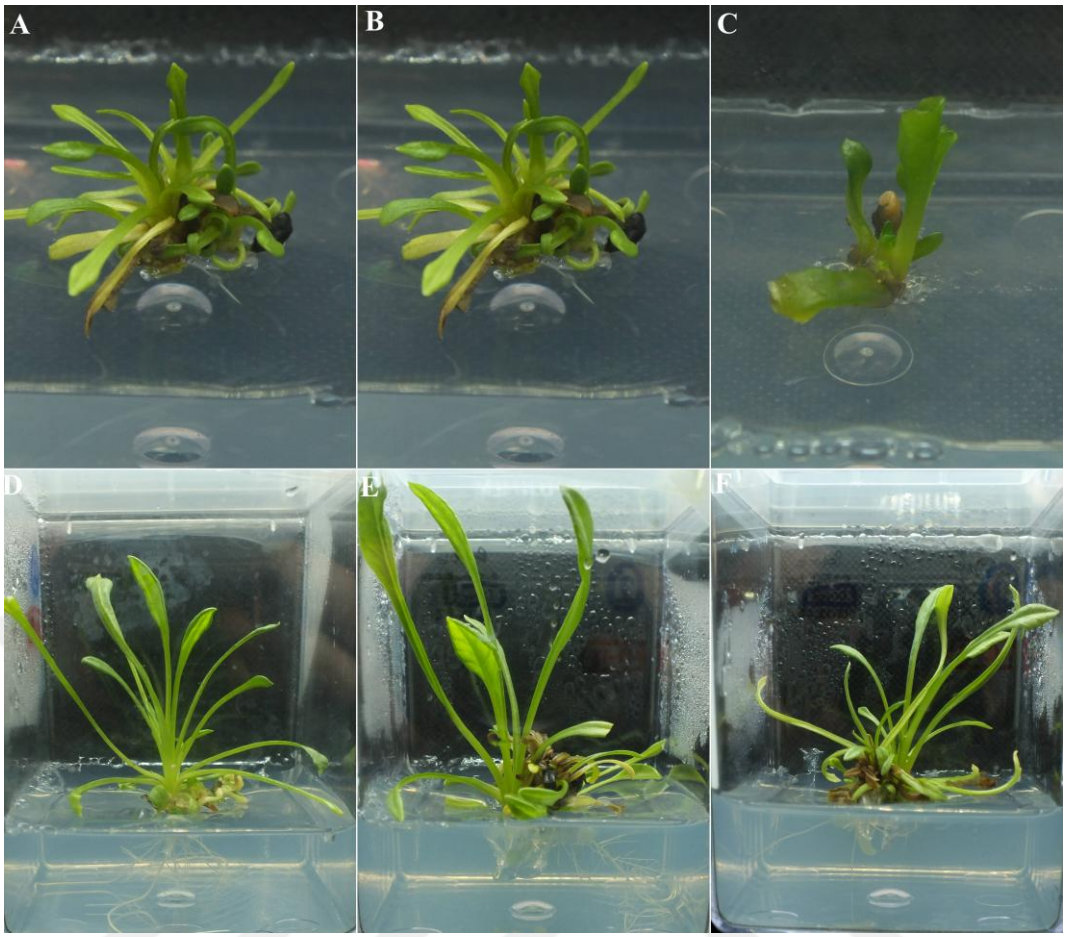


Photo 3. 15. Growth and development of putative transplastomic sugar beet plants regenerated after bombardment in 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 0.25 mg/L KIN medium. **A-C:** Development of indirectly regenerated leaf petiole explants in 0.25 mg/L KIN medium. **D-F:** Development of directly regenerated leaf petiole explants in 0.25 mg/L KIN medium

After biolistic bombardment of petiole explants, shoot regeneration was achieved in some of the calli obtained, but the shoots did not grow sufficiently and vitrification problem was observed in the obtained shoots. It was also observed that the surfaces color turned black of these calli in contact with the nutrient medium. The vitrification problem in plants was mostly seen in indirectly obtained shoots (Photo 3.16). Although the vitrification problem was largely solved by transferring the plants to standard MS plant growth medium supplemented with 0.25 mg/L kinetin after plant regeneration started, a certain portion of the explants could not show healthy shoot regeneration due to the vitrification problem that occurred especially during the callus regeneration stage. In addition to the fact that the growth of these plants almost stopped, it was observed that the plants were broken into

pieces when touched with forceps. Therefore, these calluses formed from the explants subjected to biolistic bombardment could not be used for DNA isolation and PCR screening.

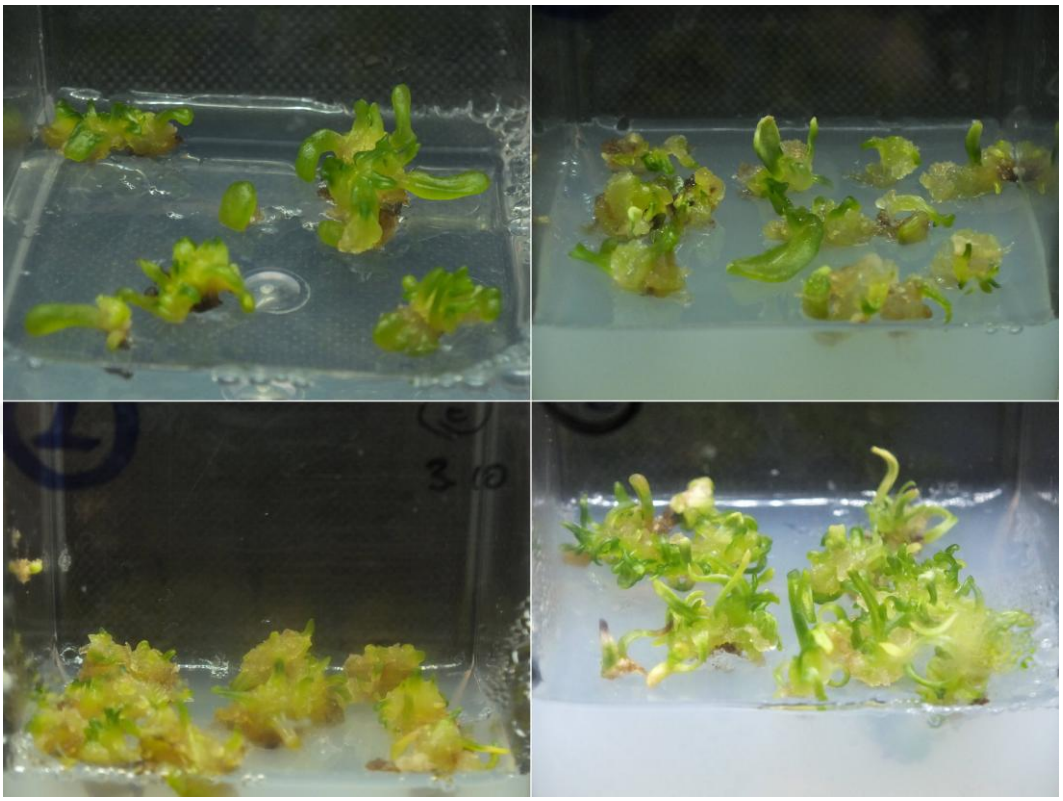


Photo 3. 16. Putative transplastomic sugar beet plants with vitrification problem grown in 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 0.25 mg/L KIN medium

When 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 0.25 mg/L KIN medium were used immediately after the shoots were formed, it significantly reduced the vitrification problem especially in plants showing direct regeneration. In addition, it was observed that the plants grown in this medium formed root (Photo 3.17).

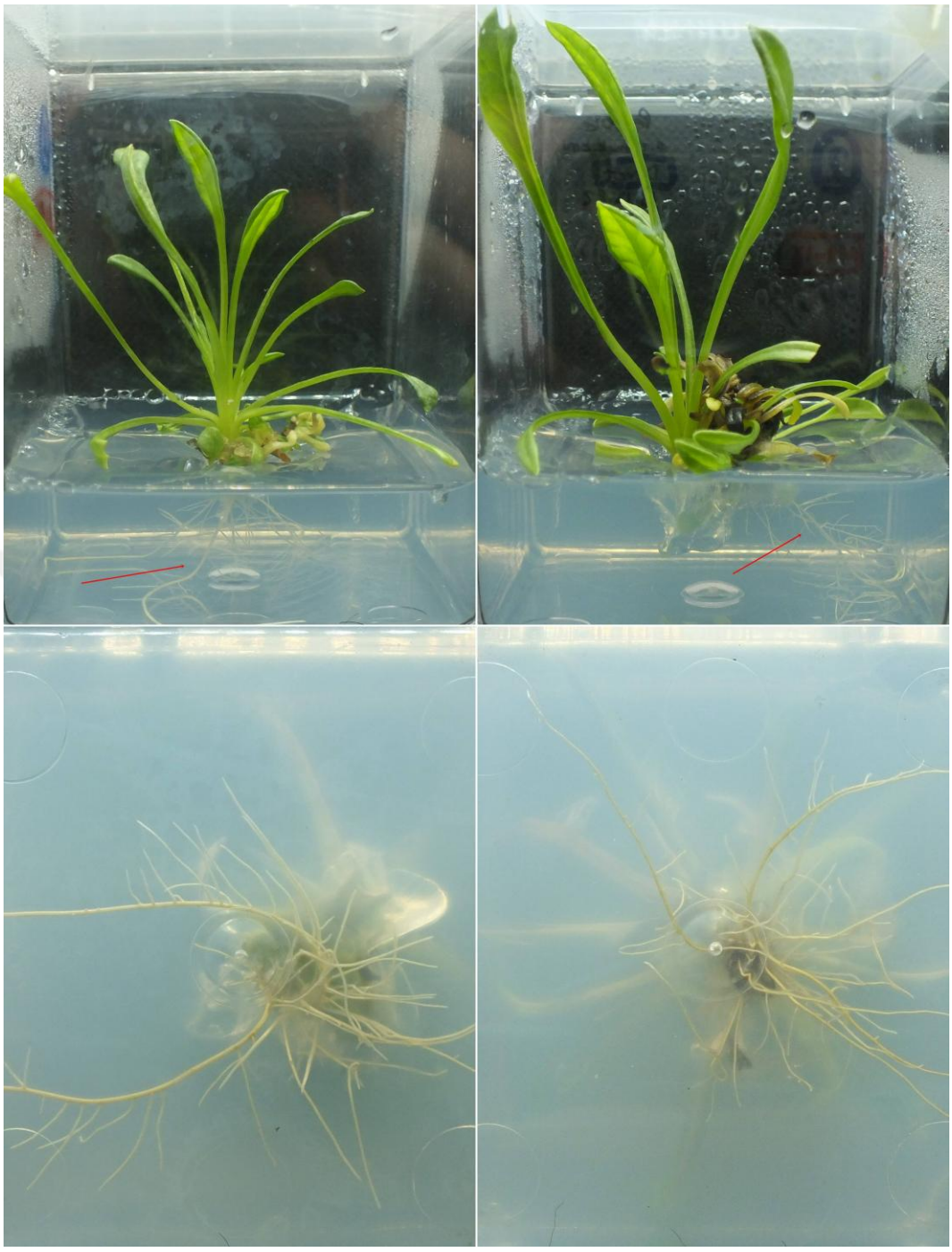


Photo 3. 17. Root formation in potentially transplastomic sugar beet plants grown on 4.4 g/L MS, 3% sucrose, 0.4% phytagel and 0.25 mg/L KIN medium for 2 months

In the first stage of the experiments, after a 1-month antibiotic selection period, callus was obtained by removing the antibiotic from the medium and it was observed that the callus also formed shoots. This selection method was used until the 19th bombardment experiments.

It was found that plants that showed regeneration with the selection method described above (1-month antibiotic selection) gave a large number of negative results in PCR studies performed to verify the integration of the pExpPN-phaA-SB vector (Photo 3.18).

The large number of non-transplastomic plants that have escaped antibiotic selection pressure has led to reconsideration and evaluation of the selection procedure.

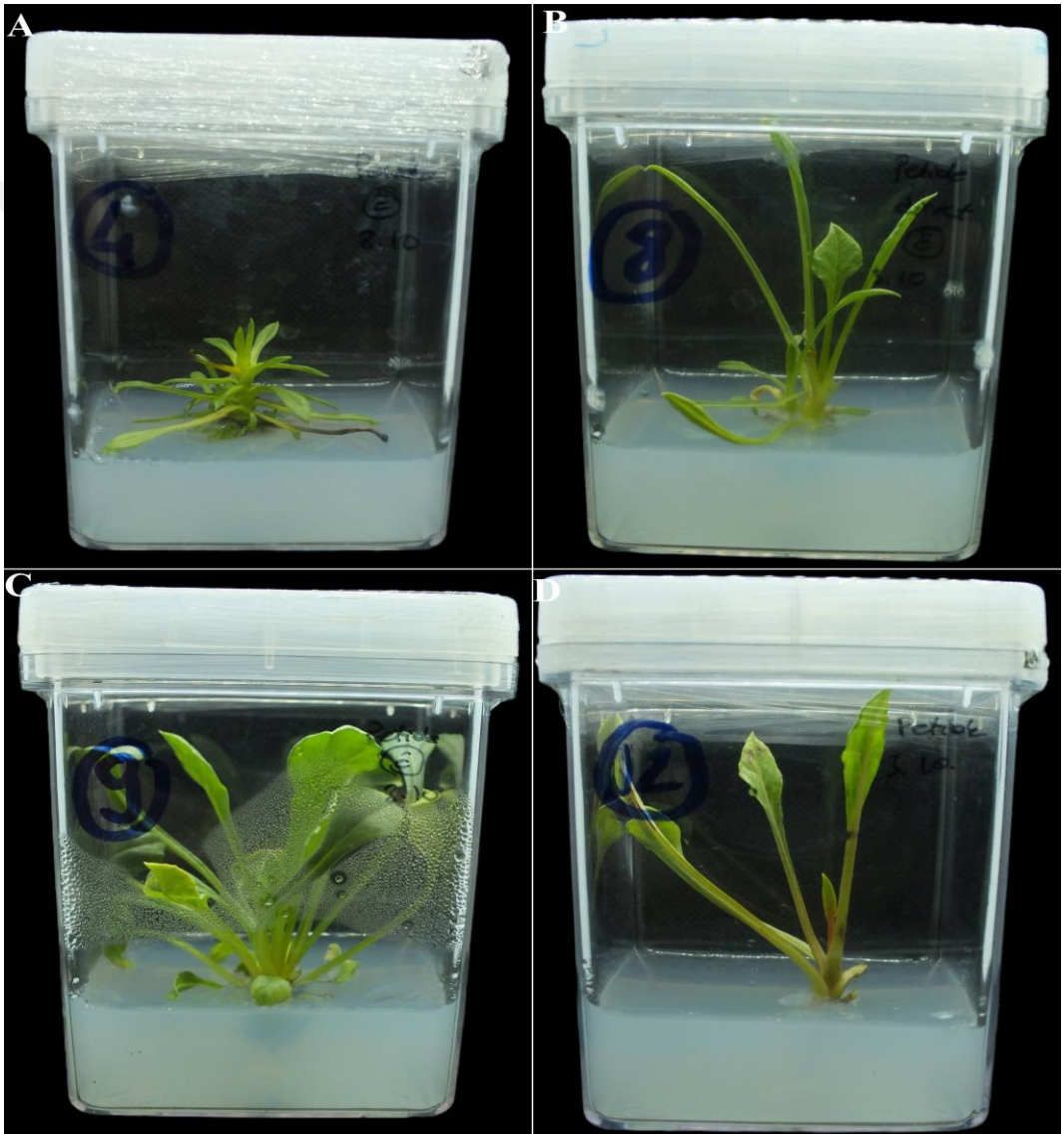


Photo 3. 18. Sugar beet plants that remained healthy in antibiotic selection but were not PCR positive. **A:** Plant grown in vitro for approximately 14 months. **B-C:** Plant grown in vitro for approximately 12 months. **D:** Plant grown in vitro for approximately 11 months

Following all these developments to prevent the growth of non-transplastomic plants, the method followed in the study of De Marchis et al., 2009, was started to be used as the antibiotic selection period. As in the study of De Marchis, the explants subjected to biolistic bombardment were kept in the dark for 2 days and then planted in MS medium containing 0.5 mg/L BAP, 0.1 mg/L NAA and 50 mg/L spectinomycin for 5 months and kept in the light for 8 hours and dark for 16 hours. This method was followed for bombardments between 19 and 38.

As seen in Table 3.1, it was observed that exposing plants to antibiotic pressure for 5 months with this new selection method negatively affected callus formation, direct and indirect regeneration efficiency and significantly reduced the amount of plant formation (Photo 3.19).

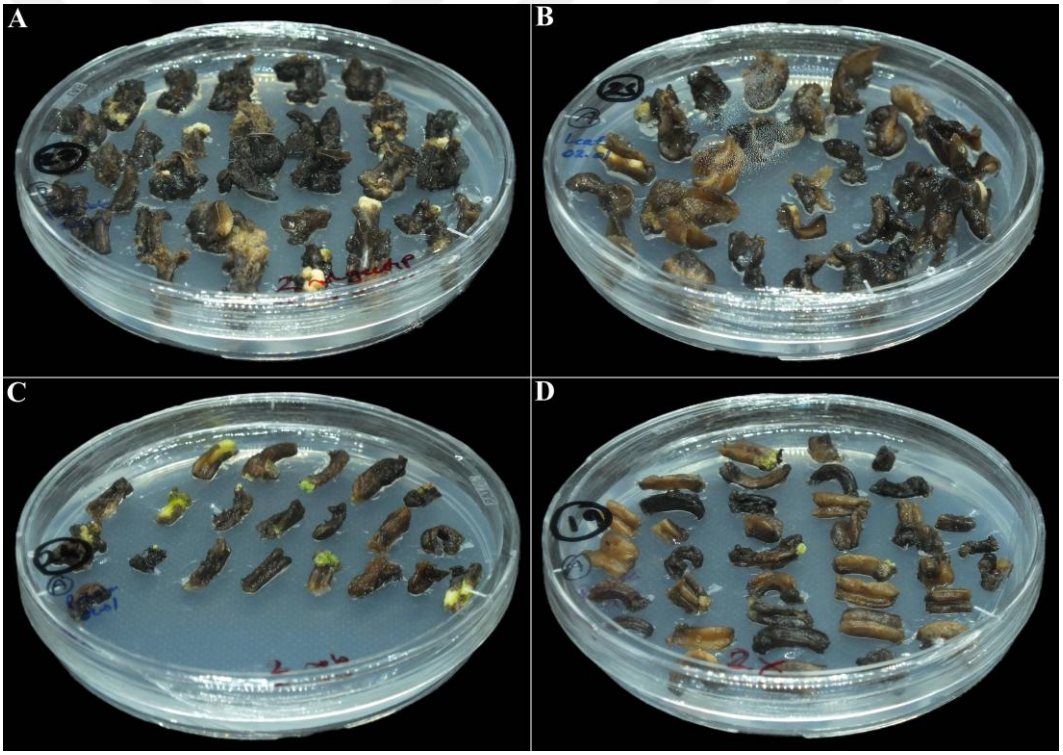


Photo 3. 19. Plants exposed to antibiotic selection for 5 months after biolistic bombardment

In bombardment experiment number 20, one of the petiole explants started to form callus at the beginning of antibiotic selection and despite being exposed to selection medium for 3.5 months, it was observed that it increased in volume. Later, the plant was transferred to MS medium containing 0.5 mg/L BAP, 0.1 mg/L NAA without antibiotic selection and then to regeneration medium without antibiotics

(MS medium containing 1.0 mg/L BAP and 0.5 mg/L ABA), but this callus could not show shoot regeneration (Photo 3.20).

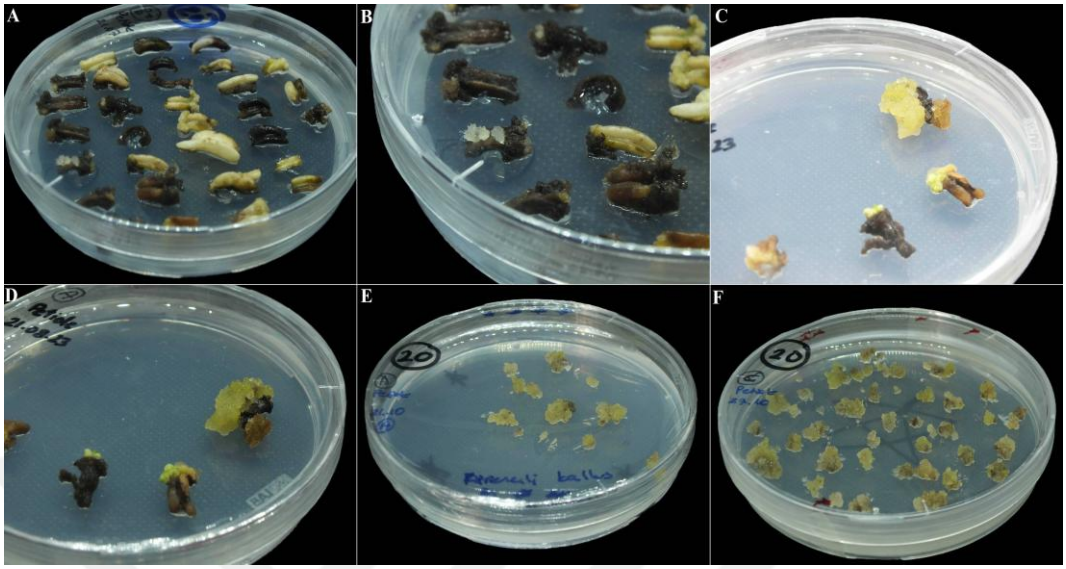


Photo 3. 20. Petiole explant forming callus in spectinomycin antibiotic medium. **A-B:** 2nd month in antibiotic medium. **C-D:** 1st month in antibiotic-free medium. **E:** 2nd month in antibiotic-free medium. **F:** 2nd month in regeneration medium

These results caused the selection issue to be reconsidered. Because exposure to long-term selection environment significantly reduced the regeneration capacity of the plants, the fact that the callus formed was not a transplastomic plant showed that the spectinomycin antibiotic was not effective enough. To understand the effect of antibiotic selection period on callus formation and regeneration capacity with the data obtained from a total of 39 bombardments, statistical analysis was performed Fisher Exact and Chi-square tests were analyzed with GraphPad Prism 10.4.1 (trial version) application. SG-12 genotype was not included in this analysis due to insufficient sample size.

When considered the SG-DH sugar beet genotype, which has a larger sample size than other genotypes and is the main explant source of our study. The 50 mg/L spectinomycin selection period of both leaf and petiole explants is kept for 1 month, it has a statistically significant better percentage of callus formation, direct and indirect regeneration formation compared to the 5-month period (Figure 3.3). It is clearly seen that the 5-month selection period has a negative effect on leaf explants.

Selection Period	SG-DH, Leaf Explant			SG-DH, Petiole Explant		
	Callus Formation (N/n,%)	Direct Regeneration (N/n,%)	Indirect Regeneration (N/n,%)	Callus Formation (N/n,%)	Direct Regeneration (N/n,%)	Indirect Regeneration (N/n,%)
1 Month Selection	282/122; %43*	282/15; %5,31**	122/10; %8,19*	3051/1207; %39,5****	3051/258; %8,46****	1207/135; %11,18***
5 Month Selection	177/57; %32	177/1; %0,56	57/0; %0,0	732/158; %21,6	732/12; %4,44	158/12; %3,16

Figure 3. 3. Selection period analysis results of SG-DH genotype. (N: sample number, n: callus and regeneration number, P<0.05=*, P<0.01= **, P<0.001= ***, P<0.0001= ****)

As a result of the analysis performed on SG-13, SG-6M and ELK345 genotypes, no significant difference was observed in the selection period on callus formation and regeneration under *in vitro* conditions, except for the callus formation percentage when the 50 mg/L spectinomycin selection period of the petiole explant of the SG-13 genotype was kept for 1 month compared to the 5-month period. On the other hand, except for the callus formation percentage of the leaf explant of the SG-13 genotype and the indirect regeneration percentage of the SG-6M genotype, the 5-month antibiotic selection period decreased the callus formation, direct and indirect regeneration percentages in all other data (Figure 3.4).

Selection Period	SG-13, Leaf Explant			SG-13, Petiole Explant		
	Callus Formation (N/n,%)	Direct Regeneration (N/n,%)	Indirect Regeneration (N/n,%)	Callus Formation (N/n,%)	Direct Regeneration (N/n,%)	Indirect Regeneration (N/n,%)
1 Month Selection	37/11; %29,7	37/1; %2,7	11/0; %0,0	229/77; %33,6*	229/6; %2,62	77/2; %2,60
5 Month Selection	53/17; %32	53/0; %0,0	17/0; %0,0	408/107; %26,2	408/7; %1,72	107/2; %1,83
Selection Period	**SG-6M, Leaf Explant**			**SG-6M, Petiole Explant**		
	Callus Formation (N/n,%)	Direct Regeneration (N/n,%)	Indirect Regeneration (N/n,%)	Callus Formation (N/n,%)	Direct Regeneration (N/n,%)	Indirect Regeneration (N/n,%)
1 Month Selection	21/1; %47,62	21/2; %9,52	10/0; %0,0	169/53; %31,3	169/16; %9,47	53/1; %1,89
5 Month Selection	24/9; %37,5	24/1; %4,17	9/0; %0,0	114/25; %21,9	114/4; %3,51	25/4; %8,0
Selection Period	**ELK345, Leaf Explant**			**ELK345, Petiole Explant**		
	Callus Formation (N/n,%)	Direct Regeneration (N/n,%)	Indirect Regeneration (N/n,%)	Callus Formation (N/n,%)	Direct Regeneration (N/n,%)	Indirect Regeneration (N/n,%)
1 Month Selection	11/2; %18,18	11/0; %0,0	2/0; %0,0	166/26; %15,6	166/5; %3,16	26/2; %7,69
5 Month Selection	11/3; %25,0	11/1; %9,09	3/0; %0,0	129/14; %13,18	129/0; %0,0	17/0; %0,0

Figure 3. 4. Results of selection period analysis in SG-13, SG-6M and ELK345 genotypes. (N: sample number, n: callus and regeneration number, P<0.05=*, P<0.01= **, P<0.001= ***, P<0.0001= ****)

In light of all these data, it was thought that long-term selection pressure would prevent the regeneration of possible transplastomic plants. Therefore, after bombardment number 39, selection pressure was again limited to 50 mg/L spectinomycin antibiotic for 1 month.

A total of **66** independent bombardment experiments were conducted, and bombardment experiments were carried out on a total of **5846** explant sources, including leaves, callus and petioles. Table 3.1 shows the total number of bombardments, the type and number of explant species, and the callus, direct and indirect shoot formation numbers. Under *in vitro* conditions, **2167** calli were formed and **376** direct shoot regeneration and **155** indirect shoot regeneration resulted in a total of **531** plants. Unfortunately, some of these shoots could not form full plants due to vitrification problems. The performance of the SG-DH genotype, which is the main sugar beet genotype of our study, was low under *in vitro* conditions. The SG-DH genotype showed the highest callus formation percentage of **43%** in leaf explants and **8.46%** direct regeneration and **11.18%** direct regeneration in petiole explants (Figure 3.3). When these data are examined, the difficulty of growing sugar beet plants under *in vitro* conditions becomes apparent.

3.6 gDNA Isolation of Regenerated Plants

The gDNA isolation of the regenerated plants was performed according to the manual isolation method described in the material method section. gDNA isolations were performed using the Ankara Turkish Sugar Institute laboratories. No problem was encountered with gDNA isolations.

3.6.1 Confirmation of isolated gDNA samples by gel electrophoresis

The gDNAs isolated from potential transplastomic plants were electrophoresed with a 1% prepared agarose gel to confirm DNA isolation (Photo 3.21). The gel electrophoresis results showed that there was no problem in obtaining gDNA from the plants.

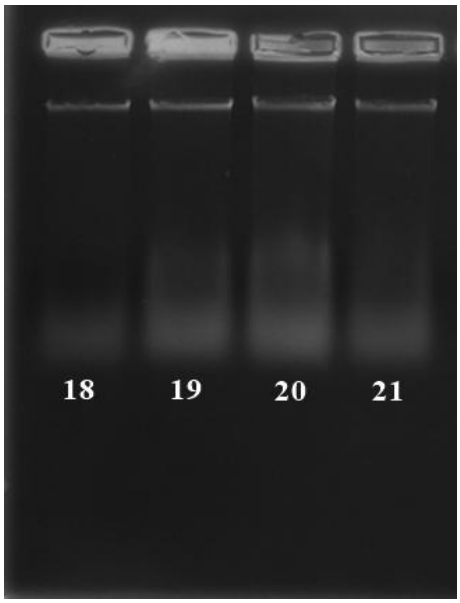


Photo 3. 21. Images of gDNA isolated samples on 1% agarose gel

3.6.2 Determination of purity and concentration of isolated gDNA samples

For all 516 plants from which gDNA was isolated, concentrations and purities were measured using a micro-drop (Thermo Scientific/ Multiskan SkyHigh) device. As a result of the measurements, no problems were encountered in most of the isolated gDNA.

Another purpose of the measurement is to ensure that all gDNA concentrations to be used in PCR experiments are equal. In this way, PCR reaction results became more meaningful. Purity and concentration measurements of 516 samples and the dilution calculation required to adjust to 100 ng are given in Appendix 1.

The pExpPN-phaA-SB vector used in biolistic bombardment was multiplied by growing it in *E. coli* (Dh5alpha) bacterial culture and then isolating the plasmid. The concentration measurement of this plasmid for use in biolistic bombardment was again done by micro-drop (Table 3.3).

Table 3. 6. DNA purity and concentration of pExpPN-phaA-SB vector isolated from *E. coli.*

Plasmid No	DNA Purity	DNA Concentration (ng)
1	2,02	157,1
2	1,997	110,6
3	1,987	195
4	1,998	118,3
5	2,01	102,5
6	1,98	106,6
7	2,01	125,6
8	1,994	137,7
9	1,991	90,3
10	1,998	102,6
11	2,03	98,73

3.7 Confirmation of Chloroplast Integration of pExpPN-phaA-SB vector by PCR

To verify the plastid genome integration of the pExpPN-PhaA-SB vector in putative transformed plants, gDNA samples were isolated and adjusted to 100 ng/µl were used. Different primers were designed (Table 2.1) and these were used in PCR screenings. The binding regions of these designed primers on the vector and the product sizes to be formed as a result of PCR are given in detail in the material method section. The *trnI* full/partial and *trnA* full/partial primers were used to show that the homologous recombination regions were correct both in the sugar beet plant and in our vector. Validation was shown using these primers in 300 plant samples.

With these designed primers, **5453** PCR reactions were performed during the screenings. Examples of agarose gel photographs of PCR reactions performed with these primers are given below.

In Photo 3.22, four gel images are given as examples of the results of PCR reactions performed with the optimized *PhaA* gene primer. As can be seen from the given gel images, the positive control in each PCR reaction set worked successfully (1449 bp), while no band was observed in the negative control as expected. In the PCR done at 53°C using the optimized *phaA* gene primer to identify and confirm putative transplastomic plants, no positive results were found.

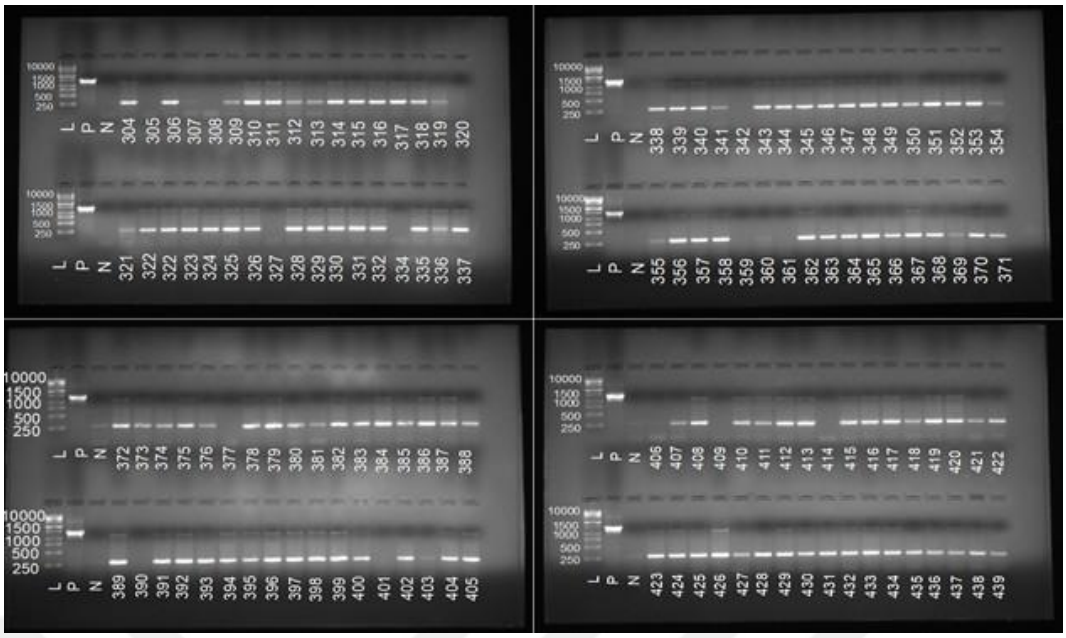


Photo 3. 22. Example gel images of gel electrophoresis analysis of PCR products made with the optimized *PhaA* gene primer (1449 bp)

In Photo 3.23, four gel images are given as examples of PCR reactions performed with the *adaA* full primer. As can be seen from the gel images given, the positive control in each PCR reaction set worked successfully (795 bp), while the expected band was not seen in negative control. To determine and confirm potential transplastomic plants with PCR reactions, no positive results were found in PCR experiments performed at 52˚C with the *adaA* full primer.

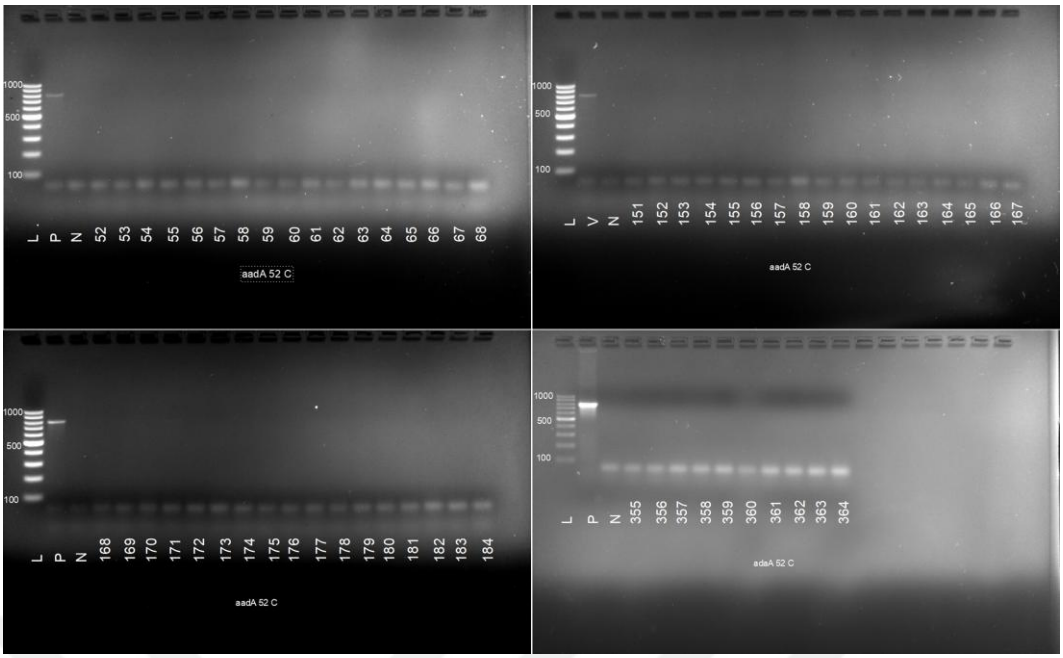


Photo 3. 23. Example gel images of gel electrophoresis analysis of PCR products made with the *adaA* full primer (795 bp)

In Photo 3.24, four gel images are given as examples of PCR reactions performed with the *ampR* full primer. As can be seen from the given gel images, the positive control in each PCR reaction set worked successfully (792 bp), while no band was observed in the negative control as expected. In the PCR experiments performed at 54 ˚C with the *ampR* full primer for the determination and confirmation of putative transplastomic plants with PCR reactions, no result aligned with the positive control was found. **However, bands slightly above the positive control were obtained in some plant samples.** These bands were sent for sequence analysis.

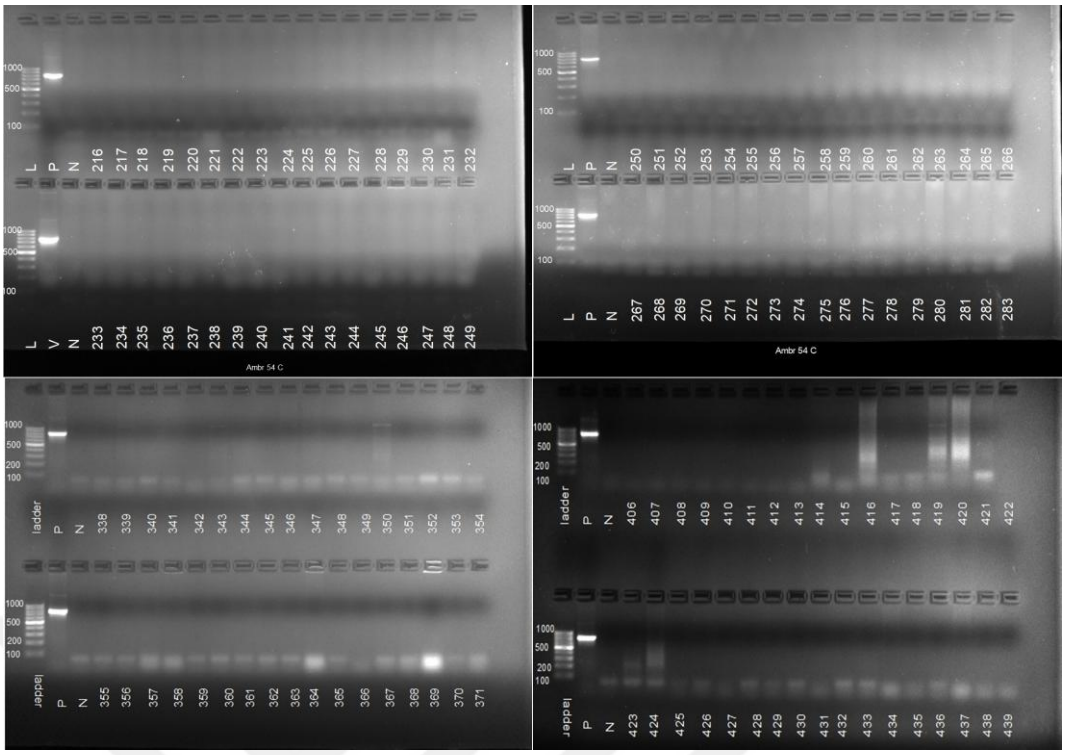


Photo 3. 24. Example gel images of gel electrophoresis analysis of PCR products made with the *ampR* full primer (792 bp)

In Photo 3.25, four gel images are given as examples of PCR reactions performed with the *phaA* full primer. As can be seen from the gel images given, the positive control in each PCR reaction set worked successfully (1179 bp), while no band was observed in the negative control as expected. To identify and confirm putative transplastomic plants with PCR reactions, no positive results were observed in PCR experiments performed at 54 ˚C with the *phaA* full primer.

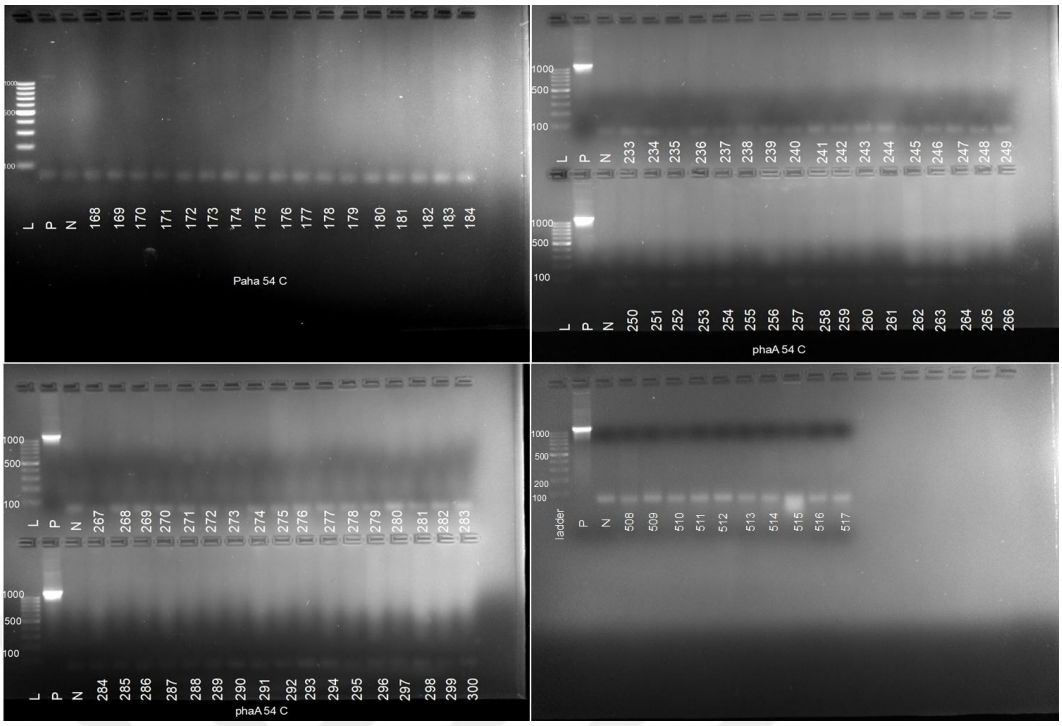


Photo 3. 25. Example gel images of gel electrophoresis analysis of PCR products made with the *phaA* full primer (1179 bp)

In Photo 3.26, four gel images are given as examples of PCR reactions performed with the *trnI* full primer. As can be seen from the given gel images, the positive control in each PCR reaction set worked successfully (626 bp), while no band was observed in the negative control as expected. To identify and confirm putative transplastomic plants with PCR reactions, **positive results were obtained in PCR experiments performed at 54 ˚C with the *trnI* full primer**.

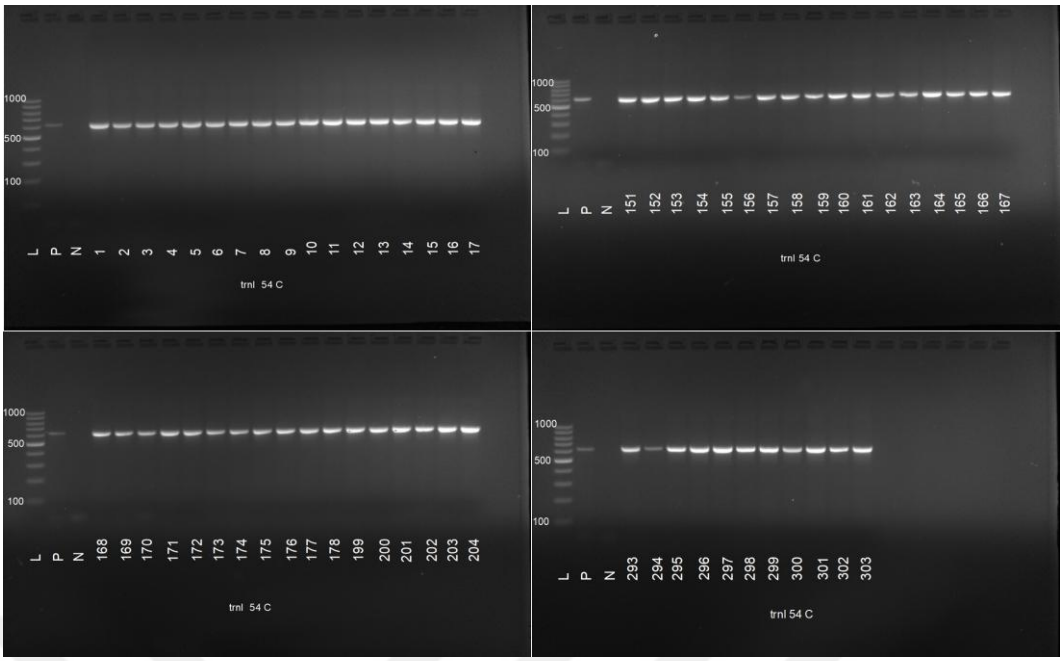


Photo 3. 26. Example gel images of gel electrophoresis analysis of PCR products made with *trnI* full primer (626 bp)

In Photo 3.27, four gel images are given as examples of PCR reactions performed with the *trnA* full primer. As can be seen from the given gel images, the positive control in each PCR reaction set worked successfully (908 bp), while no band was observed in the negative control as expected. **Positive results were obtained in PCR experiments performed at 57 ˚C with the *trnA* full primer**.

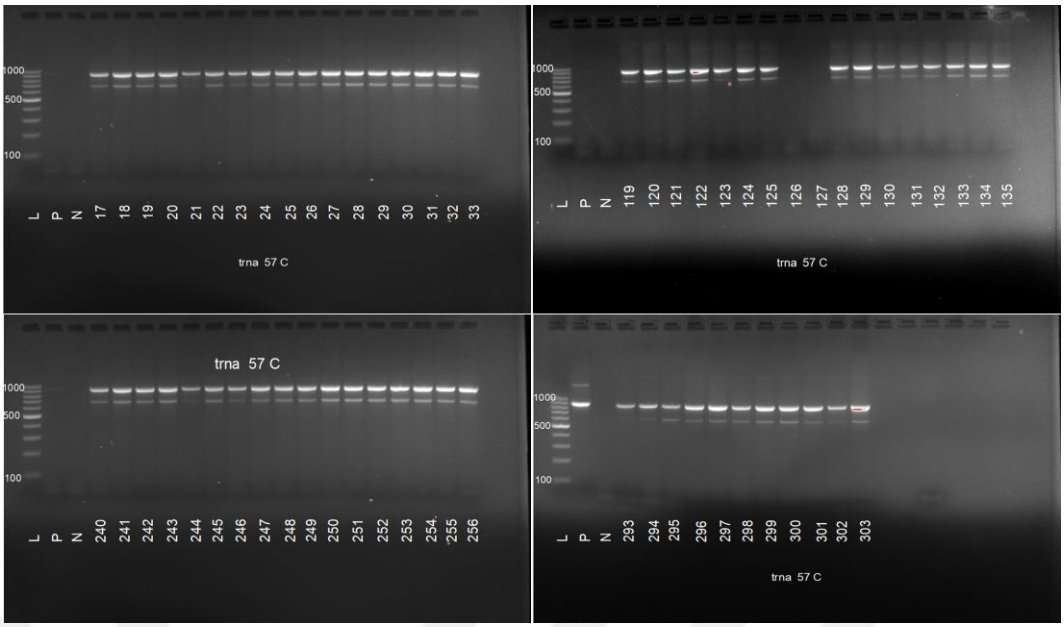


Photo 3. 27. Example gel images of gel electrophoresis analysis of PCR products made with the *trnA* full primer (908 bp)

In Photo 3.28, two gel images are given as examples of PCR reactions performed with *trnI* partial primer. As can be seen from the given gel images, the positive control in each PCR reaction set worked successfully (419 bp) and no band was observed in the negative control as expected **Positive results were obtained in PCR experiments performed with *trnI* partial primer at 54 ˚C**.

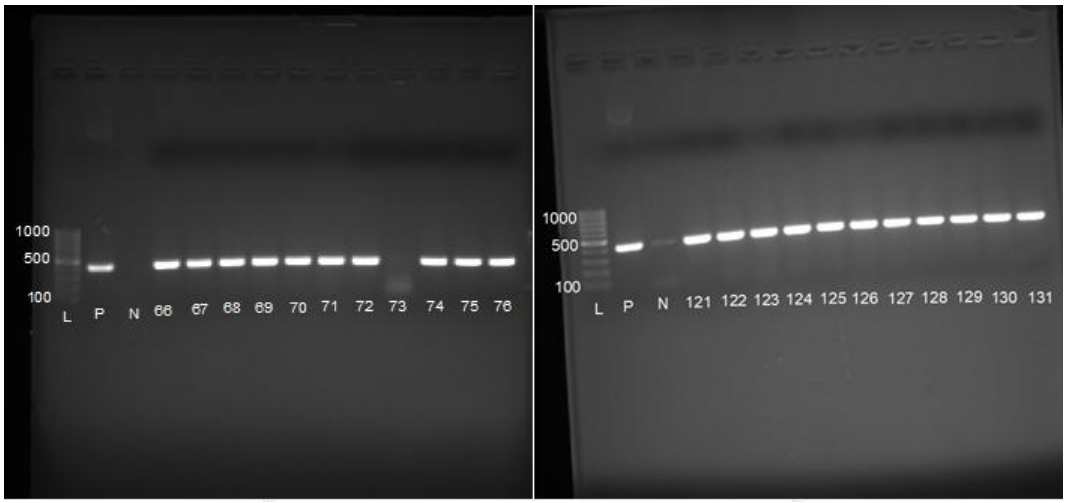


Photo 3. 28. Example gel image of gel electrophoresis analysis of PCR products made with *trnI* partial primer (491 bp)

In Photo 3.29, two gel images are given as examples of PCR reactions performed with the *trnA* partial primer. As can be seen from the given gel images, the positive control in each PCR reaction set worked successfully (518 bp), while no band was observed in the negative control as expected. Positive results were obtained in PCR experiments performed at 55 ˚C with the *trnA* partial primer.

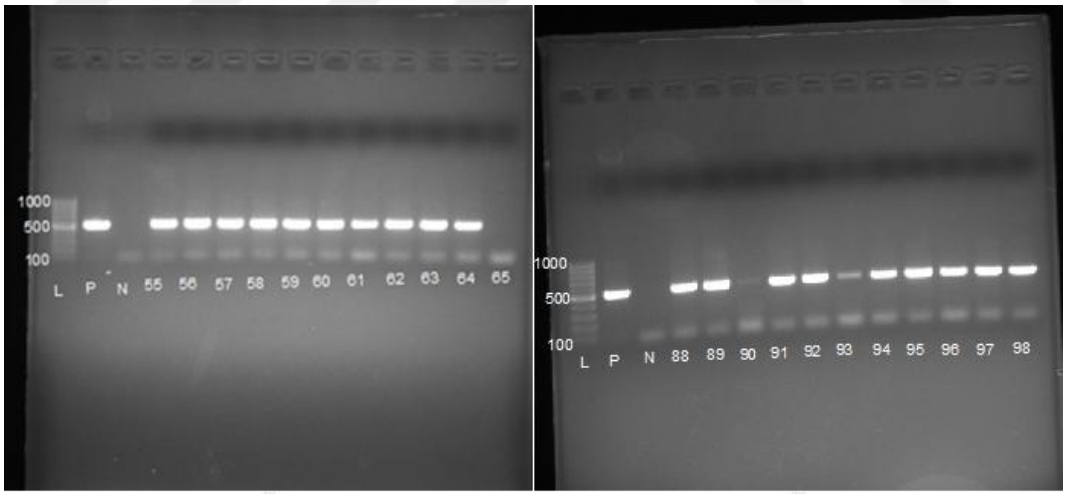


Photo 3. 29. Example gel photographs of gel electrophoresis analysis of PCR products made with *trnA* partial primer (518 bp)

As is known, gene transfer by chloroplast transformation studies is achieved through the presence of homolog recombination regions. As seen in the gel photographs between Photos 3.26 and 3.29, positive bands were observed in the scanned sugar beet plants with *trnI* and *trnA* full and partial primers. These results

show that the *trnI* and *trnA* regions found in sugar beet are compatible with the *trnI* and *trnA* regions of the pExpPN-PhaA-SB vector used for chloroplast gene transfer.

Photo 3.30 shows four gel images as examples of PCR reactions performed with the *phaA* partial primer. As can be seen from the gel images, the positive control in each PCR reaction set worked successfully (381 bp), while no band was observed in the negative control as expected. To identify and confirm potential transplastomic plants with PCR reactions, **positive bands were obtained in some plants in PCR experiments performed at 50 ˚C with *phaA* partial**. These bands were sent for sequence analysis.

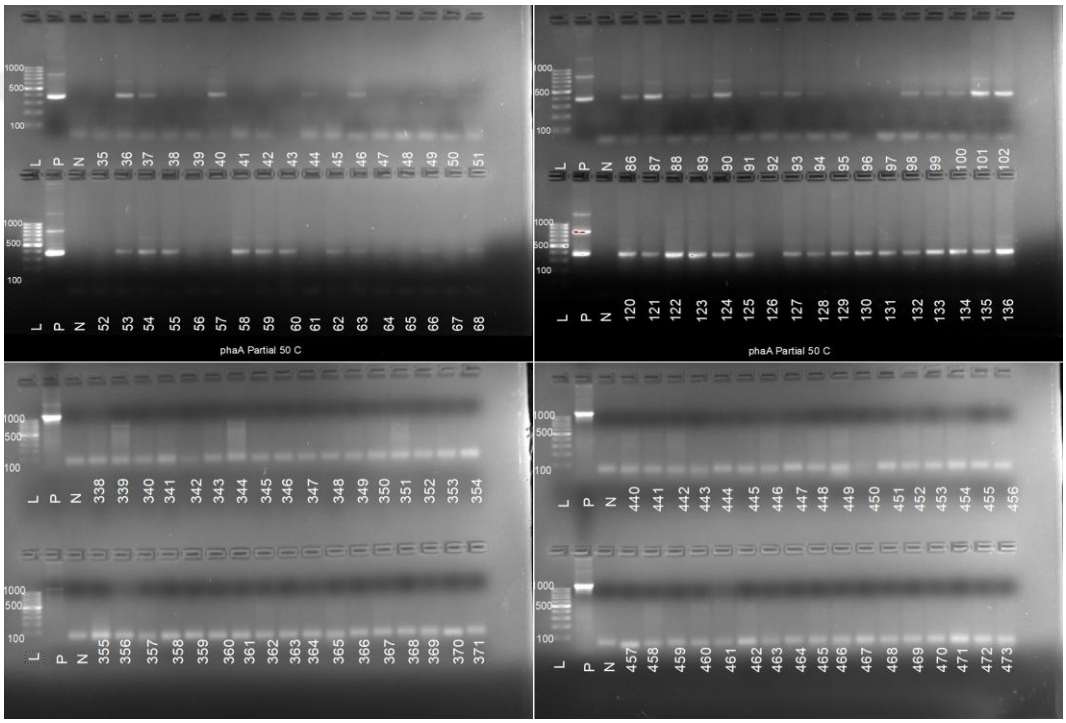


Photo 3. 30. Example gel images of gel electrophoresis analysis of PCR products made with the *phaA* partial primer (381 bp)

Photo 3.31 shows four gel images as examples of PCR reactions performed with the *ampR* partial primer. As can be seen from the gel images, the positive control in each PCR reaction set worked successfully (210 bp), but no bands were observed in the negative control as expected. In order to identify and confirm potential transplastomic plants with PCR reactions, **positive bands were obtained in some plants in PCR experiments performed with *ampR* partial at 56 ˚C**.

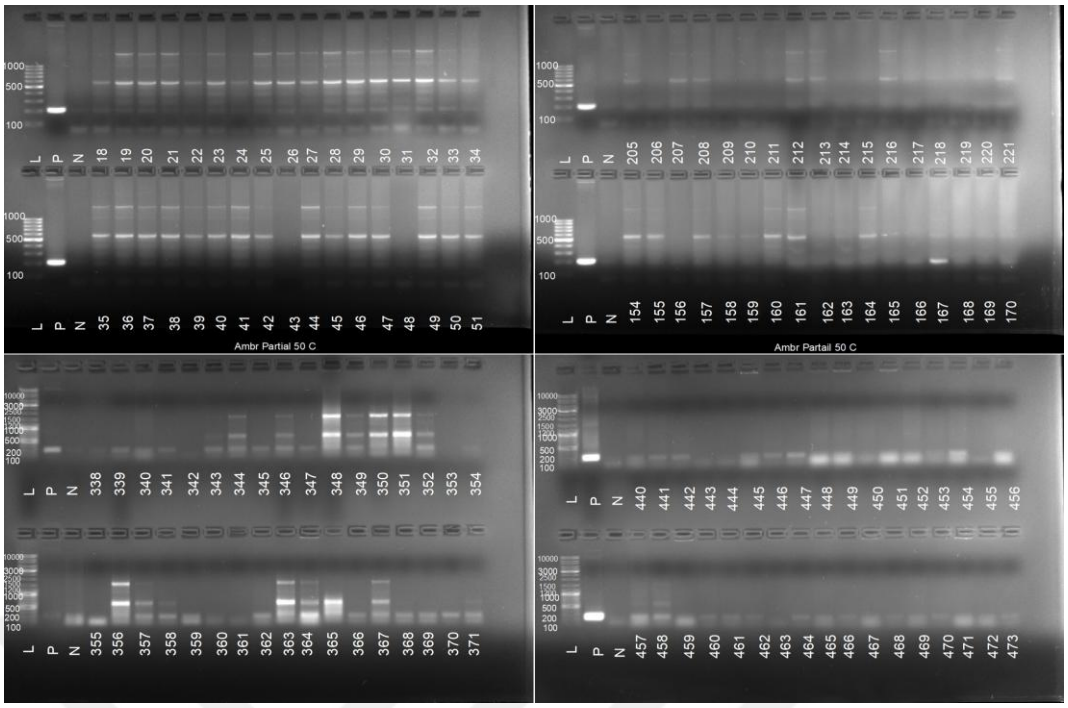


Photo 3. 31. Example gel images of gel electrophoresis analysis of PCR products made with the *ampR* partial primer (210 bp)

In Photo 3.32, four gel images are given as examples of PCR reactions performed with the *adaA* partial primer. As can be seen from the gel images, the positive control in each PCR reaction set worked successfully (341 bp), while no band was observed in the negative control as expected. To identify and confirm potential transplastomic plants with PCR reactions, **positive bands were obtained in some plants in PCR experiments performed with *adaA* partial at 50 ˚C**. These bands were sent for sequence analysis.

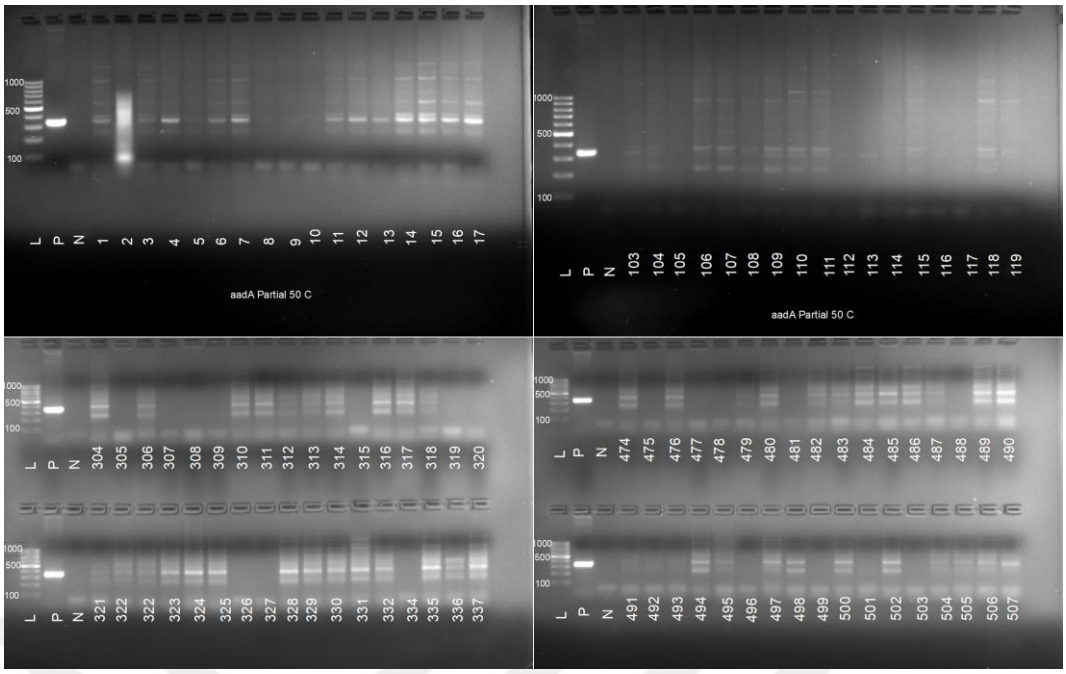


Photo 3. 32. Example gel photographs of gel electrophoresis analysis of PCR products made with the *adaA* partial primer (341 bp)

A total of **5846** explants were used in the **66** independent biolistic bombardment experiments. Despite all these disruptions in plant growth, PCR screening was performed on **516** plants showing healthy shoot regeneration with 11 primers. In these screenings, approximately **5453** PCR products were visualized on the gel with positive and negative controls. In the screenings, it was observed that optimized *phaA*, *adaA* full and *phaA* full primers did not give any positive bands. However, positive bands were obtained from *trnI* full/partial and *trnA* full/partial and partial primers as mentioned above.

As a result of PCR studies performed with primers containing a part of the designed *adaA*, *phaA* and *ampR* elements (partial primers), bands of the desired size were obtained in some of the putative transplastomic sugar beet samples. Bands were seen in some plants screened with *ampR* full primers, but these bands remained above the positive control. These positive bands were subjected to a repeat PCR reaction and gel electrophoresis, and specific bands were isolated from the gel and sent to SentebioLab and Intergen for sequence analysis. Bioinformatics analyses were performed using the obtained sequence results.

3.7.1 Sequence analysis of samples giving PCR positive

It has been stated that approximately 14 months is needed for shoot regeneration in biolistic bombarded plants. Indirect shoots regeneration takes longer than direct regeneration. Therefore, the different growth periods of the plants caused a certain period between the sequence experiments.

In the first stage, PCR products of PCR positive plants were isolated as described in the material method section and sent to SentebioLab. SentebioLab used the Sanger sequencing method in the samples. Sequence analyses and bioinformatic analyses are explained in detail below.

In PCR experiments performed with *phaA* partial primer, gDNA number 69, 154 and 201 samples were selected and re-studied. Samples were analyzed in 1% agarose gel containing RedSafe (INtRON) and the desired bands were extracted from the gel under UV light. The extracted bands are shown in Photo 3.33.

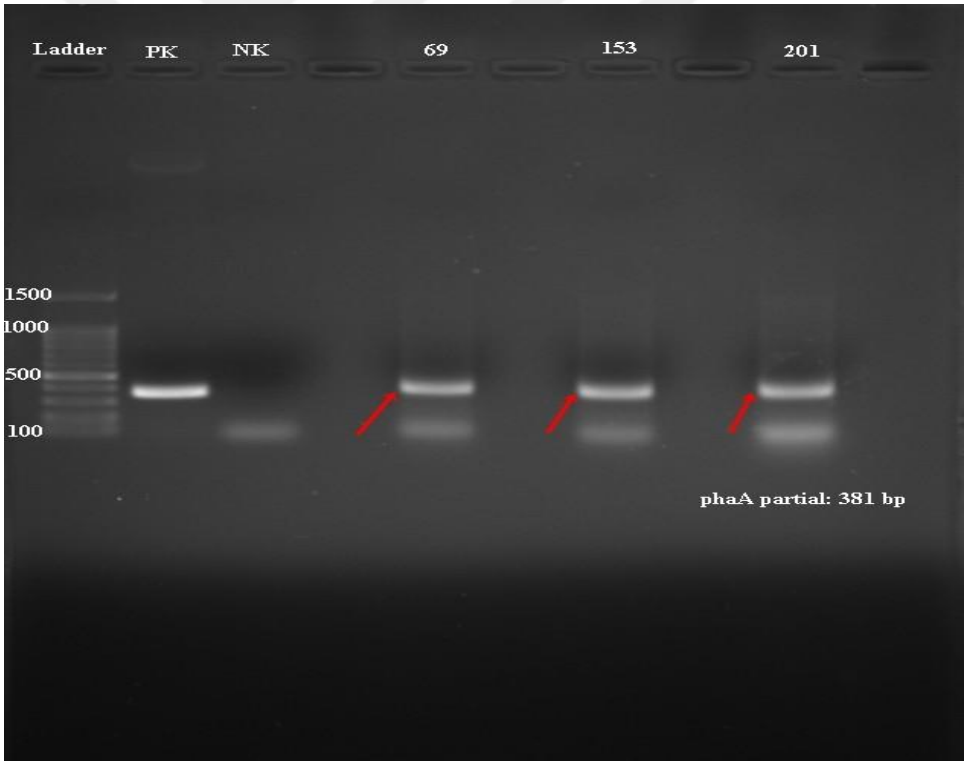


Photo 3. 33. Gel images of plants giving PCR positive bands with the *phaA* partial primer

Bioinformatics analyses of the sequences resulting from Sanger sequencing were performed using Geneious Prime, SnapGene Viewer, NCBI/BLAST, and Clustal Omega. First, alignment of forward and reverse reads of the sequences was

performed using Clustal Omega (Figure 3.5). In the second step, blast analysis of the sequences was performed using NCBI (Figure 3.6). In the last stage, the sequences obtained were matched with the pExpPN-phaA-SB vector using the Geneious prime program (Figure 3.7). In the bioinformatics analysis, it was understood that the nucleotide sequences of the bands obtained matched the sugar beet genome. Therefore, it was observed that these plants were not transplastomic sugar beet plants.

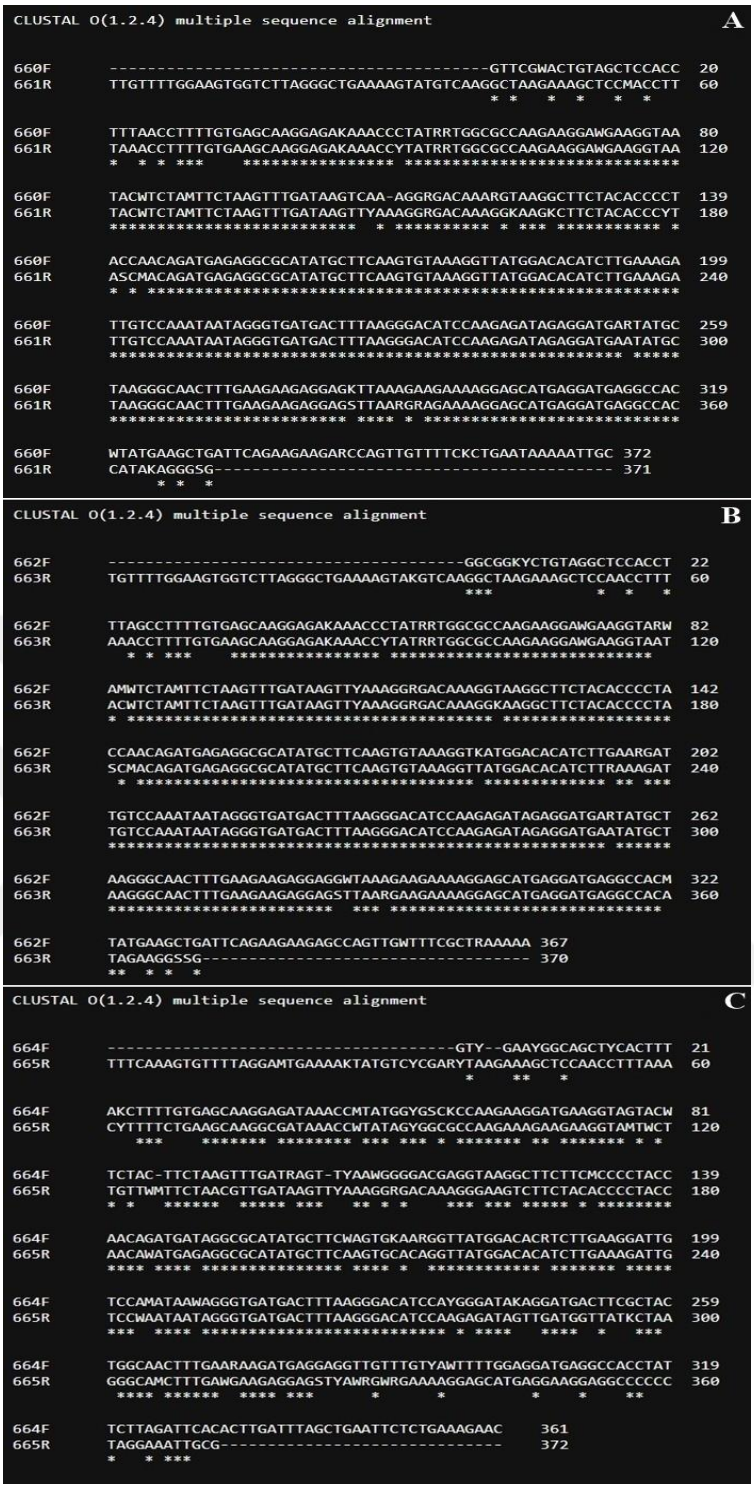


Figure 3. 5. Gene region sequence amplified with the *phaA* partial primer as a result of forward and reverse primer alignment of the sequences obtained as a result of Sanger sequencing of plant samples. **A:** Plant sample number 69 **B:** Plant sample number 153 **C:** Plant sample number 201

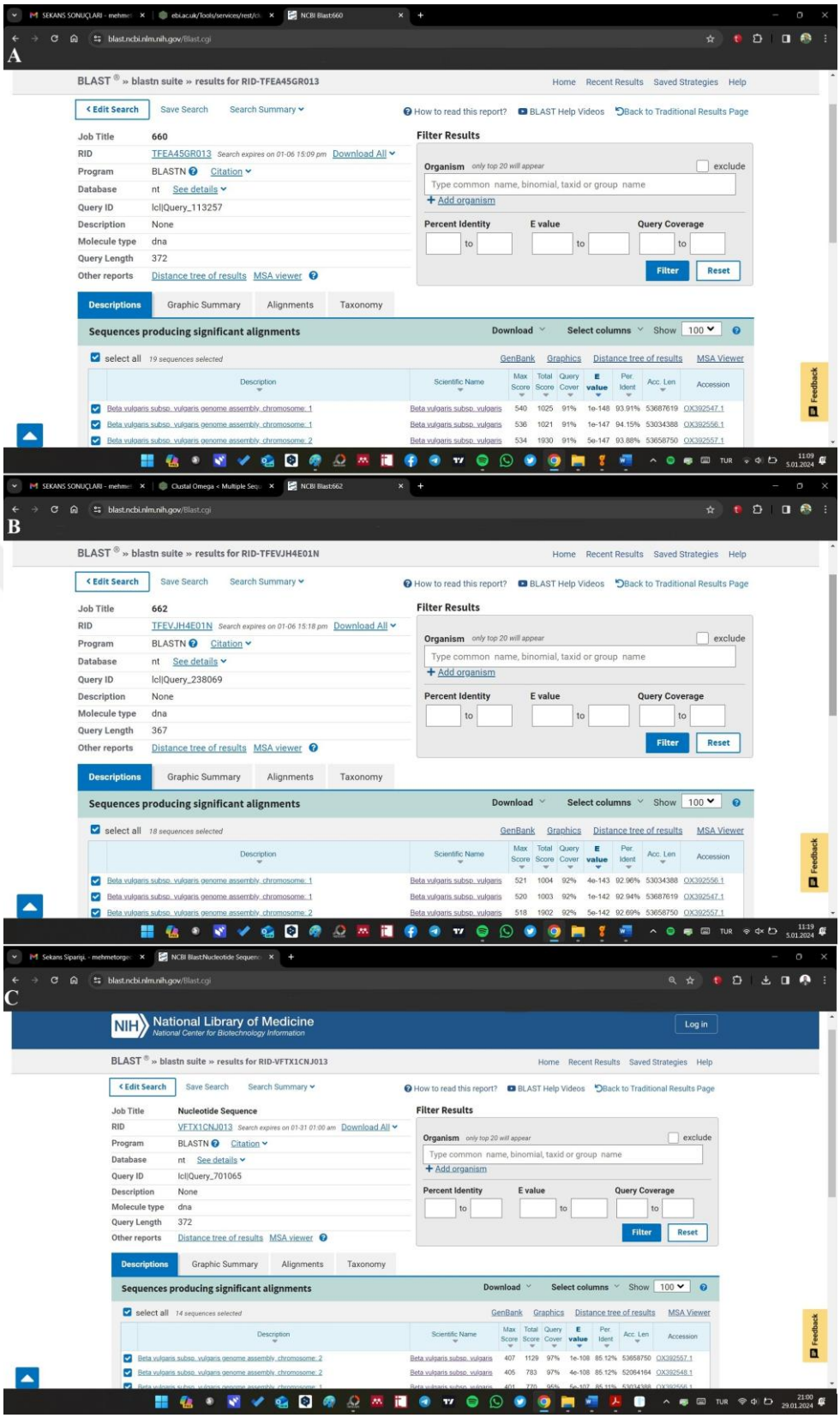


Figure 3. 6. NCBI/BLAST analysis results of plant samples. **A:** Plant sample number 69 **B:** Plant sample number 153 **C:** Plant sample number 201

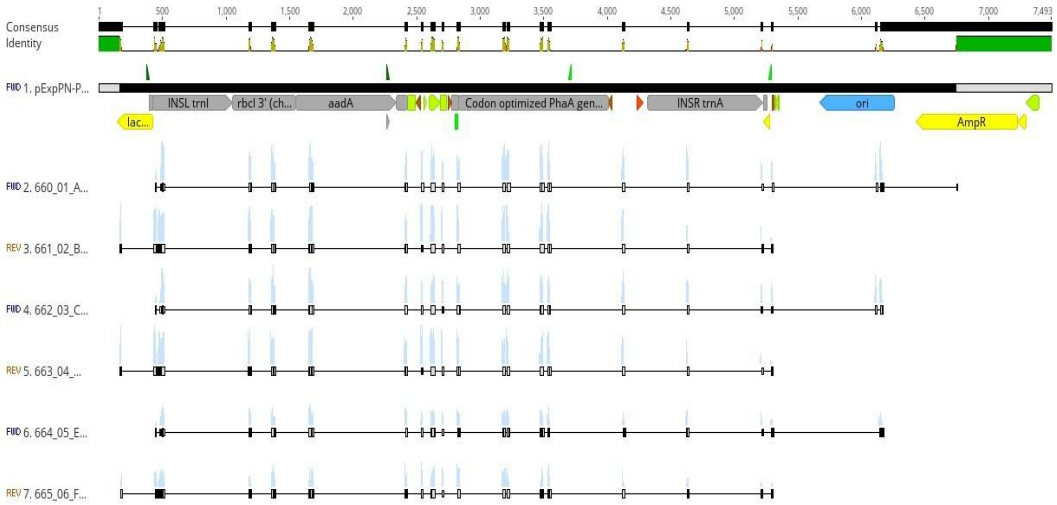


Figure 3. 7. Matching the sequences obtained as a result of Sanger sequencing of plant samples with the pExpPN-phaA-SB vector using the Geneious prime program. (660-661: plant number 69, 662-663: plant number 153 and 664-665: plant number 201)

In PCR experiments performed with *adaA* partial primer, gDNA number samples 33, 157, 165, 167 and 168 were selected and re-studied. Samples 33, 157 and 167 were selected and isolated from the gel to be sent to the sequencing company (Photo 3.34).

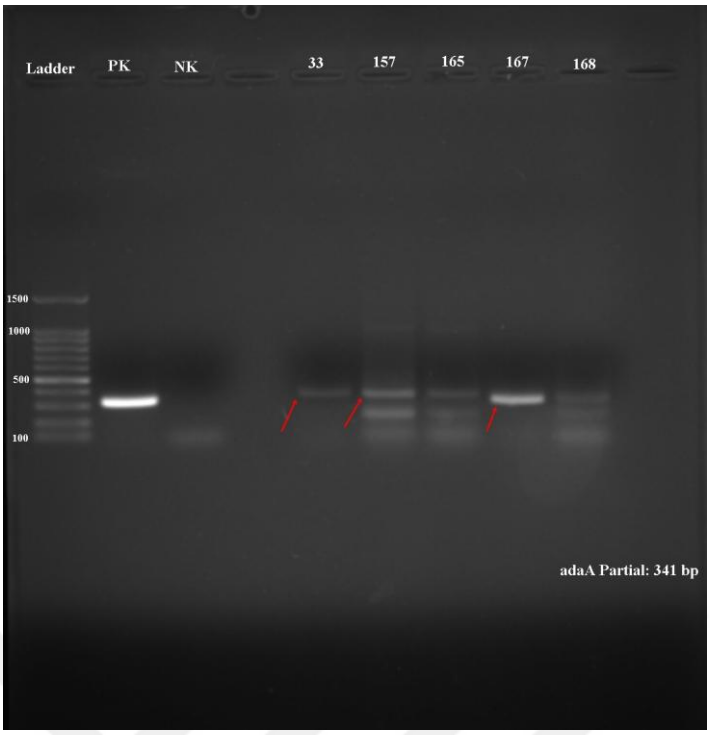


Photo 3. 34. Gel images of plants giving PCR positive bands with the *adaA* partial primer

Bioinformatics analyses of the sequences resulting from Sanger sequencing were performed using Geneious Prime, SnapGene Viewer, NCBI/BLAST, and Clustal Omega. First, the alignment of forward and reverse reads of the sequences was performed using Clustal Omega (Figure 3.8). In the second step, a blast analysis of the sequences was performed using NCBI (Figure 3.9). In the last stage, the sequences obtained were matched with the pExpPN-phaA-SB vector using the Geneious prime program (Figure 3.10). In the bioinformatics analysis of samples 33 and 157, it was found that the nucleotide sequences of the bands matched the sugar beet genome. Therefore, these plants are not transplastomic sugar beet plants. **As a result of Sanger sequencing analysis performed with both forward and reverse primers on samples from plant sample number 167, it was observed that it contained a part of the *adaA* gene region that is not found in the sugar beet genome** (Figures 3.9-3.10).

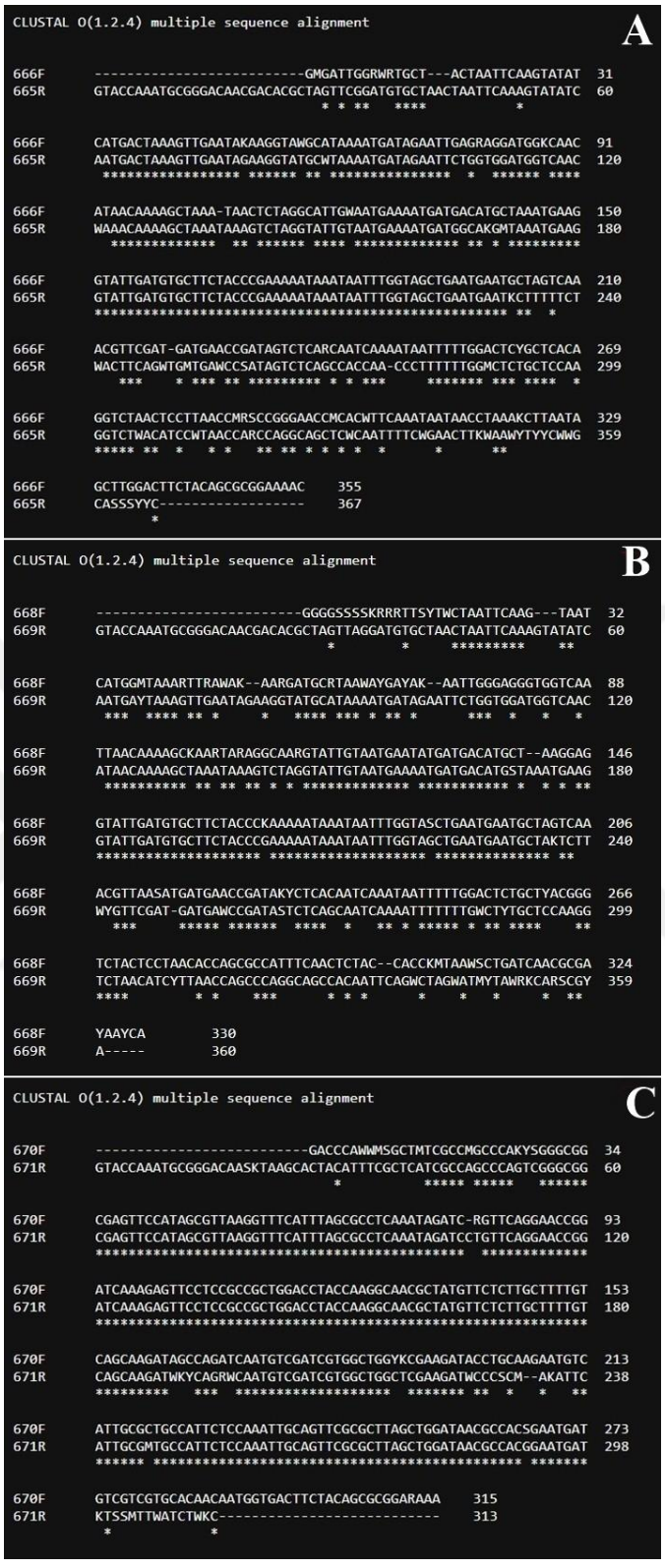


Figure 3. 8. Gene region sequence amplified with the *adaA* partial primer as a result of forward and reverse primer alignment of the sequences obtained as a result of Sanger sequencing of plant samples. **A:** Plant sample number 33 **B:** Plant sample number 157 **C:** Plant sample number 167

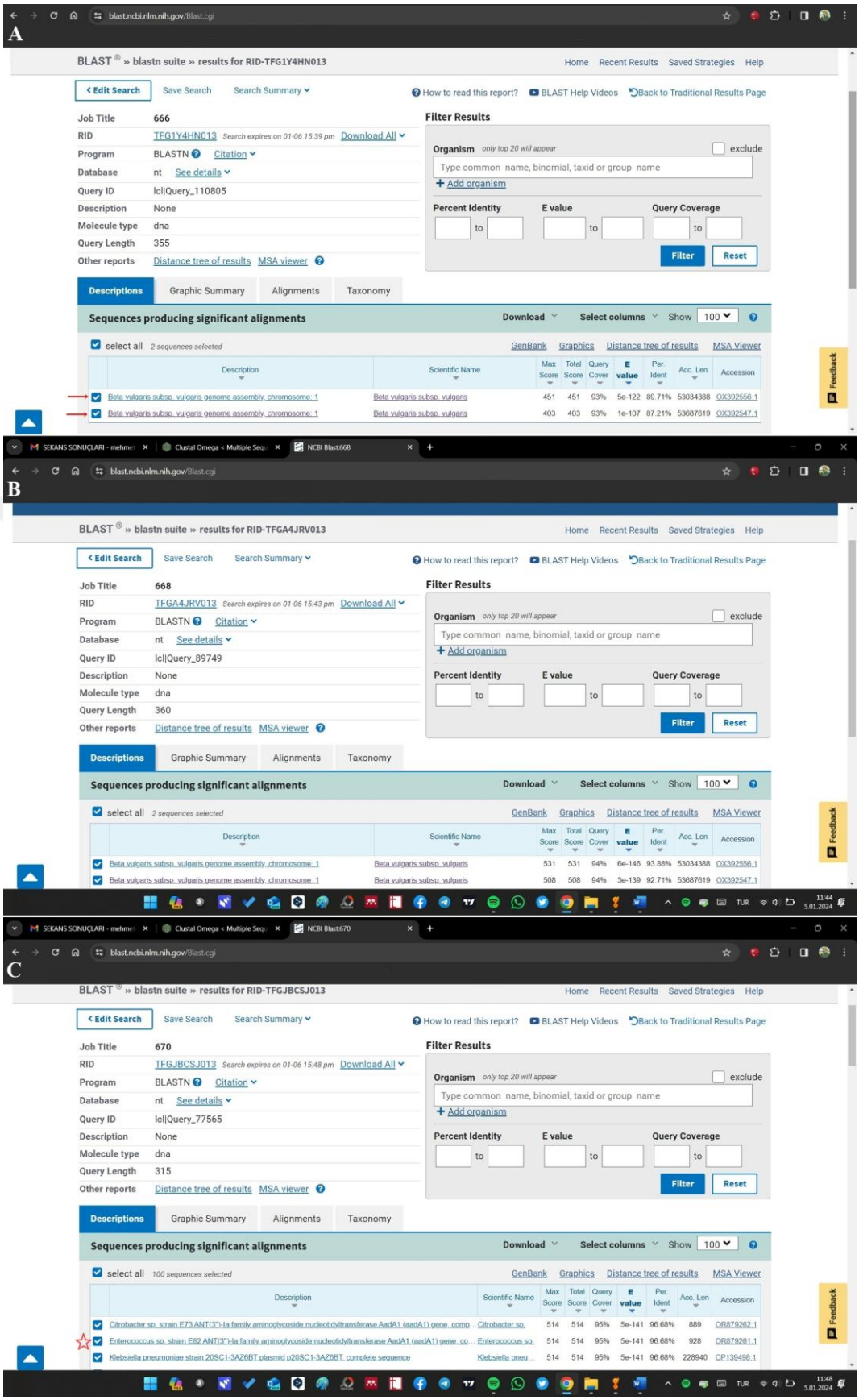


Figure 3. 9. NCBI/BLAST analysis results of plant samples. **A:** Plant sample number 33 **B:** Plant sample number 157 **C:** Plant sample number 167

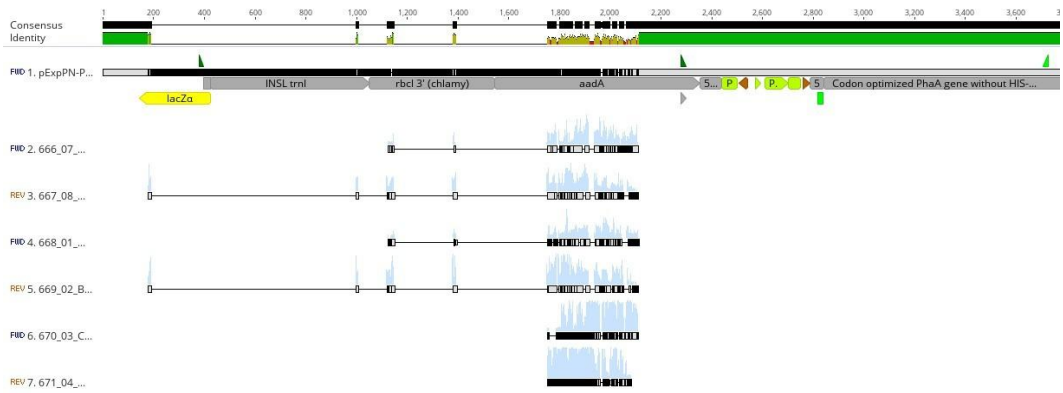


Figure 3. 10. Matching the sequences obtained as a result of Sanger sequencing of plant samples with the pExpPN-phaA-SB vector using the Geneious prime program. (666-667: plant number 33, 668-669: plant number 157 and 670-671: plant number 167)

In PCR experiments performed with the *ampR* partial primer, only 167 samples from the second set of gDNA isolation were selected, re-studied, and isolated from the gel to be sent to the sequencing company (Photo 3.35).

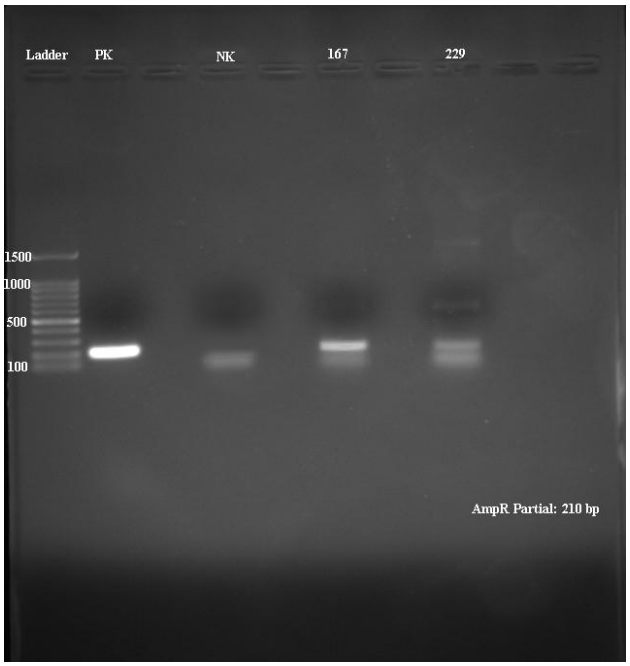


Photo 3. 35. Gel images of plants giving PCR positive bands with the *ampR* partial primer

Bioinformatics analyses of the sequences resulting from Sanger sequencing were performed using Geneious Prime, SnapGene Viewer, NCBI/BLAST, and Clustal Omega. First, the alignment of forward and reverse reads of the sequences was performed using Clustal Omega (Figure 3.11). In the second step, a blast analysis of the sequences was performed using NCBI (Figure 3.12). In the last stage, the sequences obtained were matched with the pExpPN-phaA-SB vector using the Geneious prime program (Figure 3.13). As a result of Sanger sequencing analysis performed with both forward and reverse primers on samples from plant sample number 167, it was observed that it contained a part of the *ampR* gene region that is not found in the sugar beet genome (Figures 3.12-3.13).

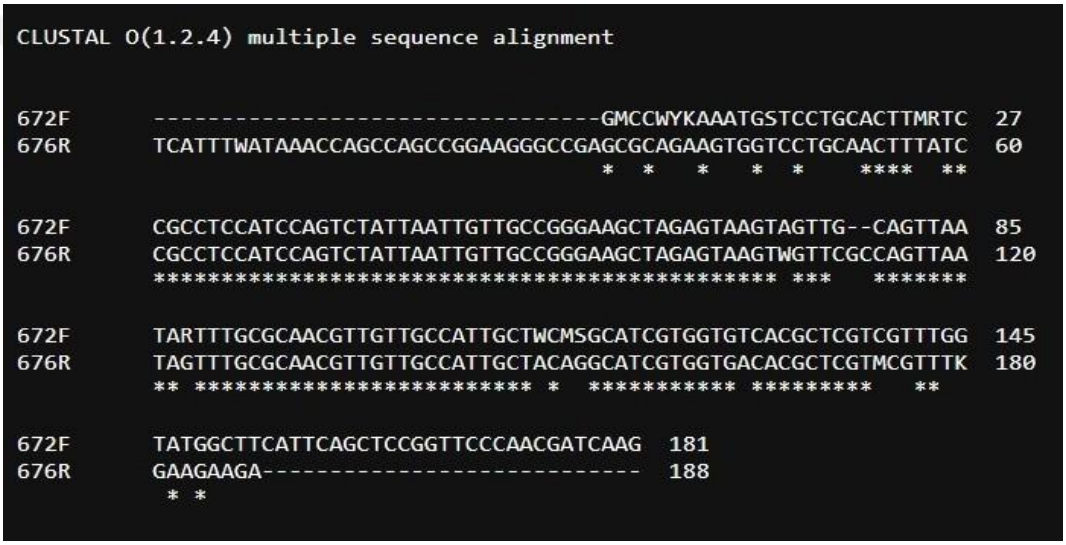


Figure 3. 11. Sequence of the *ampR* partial gene as a result of forward and reverse primer alignment of the sequences obtained by Sanger sequencing of plant sample number 167

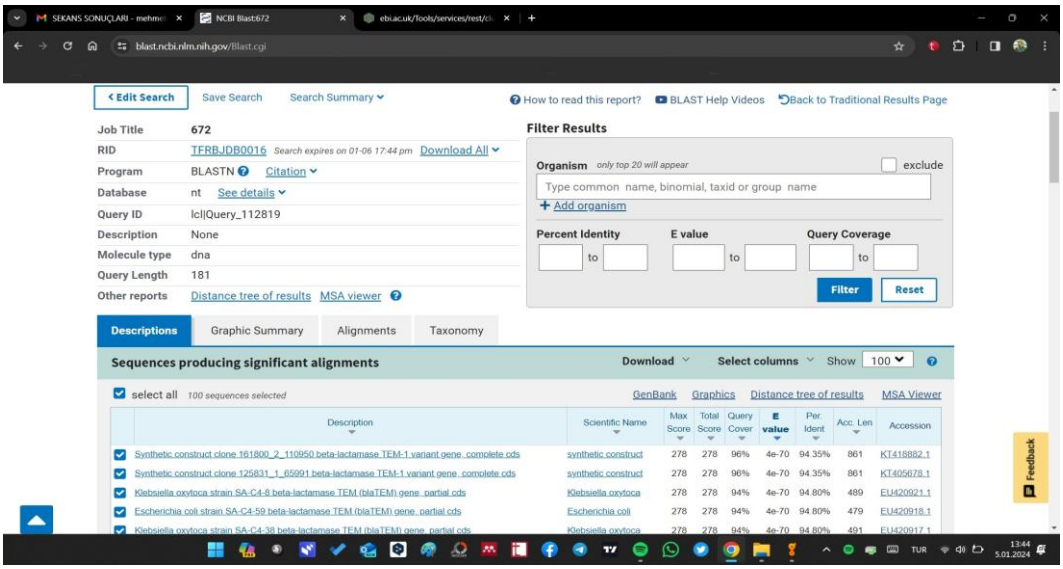


Figure 3. 12. NCBI/BLAST analysis results of plant sample 167.

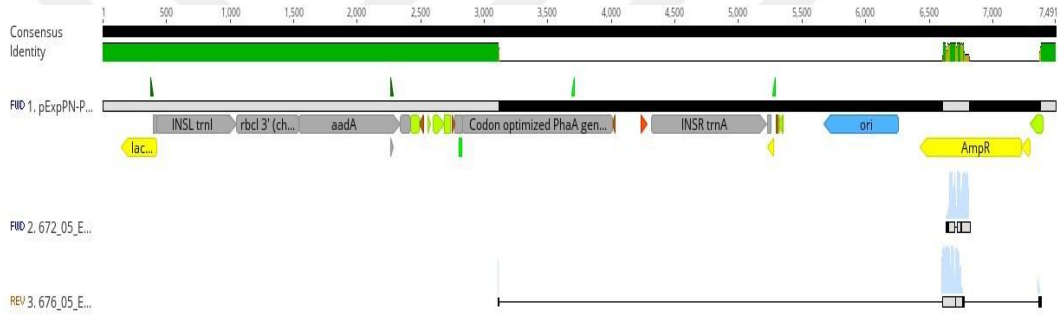


Figure 3. 13. Mapping of sequences of plant sample 167 to the pExpPN-phaA-SB vector using the Geneious prime program (672-676: plant number 167).

As a result of the analysis performed on plant sample number 167, it was revealed that some of the *adaA* and *ampR* genes in the pExpPN-phaA-SB vector were transferred to the sugar beet plant by the biolistic bombardment method. Plant number 167 was obtained by direct shoot regeneration from the petiole explant used in the 11th biolistic bombardment experiment with the SG-DH genotype. Plant number 167 was observed to grow very slowly *in vitro*. Side branches were observed to turn yellow and die much more quickly than control plants. Therefore, the side branches that were understood to have started to turn yellow were cut and stored at -80 °C for qRT-PCR and western blot experiments. Plant number 167 was divided into three (Photo 3.49).

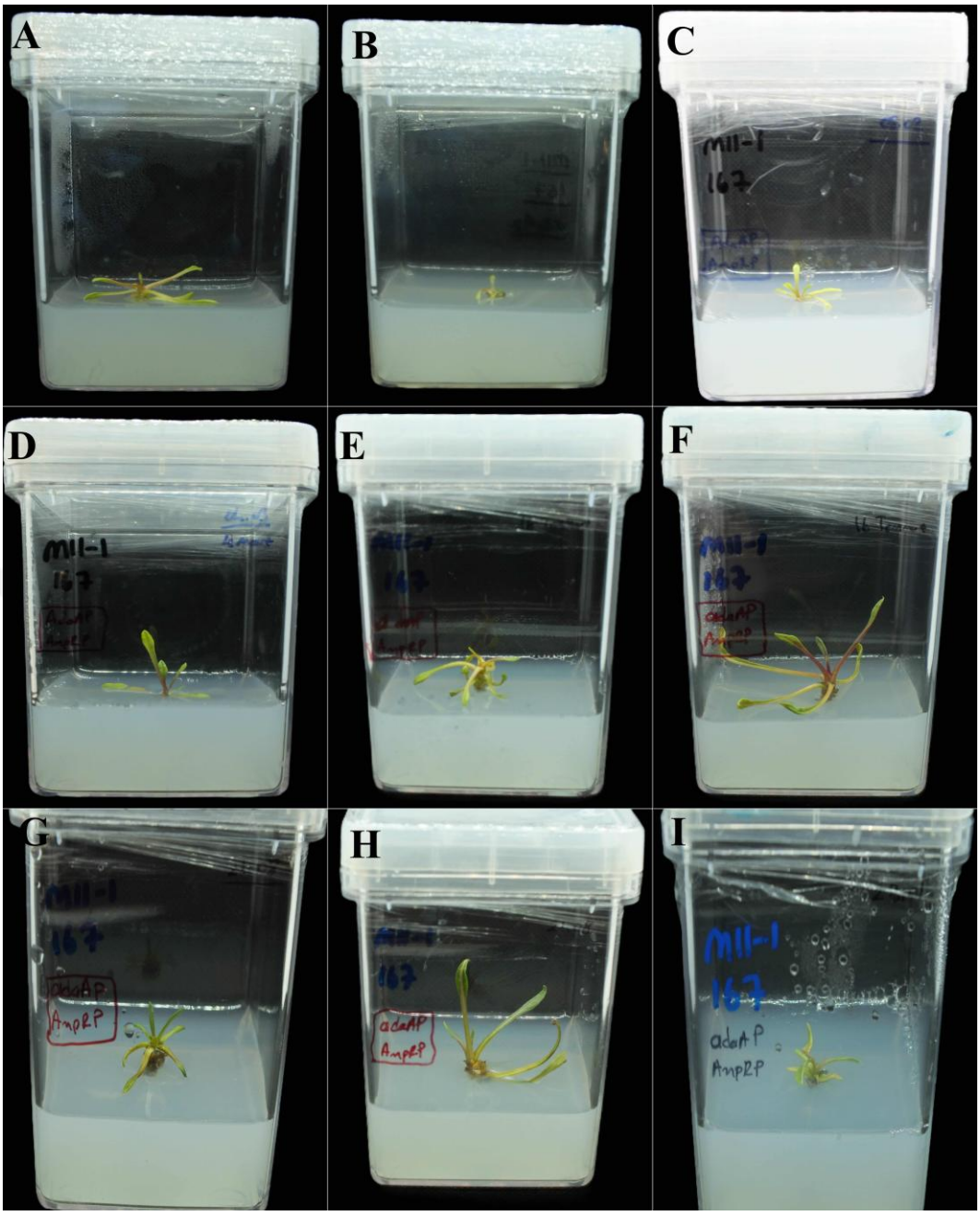


Photo 3. 36. *In vitro* development of plant number 167. **A-B:** 11th month **C:** 13th month **D:** 14th month **E-F:** 18th month **G-H:** 20th month and **I:** 25th month in plant growth medium

In the second stage of the experiments, the FavorPrep™ GEL/PCR Purification kit was used for the isolation of PCR products from PCR positive plants obtained from newly growing plants. The isolated bands were sent to Intergen company for sequencing. Intergen used the Spike-in De Novo sequencing method for the sequence analysis of the samples. The analyses of the sequences sent to Intergen are explained in detail below.

In PCR experiments performed with the *ampR* full primer, samples numbered 329, 332, 350, and 419 were selected and re-run. The samples were run on a 2% agarose gel containing RedSafe (INtRON) and the desired bands were extracted from the gel under UV light. The extracted bands are shown in Photo 3.37. The bands here are seen more clearly when viewed with the naked eye.

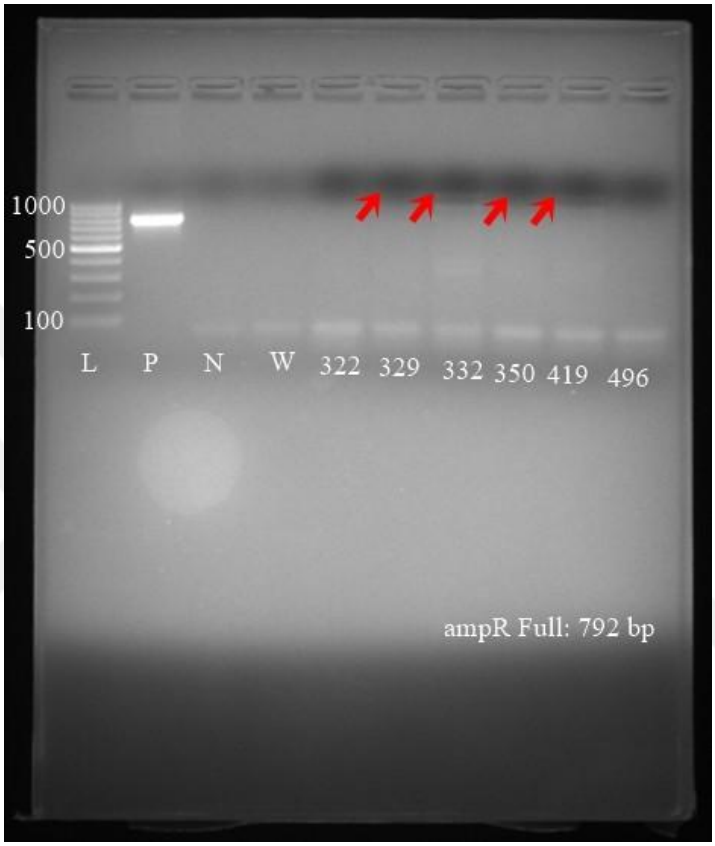


Photo 3. 37. Gel images of plants had PCR positive bands with *ampR* full primer. (L: Ladder, P: Positive control, N: Negative control and W: Wild type DNA)

Intergen company sent the sequence results for the samples screened with the *ampR* full primer in BAM file format. The relevant file was analyzed with the Integrative Genomics Viewer program. The obtained sequence results showed no similarity with the pExpPN-phaA-SB vector (Figure 3.14). Therefore, it was determined that plants numbered 329, 332, 350 and 419 were not transplastomic plants because they did not contain any sequence from the pExpPN-phaA-SB vector.

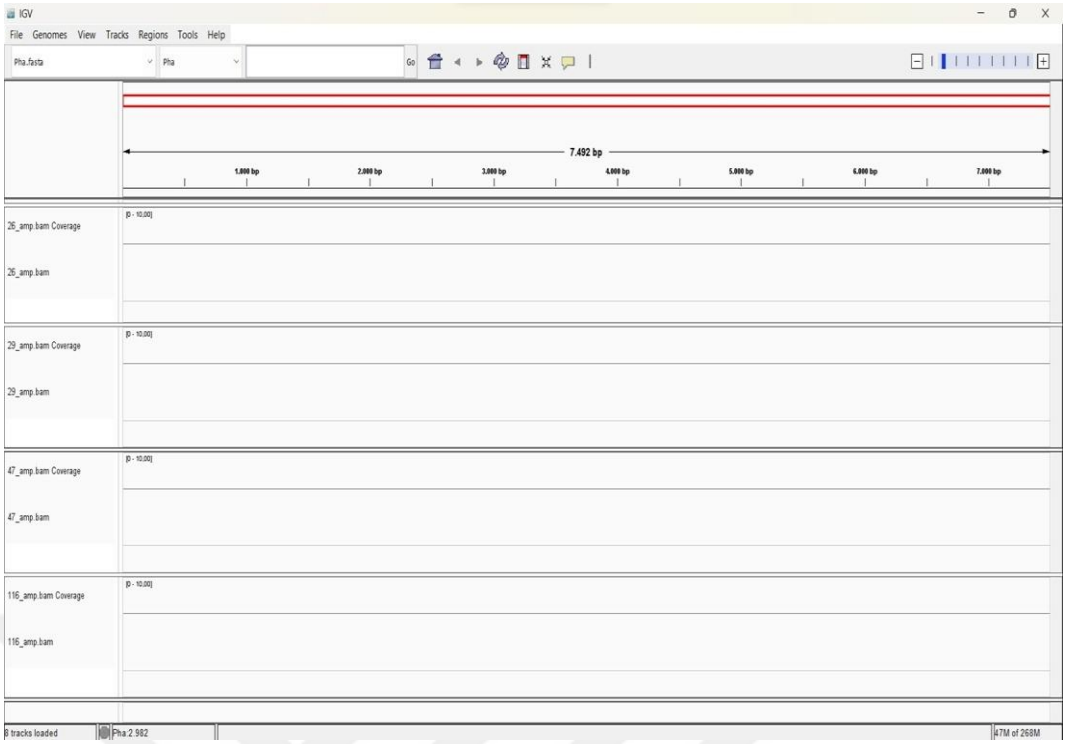


Figure 3. 14. Matching the sequences obtained from the *ampR* full primer to the pExpPN-phaA-SB vector using the Integrative Genomics Viewer program

In PCR experiments performed with the *phaA* partial primer, samples numbered 339, 370 and 405 were selected and re-run. The samples were run on a 2% agarose gel containing RedSafe and the desired bands were extracted from the gel under UV light. The extracted bands are shown in Photo 3.38.

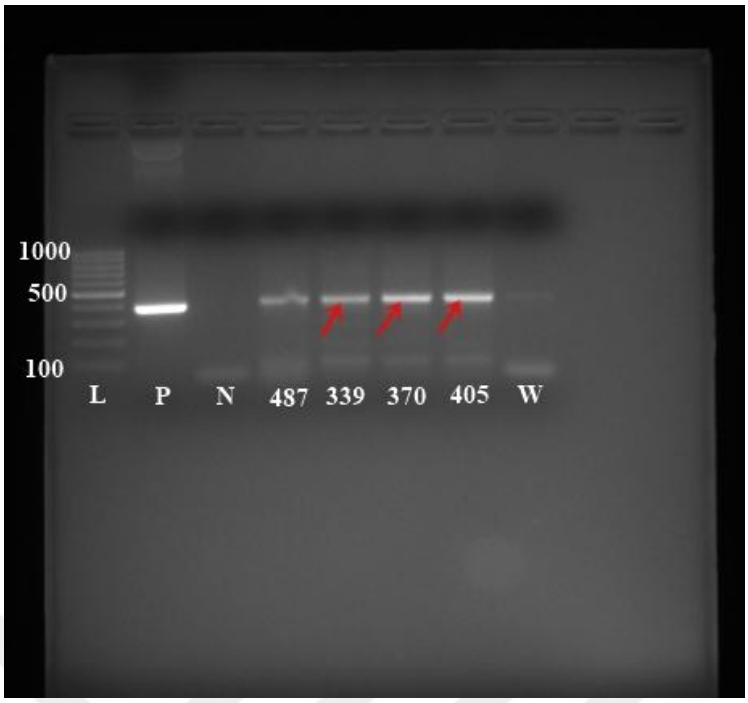


Photo 3. 38. Gel images of plants giving PCR positive bands with *phaA* partial primer. (L: Ladder, P: Positive control, N: Negative control and W: Wild type DNA)

Intergen company sent the sequence results for the samples screened with the *phaA* partial primer in BAM file format. The relevant file was analyzed with the Integrative Genomics Viewer program. The obtained sequence results showed no similarity with the pExpPN-phaA-SB vector (Figure 3.15). Therefore, it was determined that plants numbered 339, 370, 405 and 487 were not transplastomic plants because they did not contain any sequence from the pExpPN-phaA-SB vector.

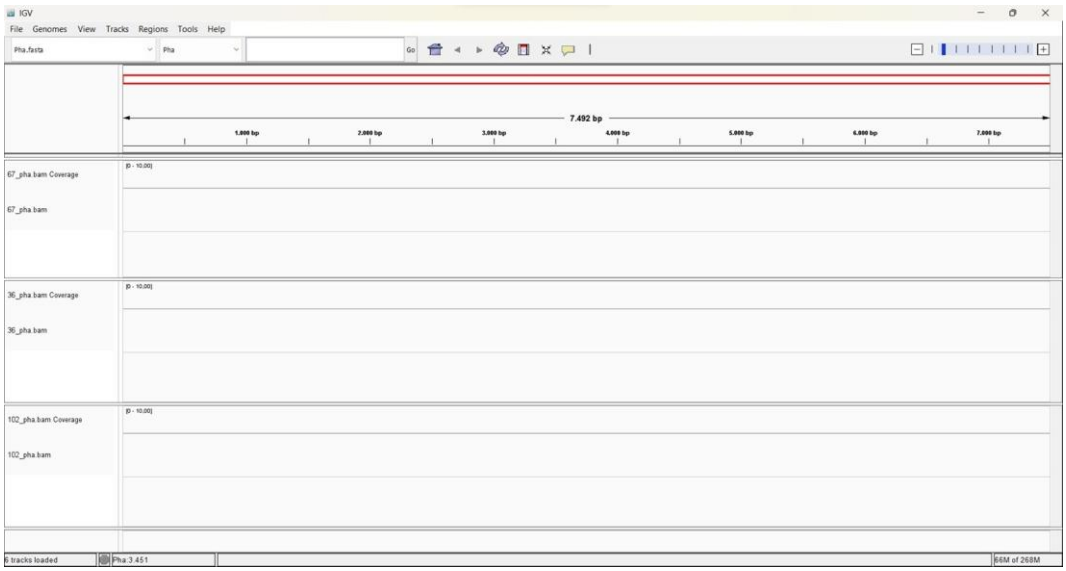


Figure 3. 15. Matching the sequences obtained from the *phaA* partial primer to the pExpPN-phaA-SB vector using the Integrative Genomics Viewer program

In PCR experiments performed with *adaA* partial primer, samples numbered 324, 335, 337, 351, 356, 354, 362, 391, 393, 394, 405, 428, 452, 460, 470 and 506 were selected and re-run. Samples were run on a 2% agarose gel containing RedSafe and the desired bands were extracted from the gel under UV light. The extracted bands are shown in Photo 3.39.

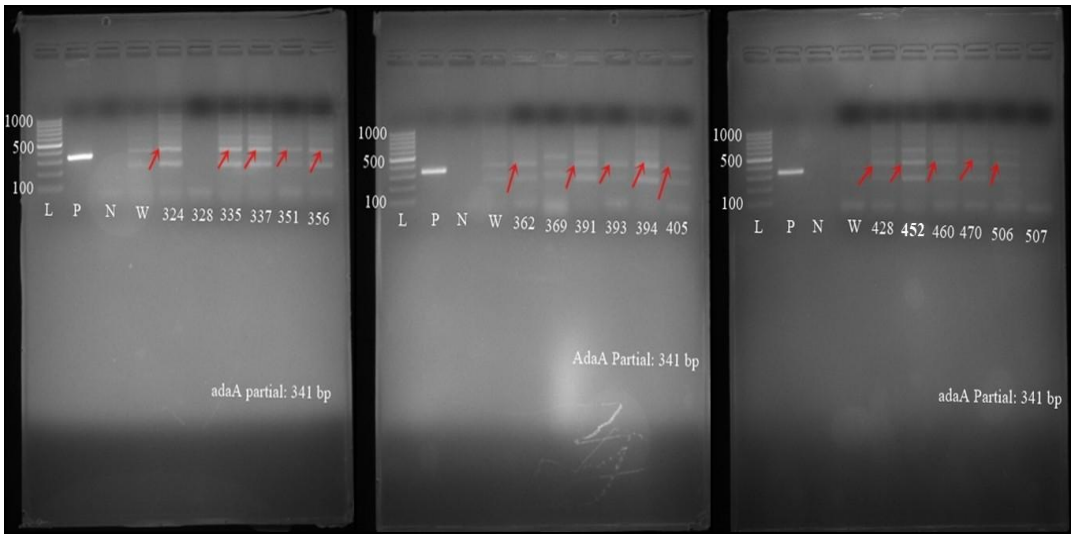


Photo 3. 39. Gel images of plants giving PCR positive bands with *adaA* partial primer. (L: Ladder, P: Positive control, N: Negative control and W: Wild type DNA)

Intergen company sent the sequence results for the samples screened with the *adaA* partial primer in BAM file format. The relevant file was analyzed with the Integrative Genomics Viewer program. The obtained sequence results showed no similarity with the pExpPN-phaA-SB vector (Figure 3.16). Therefore, it was determined that plants numbered 324, 335, 337, 351, 356, 354, 362, 391, 393, 394, 405, 428, 452, 460, 470 and 506 were not transplastomic plants because they did not contain any sequence from the pExpPN-phaA-SB vector.

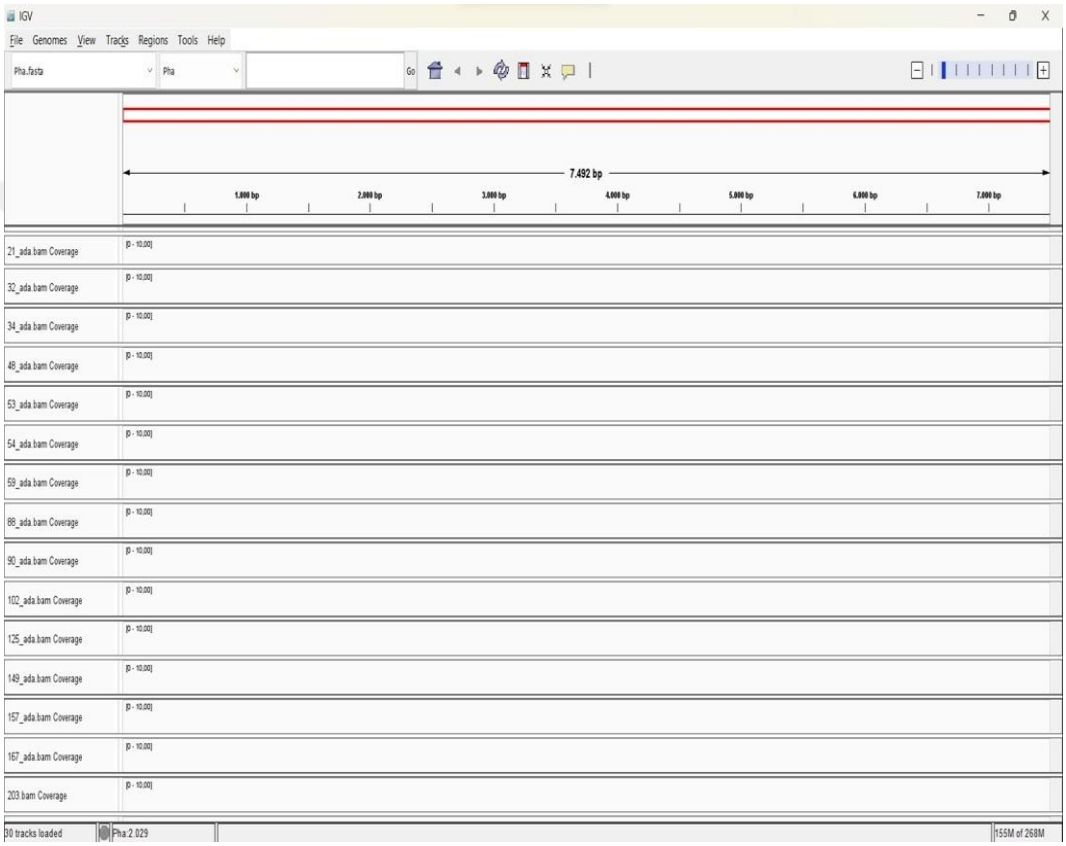


Figure 3. 16. Matching the sequences obtained from the *adaA* partial primer to the pExpPN-phaA-SB vector using the Integrative Genomics Viewer program

In PCR experiments performed with the *ampR* partial primer, samples numbered 318, 324, 335, 341, 428, 430, 470, 501, 504 and 507 were selected and re-run. The samples were run on a 2% agarose gel containing RedSafe (INtRON) and the desired bands were extracted from the gel under UV light. The extracted bands are shown in Photo 3.40.

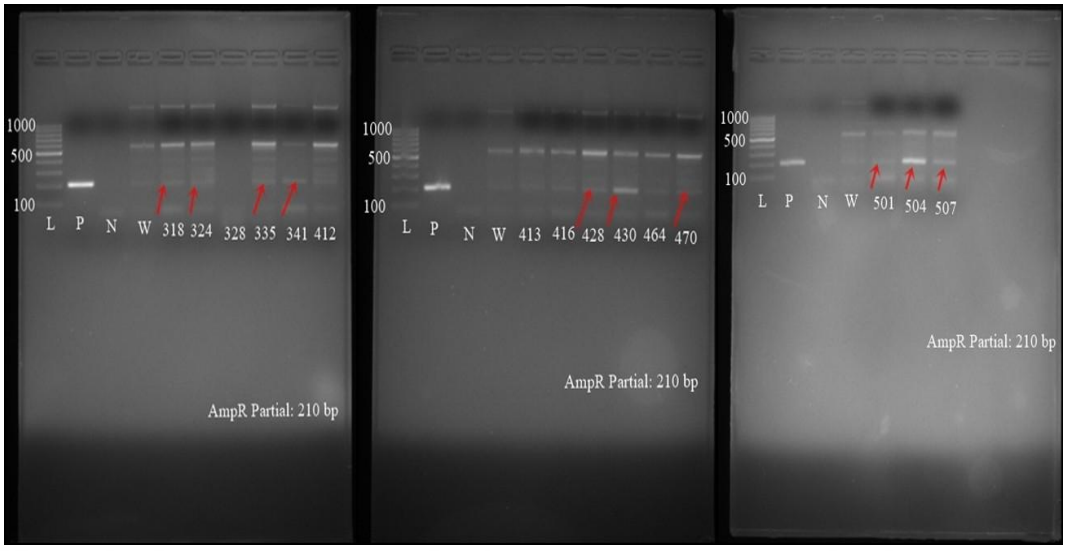


Photo 3. 40. Gel images of plants giving PCR positive bands with *ampR* partial primer. (L: Ladder, P: Positive control, N: Negative control and W: Wild type DNA)

The sequence results of the samples screened with the *ampR* partial primer were sent by Intergen in BAM file format. The relevant file was analyzed with the Integrative Genomics Viewer program. No similarity was found in sample number 324 in the matching of the sequence results with the pExpPN-phaA-SB vector (Figure 3.17).

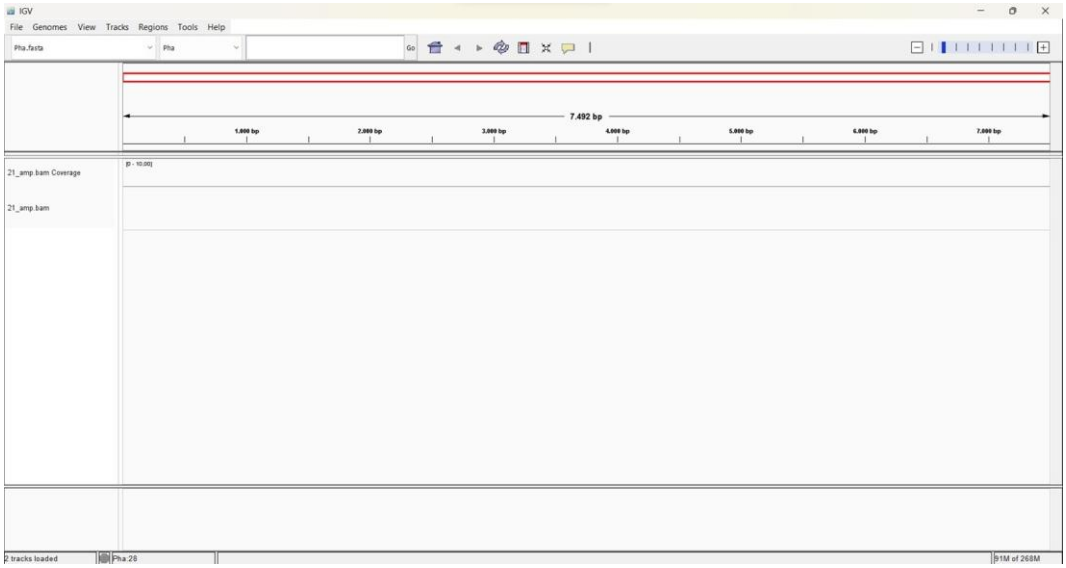


Figure 3. 17. Matching the sequences obtained as a result of sequencing of plant sample number 324 with the pExpPN-phaA-SB vector using the Integrative Genomics Viewer program

On the other hand, in the bioinformatics analysis of the sequences obtained as a result of PCR with the *ampR* partial primer of plants numbered 318, 335, 341, 428, 430, 470, 501, 504 and 507, it was found that a part of the pExpPN-phaA-SB vector was transferred to the sugar beet plant with the Integrative Genomics Viewer program.

The bioinformatic analysis shows that a portion of the pExpPN-phaA-SB vector was transferred to plant sample number 318 is shown in Figure 3.18.

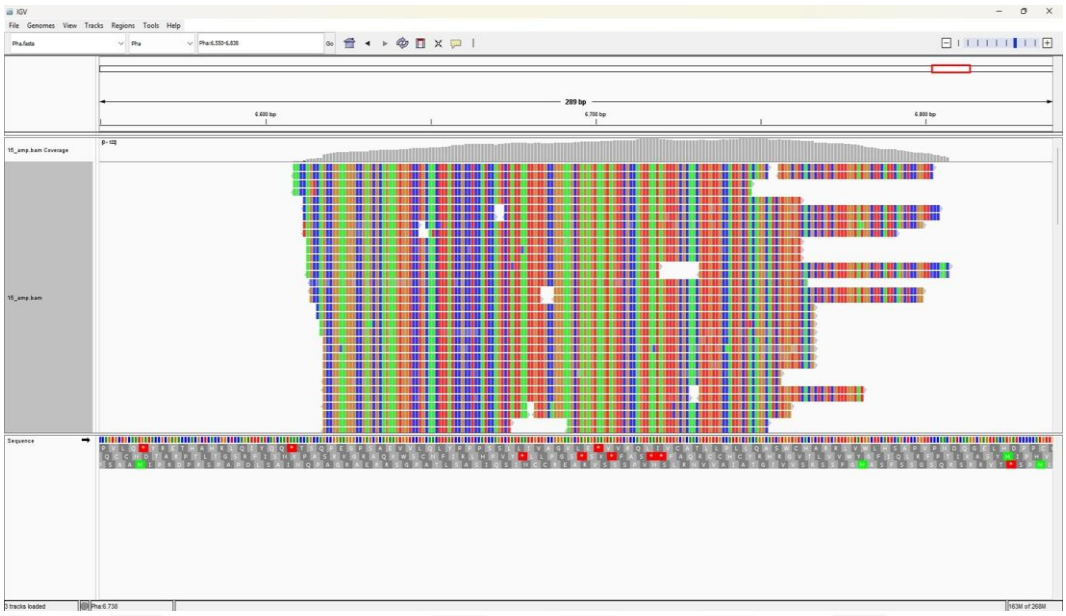
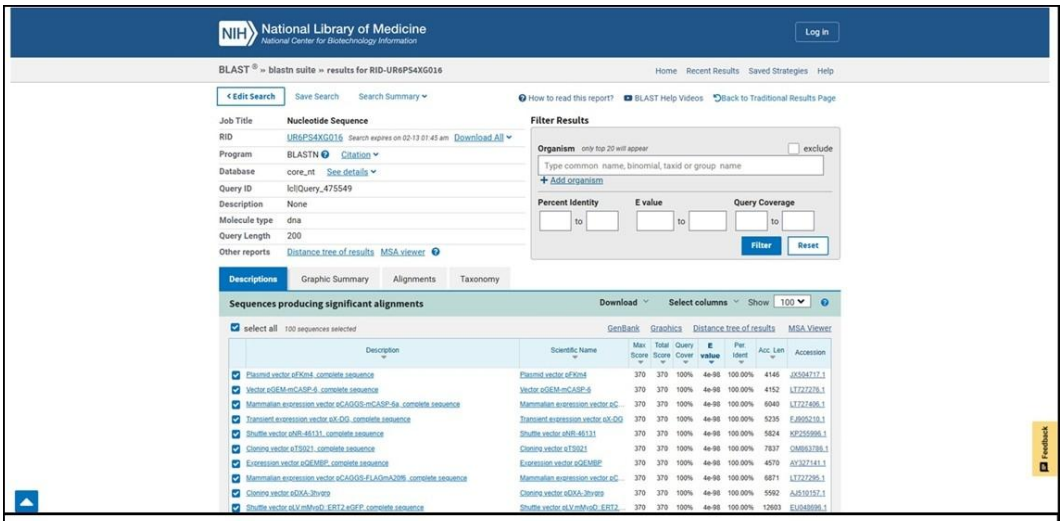


Figure 3. 18. Matching of the sequences obtained from the PCR screening of plant sample number 318 with the *ampR* partial primer to the pExpPN-phaA-SB vector using the Integrative Genomics Viewer program

Figure 3.19 shows the BLAST analysis performed using the NCBI site with the sequence of plant number 318. As a result of the BLAST analysis, it was observed that it contained a part of the *ampR* gene region that was not found in the sugar beet genome.



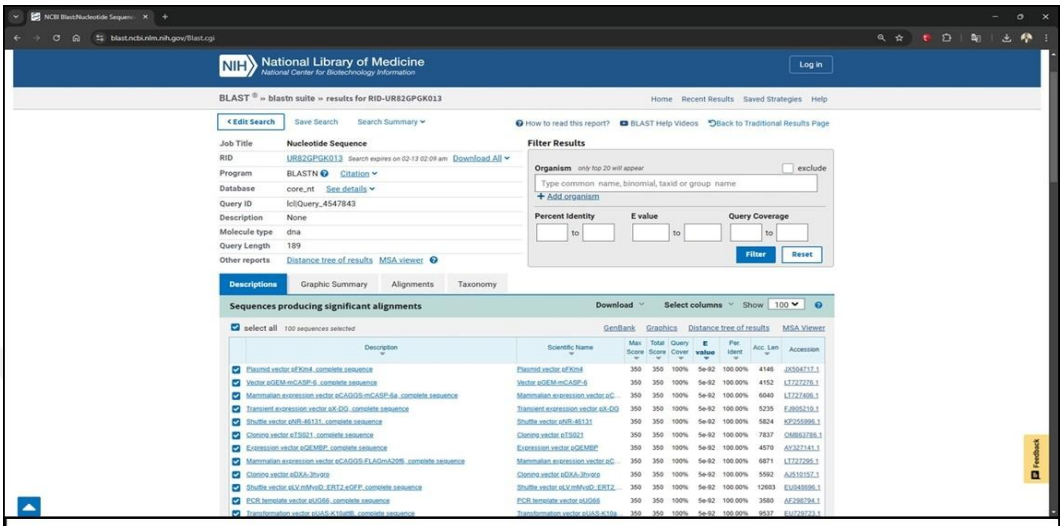
5'AACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTC
CATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTT
TGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTAT
GGCTTCATTCAGCTCCGGTTCCCAACG 3'

Figure 3. 19. NCBI/BLAST analysis results and sequence of plant sample 318

The bioinformatic analysis shows that a portion of the pExpPN-phaA-SB vector was transferred to plant sample number 335 is shown in Figure 3.20.

Figure 3. 20. Matching of the sequences obtained from the PCR screening of plant sample number 335 with the *ampR* partial primer to the pExpPN-phaA-SB vector using the Integrative Genomics Viewer program

Figure 3.21 shows the BLAST analysis performed using the NCBI site with the sequence of plant number 335. As a result of the BLAST analysis, it was observed that it contained a part of the *ampR* gene region that was not found in the sugar beet genome.



5’CCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCT
ATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACG
TTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTC
AGCTCCGGTTCCCAAC 3’

Figure 3. 21. NCBI/BLAST analysis results and sequence of plant sample 335

The bioinformatic analysis shows that a portion of the pExpPN-phaA-SB vector was transferred to plant sample number 341 is shown in Figure 3.22.

Figure 3. 22. Matching of the sequences obtained from the PCR screening of plant sample number 341 with the *ampR* partial primer to the pExpPN-phaA-SB vector using the Integrative Genomics Viewer program

Figure 3.23 shows the BLAST analysis performed using the NCBI site with the sequence of plant number 341. As a result of the BLAST analysis, it was observed that it contained a part of the *ampR* gene region that was not found in the sugar beet genome.

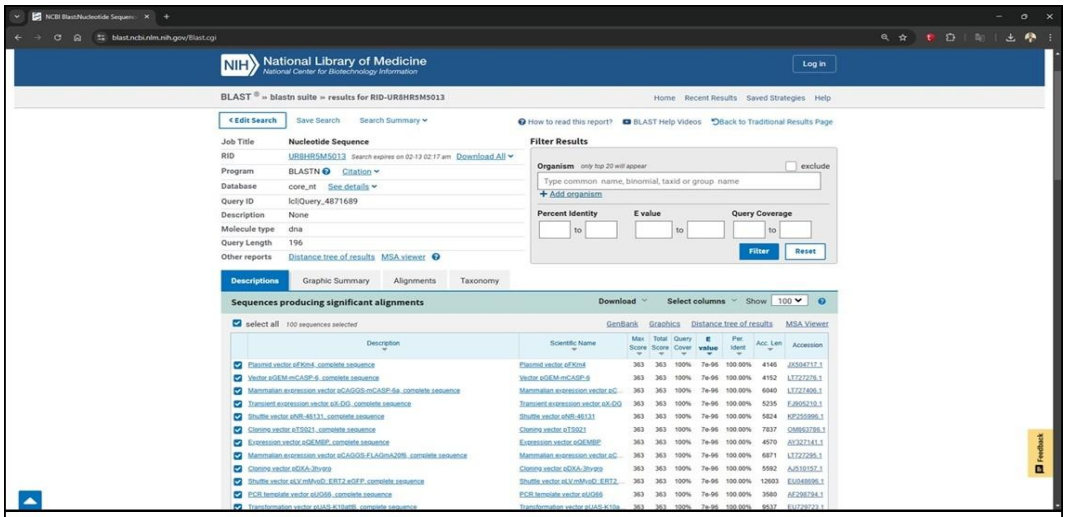


Figure 3. 23. NCBI/BLAST analysis results and sequence of plant sample 341

The bioinformatic analysis shows that a portion of the pExpPN-phaA-SB vector was transferred to plant sample number 428 is shown in Figure 3.24.

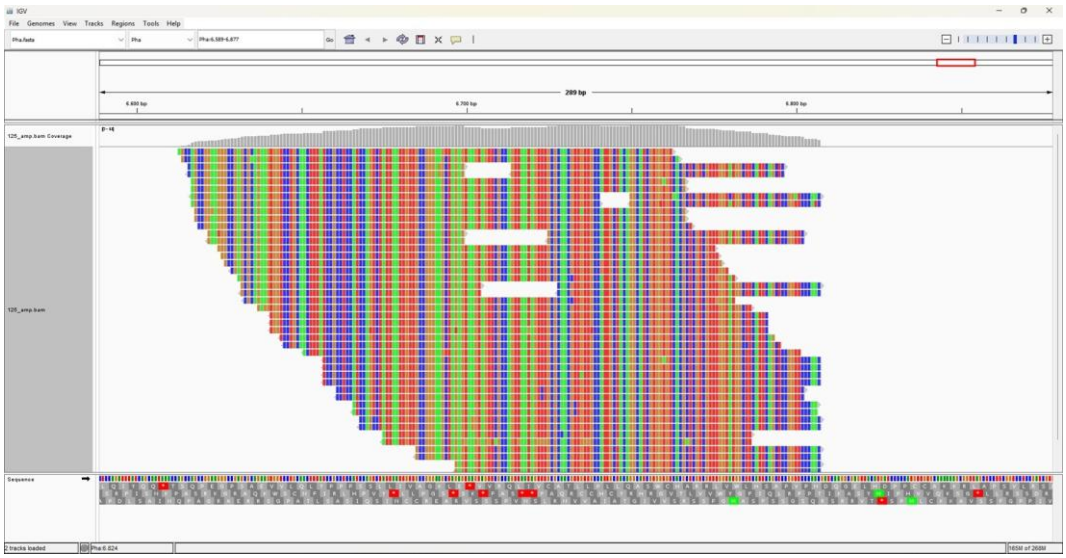


Figure 3. 24. Matching of the sequences obtained from the PCR screening of plant sample number 428 with the *ampR* partial primer to the pExpPN-phaA-SB vector using the Integrative Genomics Viewer program

Figure 3.25 shows the BLAST analysis performed using the NCBI site with the sequence of plant number 428. As a result of the BLAST analysis, it was observed that it contained a part of the *ampR* gene region that was not found in the sugar beet genome.

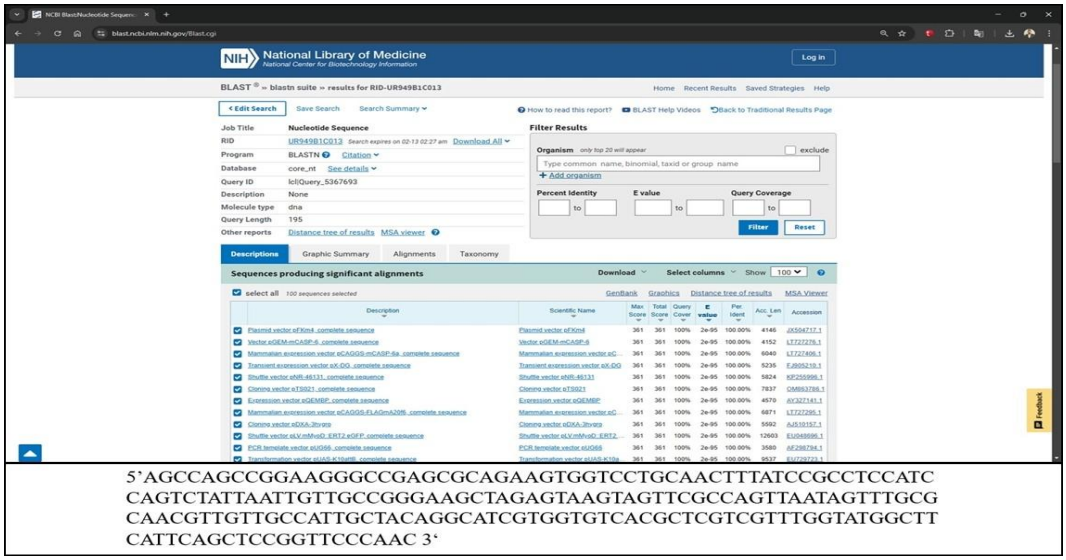


Figure 3. 25. NCBI/BLAST analysis results and sequence of plant sample 428

The bioinformatic analysis shows that a portion of the pExpPN-phaA-SB vector was transferred to plant sample number 430 is shown in Figure 3.26.

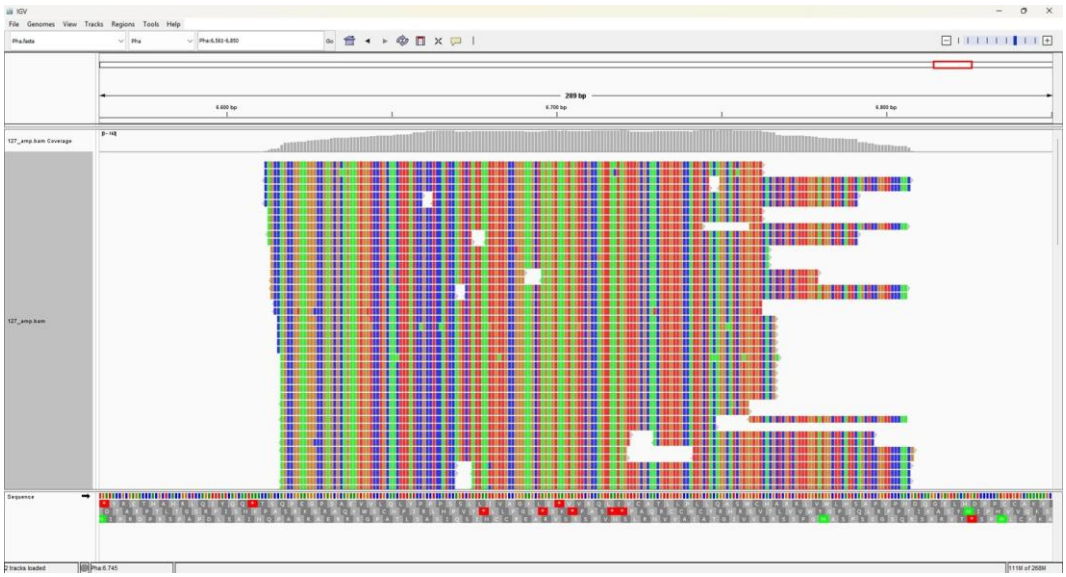


Figure 3. 26. Matching of the sequences obtained from the PCR screening of plant sample number 430 with the *ampR* partial primer to the pExpPN-phaA-SB vector using the Integrative Genomics Viewer program

Figure 3.27 shows the BLAST analysis performed using the NCBI site with the sequence of plant number 430. As a result of the BLAST analysis, it was observed that it contained a part of the *ampR* gene region that was not found in the sugar beet genome.

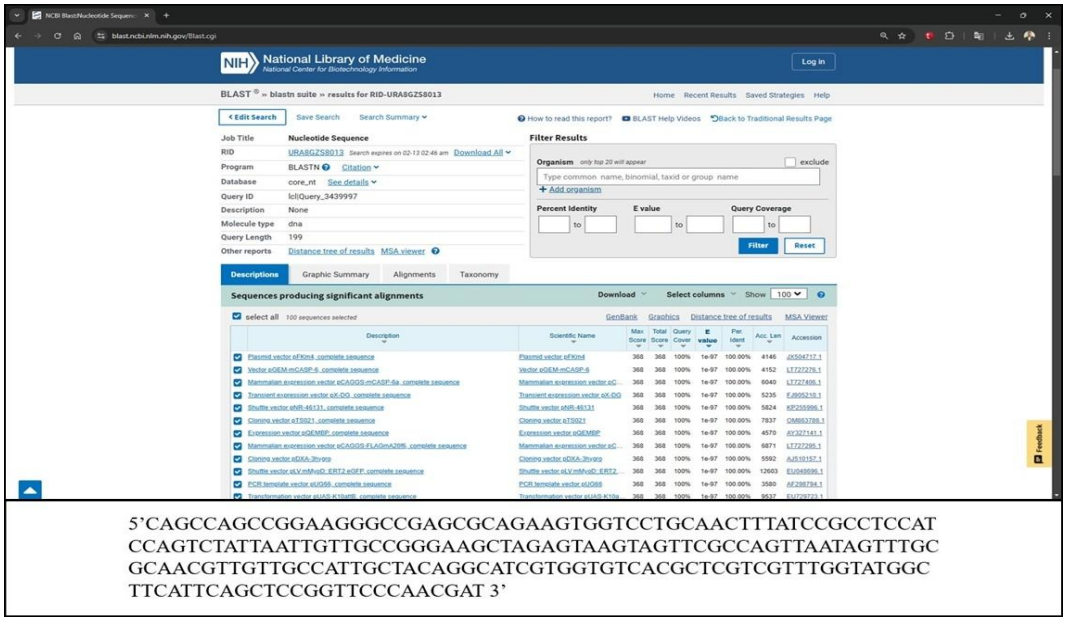
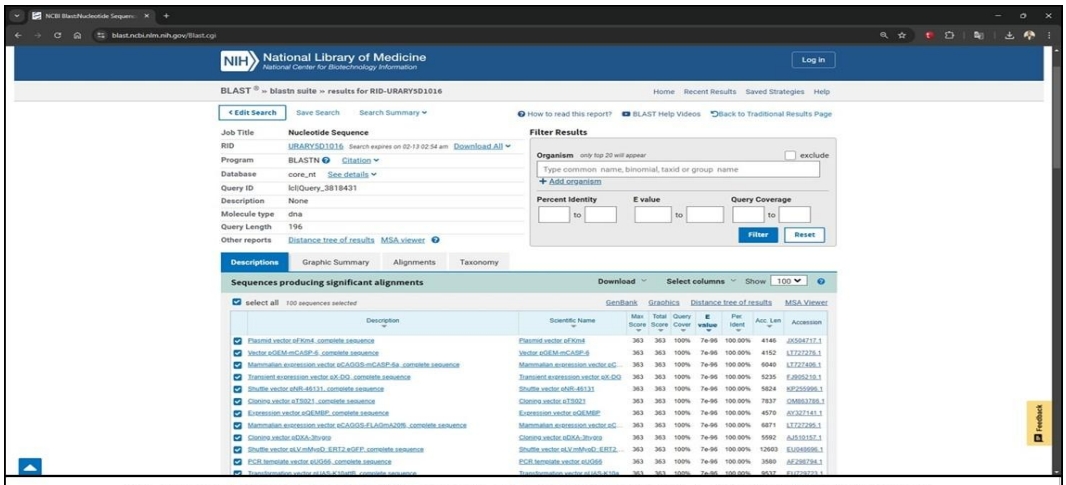


Figure 3. 27. NCBI/BLAST analysis results and sequence of plant sample 430

The bioinformatic analysis shows that a portion of the pExpPN-phaA-SB vector was transferred to plant sample number 470 is shown in Figure 3.28.

Figure 3. 28. Matching of the sequences obtained from the PCR screening of plant sample number 470 with the *ampR* partial primer to the pExpPN-phaA-SB vector using the Integrative Genomics Viewer program

Figure 3.29 shows the BLAST analysis performed using the NCBI site with the sequence of plant number 470. As a result of the BLAST analysis, it was observed that it contained a part of the *ampR* gene region that was not found in the sugar beet genome.



5’AGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATC
CAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCG
CAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTT
CATTCAGCTCCGGTTCCCAACG 3’

Figure 3. 29. NCBI/BLAST analysis results and sequence of plant sample 470

The bioinformatic analysis shows that a portion of the pExpPN-phaA-SB vector was transferred to plant sample number 501 is shown in Figure 3.30.

Figure 3. 30. Matching of the sequences obtained from the PCR screening of plant sample number 501 with the *ampR* partial primer to the pExpPN-phaA-SB vector using the Integrative Genomics Viewer program

Figure 4.31 shows the BLAST analysis performed using the NCBI site with the sequence of plant number 501. As a result of the BLAST analysis, it was

observed that it contained a part of the *ampR* gene region that was not found in the sugar beet genome.

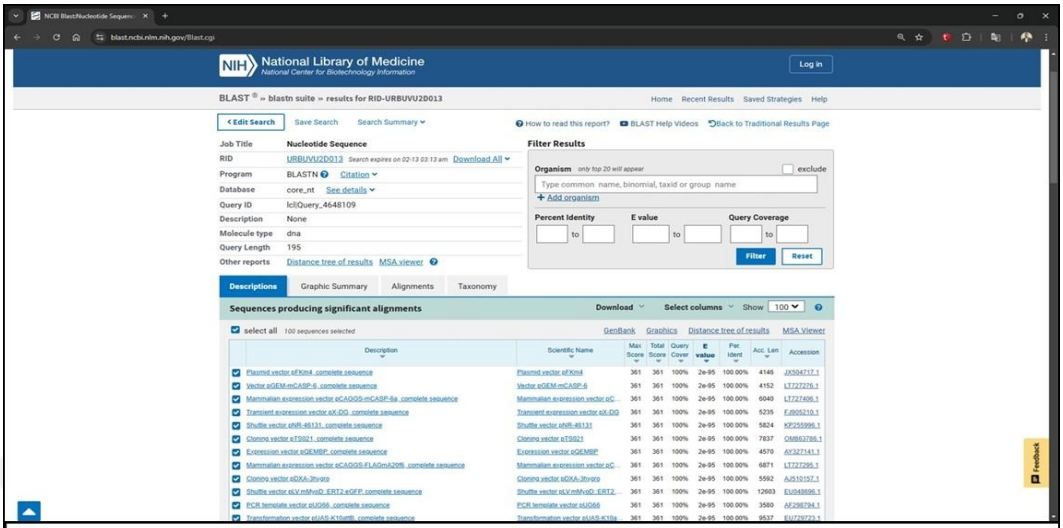
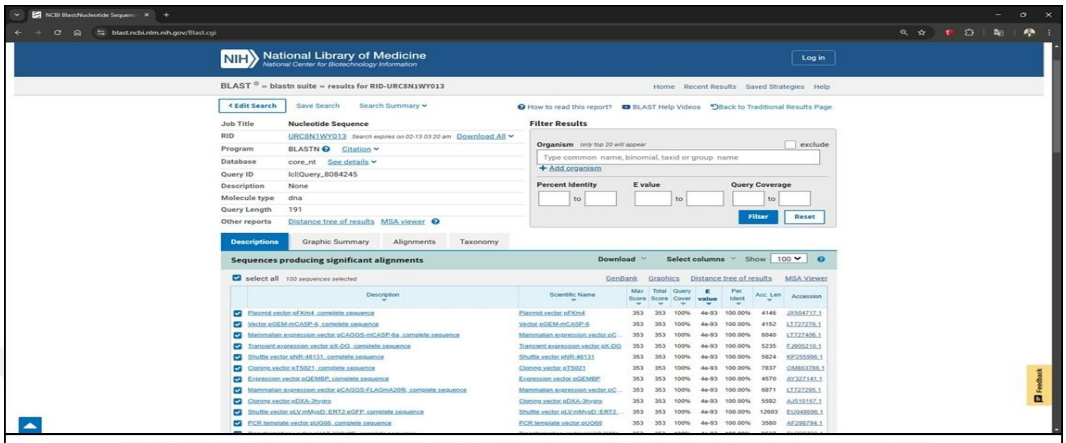


Figure 3. 31. NCBI/BLAST analysis results and sequence of plant sample 501

Figure 3. 32. Matching of the sequences obtained from the PCR screening of plant sample number 504 with the *ampR* partial primer to the pExpPN-phaA-SB vector using the Integrative Genomics Viewer program

Figure 3.33 shows the BLAST analysis performed using the NCBI site with the sequence of plant number 504. As a result of the BLAST analysis, it was observed that it contained a part of the *ampR* gene region that was not found in the sugar beet genome.



5'AGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGT
CTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAAC
GTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATT
CAGCTCCGGTTCCCAAC 3'

Figure 3. 33. NCBI/BLAST analysis results and sequence of plant sample 504

The bioinformatic analysis shows that a portion of the pExpPN-phaA-SB vector was transferred to plant sample number 507 is shown in Figure 3.34.

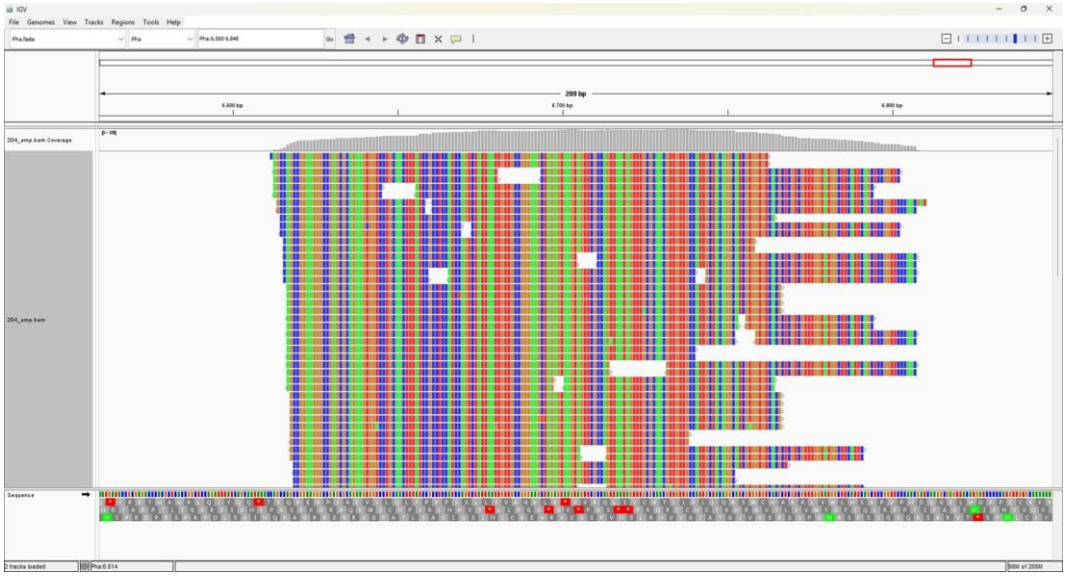
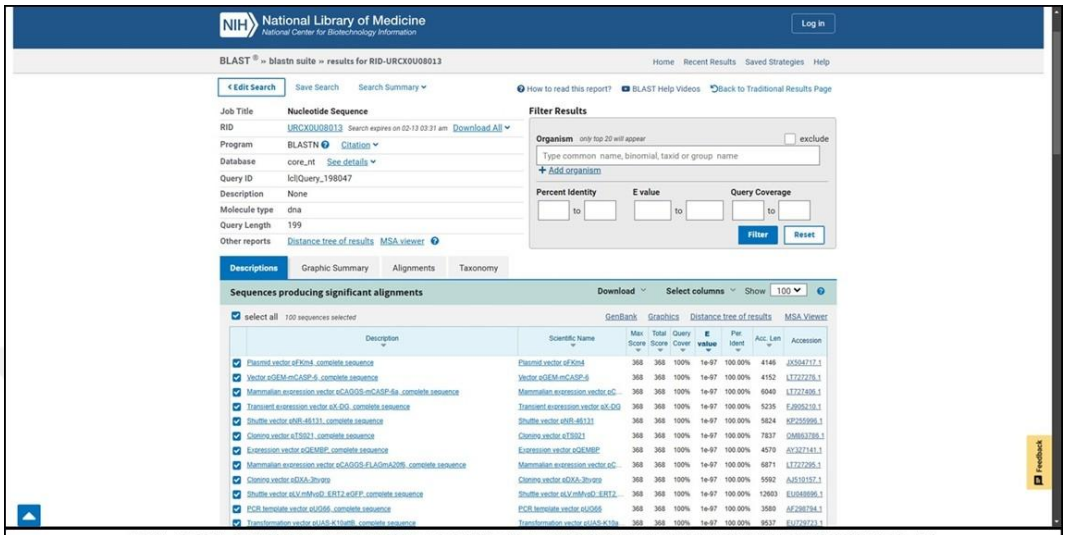


Figure 3. 34. Matching of the sequences obtained from the PCR screening of plant sample number 507 with the *ampR* partial primer to the pExpPN-phaA-SB vector using the Integrative Genomics Viewer program

Figure 3.35 shows the BLAST analysis performed using the NCBI site with the sequence of plant number 504. As a result of the BLAST analysis, it was observed that it contained a part of the *ampR* gene region that was not found in the sugar beet genome.



5'CAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCAT
CCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGC
GCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGC
TTCATTCAGCTCCGGTTCCCAACGAT 3'

Figure 3. 35. NCBI/BLAST analysis results and sequence of plant sample 507

A total of **5846** explants were used in **66** independent biolistic bombardments. A total of **5453** PCR reactions were performed using different primers with gDNA isolated from healthy regenerated plants. Based on PCR screening and sequencing results, partial transfer of the pExpPN-phaA-SB vector was detected in ten of the plant samples. When the sequence results were analyzed, it was understood that one of these plants contained both the *adaA* and *ampR* regions of the vector, while the other nine contained only part of the *ampR* region (Table 3.4, Photo 3.41.). Three of these plants remained as callus and did not show shoot regeneration. Seven of them were plants regenerated through direct regeneration (Table 3.4). None of the plants obtained by indirect regeneration gave positive results in PCR screening. Although there were five different sugar beet genotypes used, partial transfer was detected only in SG-DH and SG-12 line. Nine transplastomic plants were obtained from petiole explants and only one sample was obtained from leaf explants.

Table 3. 7. Biolistic bombardment and regeneration information of transplastomic plants.

Bombardment No	gDNA No	Sugar Beet Genotype	Explant Type	Psi	Regeneration type	Gen Region
9	318	SG-DH	Petiole	1100	Direct	*AmpR*-P
9	335	SG-DH	Petiole	1100	Direct	*AmpR*-P
11	167	SG-DH	Petiole	1100	Direct	*adaA*-P, *AmpR*-P
38	341	SG-12	Petiole	1100	Direct	*AmpR*-P
44	504	SG-DH	Leaf	1350	Callus	*AmpR*-P
47	507	SG-12	Petiole	1350	Callus	*AmpR*-P
55	428	SG-12	Petiole	1350	Direct	*AmpR*-P
56	430	SG-DH	Petiole	1350	Direct	*AmpR*-P
58	464	SG-DH	Petiole	1350	Direct	*AmpR*-P
Mix	501	SG-DH	Petiole	-	Callus	*AmpR*-P

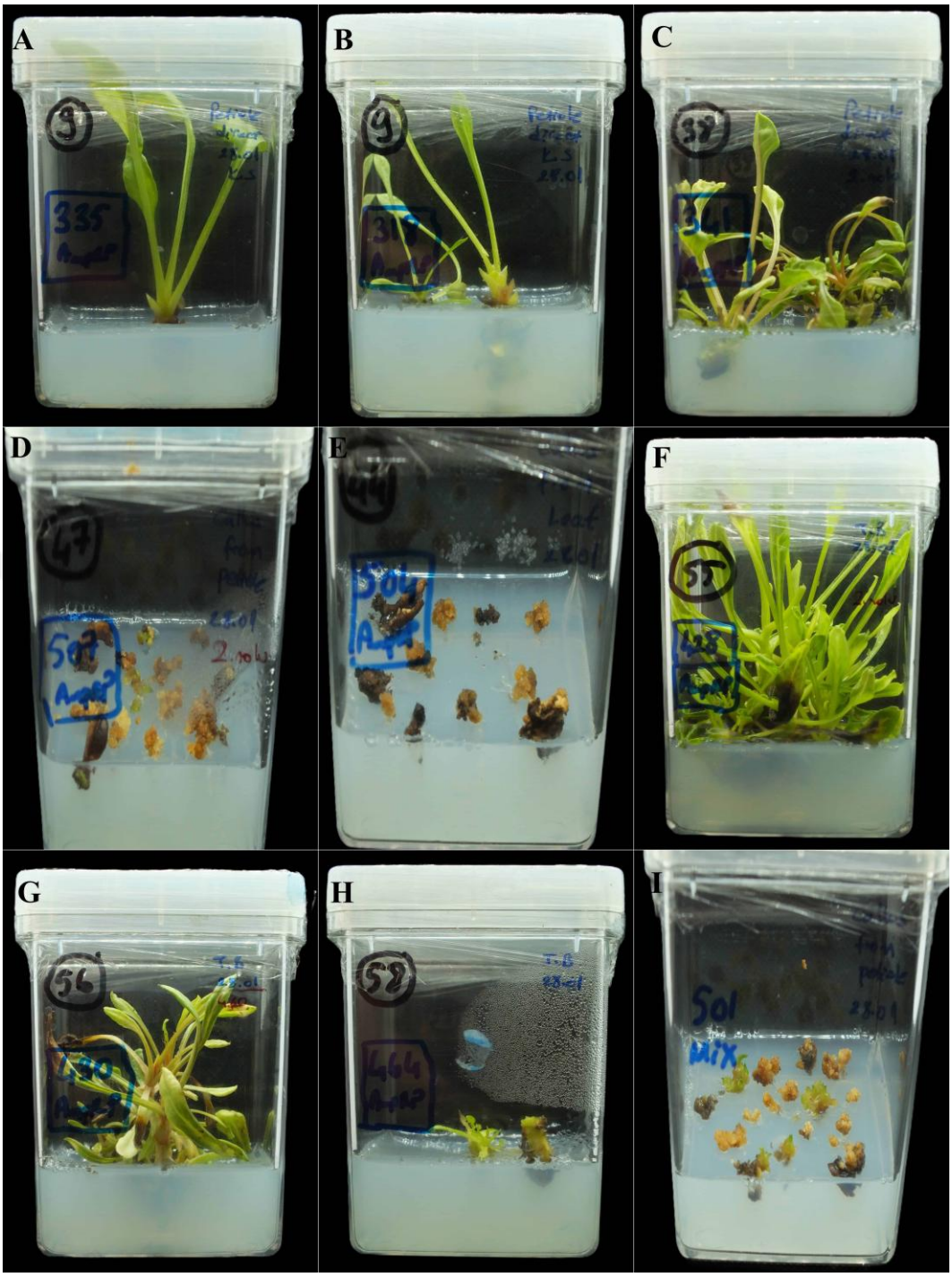


Photo 3. 41. Plants into which the *ampR* region was partially transferred by biolistic method. **A-B:** Plants 335 and 318, 26 months after bombardment. **C:** Plant 341, 12 months after bombardment. **D:** Plant 507, 7 months after bombardment. **E:** Plant 504, 8 months after bombardment. **F:** Plant 428, 6 months after the bombardment. **G:** Plant 430, 5 months after bombardment. **H:** Plant 464, 5 months after the bombardment

3.8 Expression Analysis of pExpPN-phaA-SB Vector by qRT-PCR

Total RNA was isolated from the 10 transplastomic plants specified in Table 3.4, as well as from leaf tissues previously taken from plant number 167 and the wild type of SG-DH plant. No problems were encountered during the isolation of Total RNA from transplastomic plants. To determine the accuracy, purity and RNA concentration of the isolated RNAs, both agarose gel analysis and nanodorp measurements were made (Photo 3.42, Table 3.5).

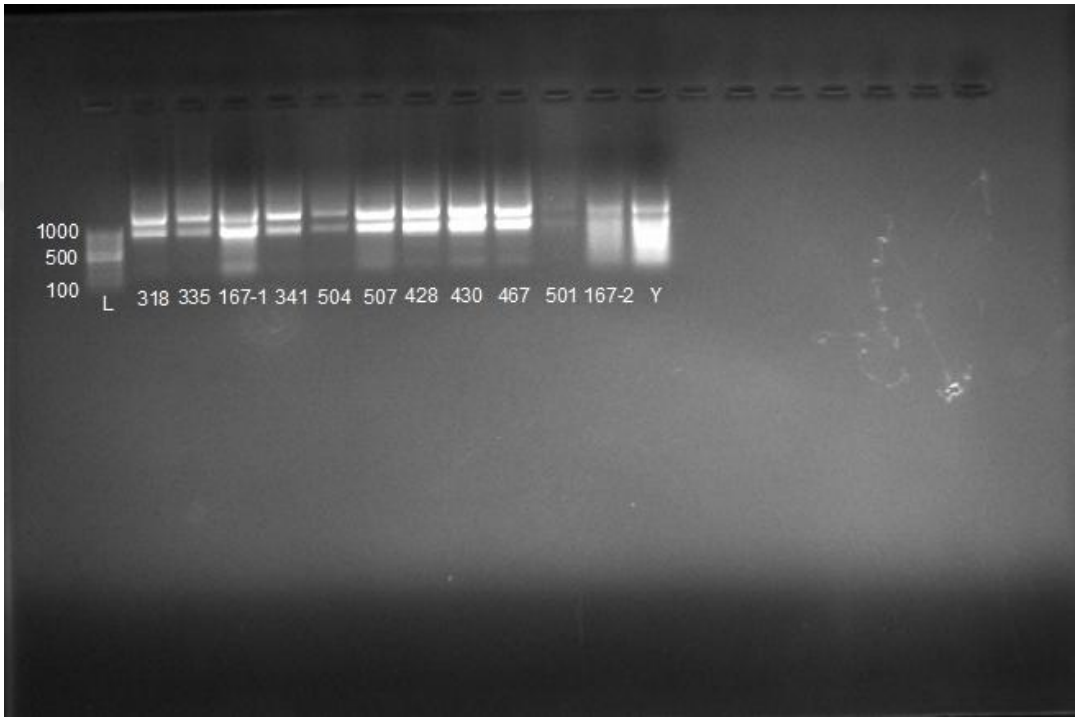


Photo 3. 42. 1% agarose gel images of total RNAs

The concentrations of the isolated RNA samples were measured using nanodorp (Thermo 2000) and the average RNA amount and purity rates were determined (Table 3.5).

Table 3. 8. Purities and RNA concentrations of isolated total RNAs.

Plant No	RNA Purity	Concentration in 1 µl	Concentration in 1 µl	Average Concentration in 1 µl
318	2.15	266.2	266.3	266.25
335	2.12	202.9	202.9	202.9
167-1	2.13	429.4	432	430.7
341	2.12	140.3	137.8	139.05
504	2.09	61.3	61.9	61.6
507	2.15	244	242.8	243.4
428	2.15	231.8	230.8	231.3
430	2.16	321	325.9	323.45
464	2.14	235.5	234.3	234.9
464	1.97	41	42.4	41.7
167-2	2.12	151.6	152.9	152.25
Wild Type	2.16	333	330.4	331.7

qRT-PCR results of samples 167-1 and 167-2, which contain a part of the *adaA* gene, are given in Figure 3.36. As a result of the analysis, it was seen that the expression level increased in plant 167-1. Samples 167-1 and 167-2 belong to the same plant. However, the tissues of sample 167-1 were taken at later stages of the plant and stored at -80 °C. When the expression level in plant 167-1 is compared to plant 167-2, there is a 10-fold increase. When the results are examined, it is seen that the partially transferred *adaA* gene continues to be expressed by increasing in later stages of plant growth.

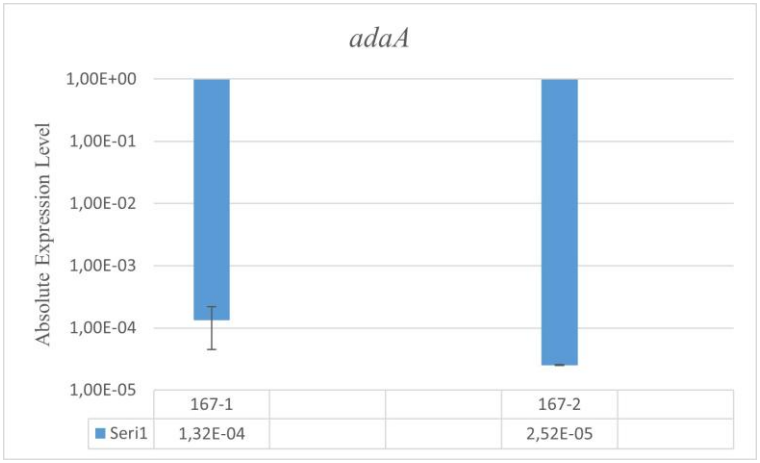


Figure 3. 36. Absolute expression results by qRT-PCR analysis of samples containing the *adaA* gene in the sequence results

The absolute qRT-PCR results of samples containing a portion of the *ampR* gene are given in Figure 3.37. As a result of the analyses, it was shown that all transplastomic plants had expression of the partially transferred *ampR* gene. The highest expression level among all plants was seen in plant number 167-2. The next highest was plant number 464. When both analyses were evaluated, the reason for the variability in expression levels can be explained by the different number of copies of the transferred gene in the plant and the age of the plant.

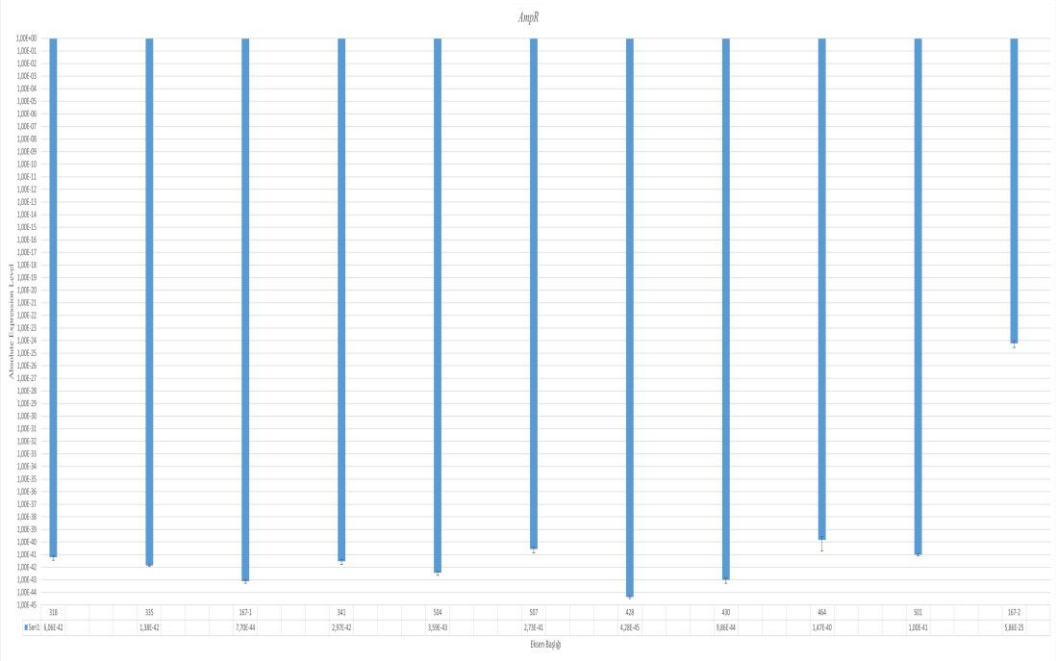


Figure 3. 37. Absolute expression results by qRT-PCR analysis of samples containing the ampR gene in the sequence results

3.9 Western-Blot Analysis of Isolated Proteins

The BSA method was used to determine the amounts of isolated proteins. BSA is used to estimate protein concentration from samples and shows a standard curve of protein concentration versus absorbance at 595 nm. As a result of spectrometer measurements, a standard BSA curve was created (Figure 3.38) and protein amounts were calculated. Protein loading was between 3 and 15 mg.

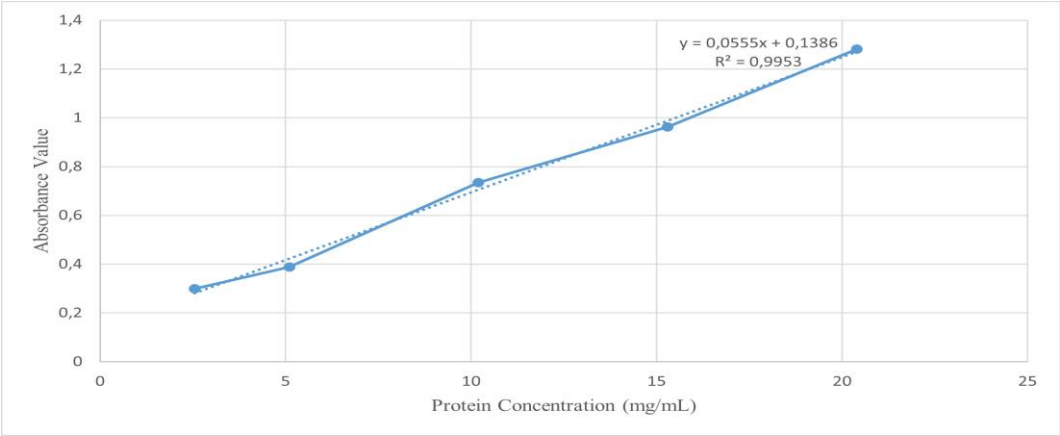


Figure 3. 38. BSA standard calibration curve

It was tested whether the *phaA* gene produces beta-ketothiolase enzyme by Western Blot method. In addition to the transplastomic plants specified in Table 3.4 in the SDS page gel, 2 bacterial samples and pellets formed in the protein isolation of plants numbered 167-1, 504, 428 and 167-2 were loaded. As a result of the analysis, it was understood that there was no protein production in the other 10 plant samples except the *E. coli* cells harboring the pExpPN-phaA-SB vector (Figure 3.39). Our primary antibody used is specific to the Beta-ketothiolase enzyme encoding the *phaA* gene. Transplastomic plants used in Western-Blot analysis contain *adaA* and *ampR* genes. Therefore, we did not expect positive bands in Western-Blot analysis except for bacterial cultures. PonceuS staining also shows that other proteins found in the plant are transferred to PVDF membranes.

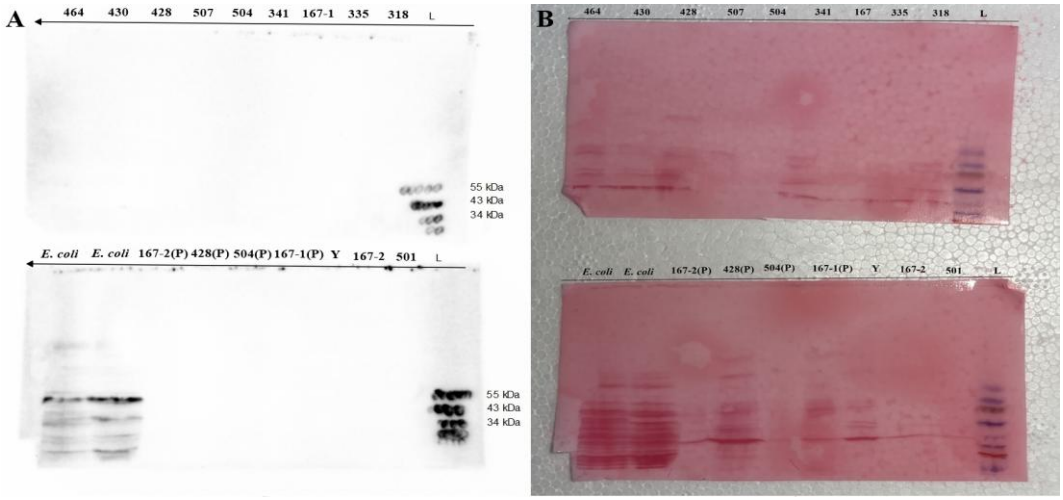


Figure 3. 39. Protein images used in Western blot analysis. A: 10% SDS gel image. B: PonceuS staining image for verification of protein transfer

4. DISCUSSION

4.1 Plant Genotypes and Explant Selection for Biolistic Bombardment

Upon evaluating the literature, it was understood that the transformation efficiency was different among plant genotypes even though the same chloroplast transformation vector was used (De Marchis et al., 2009). In a different study, researchers found that stable transformation rates were different from each other in different genotypes as a result of gene transfer experiments to sugar beet using the *Agrobacterium* method (Gurel, 2021). In a different study, they found that genotype influences sugar beet genetic transformation (Hisano et al., 2004). The SG-DH genotype was used as the main seed source in our study. To reduce and test the effect of the genotype on transformation, four different sugar beet genotypes, SG-12, SG-13, SG-6M and ELK345, were used in biolistic bombardment experiments. When the biolistic transformation results were examined, it was understood that seven partial transfers were in the SG-DH genotype and three were in the SH-12 genotype. In the PCR screenings, no positive results were found in the other three genotypes. When our study results were evaluated, it was understood that the efficiency of chloroplast transformation was different among genotypes. So, when the results were compared with the literature, our findings were consistent.

The literature review revealed it was understood that different sugar beet tissues were used for chloroplast transformation. Examples of these studies include petiole (De Marchis et al., 2009), leaf (Ivic & Smigocki, 2005), guard cells (Hall et al., 1997), callus (Snyder et al., 1999; Ivic & Smigocki, 2001), and cotyledon (Snyder et al., 1999). In our study, three different explants were selected to be used in biolistic bombardment: petiole, leaf and callus.

4.2 Determination of Antibiotic Selection Medium

The expression cassette of the sugar beet-specific pExpPN-phaA-SB vector designed within the scope of the TÜBİTAK project number 120O596 contains *adaA*, the gene that provides resistance and advantage to spectinomycin and streptomycin antibiotics for the selection of transplastomic plants, under the control of the *5'-psbA* promoter and the *3'-rbcl* (*Chlamydonas*) terminator. Since sugar beet is naturally resistant to streptomycin, the antibiotic spectinomycin was preferred for

the selection of transplastomic sugar beet plants in our experiments. The *aadA* gene was first discovered stably and expressed in the *Chlamydomonas* chloroplast genome (Goldschmidt-clermont, 1991).

An effective plastid transformation experiment requires the successful completion of more than one step. These can be briefly classified as the appropriate transfer of the vector into the plastid genome, the presence of an active homologous recombination mechanism in the plastid genome and finally a high-throughput selection and regeneration protocol for transplastomic cells. In gene transfer experiments, when foreign DNA is transferred into the chloroplast, it transforms only a few copies of the plastome. This stage of transformation experiments is called heteroplasmic. Later, by selecting these explants under *in vitro* conditions with antibiotics etc., the plastome with foreign genes gain an advantageous position and increase their numbers. Over time, it turns into a homoplasmy state in which all plastome in the plant contain foreign genes (Adem et al., 2017).

The antibiotic spectinomycin is used for the selection of transplastomic plants. Spectinomycin binds to *16S rRNA* and blocks translation in prokaryotic type 70S plastid ribosomes (Wilson, 2014; Wirmer & Westhof, 2006). Thus, it inhibits greening and shoot regeneration in cells grown in tissue culture that cannot show resistance (Svab et al., 1990). The *aadA* gene encodes the aminoglycoside-3"-adenyltransferase enzyme, which inactivates spectinomycin and streptomycin by adenylation and prevents binding to chloroplast ribosomes (Goldschmidt-clermont, 1991). As a result, it provides an advantage for the selection of transplastomic plants encoding the *aadA* gene after transformation compared to non-transplastomic plants. In a previous study, the *aadA* gene was used as a selectable marker in tobacco and was observed to increase the frequency of transformation events 100-fold more than mutant *16S rRNA* genes (Svab & Maliga, 1993). Another study reported that the *aadA* gene is a dominant marker compared to other marker genes (such as *rrnS* and *rrnL*) (Day & Goldschmidt-Clermont, 2011). In the same study, it was mentioned that the *aadA* gene was successfully used in plastid transformation in many plants such as potato, tobacco and rice. As we mentioned in the results section, when six different spectinomycin doses were tested on sugar beet, the optimum spectinomycin antibiotic concentration was found to be 50 mg/L. Spectinomycin doses of 75, 100, 150 and 200 mg/L almost completely stopped the growth of plants and prevented callus formation. These data are consistent with the

study conducted by De Marchis et al. (2009). As seen in the study of De Marchis et al., 2009, it was shown that 50 mg/L spectinomycin concentration caused chlorosis in non-transformed sugar beet petiole and prevented regeneration. In another chloroplast transformation study on potato, they showed that spectinomycin levels between 40 and 50 mg/L were sufficient to cause tissue bleaching and inhibit shoot development (Sidorov et al., 1999).

The most important point to be considered in antibiotic selection is to determine the optimum dose. Exposure to high dose antibiotic selection may cause damage to transplastomic plants. When both our own results and the literature were compared, it was deemed appropriate to use a dose of 50 mg/L spectinomycin.

4.3 Biolistic Bombardment of the pExpPN-phaA-SB Vector, Selection of Transformants and Shoot Regeneration

The most important step after biolistic bombardment experiments is to grow the plants in the selection medium and ensure healthy plant regeneration. As mentioned above, the optimum antibiotic concentration was determined as 50 mg/L based on our results and previous studies.

Therefore, 50 mg/L spectinomycin was selected as the initial concentration for the selection of transplastomic plants. As seen in the study of De Marchis et al. (2009), spectinomycin concentration of 50 mg/L was shown to cause chlorosis in non-transformed petiole plants and prevent regeneration. In addition, the same study reported that calli did not regenerate under 50 mg/L spectinomycin selection. Another study reported that callus was formed from explants whose petioles turned dark brown (De Marchis & Bellucci, 2021). When different studies in literature are examined, it has been observed that if antibiotics are present in the tissue culture medium of leaf explants, callus formation decreases and slows down, and indirect shoot regeneration does not occur (Ivic & Smigocki, 2005; Ivic & Smigocki, 2003). As seen in the study of De Marchis et al. (2009), when spectinomycin was present in the MS medium, regeneration could not be achieved from the calli formed by the leaf petiole, and when spectinomycin was removed from the nutrient medium at the end of the selection, it was reported that the calli started shoot regeneration.

It was mentioned in the result section that the selection period was reduced to one month because the bombarded plants did not grow in the selection medium and died. When this method was followed, it was understood in the PCR studies

that a large number of false positive plants grew from the selection that did not include the pExpPN-phaA-SB vector. Therefore, as mentioned in the result section, the method followed in the study of De Marchis et al. (2009) was used as the antibiotic selection period. As a result of the analyses (Figures 3.3 and 3.4), it was understood that extending the antibiotic period to five months significantly reduced the callus and regeneration capacity of petiole and leaf explants. Consequently, since long-term antibiotic selection would prevent the growth of transplastomic plants, limiting the period to one month was found to be more suitable in terms of sugar beet tissue culture possibilities.

We have previously stated that Sidorov et al. (1999) showed that spectinomycin levels between 40 and 50 mg/L were sufficient for selection in potato chloroplast transformation studies. In the same study, molecular confirmation analyses performed on plants that formed resistant shoots in the 40 mg/L spectinomycin selection medium showed that plastid transformations were not confirmed. When the amount of spectinomycin in the medium was increased up to 300 mg/L, 400 mg/L and 500 mg/L spectinomycin, it was shown that transplastomic plants could survive at these concentrations. These plants were also *GFP* positive and were identified as homoplastomic by Southern blot analysis. On the other hand, researchers stated that there were plants resistant to 500 mg/L spectinomycin but were not transplastomic.

In another sugar beet biolistic transformation study, kanamycin (Km) was used for antibiotic selection (Ivic & Smigocki, 2005). They found that when they used 10 and 30 mg/L Km, the growth of the calluses was delayed and slow. Moreover, they reported that at the end of seven weeks there was no indirect regeneration, and that callus did not even form when they used 100 mg/L Km. They reported that the calli obtained from the four GUS^{+} sugar beet plants without exposing them to antibiotic selection after bombardment regenerated in 7-11 weeks.

When we look at the results of our own study and the studies in the literature, it is understood how important it is to have reporter genes (*GUS*, *GFP*, etc.) in addition to antibiotic selection marker genes in the vectors used in genetic transformation studies of plants that are difficult to grow in selection environments and are recalcitrant in tissue culture, such as sugar beet.

These callus-forming plants and callus explants were planted in MS media containing 1.0 mg/L BA and 0.5 mg/L ABA hormones to ensure regeneration in the

later stages. In a previous study, the environments with the highest regeneration capacity of morphogenic calli were those in which 1.0 mg/L BAP and 0.5 mg/L ABA or 1.0 mg/L ABA hormones were used (Mishutkina & Gaponenko, 2006). Callus formation in sugar beet is a difficult and long process. In particular, the amount of antibiotic applied, and exposure time had a significant effect on callus formation (Figures 3.3 and 3.4).

Shoot regeneration was achieved in some of the calluses obtained after biolistic bombardment of petiole explants, but the shoots did not grow sufficiently and hyperhydricity problem was observed in the obtained shoots. The vitrification problem is mostly seen in plants that are regenerated indirectly and is mentioned in the results section. When we look at the literature, hypocotyl and cotyledon explants were used in the biolistic transformation of sugar beet in the study. Although the calluses formed shoots, it was reported that healthy plant development could not be achieved due to the vitrification problem (Ivic and Smigocki, 2001). After shoot formation, planting the plants in MS medium containing 0.25 mg/L KIN primarily decreased vitrification problem. However, vitrification of the plants at the callus stage negatively affected regeneration. In our study, which is similar to the studies in literature, the plants that showed vitrification did not mostly develop in our study and broke into pieces even with the slightest touch. The vitrification problem has significantly negatively affected the selection and regeneration of plants in antibiotic selection medium. Thus, our outcome aligns with previous research. In addition, compact calluses did not produce shoot regeneration even though they formed green dots. This result is similar to the study conducted by Pedersen et al. in 1993.

According to the literature, in gene transfer studies conducted specifically for sugar beet, vitrification problems encountered by plants during the regeneration phase have been stated as a major problem. (Mukherjee & Gantait, 2023). In the genetic transformation study conducted on sugar beet, only three of the genotypes showed shoot regeneration from the callus stage. These samples could not return to normal and healthy plants due to vitrification (Ivic & Smigocki, 2001). In another study, guard cells in sugar beet stomata were used for gene transfer. As a result of the study, 7% of the herbicide resistant callus was obtained and only 2% of the obtained callus showed regeneration and formed plantlets. The remaining callus did not develop either or vitrified. Moreover, in the same research article, it was

mentioned that it took approximately two years to obtain a transgenic sugar beet plant (Hall et al., 1996).

SG-DH genotype showed the highest callus formation percentage with 43% in leaf explants, 8.46% direct regeneration, and 11.18% direct regeneration in petiole explants (Figure 3.3). In light of the data we obtained, the difficulty of growing sugar beet plants under *in vitro* conditions becomes apparent. During our experiments, some explants in medium containing the spectinomycin produced albino plants. This situation is similar to the study conducted on canola (*Brassica napus*) (Zubko & Day, 1998).

In the study conducted among different sugar beet genotypes, they found the morphogenic callus formation percentage to be between 11.5% and 44.3% (Mishutkina & Gaponenko, 2006). In the same study, they stated that the best callus-forming tissue culture medium in four different sugar beet genotypes was MS medium containing 0.5 mg/L BAP and 0.1 NAA. The callus formation percentages in our study are similar to the results obtained in the study by Mishutkina and Gaponenko (2006). In another study, the transfer of the *uidA* gene to sugar beet stomatal cells was attempted by the PEG transformation method. As a result of this study, 7% of the calli showed regeneration, while only 2% of them formed full plants (Hall et al., 1996). In another study conducted by Kishchenko et al., 2005, a genetic transformation study was conducted using sugar beet suspension culture cells using the *A. tumefaciens* method. The transgenic calli obtained in this study could not be regenerated. This study shows that the regeneration ability of the sugar beet plant, especially in genetic transformation studies, is very limited. The most important step in obtaining homoplastomic plants, especially in genetic transformation studies, is that callus formation and indirect regeneration protocols work with high efficiency.

In obtaining homoplastomic plants after the transformation stage, it is very important to obtain plants through indirect plant regeneration after the explant forms callus. In addition, *in vitro* callus formation and regeneration of sugar beet is quite slow and difficult. As mentioned above, studies have shown that a period of 14 months to two years is needed. Tissue culture is directly related to different parameters such as plant growth medium, growth conditions, explant type and especially genotype (Örgeç et al., 2021). Sugar beet genotypes generally respond differently to *in vitro* culture in callus formation and plant regeneration. This

finding suggests that callus formation and plant regeneration may be heritable traits and genes involved in these traits may be rare in sugar beet populations (Kagami et al., 2016). In a study on this subject, 61 sugar beet lines were investigated for their responses to *in vitro* growth conditions and variation was found in the frequency of callus formation and somatic embryo formation (Tomita et al., 2013). Unfortunately, there is no tissue culture protocol in the literature that works equally successfully in all sugar beet genotypes. As is known, the success rate in scientific studies carried out with plant tissue culture is directly related to the effective performance of tissue culture. Sugar beet has a resistant structure against tissue culture and also, as mentioned, callus formation is quite slow. In our study, the tissue culture performance of sugar beet played a key role.

4.4 gDNA Isolation of Regenerated Plants

Since the nucleotides that make up DNA and RNA show absorbance at 260 nm, this value is used in DNA purity measurements. Proteins absorb light at 280 nm wavelength. Similarly, absorbances obtained at 230 nm wavelength are considered as contamination since it is the absorbance value of most organic molecules. In the measurements made as a result of the isolation of nucleic acids, the 260/280 ratio provides information about protein contamination. As protein contamination increases, the 260/280 ratio begins to decrease. The 260/230 ratio is used to measure the presence of organic contamination in DNA. The ratio of pure DNA is considered to be between 1.8 and 2.2. The purity rates of 97% of our isolated gDNA samples are between 1.8 and 2.2, which is suitable in terms of purity values.

4.5 Confirmation of Chloroplast Integration of pExpPN-phaA-SB Vector by PCR and Expression Analyses

As a result of 5846 explants used in 66 independent bombardments, partial transfer of the pExpPN-phaA-SB vector was detected in 10 plants. When the sequence results were analyzed, it was understood that one of these plants contained both the *adaA* and *ampR* regions of the vector, while the other nine contained only part of the *ampR* region. Three of these plants remained as callus and did not show shoot regeneration, while seven plants were regenerated through direct regeneration. None of the plants obtained through indirect regeneration gave

positive results in the PCR screenings. When the sugar beet genotypes were evaluated, partial transfer was detected only in the SG-DH and SG-12 sugar beet genotypes.

The pExpPN-phaA-SB vector used in our study contains the *phaA* gene (1,179 bp) encoding ß-ketothiolase from *Acinetobacter* sp. (accession no. L37761), which is controlled by the *PrrnPEP+NEP* promoter and the *3′ tobacco rbcL* terminator, as well as the *adaA* and *ampR* genes.

Polyhydroxyalkanoates (PHAs) are a class of aliphatic microbial polyesters and are renewable sources of biodegradable thermoplastics. The most common PHA, poly-3-hydroxybutyrate (PHB), is produced and stored by a variety of bacteria (Nakashita et al., 2001) and is stored (Poirier et al., 1992). PHB and PHA are renewable and biodegradable thermoplastics and elastomers. PHB is synthesized from acetyl-CoA by the sequential action of three enzymes, 3-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase, which are encoded by the *phbA*, *phbB*, and *phbC* genes (Nawrath et al., 1994). The *phaA* gene has been used to induce male sterility in plants.

PHBs have also been produced in plants by gene transfer. When we look at literature, PHB production has been achieved by transferring genes to more than one plant, such as *Brassica napus* (Houmiel et al., 1999) *Arabidopsis thaliana* (Nawrath et al., 1994), using the *Agrobacterium tumefaciens* method. In the study conducted on *Arabidopsis thaliana*, although no negative effects were observed on germination, they reported that chlorosis effect was observed in the leaves of 60-day-old plants (Houmiel et al., 1999). In another study, they designed a vector containing the three genes that make up PHB and transferred it to *Arabidopsis thaliana* using the *Agrobacterium tumefaciens* method. In their study, they found a negative correlation between the amount of PHB produced and plant growth. They found that some plants did not produce seeds and had delays in their growth (Bohmert et al., 2000). In another study on the *phbA* gene, it was stated that constitutive expression of the *phbA* gene, one of the PHB synthesis genes transferred by the *Agrobacterium tumefaciens* method in *Arabidopsis*, *tobacco* and potato, led to a significant decrease in genetic transformation efficiency, prevented the recovery of transgenic lines and prevented the analysis of plants expressing the b-ketothiolase gene. Moreover, they emphasized that it prevents the regeneration of calluses (Bohmert et al., 2002). In the study conducted on tobacco, *phbA*, *phbB* and

phbC were transferred to leaf chloroplasts by biolistic bombardment. At the end of the biolistic bombardment, five different transplastomic plants were obtained. It was reported that the regenerated transformants grew slowly compared to wild tobacco plants and shed approximately 95% of their leaves and turned into male sterile plants. The researchers also concluded that significant levels of PHB in tobacco can only be achieved if sufficient amounts of acetyl-CoA precursor are produced (Lössl et al., 2003). In the study where the vector containing the *phaA*, *phaB* and *phaC* genes was transferred to the poplar plant with *Agrobacterium tumefaciens*, it was determined that the growth of the resulting plants slowed down (Dalton et al., 2011). In another study on sugar beet, hairy roots were formed using *Agrobacterium tumefaciens* and electroporated with the pBI ABC plasmid harboring three bacterial genes, *phaA*, *phaB* and *phaC*, fused to the coding region of the plastid transit peptide of the small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco). Researchers have achieved PHB accumulation in root plastid cells. However, researchers claimed that the *phaA* gene negatively affects the molecular and physiological processes involved in shoot regeneration and causes growth retardation (Menzel et al., 2003).

In another study conducted in tobacco, a vector containing the *phaA*, *PhaB* and *phaC* genes under the control of the *psbA* promoter was transferred to plastids by biolistic bombardment. As a result of the study, they found that transplastomic tobacco plants grew slower than wild tobacco plants, but unlike other studies, the resulting T_0 and T_1 plants were fertile. In addition, they stated that if the homologous recombination sites of the vector can be located in more than one way at different locations in the plant plastome sequences, it leads to undesirable homologous recombination (Bohmert-Tatarev et al., 2011).

As mentioned above, a total of 5846 explants were subjected to biolistic bombardment throughout the study, and partial transfer was detected in only 10 of plant. The vast majority of regenerated plants consist of direct shoot regeneration. It is stated in the results section that most of the calluses do not show regeneration and face the problem of vitrification. This situation also made it difficult to perform DNA isolation and PCR screening in explants that remained as callus. Three of the 10 transplastomic sugar beet plants identified remained as callus and did not show shoot regeneration. This situation is similar to the study of Ivic and Smigocki (2001). The fact that growing sugar beet plants in tissue culture is a difficult process

and that there is no effective tissue culture protocol independent of the genotype effect specific to sugar beet has negatively affected our study. The fact that PCR studies could not be performed due to insufficient tissue amount of plants that could not be grown in tissue culture medium or whose development remained in the callus stage due to vitrification may indicate that transplastomic sugar beet plants could not be captured.

In another plastid transformation study on *Lesquerella fendleri*, they used the *adaA* gene for antibiotic selection. As a result of the analysis, the researchers revealed that many leaf explants produced shoot regeneration during the selection phase under the antibiotic spectinomycin but gave negative results in PCR screenings. Researchers explained that the reason for this situation is spontaneously occurring spectinomycin-resistant mutants. Point mutations in plastid *16S* rRNA frequently eliminate an *AatII* recognition sequence in the *rrn16* gene and prevent binding to spectinomycin (Skarjinskaia et al., 2003). In another plastid transformation study in canola plants, similar results were obtained (Hou et al., 2003). Considering our own study, the fact that only 10 partially transferred plants were obtained out of 516 regenerated plants suggests that there may be point mutations providing resistance to spectinomycin in sugar beet.

In another genetic transformation study conducted on sugar beet, they tried to transfer the *1-sst* gene to the protoplasts in the stomata guard cells of sugar beet using the PEG method. In the study, $1.6x10^6$ protoplast cells were used and 174 resistant calli were formed after selection. While 18 of these calluses showed shoot regeneration, only five of them could be grown. Although the size of the vector that was attempted to be transferred to sugar beet was 3.1 kb, verification studies reported that a portion of the vector was transferred into three samples (Sévenier et al., 1998). In our study, partial transfer of the pExpPN-phaA-SB vector was achieved in 10 sugar beet plants. These findings are similar to the study of Sevenier et al. (1998).

In another study conducted on sugar beet plants, they tried to transfer the pSB1-*bar* vector containing the bar gene into the chloroplast of sugar beet using the biolistic bombardment method. The pSB1-*bar* vector also contains the *adaA* and *GFP* genes. Researchers have stated that calluses formed after biolistic bombardment did not show regeneration when there was antibiotic pressure in the environment. This finding is consistent with our study. In addition, in the same

study, *bar* genes were not found in 3 of 7 callus that were *GFP* positive in PCR screening and determined as transplastomic. Although they detected *bar* genes in 4 callus in screening, they reported that 4 calli lost this gene after a while (De Marchis et. al., 2014). If we look at our study, we only detected the genes present in the vector for selection by PCR screening and we could not detect the *phaA* gene. These two studies are similar to each other.

According to the literature, there is only one research article showing that the *phaA* gene can be transferred to the chloroplast organelle by the biolistic method without any problems and without adversely affecting plant growth (Ruiz & Daniell, 2005). On the other hand, it has been shown in many research articles that when the *phaA* gene is transferred to the plant, this gene has a negative effect on plant growth (Bohmert et al., 2000; Bohmert et al., 2002; Lössl et al., 2003; Menzel et al., 2003; Bohmert-Tatarev et al., 2011; Dalton et al., 2011). It has been mentioned that the *phaA* gene is transferred to plants or that there is a negative effect on the growth of the plants after transfer and their response to tissue culture. In particular, Bohmert et al. (2002) stated that the *phaA* gene has a negative effect on genetic transformation.

In our study, more biolistic bombardment was carried out than planned. Despite this, in 5453 PCR screenings, only partial transfer of *adaA* and *ampR* genes was detected, but no plant containing all or part of the *phaA* gene was found. Therefore, the fact that the majority of the 5846 explants did not show regeneration may be because the potential plants containing the *phaA* gene could not grow or the transformation effect of the *phaA* gene may have been reduced and therefore could not be detected. However, despite all this, partial transfer of the pExpPN-phaA-SB vector was achieved, and transplastomic sugar beet plants were obtained.

5. CONCLUSION

Sugar beet, which alone provides 22% of the world's sugar needs, is a strategic product in terms of both economic and political aspects. Turkey ranks 5th in the world and 4th in Europe in terms of sugar beet production. In recent years, Turkey has been making serious investments to produce local and national sugar beet seeds.

In this study, it was aimed to obtain transplastomic sugar beet plants by using the pExpPN-phaA-SB vector developed within the scope of the TÜBİTAK project number 120O596 by using the biolistic bombardment method. As a result of 66 independent biolistic bombardments, a portion of the pExpPN-phaA-SB vector was transferred to 10 sugar beet plants and transplastomic plants were obtained. Our results allow us to speculate that the reason for the failure to detect the *phaA* gene is that its transformation efficiency is reduced or that it negatively affects plant growth. On the other hand, among the antibiotic concentrations tested, 50 mg/L spectinomycin concentration was found to be optimum. In addition, it has been determined that the long-term presence of antibiotics in the growth medium negatively affects the callus formation of explants and especially indirect regeneration. It has also been understood that antibiotic selection delays the growth of the plant. When all these results on antibiotic selection were evaluated, it became clear that antibiotic selection alone was not sufficient in sugar beet genetic transformation studies. It has been understood that the vitrification problem, which is one of the biggest difficulties encountered in sugar beet plants grown *in vitro*, can be minimized with MS medium containing 0.25 mg/L KIN hormone.

Within the scope of our research, the feasibility of chloroplast transformation in sugar beet was tested. The findings obtained are of great importance for the development of genetic engineering methods, plant breeding and biotechnological innovations. These findings will form a critical basis for new studies in the field of plant biotechnology and shed light on future research.

According to the results obtained in our study, we can summarize our recommendations as follows:

Plant genetic transformation studies are scientific studies that are intertwined with plant tissue culture and their success largely depends on the tissue culture protocol. Therefore, the creation of a tissue culture protocol that is specific

to sugar beet and can be effectively applied independently of genotype is very important for sugar beet genetic transformation studies. Therefore, an effective tissue culture protocol that works in sugar beet should be determined.

Antibiotic selection applied to sugar beet is not sufficient alone and negatively affects plant growth, as explained in both the results and discussion sections. Therefore, the presence of reporter genes such as *GUS*, *GFP*, *LUC* etc. in the expression vector can be used as an important tool for the early detection of possible transplastomic plants.

Chloroplast transformation is performed based on homologous recombination system. pExpPN-phaA-SB vector uses *trnA* and *trnI* region for homologous recombination. Testing and using other conserved regions found in sugar beet may increase the transformation percentage.

Considering the difficulty of directly transferring the *phaA* gene to the chloroplast and the fact that this method has been reported in only one publication in the literature it can be suggested that CMS plants can be obtained in future studies by transferring the *phaA* gene using the *Agrobacterium* method and ensuring that the protein produced by adding a polypeptide sequence to the vector affects the chloroplast.

REFERENCES

Adem, M., Beyene, D., & Feyissa, T. (2017). Recent achievements obtained by chloroplast transformation. Plant Methods, 13(1), 1–11. https://doi.org/10.1186/s13007-017-0179-1

Altpeter, F., Baisakh, N., Beachy, R., Bock, R., Capell, T., Christou, P., Daniell, H., Datta, K., Datta, S., Dix, P. J., Fauquet, C., Huang, N., Kohli, A., Mooibroek, H., Nicholson, L., Nguyen, T. T., Nugent, G., Raemakers, K., Romano, A., Visser, R. (2005). Particle bombardment and the genetic enhancement of crops: Myths and realities. Molecular Breeding, 15(3), 305–327. https://doi.org/10.1007/s11032-004-8001-y

Altpeter, F., Springer, N. M., Bartley, L. E., Blechl, A. E., Brutnell, T. P., Citovsky, V., Conrad, L. J., Gelvin, S. B., Jackson, D. P., Kausch, A. P., Lemaux, P. G., Medford, J. I., Orozco-Cárdenas, M. L., Tricoli, D. M., Van Eck, J., Voytas, D. F., Walbot, V., Wang, K., Zhang, Z. J., & Neal Stewart, C. (2016). Advancing crop transformation in the era of genome editing. Plant Cell, 28(7), 1510–1520. https://doi.org/10.1105/tpc.16.00196

Anjanappa, R. B., & Gruissem, W. (2021). Current progress and challenges in crop genetic transformation. Journal of Plant Physiology, 261, 153411. https://doi.org/10.1016/j.jplph.2021.153411

Arakawa, T., Matsunaga, M., Matsui, K., Itoh, K., Kuroda, Y., Matsuhira, H., Kitazaki, K., & Kubo, T. (2020). The molecular basis for allelic differences suggests Restorer-of-fertility 1 is a complex locus in sugar beet (*Beta vulgaris* L.). BMC Plant Biology, 20(1), 1–11. https://doi.org/10.1186/s12870-020-02721-9

Arumuganathan, K., & Earle, E. D. (1991). Nuclear DNA Content of Some Important Plant Species Nuclear DNA Content Material and Methods. Plant Molecular Biology Reporter, 9(3), 208–218.

Aşkın, B., & Küçüköner, E. (2020). Turkish journal of agriculture - food science and technology. Turkish Journal of Agriculture - Food Science and Technology, 8(11), 2300–2306. http://agrifoodscience.com/index.php/TURJAF/article/view/2298/1070

Barton, K. A., Binns, A. N., Matzke, A. J. M., & Chilton, M. D. (1983). Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. Cell, 32(4), 1033–1043. https://doi.org/10.1016/0092-8674(83)90288-X

Bartsch, D. (2010). Gene Flow in Sugar Beet. Sugar Tech, 12(3–4), 201–206. https://doi.org/10.1007/s12355-010-0053-1

Basso, M. F., Arraes, F. B. M., Grossi-de-Sa, M., Moreira, V. J. V., Alves-Ferreira, M., & Grossi-de-Sa, M. F. (2020). Insights Into Genetic and Molecular Elements for Transgenic Crop Development. Frontiers in Plant Science, 11(May), 1–24. https://doi.org/10.3389/fpls.2020.00509

Basso, M. F., da Cunha, B. A. D. B., Ribeiro, A. P., Martins, P. K., de Souza, W. R., de Oliveira, N. G., Nakayama, T. J., Augusto das Chagas Noqueli Casari, R., Santiago, T. R., Vinecky, F., Cançado, L. J., de Sousa, C. A. F., de Oliveira, P. A., de Souza, S. A. C. D., Cançado, G. M. de A., Kobayashi, A. K., & Molinari, H. B. C. (2017). Improved Genetic Transformation of Sugarcane (*Saccharum* spp.) Embryogenic Callus Mediated by *Agrobacterium tumefaciens*. Current Protocols in Plant Biology, 2(3), 221–239. https://doi.org/10.1002/cppb.20055

Biancardi, E., McGrath, J. M., Panella, L. W., Lewellen, R. T., & Stevanato, P. (2010a). Sugar Beet. In J. E. Bradshaw (Ed.), Molecular Breeding (Vol. 35, Issue 1, pp. 173–219). Springer New York. https://doi.org/10.1007/978-0-387-92765-7_6

Biancardi, E., McGrath, J. M., Panella, L. W., Lewellen, R. T., & Stevanato, P. (2010b). Sugar Beet. In J. Bradshaw (Ed.), Root and Tuber Crops. Handbook of Plant Breeding (7th ed., Vol.

35, Issue 1, pp. 173–219). Springer New York. https://doi.org/10.1007/978-0-387-92765-7_6

Bock, R. (2001). Transgenic plastids in basic research and plant biotechnology. Journal of Molecular Biology, 312(3), 425–438. https://doi.org/10.1006/jmbi.2001.4960

Bock, R. (2015). Engineering plastid genomes: Methods, tools, and applications in basic research and biotechnology. Annual Review of Plant Biology, 66, 211–241. https://doi.org/10.1146/annurev-arplant-050213-040212

Bohmert, K., Balbo, I., Kopka, J., Mittendorf, V., Nawrath, C., Poirier, Y., Tischendorf, G., Trethewey, R. N., & Willmitzer, L. (2000). Transgenic *Arabidopsis* plants can accumulate polyhydroxybutyrate to up to 4% of their fresh weight. Planta, 211(6), 841–845. https://doi.org/10.1007/s004250000350

Bohmert, K., Balbo, I., Steinbüchel, A., Tischendorf, G., & Willmitzer, L. (2002). Constitutive expression of the β-ketothiolase gene in transgenic plants. A major obstacle for obtaining polyhydroxybutyrate-producing plants. Plant Physiology, 128(4), 1282–1290. https://doi.org/10.1104/pp.010615

Bohmert-Tatarev, K., McAvoy, S., Daughtry, S., Peoples, O. P., & Snell, K. D. (2011). High levels of bioplastic are produced in fertile transplastomic tobacco plants engineered with a synthetic operon for the production of polyhydroxybutyrate. Plant Physiology, 155(4), 1690–1708. https://doi.org/10.1104/pp.110.169581

Bojović, R., Popović, V., Popović, D., Prodanović, R., Đukić, R., Bošković, J., Ćirić, M., & Filipović, V. (2024). Economical Sugar Beet Production: Biotechnological Advances to Improve Yield in Conditions of Abiotic and Biotic Stress. Sugar Tech, 0123456789. https://doi.org/10.1007/s12355-024-01461-6

Bosemark, N. O. (2007). Genetics and Breeding. In A.P. Draycott (Ed.), Sugar Beet (pp. 50–88). https://doi.org/10.1002/9780470751114.ch4

Boyhan, D., & Daniell, H. (2011). Low-cost production of proinsulin in tobacco and lettuce chloroplasts for injectable or oral delivery of functional insulin and C-peptide. Plant Biotechnology Journal, 9(5), 585–598. https://doi.org/10.1111/j.1467-7652.2010.00582.x

Boynton, J. E., Gillham, N. W., Harris, E. H., Hosler, J. P., Johnson, A. M., Jones, A. R., Randolph-Anderson, B. L., Robertson, D., Klein, T. M., Shark, K. B., & Sanford, J. C. (1988). Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science, 240(4858), 1534–1538. https://doi.org/10.1126/science.2897716

Bradford M. M. (1976.) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Brar, N. S., Dhillon, B. S., Saini, K. S., & Sharma, P. K. (2015). Agronomy of sugar beet cultivation-A review. Agricultural Reviews, 36(3), 184. https://doi.org/10.5958/0976-0741.2015.00022.7

Carrer, H., Hockenberry, T. N., Svab, Z., & Maliga, P. (1993). Kanamycin resistance as a selectable marker for plastid transformation in tobacco. MGG Molecular & General Genetics, 241(1–2), 49–56. https://doi.org/10.1007/BF00280200

Chandimali, N., Park, E. H., Bak, S. G., Lim, H. J., Won, Y. S., & Lee, S. J. (2024). Seaweed callus culture: A comprehensive review of current circumstances and future perspectives. Algal Research, 77. https://doi.org/10.1016/j.algal.2023.103376

Chase, C. D. (2007). Cytoplasmic male sterility: a window to the world of plant mitochondrial-nuclear interactions. Trends in Genetics, 23(2), 81–90. https://doi.org/10.1016/j.tig.2006.12.004

Chen, L., & Liu, Y. G. (2014). Male sterility and fertility restoration in crops. Annual Review of Plant Biology, 65(5), 579–606. https://doi.org/10.1146/annurev-arplant-050213-040119

Cheng, L., Li, H. P., Qu, B., Huang, T., Tu, J. X., Fu, T. D., & Liao, Y. C. (2010). Chloroplast transformation of rapeseed (*Brassica napus*) by particle bombardment of cotyledons. Plant Cell Reports, 29(4), 371–381. https://doi.org/10.1007/s00299-010-0828-6

Ćirić, M., Popović, V., Prodanović, S., Živanović, T., Ikanović, J., & Bajić, I. (2024). Sugar Beet: Perspectives for the Future. Sugar Tech, Biancardi 2010. https://doi.org/10.1007/s12355-024-01462-5

Clarke, J. L., & Daniell, H. (2011). Plastid biotechnology for crop production: Present status and future perspectives. Plant Molecular Biology, 76(3–5), 211–220. https://doi.org/10.1007/s11103-011-9767-z

Dalton, D. A., Ma, C., Shrestha, S., Kitin, P., & Strauss, S. H. (2011). Trade-offs between biomass growth and inducible biosynthesis of polyhydroxybutyrate in transgenic poplar. Plant Biotechnology Journal, 9(7), 759–767. https://doi.org/10.1111/j.1467-7652.2010.00585.x

Daniell, H., Datta, R., Varma, S., Gray, S., & Lee, S. B. (1998). Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nature Biotechnology, 16(4), 345–348. https://doi.org/10.1038/nbt0498-345

Daniell, H., Lin, C. S., Yu, M., & Chang, W. J. (2016). Chloroplast genomes: Diversity, evolution, and applications in genetic engineering. Genome Biology, 17(1), 1–29. https://doi.org/10.1186/s13059-016-1004-2

Daniell, H., Ruiz, G., Denes, B., Sandberg, L., & Langridge, W. (2009). Optimization of codon composition and regulatory elements for expression of human insulin like growth factor-1 in transgenic chloroplasts and evaluation of structural identity and function. BMC Biotechnology, 9. https://doi.org/10.1186/1472-6750-9-33

Day, A., & Goldschmidt-Clermont, M. (2011). The chloroplast transformation toolbox: Selectable markers and marker removal. Plant Biotechnology Journal, 9(5), 540–553. https://doi.org/10.1111/j.1467-7652.2011.00604.x

De Marchis, F., & Bellucci, M. (2021). Plastid transformation in sugar beet: an important industrial crop. Methods in Molecular Biology, 2317, 283–290. https://doi.org/10.1007/978-1-0716-1472-3_16

De Marchis, F., Wang, Y., Stevanato, P., Arcioni, S., & Bellucci, M. (2009). Genetic transformation of the sugar beet plastome. Transgenic Research, 18(1), 17–30. https://doi.org/10.1007/s11248-008-9193-4

Dobrogojski, J., Spychalski, M., Luciński, R., & Borek, S. (2018). Transgenic plants as a source of polyhydroxyalkanoates. Acta Physiologiae Plantarum, 40(9). https://doi.org/10.1007/s11738-018-2742-4

Draycott, A. P. (2007). Introduction. Sugar Beet, 1–8. https://doi.org/10.1002/9780470751114.ch1

Elliott, M. C., & Weston, G. D. (1993). Biology and physiology of the sugar-beet plant. In R. K. Cooke, D.A., Scott (Ed.), The Sugar Beet Crop (pp. 37–66). Springer Dordrecht. https://doi.org/10.1007/978-94-009-0373-9_2

Felix L. Sandell, Stralis-Pavese, N., McGrath, J. M., Schulz, B., Himmelbauer, H., & Dohm, J. C. (2022). Genomic distances reveal relationships of wild and cultivated beets. Nature Communications, 13(1), 1–13. https://doi.org/10.1038/s41467-022-29676-9

Filipenko, E. A., Sidorchuk, Y. V., Titov, I. I., Maltsev, V. P., & Deineko, E. V. (2011). Spontaneous spectinomycin resistance mutations detected after biolistic transformation of *Daucus carota* L. Physiology and Molecular Biology of Plants, 17(1), 79–86. https://doi.org/10.1007/s12298-011-0051-0

Finkenstadt, V. L. (2014). A Review on the Complete Utilization of the Sugar beet. Sugar Tech, 16(4), 339–346. https://doi.org/10.1007/s12355-013-0285-y

Food and Agriculture Organization of the United Nations. (2024). Sugar beet. https://www.fao.org/faostat/en/#data/QCL/visualize Retrieved from the address on 2024 November 15.

Food and Agriculture Organization of the United Nations. (2024). https://www.fao.org/faostat/en/#data/QCL/visualize Retrieved from the address on 2024 November 15.

Francis, S. A. (2007). Development of Sugar Beet. In A. P. Draycott (Ed.), Sugar Beet (pp. 9–29). https://doi.org/10.1002/9780470751114.ch2

Fu, X., Duc, L. T., Fontana, S., Bong, B. B., Tinjuangjun, P., Sudhakar, D., Twyman, R. M., Christou, P., & Kohli, A. (2000). Linear transgene constructs lacking vector backbone sequences generate low-copy-number transgenic plants with simple integration patterns. Transgenic Research, 9(1), 11–19. https://doi.org/10.1023/A:1008993730505

Galewski, P., & McGrath, J. M. (2020). Genetic diversity among cultivated beets (*Beta vulgaris*) assessed via population-based whole genome sequences. BMC Genomics, 21(1), 1–14. https://doi.org/10.1186/s12864-020-6451-1

Goldman, I. L., & Janick, J. (2021). Evolution of Root Morphology in Table Beet: Historical and Iconographic. Frontiers in Plant Science, 12(August), 1–13. https://doi.org/10.3389/fpls.2021.689926

Goldschmidt-clermont, M. (1991). Transgenic expression of aminoglycoside adenine transferase in the chloroplast: A selectable marker for site-directed transformation of *Chlamydomonas*. Nucleic Acids Research, 19(15), 4083–4089. https://doi.org/10.1093/nar/19.15.4083

Gosal, S. S., & Kang, M. S. (2012). Plant Tissue Culture and Genetic Transformation for Crop Improvement. Improving Crop Resistance to Abiotic Stress, 1, 357–397. https://doi.org/10.1002/9783527632930.ch16

Gould, S. B., Waller, R. F., & McFadden, G. I. (2008). Plastid evolution. Annual Review of Plant Biology, 59, 491–517. https://doi.org/10.1146/annurev.arplant.59.032607.092915

Gruska, R. M., Baryga, A., Kunicka-Styczyńska, A., Brzeziński, S., Rosicka-Kaczmarek, J., Miśkiewicz, K., & Sumińska, T. (2022). Fresh and Stored Sugar Beet Roots as a Source of Various Types of Mono- and Oligosaccharides. Molecules, 27(16). https://doi.org/10.3390/molecules27165125

Gurel, E., Gurel, S., & Lemaux, P. G. (2008). Biotechnology applications for sugar beet. Critical Reviews in Plant Sciences, 27(2), 108–140. https://doi.org/10.1080/07352680802202000

Gurel, S. (2021). Sand-Wounding of Shoot and Petiole Explants Enhances Transformation Efficiency in Sugar Beet (*Beta vulgaris* L.) by Agrobacterium-mediated Transformation. Sugar Tech, 23(2), 415–427. https://doi.org/10.1007/s12355-020-00893-0

Gürel, S., Oğuz, M. Ç., Turan, F., Kazan, K., Özmen, C. Y., Gürel, E., & Ergül, A. (2019). Utilization of sucrose during cocultivation positively affects agrobacterium-mediated transformation efficiency in sugar beet (*Beta vulgaris* L.). Turkish Journal of Agriculture and Forestry, 43(6), 509–517. https://doi.org/10.3906/tar-1812-90

Hagemann, R. (2007). The Sexual Inheritance of Plant Organelles. Molecular Biology and Biotechnology of Plant Organelles, 93–113. https://doi.org/10.1007/978-1-4020-3166-3_4

Hall, R. D., Riksen-Bruinsma, T., Weyens, G. J., Rosquin, I. J., Denys, P. N., Evans, I. J., Lathouwers, J. E., Lefèbvre, M. P., Dunwell, J. M., van Tunen, A., & Krens, F. A. (1996). A high efficiency technique for the generation of transgenic sugar beets from stomatal

guard cells. Nature Biotechnology, 14(9), 1133–1138. https://doi.org/10.1038/nbt0996-1133

Hall, R. D., Riksen-Bruinsma, T., Weyens, G., Lefèbvre, M., Dunwell, J. M., Van Tunen, A., & Krens, F. A. (1997). Sugar beet guard cell protoplasts demonstrate a remarkable capacity for cell division enabling applications in stomatal physiology and molecular breeding. Journal of Experimental Botany, 48(307), 255–263. https://doi.org/10.1093/jxb/48.2.255

Hanson, M. R., Gray, B. N., & Ahner, B. A. (2013). Chloroplast transformation for engineering of photosynthesis. Journal of Experimental Botany, 64(3), 731–742. https://doi.org/10.1093/jxb/ers325

Harlow, E., Lane, D. 1988. "Antibodies, a laboratory manual", Cold Spring Harbor Laboratory.

Havey, M. J. (2007). The Use of Cytoplasmic Male Sterility for Hybrid Seed Production. Molecular Biology and Biotechnology of Plant Organelles, 1943, 623–634. https://doi.org/10.1007/978-1-4020-3166-3_23

Hisano, H., Kimoto, Y., Hayakawa, H., Takeichi, J., Domae, T., Hashimoto, R., Abe, J., Asano, S., Kanazawa, A., & Shimamoto, Y. (2004). High frequency *Agrobacterium*-mediated transformation and plant regeneration via direct shoot formation from leaf explants in Beta vulgaris and Beta maritima. Plant Cell Reports, 22(12), 910–918. https://doi.org/10.1007/s00299-004-0773-3

Hou, B. K., Zhou, Y. H., Wan, L. H., Zhang, Z. L., Shen, G. F., Chen, Z. H., & Hu, Z. M. (2003). Chloroplast transformation in oilseed rape. Transgenic Research, 12(1), 111–114. https://doi.org/10.1023/A:1022180315462

Houmiel, K. L., Slater, S., Broyles, D., Casagrande, L., Colburn, S., Gonzalez, K., Mitsky, T. A., Reiser, S. E., Shah, D., Taylor, N. B., Tran, M., Valentin, H. E., & Gruys, K. J. (1999). Poly(β-hydroxybutyrate) production in oilseed leukoplasts of *Brassica napus*. Planta, 209(4), 547–550. https://doi.org/10.1007/s004250050760

Huang, F. C., Klaus, S., Herz, S., Zou, Z., Koop, H. U., & Golds, T. (2002). Efficient plastid transformation in tobacco using the aphA-6 gene and kanamycin selection. Molecular Genetics and Genomics, 268(1), 19–27. https://doi.org/10.1007/s00438-002-0738-6

Ivic, S. D., & Smigocki, A. C. (2003). Transformation of sugar beet cell suspension cultures. In Vitro Cellular and Developmental Biology - Plant, 39(6), 573–577. https://doi.org/10.1079/IVP2003433

Ivic, SD., & Smigocki, AC. (2001). Evaluation of the biolistic transformation method for commercially important sugar beet breeding lines. In: Proc 31st Meet Am Soc Sugar Beet Technol, Denver,p 20.

Ivic-Haymes, S. D., & Smigocki, A. C. (2005). Biolistic transformation of highly regenerative sugar beet (*Beta vulgaris* L.) leaves. Plant Cell Reports, 23(10–11), 699–704. https://doi.org/10.1007/s00299-004-0873-0

Kaffka, S. R., & Grantz, D. A. (2014). Sugar Crops. Encyclopedia of Agriculture and Food Systems, 5, 240–260. https://doi.org/10.1016/B978-0-444-52512-3.00150-9

Kagami, H., Taguchi, K., Arakawa, T., Kuroda, Y., Tamagake, H., & Kubo, T. (2016). Efficient callus formation and plant regeneration are heritable characters in sugar beet (*Beta vulgaris* L.). Hereditas, 153, 12. https://doi.org/10.1186/s41065-016-0015-z

Kang, T. J., Seo, J. E., Loc, N. H., & Yang, M. S. (2003). Herbicide resistance of tobacco chloroplasts expressing the bar gene. Molecules and Cells, 16(1), 60–66. https://doi.org/10.1016/s1016-8478(23)13766-6

Karaağaç, 0., & Balkaya., A. (2009). Sebzelerde erkek kisirliği mekanizmasi da yararlanılarak F_1 hibrit tohum üretimi. Anadolu Tarım Bilimleri Dergisi, 24(2), 114–123. https://doi.org/doi.org/10.7161/anajas.2009.24.2.114-123

Kawaguchi, R., & Bailey-Serres, J. (2005). mRNA sequence features that contribute to translational regulation in *Arabidopsis*. Nucleic Acids Research, 33(3), 955–965. https://doi.org/10.1093/nar/gki240

Keshavareddy, G., Kumar, A. R. V., & S. Ramu, V. (2018). Methods of Plant Transformation- A Review. International Journal of Current Microbiology and Applied Sciences, 7(07), 2656–2668. https://doi.org/10.20546/ijcmas.2018.707.312

Kikkert, J. R., Vidal, J. R., & Reisch, B. I. (2005). Stable transformation of plant cells by particle bombardment/biolistics. Methods in Molecular Biology (Clifton, N.J.), 286, 61–78. https://doi.org/10.1385/1-59259-827-7:061

Kishchenko, E. M., Komarnitskii, I. K., & Kuchuk, N. V. (2005). Production of transgenetic sugar beet (*Beta vulgaris* L.) plants resistant to phosphinothricin. Cell Biology International, 29(1), 15–19. https://doi.org/10.1016/j.cellbi.2004.11.003

Kong, F., Zhao, H., Liu, W., Li, N., & Mao, Y. (2017). Construction of Plastid Expression Vector and Development of Genetic Transformation System for the Seaweed *Pyropia yezoensis*. Marine Biotechnology, 19(2), 147–156. https://doi.org/10.1007/s10126-017-9736-x

Koya, V., & Daniell, H. (2005). OBPC symposium: Maize 2004 & beyond - Recent advances in chloroplast genetic engineering. In Vitro Cellular and Developmental Biology - Plant, 41(4), 388–404. https://doi.org/10.1079/IVP2005660

Kumar, A. U., & Ling, A. P. K. (2021). Gene introduction approaches in chloroplast transformation and its applications. Journal of Genetic Engineering and Biotechnology, 19(1). https://doi.org/10.1186/s43141-021-00255-7

Kumar, S., Dhingra, A., & Daniell, H. (2004). Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. Plant Molecular Biology, 56(2), 203–216. https://doi.org/10.1007/s11103-004-2907-y

Kusnetsov, V. V. (2018). Chloroplasts: Structure and Expression of the Plastid Genome. Russian Journal of Plant Physiology, 65(4), 465–476. https://doi.org/10.1134/S1021443718030044

Lange, W., Brandenburg, W. A., & De Bock, T. S. M. (1999). Taxonomy and cultonomy of beet (*Beta vulgaris* L.). Botanical Journal of the Linnean Society, 130(1), 81–96. https://doi.org/10.1006/bojl.1998.0250

Leister, D. (2003). Chloroplast research in the genomic age. Trends in Genetics, 19(1), 47–56. https://doi.org/10.1016/S0168-9525(02)00003-3

Lelivelt, C. L. C., McCabe, M. S., Newell, C. A., De Snoo, C. B., Van Dun, K. M. P., Birch-Machin, I., Gray, J. C., Mills, K. H. G., & Nugent, J. M. (2005). Stable plastid transformation in lettuce (Lactuca sativa L.). Plant Molecular Biology, 58(6), 763–774. https://doi.org/10.1007/s11103-005-7704-8

Lin, C., Zhang, C., Zhao, H., Xing, S., Wang, Y., Liu, X., Yuan, C., Zhao, L., & Dong, Y. (2014). Sequencing of the chloroplast genomes of cytoplasmic male-sterile and male-fertile lines of soybean and identification of polymorphic markers. Plant Science, 229, 208–214. https://doi.org/10.1016/j.plantsci.2014.09.005

Liu, C. W., Lin, C. C., Chen, J. J. W., & Tseng, M. J. (2007). Stable chloroplast transformation in cabbage (*Brassica oleracea* L. var. *capitata* L.) by particle bombardment. Plant Cell Reports, 26(10), 1733–1744. https://doi.org/10.1007/s00299-007-0374-z

Long, Y., Yang, Y., Pan, G., & Shen, Y. (2022). New Insights into Tissue Culture Plant-Regeneration Mechanisms. Frontiers in Plant Science, 13. https://doi.org/10.3389/fpls.2022.926752

Lössl, A., Eibl, C., Harloff, H. J., Jung, C., & Koop, H. U. (2003). Polyester synthesis in transplastomic tobacco (*Nicotiana tabacum* L.): Significant contents of polyhydroxybutyrate are associated with growth reduction. Plant Cell Reports, 21(9), 891–899. https://doi.org/10.1007/s00299-003-0610-0

Loyola-Vargas, V. M., & Ochoa-Alejo, N. (2018). An Introduction to Plant Tissue Culture: Advances and Perspectives. Methods in Molecular Biology, 1815, 3–13. https://doi.org/10.1007/978-1-4939-8594-4_1

Lutz, K. A., Azhagiri, A. K., Tungsuchat-Huang, T., & Maliga, P. (2007). A guide to choosing vectors for transformation of the plastid genome of higher plants. Plant Physiology, 145(4), 1201–1210. https://doi.org/10.1104/pp.107.106963

Maliga, P. (2004). Plastid transformation in higher plants. Annual Review of Plant Biology, 55, 289–313. https://doi.org/10.1146/annurev.arplant.55.031903.141633

Martin, W., Rujan, T., Richly, E., Hansen, A., Cornelsen, S., Lins, T., Leister, D., Stoebe, B., Hasegawa, M., & Penny, D. (2002). Evolutionary analysis of Arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. Proceedings of the National Academy of Sciences of the United States of America, 99(19), 12246–12251. https://doi.org/10.1073/pnas.182432999

Mazuś, B., Krysiak, C., & Buchowicz, J. (2000). Tungsten particle-induced nicking of supercoiled plasmid DNA. Plasmid, 44(1), 89–93. https://doi.org/10.1006/plas.2000.1475

McBride, K. E., Svab, Z., Schaaf, D. J., Hogan, P. S., Stalker, D. M., & Maliga, P. (1995). Amplification of a chimeric bacillus gene in chloroplasts leads to an extraordinary level of insecticidal protein in tobacco. Bio/Technology, 13(4), 362–365. https://doi.org/10.1038/nbt0495-362

McElroy, D., Zhang, W., Cao, J., & Wu, R. (1990). Isolation of an efficient actin promoter for use in rice transformation. Plant Cell, 2(2), 163–171. https://doi.org/10.1105/tpc.2.2.163

McGrath, J. M., Funk, A., Galewski, P., Ou, S., Townsend, B., Davenport, K., Daligault, H., Johnson, S., Lee, J., Hastie, A., Darracq, A., Willems, G., Barnes, S., Liachko, I., Sullivan, S., Koren, S., Phillippy, A., Wang, J., Liu, T., Dorn, K. M. (2023). A contiguous de novo genome assembly of sugar beet EL10 (*Beta vulgaris* L.). DNA Research, 30(1), 1–14. https://doi.org/10.1093/dnares/dsac033

Menzel, G., Harloff, H. J., & Jung, C. (2003). Expression of bacterial poly(3-hydroxybutyrate) synthesis genes in hairy roots of sugar beet (*Beta vulgaris* L.). Applied Microbiology and Biotechnology, 60(5), 571–576. https://doi.org/10.1007/s00253-002-1152-z

Mikami, T., P. Yamamoto, M., Matsuhira, H., Kitazaki, K., & Kubo, T. (2011). Molecular basis of cytoplasmic male sterility in beets: An overview. Plant Genetic Resources: Characterisation and Utilisation, 9(2), 284–287. https://doi.org/10.1017/S1479262111000177

Mishutkina, Y. V., & Gaponenko, A. K. (2006). Sugar beet (*Beta vulgaris* L.) morphogenesis in vitro: Effects of phytohormone type and concentration in the culture medium, type of explants, and plant genotype on shoot regeneration frequency. Russian Journal of Genetics, 42(2), 150–157. https://doi.org/10.1134/S1022795406020086

Mitchell Mcgrath, J., & Panella, L. (2019). Sugar Beet Breeding. Plant Breeding Reviews, 42, 167–218. https://doi.org/10.1002/9781119521358.ch5

Moore, P. H. (2016). Sugarcane and Sugar beet. In Encyclopedia of Applied Plant Sciences (Second Edi, Vol. 3, Issue November 2013). Elsevier. https://doi.org/10.1016/B978-0-12-394807-6.00007-1

Mujtaba, M. A., Muk Cho, H., Masjuki, H. H., Kalam, M. A., Ong, H. C., Gul, M., Harith, M. H., & Yusoff, M. N. A. M. (2020). Critical review on sesame seed oil and its methyl ester on cold flow and oxidation stability. Energy Reports, 6, 40–54. https://doi.org/10.1016/j.egyr.2019.11.160

Mukherjee, E. (2023). Genetic Transformation in Sugar Beet (*Beta vulgaris* L.): Technologies and Applications. Sugar Tech, 25(2), 269–281. https://doi.org/10.1007/s12355-022-01176-6

Mukherjee, E., & Gantait, S. (2023). Genetic Transformation in Sugar Beet (*Beta vulgaris* L.): Technologies and Applications. Sugar Tech, 25(2), 269–281. https://doi.org/10.1007/s12355-022-01176-6

Murashige, T., & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum, 15(3), 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nakashita, H., Arai, Y., Shikanai, T., Yoshiharu, D., & Yamaguchi, I. (2001). Introduction of bacterial metabolism into higher plants by polycistronic transgene expression. Bioscience, Biotechnology and Biochemistry, 65(7), 1688–1691. https://doi.org/10.1271/bbb.65.1688

Nawrath, C., Poirier, Y., & Somerville, C. (1994). Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. Proceedings of the National Academy of Sciences of the United States of America, 91(26), 12760–12764. https://doi.org/10.1073/pnas.91.26.12760

Okumura, S., Sawada, M., Park, Y. W., Hayashi, T., Shimamura, M., Takase, H., & Tomizawa, K. I. (2006). Transformation of poplar (*Populus alba*) plastids and expression of foreign proteins in tree chloroplasts. Transgenic Research, 15(5), 637–646. https://doi.org/10.1007/s11248-006-9009-3

Örgeç, M., Verma, S. K., Şahin, G., Zencirci, N., & Gürel, E. (2021). *In vitro* tissue culture protocol of ancient einkorn (*Triticum monococcum* ssp. *monococum*) wheat via indirect shoot regeneration. In Vitro Cellular and Developmental Biology - Plant, 57(1), 143–151. https://doi.org/10.1007/s11627-020-10122-8

Ozyigit, I. I., & Yucebilgili Kurtoglu, K. (2020). Particle bombardment technology and its applications in plants. Molecular Biology Reports, 47(12), 9831–9847. https://doi.org/10.1007/s11033-020-06001-5

Pathak, A. D., Kapur, R., Solomon, S., Kumar, R., Srivastava, S., & Singh, P. R. (2014). Sugar Beet: A Historical Perspective in Indian Context. Sugar Tech, 16(2), 125–132. https://doi.org/10.1007/s12355-014-0304-7

Petrillo, C. P., Carneiro, N. P., Purcino, A. Á. C., Carvalho, C. H. S., Alves, J. D., & Carneiro, A. A. (2008). Optimization of particle bombardment parameters for the genetic transformation of Brazilian maize inbred lines. Pesquisa Agropecuaria Brasileira, 43(3), 371–378. https://doi.org/10.1590/S0100-204X2008000300012

Poirier, Y., Dennis, D. E., Klomparens, K., & Somerville, C. (1992). Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. Science, 256(5056), 520–523. https://doi.org/10.1126/science.256.5056.520

Purwantoro, A., Purwestri, Y. A., Lawrie, M. D., & Semiarti, E. (2022). Genetic transformation via plant tissue culture techniques: Current and future approaches. Advances in Plant Tissue Culture: Current Developments and Future Trends, 131–156. https://doi.org/10.1016/B978-0-323-90795-8.00001-1

Rana, A. K., Gupta, V. K., Newbold, J., Roberts, D., Rees, R. M., Krishnamurthy, S., & Thakur, V. K. (2022). Sugar beet pulp: Resurgence and trailblazing journey towards a circular bioeconomy. Fuel, 312. https://doi.org/10.1016/j.fuel.2021.122953

Rasco-Gaunt, S., Riley, A., Barcelo, P., & Lazzeri, P. A. (1999). Analysis of particle bombardment parameters to optimise DNA delivery into wheat tissues. Plant Cell Reports, 19(2), 118–127. https://doi.org/10.1007/s002990050721

Rascón-Cruz, Q., González-Barriga, C. D., Iglesias-Figueroa, B. F., Trejo-Muñoz, J. C., Siqueiros-Cendón, T., Sinagawa-García, S. R., Arévalo-Gallegos, S., & Espinoza-Sánchez, E. A. (2021). Plastid transformation: Advances and challenges for its implementation in agricultural crops. Electronic Journal of Biotechnology, 51, 95–109. https://doi.org/10.1016/j.ejbt.2021.03.005

Reed, M. L., Wilson, S. K., Sutton, C. A., & Hanson, M. R. (2001). High-level expression of a synthetic red-shifted GFP coding region incorporated into transgenic chloroplasts. Plant Journal, 27(3), 257–265. https://doi.org/10.1046/j.1365-313X.2001.01088.x

Rivera, A. L., Gómez-Lim, M., Fernández, F., & Loske, A. M. (2012). Physical methods for genetic plant transformation. Physics of Life Reviews, 9(3), 308–345. https://doi.org/10.1016/j.plrev.2012.06.002

Rommens, C. M. (2004). All-native DNA transformation: A new approach to plant genetic engineering. Trends in Plant Science, 9(9), 457–464. https://doi.org/10.1016/j.tplants.2004.07.001

Ruf, S., Hermann, M., Berger, I. J., Carrer, H., & Bock, R. (2001). Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. Nature Biotechnology, 19(9), 870–875. https://doi.org/10.1038/nbt0901-870

Ruiz, O. N., & Daniell, H. (2005). Engineering cytoplasmic male sterility via the chloroplast genome by expression of β-ketothiolase. Plant Physiology, 138(3), 1232–1246. https://doi.org/10.1104/pp.104.057729

Sadzık, G. (2024). Cytoplasmic male sterility in plants with special emphasis on sugar beet. Folia Biologica et Oecologica 18:, 18, 142–147.

Saeed, T., & Shahzad, A. (2015). Basic principles behind genetic transformation in plants. Biotechnological Strategies for the Conservation of Medicinal and Ornamental Climbers, 327–350. https://doi.org/10.1007/978-3-319-19288-8_13

Scotti, N., Rigano, M. M., & Cardi, T. (2012). Production of foreign proteins using plastid transformation. Biotechnology Advances, 30(2), 387–397. https://doi.org/10.1016/j.biotechadv.2011.07.019

Sehgal, D., & Khan, T. (2020). Plant Tissue Culture: Beyond Being a Tool for Genetic Engineering. Environmental Microbiology and Biotechnology: Volume 1, Biovalorization of Solid Wastes and Wastewater Treatment, 1, 175–200. https://doi.org/10.1007/978-981-15-6021-7_9

Sévenier, R., Hall, R. D., Van der Meer, I. M., Hakkert, H. J. C., Van Tunen, A. J., & Koops, A. J. (1998). High level fructan accumulation in a transgenic sugar beet. Nature Biotechnology, 16(9), 843–846. https://doi.org/10.1038/nbt0998-843

Sharma, K. K., Bhatnagar-Mathur, P., & Thorpe, T. A. (2005). Genetic transformation technology: Status and problems. In Vitro Cellular and Developmental Biology - Plant, 41(2), 102–112. https://doi.org/10.1079/IVP2004618

Sidorenko, L. V., Lee, T. F., Woosley, A., Moskal, W. A., Bevan, S. A., Merlo, P. A. O., Walsh, T. A., Wang, X., Weaver, S., Glancy, T. P., Wang, P., Yang, X., Sriram, S., & Meyers, B. C. (2017). GC-rich coding sequences reduce transposon-like, small RNA-mediated transgene silencing. Nature Plants, 3(11), 875–884. https://doi.org/10.1038/s41477-017-0040-6

Sidorov, V. A., Kasten, D., Pang, S. Z., Hajdukiewicz, P. T. J., Staub, J. M., & Nehra, N. S. (1999). Stable chloroplast transformation in potato: Use of green fluorescent protein as a plastid marker. Plant Journal, 19(2), 209–216. https://doi.org/10.1046/j.1365-313X.1999.00508.x

Skarjinskaia, M., Svab, Z., & Maliga, P. (2003). Plastid transformation in Lesquerella fendleri, an oilseed *Brassicacea*. Transgenic Research, 12(1), 115–122. https://doi.org/10.1023/A:1022110402302

Snyder, G. W., Ingersoll, J. C., Smigocki, A. C., & Owens, L. D. (1999). Introduction of pathogen defense genes and a cytokinin biosynthesis gene into sugar beet (*Beta vulgaris* L.) by Agrobacterium or particle bombardment. Plant Cell Reports, 18(10), 829–834. https://doi.org/10.1007/s002990050669

Sofi, P. A., Rather, A. G., & Wani, S. A. (2007). Genetic and molecular basis of cytoplasmic male sterility in maize. Communications in Biometry and Crop Science, 2(1), 49–60.

Staub, J. M., & Maliga, P. (1992). Long regions of homologous DNA are incorporated into the tobacco plastid genome by transformation. Plant Cell, 4(1), 39–45. https://doi.org/10.1105/tpc.4.1.39

Staub, J. M., & Maliga, P. (1993). Accumulation of D1 polypeptide in tobacco plastids is regulated via the untranslated region of the psbA mRNA. The EMBO Journal, 12(2), 601–606. https://doi.org/10.1002/j.1460-2075.1993.tb05692.x

Stevanato, P., Chiodi, C., Broccanello, C., Concheri, G., Biancardi, E., Pavli, O., & Skaracis, G. (2019). Sustainability of the Sugar Beet Crop. Sugar Tech, 21(5), 703–716. https://doi.org/10.1007/s12355-019-00734-9

Su, W., Xu, M., Radani, Y., & Yang, L. (2023). Technological Development and Application of Plant Genetic Transformation. International Journal of Molecular Sciences, 24(13). https://doi.org/10.3390/ijms241310646

Subrahmanyeswari, T., & Gantait, S. (2022). Advancements and prospectives of sugar beet (*Beta vulgaris* L.) biotechnology. Applied Microbiology and Biotechnology, 106(22), 7417–7430. https://doi.org/10.1007/s00253-022-12226-0

Svab, Z., & Maliga, P. A. L. (1993). High-frequency plastid transformation in tobacco. Genetics, 90(February), 913–917.

Svab, Z., Hajdukiewicz, P., & Maliga, P. (1990). Stable transformation of plastids in higher plants. Proceedings of the National Academy of Sciences of the United States of America, 87(21), 8526–8530. https://doi.org/10.1073/pnas.87.21.8526

Swamy, B. N., Hedau, N. K., Chaudhari, G. V., Kant, L., & Pattanayak, A. (2017). CMS system and its stimulation in hybrid seed production of *Capsicum annuum* L. Scientia Horticulturae, 222(May), 175–179. https://doi.org/10.1016/j.scienta.2017.05.023

Tayyab, M., Wakeel, A., Mubarak, M. U., Artyszak, A., Ali, S., Hakki, E. E., Mahmood, K., Song, B., & Ishfaq, M. (2023a). Sugar Beet Cultivation in the Tropics and Subtropics : Challenges and Sugar Beet Cultivation in the Tropics and Subtropics : Challenges and Opportunities. April. https://doi.org/10.3390/agronomy13051213

Tayyab, M., Wakeel, A., Mubarak, M. U., Artyszak, A., Ali, S., Hakki, E. E., Mahmood, K., Song, B., & Ishfaq, M. (2023b). Sugar Beet Cultivation in the Tropics and Subtropics: Challenges and Opportunities. Agronomy, 13(5). https://doi.org/10.3390/agronomy13051213

Tissot, G., Canard, H., Nadai, M., Martone, A., Botterman, J., & Dubald, M. (2008). Translocation of aprotinin, a therapeutic protease inhibitor, into the thylakoid lumen of genetically engineered tobacco chloroplasts. Plant Biotechnology Journal, 6(3), 309–320. https://doi.org/10.1111/j.1467-7652.2008.00321.x

Tomita, K. I., Hiura, S., & Tamagake, H. (2013). Evaluation of the potential for somatic embryogenesis in sugar beet (*Beta vulgaris* L.) breeding lines and improvement of regeneration efficiency. Plant Biotechnology, 30(5), 479–487. https://doi.org/10.5511/plantbiotechnology.13.0816a

Türkiye İstatistik Kurumu. (2024). https://data.tuik.gov.tr/Bulten/Index?p=Bitkisel-Uretim-Istatistikleri-2024-53447 Retrieved from the address on 2024 December 29.

Twyman, R. M., & Christou, P. (2004). Plant Transformation Technology: Particle Bombardment. Handbook of Plant Biotechnology. https://doi.org/10.1002/0470869143.kc015

Valkov, V. T., Gargano, D., Manna, C., Formisano, G., Dix, P. J., Gray, J. C., Scotti, N., & Cardi, T. (2011). High efficiency plastid transformation in potato and regulation of transgene expression in leaves and tubers by alternative 5′ and 3′ regulatory sequences. Transgenic Research, 20(1), 137–151. https://doi.org/10.1007/s11248-010-9402-9

van Wijk, K. J., & Baginsky, S. (2011). Plastid proteomics in higher plants: Current state and future goals. Plant Physiology, 155(4), 1578–1588. https://doi.org/10.1104/pp.111.172932

Venkatesh, J., & Park, S. W. (2012). Plastid genetic engineering in Solanaceae. Protoplasma, 249(4), 981–999. https://doi.org/10.1007/s00709-012-0391-9

Verma, D., & Daniell, H. (2007). Chloroplast vector systems for biotechnology applications. Plant Physiology, 145(4), 1129–1143. https://doi.org/10.1104/pp.107.106690

Verma, D., Samson, N. P., Koya, V., & Daniell, H. (2008). A protocol for expression of foreign genes in chloroplasts. Nature Protocols, 3(4), 739–758. https://doi.org/10.1038/nprot.2007.522

Wang, L., Zhang, Z., Han, P., Liang, Y., Zhang, H., Fu, Z., Zhao, S., Yuanyuan, E., Zhang, H., Wu, X., Zhang, B., Chang, Y., Tang, K., Zheng, W., Chen, L., Wang, R., Gao, W., Hasi, A., Li, X., & Bai, C. (2023). Association analysis of agronomic traits and construction of genetic networks by resequencing of 306 sugar beet (*Beta vulgaris* L.) lines. Scientific Reports, 13(1), 1–15. https://doi.org/10.1038/s41598-023-42182-2

Wilson, D. N. (2014). Ribosome-targeting antibiotics and mechanisms of bacterial resistance. Nature Reviews Microbiology, 12(1), 35–48. https://doi.org/10.1038/nrmicro3155

Winner, C. (1993). History of the crop. In D. A. Cooke & R. K. Scott (Eds.), The Sugar Beet Crop (World Crop, pp. 1–35). Springer Dordrecht. https://doi.org/10.1007/978-94-009-0373-9_1

Wirmer, J., & Westhof, E. (2006). Molecular Contacts Between Antibiotics and the 30S Ribosomal Particle. Methods in Enzymology, 415, 180–202. https://doi.org/10.1016/S0076-6879(06)15012-0

Yancheva, S., & Kondakova, V. (2018). Plant Tissue Culture Technology: Present and Future Development. Reference Series in Phytochemistry, 39–63. https://doi.org/10.1007/978-3-319-54600-1_16

Yu, Y., Yu, P. C., Chang, W. J., Yu, K., & Lin, C. S. (2020). Plastid transformation: How does it work? can it be applied to crops? what can it offer? International Journal of Molecular Sciences, 21(14), 1–21. https://doi.org/10.3390/ijms21144854

Yue, F., Cui, L., dePamphilis, C. W., Moret, B. M. E., & Tang, J. (2008). Gene rearrangement analysis and ancestral order inference from chloroplast genomes with inverted repeat. BMC Genomics, 9(SUPPL. 1). https://doi.org/10.1186/1471-2164-9-S1-S25

Zicari, S., Zhang, R., & Kaffka, S. (2019). Sugar Beet. In S. Z. Zhongli Pan, Ruihong Zhang (Ed.), Integrated Processing Technologies for Food and Agricultural By-Products (pp. 331–351). Elsevier Inc. https://doi.org/10.1016/B978-0-12-814138-0.00013-7

Zorrilla-López, U., Masip, G., Arjó, G., Bai, C., Banakar, R., Bassie, L., Berman, J., Farré, G., Miralpeix, B., Pérez-Massot, E., Sabalza, M., Sanahuja, G., Vamvaka, E., Twyman, R. M., Christou, P., Zhu, C., & Capell, T. (2013). Engineering metabolic pathways in plants by multigene transformation. International Journal of Developmental Biology, 57(6–8), 565–576. https://doi.org/10.1387/ijdb.130162pc

Zoubenko, O. V., Allison, L. A., Svab, Z., & Maliga, P. (1994). Efficient targeting of foreign genes into the tobacco plastid genome. Nucleic Acids Research, 22(19), 3819–3824. https://doi.org/10.1093/nar/22.19.3819

Zubko, M. K., & Day, A. (1998). Stable albinism induced without mutagenesis: A model for ribosome-free plastid inheritance. Plant Journal, 15(2), 265–271. https://doi.org/10.1046/j.1365-313X.1998.00195.x

APPENDICES

APPENDIX 1 Genomic gDNA samples with purity and DNA concentrations measured.

Sample No	gDNA Purity	gDNA Con. (ng)	Amount of gDNA to be taken (µl)	Last Con. (ng/µl)	Final Volume (µl)	Amount of NFW to be taken (µl)
1	1,932	2004	2,5	100	50	47,5
2	1,884	1319	3,8	100	50	46,2
3	1,967	1914	2,6	100	50	47,4
4	1,895	1971	2,5	100	50	47,5
5	1,617	3357	1,5	100	50	48,5
6	2,039	1279	3,9	100	50	46,1
7	1,835	3262	1,5	100	50	48,5
8	2,098	1068	4,7	100	50	45,3
9	1,893	1421	3,5	100	50	46,5
10	2,134	1223	4,1	100	50	45,9
11	2,116	1065	4,7	100	50	45,3
12	2,052	1448	3,5	100	50	46,5
13	1,864	1272	3,9	100	50	46,1
14	1,934	1069	4,7	100	50	45,3
15	2,135	2221	2,3	100	50	47,7
16	2,019	3067	1,6	100	50	48,4
17	2,019	2526	2,0	100	50	48,0
18	1,674	552,6	9,0	100	50	41,0
19	2,163	2337	2,1	100	50	47,9
20	2,174	2120	2,4	100	50	47,6
21	2,068	358,9	13,9	100	50	36,1
22	2,073	1173	4,3	100	50	45,7
23	2,114	2291	2,2	100	50	47,8
24	2,052	1211	4,1	100	50	45,9
25	2,093	688,1	7,3	100	50	42,7
26	2,092	1492	3,4	100	50	46,6
27	2,004	1317	3,8	100	50	46,2
28	2,014	3075	1,6	100	50	48,4
29	2,173	1968	2,5	100	50	47,5
30	1,928	821,6	6,1	100	50	43,9
31	2,130	2149	2,3	100	50	47,7
32	2,029	568,8	8,8	100	50	41,2
33	2,104	2337	2,1	100	50	47,9
34	2,021	1497	3,3	100	50	46,7
35	2,081	890,7	5,6	100	50	44,4
36	2,029	369,6	13,5	100	50	36,5
37	1,921	2534	2,0	100	50	48,0
38	1,962	1084	4,6	100	50	45,4
39	1,933	1404	3,6	100	50	46,4
40	2,018	2347	2,1	100	50	47,9

APPENDIX 1 (Continued)

Sample No	gDNA Purity	gDNA Con. (ng)	Amount of gDNA to be taken (µl)	Last Con. (ng/µl)	Final Volume (µl)	Amount of NFW to be taken (µl)

41	2,044	1476	3,4	100	50	46,6
42	1,890	1419	3,5	100	50	46,5
43	1,843	1505	3,3	100	50	46,7
44	1,980	2131	2,3	100	50	47,7
45	1,921	983,1	5,1	100	50	44,9
46	1,914	2699	1,9	100	50	48,1
47	1,860	835,8	6,0	100	50	44,0
48	1,914	1370	3,6	100	50	46,4
49	2,033	1699	2,9	100	50	47,1
50	1,975	2300	2,2	100	50	47,8
51	2,046	1964	2,5	100	50	47,5
52	2,004	1184	4,2	100	50	45,8
53	1,913	1510	3,3	100	50	46,7
54	1,998	1393	3,6	100	50	46,4
55	2,005	872	5,7	100	50	44,3
56	1,884	1638	3,1	100	50	46,9
57	1,909	1132	4,4	100	50	45,6
58	2,036	1136	4,4	100	50	45,6
59	1,995	1252	4,0	100	50	46,0
60	1,886	158,4	31,6	100	50	18,4
61	2,052	2020	2,5	100	50	47,5
62	1,954	74,51	53,7	100	40	-13,7
63	2,049	313,8	15,9	100	50	34,1
64	2,117	772,4	6,5	100	50	43,5
65	1,963	1217	4,1	100	50	45,9
66	1,845	1301	3,8	100	50	46,2
67	1,990	1792	2,8	100	50	47,2
68	2,030	1375	3,6	100	50	46,4
69	2,090	1348	3,7	100	50	46,3
70	2,116	1533	3,3	100	50	46,7
71	2,028	666,1	7,5	100	50	42,5
72	2,139	2176	2,3	100	50	47,7
73	2,049	200,1	25,0	100	50	25,0
74	2,181	1371	3,6	100	50	46,4
75	2,139	1088	4,6	100	50	45,4
76	2,083	587	8,5	100	50	41,5
77	2,079	777,1	6,4	100	50	43,6
78	1,994	1098	4,6	100	50	45,4
79	1,344	3401	1,5	100	50	48,5
80	1,956	1102	4,5	100	50	45,5
81	2,086	2441	2,0	100	50	48,0
82	1,954	1823	2,7	100	50	47,3

APPENDIX 1 (Continued)

Sample No	gDNA Purity	gDNA Con. (ng)	Amount of gDNA to be taken (µl)	Last Con. (ng/µl)	Final Volume (µl)	Amount of NFW to be taken (µl)

83	1,635	3335	1,5	100	50	48,5
84	1,074	3392	1,5	100	50	48,5
85	1,952	1476	3,4	100	50	46,6
86	2,120	1529	3,3	100	50	46,7
87	2,108	1382	3,6	100	50	46,4
88	2,006	2326	2,1	100	50	47,9
89	1,987	2687	1,9	100	50	48,1
90	1,970	1714	2,9	100	50	47,1
91	2,024	939,9	5,3	100	50	44,7
92	2,078	2323	2,2	100	50	47,8
93	1,990	2224	2,2	100	50	47,8
94	1,095	3389	1,5	100	50	48,5
95	1,717	3437	1,5	100	50	48,5
96	1,992	2957	1,7	100	50	48,3
97	2,052	1341	3,7	100	50	46,3
98	1,744	3262	1,5	100	50	48,5
99	1,890	2043	2,4	100	50	47,6
100	1,949	1291	3,9	100	50	46,1
101	1,249	3385	1,5	100	50	48,5
102	2,075	1921	2,6	100	50	47,4
103	1,780	1482	3,4	100	50	46,6
104	2,044	2296	2,2	100	50	47,8
105	2,127	2627	1,9	100	50	48,1
106	2,111	1321	3,8	100	50	46,2
107	2,104	2398	2,1	100	50	47,9
108	1,937	184	27,2	100	50	22,8
109	2,151	965,1	5,2	100	50	44,8
110	2,085	845,1	5,9	100	50	44,1
111	1,990	1866	2,7	100	50	47,3
112	2,045	1877	2,7	100	50	47,3
113	2,082	1231	4,1	100	50	45,9
114	2,017	1427	3,5	100	50	46,5
115	1,947	844,7	5,9	100	50	44,1
116	2,050	938,4	5,3	100	50	44,7
117	2,080	1279	3,9	100	50	46,1
118	2,093	1606	3,1	100	50	46,9
119	2,151	1997	2,5	100	50	47,5
120	2,121	2168	2,3	100	50	47,7
121	2,036	2488	2,0	100	50	48,0
122	1,474	3390	1,5	100	50	48,5
123	1,946	2703	1,8	100	50	48,2
124	2,067	2376	2,1	100	50	47,9

APPENDIX 1 (Continued)

Sample No	gDNA Purity	gDNA Con. (ng)	Amount of gDNA to be taken (µl)	Last Con. (ng/µl)	Final Volume (µl)	Amount of NFW to be taken (µl)

125	2,106	2179	2,3	100	50	47,7
126	1,987	1664	3,0	100	50	47,0
127	2,123	1063	4,7	100	50	45,3
128	2,087	876,4	5,7	100	50	44,3
129	2,093	802,7	6,2	100	50	43,8
130	2,043	2620	1,9	100	50	48,1
131	2,088	2589	1,9	100	50	48,1
132	1,934	3148	1,6	100	50	48,4
133	2,081	2569	1,9	100	50	48,1
134	2,224	1248	4,0	100	50	46,0
135	2,126	1863	2,7	100	50	47,3
136	2,077	1265	4,0	100	50	46,0
137	2,139	1455	3,4	100	50	46,6
138	2,123	782,3	6,4	100	50	43,6
139	1,965	1084	4,6	100	50	45,4
140	4,227	9118	0,4	100	40	39,6
141	2,123	1721	2,9	100	50	47,1
142	2,119	1532	3,3	100	50	46,7
143	2,115	2162	2,3	100	50	47,7
144	2,072	1485	3,4	100	50	46,6
145	2,107	1358	3,7	100	50	46,3
146	2,082	716,2	7,0	100	50	43,0
147	1,980	2410	2,1	100	50	47,9
148	2,042	2897	1,7	100	50	48,3
149	2,017	3040	1,6	100	50	48,4
150	2,108	1607	3,1	100	50	46,9
151	2,135	1039	4,8	100	50	45,2
152	2,033	1672	3,0	100	50	47,0
153	2,901	1215	4,1	100	50	45,9
154	1,503	1427	3,5	100	50	46,5
155	1,961	3369	1,5	100	50	48,5
156	2,075	567,8	8,8	100	50	41,2
157	1,942	1416	3,5	100	50	46,5
158	1,731	1625	3,1	100	50	46,9
159	2,085	792,6	6,3	100	50	43,7
160	2,144	1894	2,6	100	50	47,4
161	2,020	2082	2,4	100	50	47,6
162	2,074	830,9	6,0	100	50	44,0
163	2,087	1424	3,5	100	50	46,5
164	2,039	1423	3,5	100	50	46,5
165	1,917	996,7	5,0	100	50	45,0
166	2,086	1130	4,4	100	50	45,6

APPENDIX 1 (Continued)

Sample No	gDNA Purity	gDNA Con. (ng)	Amount of gDNA to be taken (µl)	Last Con. (ng/µl)	Final Volume (µl)	Amount of NFW to be taken (µl)

167	2,029	632,1	7,9	100	50	42,1
168	1,916	2060	2,4	100	50	47,6
169	2,093	855,5	5,8	100	50	44,2
170	2,061	1246	4,0	100	50	46,0
171	2,029	254,4	19,7	100	50	30,3
172	2,118	1364	3,7	100	50	46,3
173	2,011	2882	1,7	100	50	48,3
174	2,046	1593	3,1	100	50	46,9
175	2,158	990,9	5,0	100	50	45,0
176	2,039	1322	3,8	100	50	46,2
177	1,934	1834	2,7	100	50	47,3
178	2,058	794,3	6,3	100	50	43,7
179	1,856	1747	2,9	100	50	47,1
180	1,863	2224	2,2	100	50	47,8
181	1,741	3287	1,5	100	50	48,5
182	2,029	1379	3,6	100	50	46,4
183	2,008	1649	3,0	100	50	47,0
184	2,055	2671	1,9	100	50	48,1
185	1,801	3205	1,6	100	50	48,4
186	2,049	1360	3,7	100	50	46,3
187	2,074	2546	2,0	100	50	48,0
188	2,125	847	5,9	100	50	44,1
189	1,944	2954	1,7	100	50	48,3
190	2,407	2448	2,0	100	50	48,0
191	1,764	3083	1,6	100	50	48,4
192	2,136	1002	5,0	100	50	45,0
193	1,127	1644	3,0	100	50	47,0
194	1,928	3043	1,6	100	50	48,4
195	2,093	1026	4,9	100	50	45,1
196	2,053	1660	3,0	100	50	47,0
197	2,160	1053	4,7	100	50	45,3
198	2,102	1027	4,9	100	50	45,1
199	1,892	1321	3,8	100	50	46,2
200	2,014	936,5	5,3	100	50	44,7
201	2,018	381,8	13,1	100	50	36,9
202	2,124	576,1	8,7	100	50	41,3
203	2,018	1620	3,1	100	50	46,9
204	2,001	526,3	9,5	100	50	40,5
205	1,872	1687	3,0	100	50	47,0
206	2,023	1208	4,1	100	50	45,9
207	2,078	1568	3,2	100	50	46,8
208	2,072	1015	4,9	100	50	45,1

APPENDIX 1 (Continued)

Sample No	gDNA Purity	gDNA Con. (ng)	Amount of gDNA to be taken (µl)	Last Con. (ng/µl)	Final Volume (µl)	Amount of NFW to be taken (µl)
209	1,744	591,8	8,4	100	50	41,6

210	1,999	1658	3,0	100	50	47,0
211	1,722	646,2	7,7	100	50	42,3
212	1,985	1776	2,8	100	50	47,2
213	2,083	1680	3,0	100	50	47,0
214	1,965	1686	3,0	100	50	47,0
215	2,040	1200	4,2	100	50	45,8
216	2,022	1738	2,9	100	50	47,1
217	2,089	834	6,0	100	50	44,0
218	1,939	588,1	8,5	100	50	41,5
219	2,075	942,7	5,3	100	50	44,7
220	1,091	1897	2,6	100	50	47,4
221	1,079	2057	2,4	100	50	47,6
222	1,436	2021	2,5	100	50	47,5
223	1,946	1796	2,8	100	50	47,2
224	2,007	1802	2,8	100	50	47,2
225	2,133	1176	4,3	100	50	45,7
226	1,524	2007	2,5	100	50	47,5
227	1,991	1029	4,9	100	50	45,1
228	1,686	2005	2,5	100	50	47,5
229	2,026	974,7	5,1	100	50	44,9
230	1,910	185,2	27,0	100	50	23,0
231	1,214	2047	2,4	100	50	47,6
232	2,135	642,5	7,8	100	50	42,2
233	2,033	1002	5,0	100	50	45,0
234	2,059	1685	3,0	100	50	47,0
235	1,970	561,5	8,9	100	50	41,1
236	1,485	2030	2,5	100	50	47,5
237	1,424	2018	2,5	100	50	47,5
238	1,992	1004	5,0	100	50	45,0
239	1,945	1855	2,7	100	50	47,3
240	1,458	2052	2,4	100	50	47,6
241	2,007	1680	3,0	100	50	47,0
242	1,804	1945	2,6	100	50	47,4
243	1,862	1923	2,6	100	50	47,4
244	1,902	1832	2,7	100	50	47,3
245	1,682	1743	2,9	100	50	47,1
246	1,700	1459	3,4	100	50	46,6
247	2,014	1150	4,3	100	50	45,7
248	2,003	1493	3,3	100	50	46,7
249	1,993	855,4	5,8	100	50	44,2
250	1,585	1996	2,5	100	50	47,5

APPENDIX 1 (Continued)

Sample No	gDNA Purity	gDNA Con. (ng)	Amount of gDNA to be taken (µl)	Last Con. (ng/µl)	Final Volume (µl)	Amount of NFW to be taken (µl)
251	1,963	1250	4,0	100	50	46,0

252	2,065	1126	4,4	100	50	45,6
253	1,969	408,9	12,2	100	50	37,8
254	2,005	1816	2,8	100	50	47,2
255	2,115	1235	4,0	100	50	46,0
256	1,911	857,6	5,8	100	50	44,2
257	1,992	1046	4,8	100	50	45,2
258	1,721	1893	2,6	100	50	47,4
259	1,718	1731	2,9	100	50	47,1
260	2,017	1491	3,4	100	50	46,6
261	1,401	2019	2,5	100	50	47,5
262	1,668	1978	2,5	100	50	47,5
263	1,263	2026	2,5	100	50	47,5
264	2,022	1371	3,6	100	50	46,4
265	2,039	1242	4,0	100	50	46,0
266	1,845	1906	2,6	100	50	47,4
267	1,884	1495	3,3	100	50	46,7
268	2,071	1079	4,6	100	50	45,4
269	1,905	1812	2,8	100	50	47,2
270	2,081	997	5,0	100	50	45,0
271	1,972	1305	3,8	100	50	46,2
272	2,031	1008	5,0	100	50	45,0
273	1,985	1093	4,6	100	50	45,4
274	1,732	1805	2,8	100	50	47,2
275	2,006	1728	2,9	100	50	47,1
276	2,006	1204	4,2	100	50	45,8
277	1,915	312	16,0	100	50	34,0
278	1,925	1409	3,5	100	50	46,5
279	1,862	1411	3,5	100	50	46,5
280	2,039	1191	4,2	100	50	45,8
281	2,038	836,7	6,0	100	50	44,0
282	2,001	967,1	5,2	100	50	44,8
283	1,914	1760	2,8	100	50	47,2
284	1,858	1930	2,6	100	50	47,4
285	1,686	1984	2,5	100	50	47,5
286	1,627	1992	2,5	100	50	47,5
287	1,878	1689	3,0	100	50	47,0
288	1,977	1812	2,8	100	50	47,2
289	1,567	1991	2,5	100	50	47,5
290	1,999	1714	2,9	100	50	47,1
291	1,653	1990	2,5	100	50	47,5
292	1,968	1720	2,9	100	50	47,1

APPENDIX 1 (Continued)

Sample No	gDNA Purity	gDNA Con. (ng)	Amount of gDNA to be taken (µl)	Last Con. (ng/µl)	Final Volume (µl)	Amount of NFW to be taken (µl)
293	1,855	1912	2,6	100	50	47,4

294	2,112	1080	4,6	100	50	45,4
295	2,056	953	5,2	100	50	44,8
296	2,033	1298	3,9	100	50	46,1
297	2,042	1182	4,2	100	50	45,8
298	1,981	1951	2,6	100	50	47,4
299	1,804	1802	2,8	100	50	47,2
300	2,017	1780	2,8	100	50	47,2
301	1,980	478,2	10,5	100	50	39,5
302	2,050	1634	3,1	100	50	46,9
303	1,489	2015	2,5	100	50	47,5
304	2,094	2347	2,1	100	50	47,9
305	2,110	1270	3,9	100	50	46,1
306	2,098	2676	1,9	100	50	48,1
307	2,217	1895	2,6	100	50	47,4
308	2,127	847,5	5,9	100	50	44,1
309	1,995	3117	1,6	100	50	48,4
310	1,296	3134	1,6	100	50	48,4
311	1,090	3361	1,5	100	50	48,5
312	2,182	2624	1,9	100	50	48,1
313	2,067	3077	1,6	100	50	48,4
314	2,087	894,9	5,6	100	50	44,4
315	1,028	3387	1,5	100	50	48,5
316	2,030	1445	3,5	100	50	46,5
317	1,840	3215	1,6	100	50	48,4
318	2,135	2403	2,1	100	50	47,9
319	2,225	798,5	6,3	100	50	43,7
320	1,725	283,1	17,7	100	50	32,3
321	2,211	991,88	5,0	100	50	45,0
322	2,170	579,9	8,6	100	50	41,4
323	2,061	700,4	7,1	100	50	42,9
324	2,139	1604	3,1	100	50	46,9
325	2,180	1298	3,9	100	50	46,1
326	1,648	3259	1,5	100	50	48,5
327	1,210	3395	1,5	100	50	48,5
328	1,316	3354	1,5	100	50	48,5
329	1,050	3405	1,5	100	50	48,5
330	1,091	3420	1,5	100	50	48,5
331	1,405	3347	1,5	100	50	48,5
332	1,092	3396	1,5	100	50	48,5
333	1,082	3408	1,5	100	50	48,5
334	1,779	3169	1,6	100	50	48,4

APPENDIX 1 (Continued)

Sample No	gDNA Purity	gDNA Con. (ng)	Amount of gDNA to be taken (µl)	Last Con. (ng/µl)	Final Volume (µl)	Amount of NFW to be taken (µl)
335	1,309	3400	1,5	100	50	48,5

336	2,130	2727	1,8	100	50	48,2
337	1,121	3226	1,5	100	50	48,5
338	2,093	1709	2,9	100	50	47,1
339	1,608	3358	1,5	100	50	48,5
340	1,989	3380	1,5	100	50	48,5
341	2,113	1601	3,1	100	50	46,9
342	1,619	3326	1,5	100	50	48,5
343	1,505	3354	1,5	100	50	48,5
344	1,699	3283	1,5	100	50	48,5
345	1,865	3162	1,6	100	50	48,4
346	1,966	2764	1,8	100	50	48,2
347	1,956	2937	1,7	100	50	48,3
348	1,716	3282	1,5	100	50	48,5
349	2,041	2500	2,0	100	50	48,0
350	1,934	2568	1,9	100	50	48,1
351	2,100	2172	2,3	100	50	47,7
352	2,215	374,3	13,4	100	50	36,6
353	1,879	2160	2,3	100	50	47,7
354	1,138	3435	1,5	100	50	48,5
355	2,044	2173	2,3	100	50	47,7
356	1,634	3037	1,6	100	50	48,4
357	1,997	1039	4,8	100	50	45,2
358	2,022	1163	4,3	100	50	45,7
359	1,164	3370	1,5	100	50	48,5
360	1,032	3442	1,5	100	50	48,5
361	2,067	1121	4,5	100	50	45,5
362	1,035	3377	1,5	100	50	48,5
363	1,869	3224	1,6	100	50	48,4
364	1,831	1186	4,2	100	50	45,8
365	2,007	1097	4,6	100	50	45,4
366	1,671	3306	1,5	100	50	48,5
367	2,038	1730	2,9	100	50	47,1
368	1,570	3342	1,5	100	50	48,5
369	1,890	2462	2,0	100	50	48,0
370	1,370	3382	1,5	100	50	48,5
371	1,949	2733	1,8	100	50	48,2
372	1,600	3038	1,6	100	50	48,4
373	1,048	3409	1,5	100	50	48,5
374	1,687	3296	1,5	100	50	48,5
375	2,031	1992	2,5	100	50	47,5
376	1,954	958,5	5,2	100	50	44,8

APPENDIX 1 (Continued)

Sample No	gDNA Purity	gDNA Con. (ng)	Amount of gDNA to be taken (µl)	Last Con. (ng/µl)	Final Volume (µl)	Amount of NFW to be taken (µl)
377	1,899	3026	1,7	100	50	48,3

378	1,275	3414	1,5	100	50	48,5
379	1,523	3324	1,5	100	50	48,5
380	1,938	3061	1,6	100	50	48,4
381	2,124	1001	5,0	100	50	45,0
382	1,840	3198	1,6	100	50	48,4
383	2,060	2383	2,1	100	50	47,9
384	1,100	3371	1,5	100	50	48,5
385	1,049	3291	1,5	100	50	48,5
386	2,068	1706	2,9	100	50	47,1
387	2,088	320,2	15,6	100	50	34,4
388	1,944	2219	2,3	100	50	47,7
389	2,050	2584	1,9	100	50	48,1
390	2,119	1464	3,4	100	50	46,6
391	1,700	3304	1,5	100	50	48,5
392	2,157	1821	2,7	100	50	47,3
393	2,089	1007	5,0	100	50	45,0
394	2,117	1728	2,9	100	50	47,1
395	2,210	779,1	6,4	100	50	43,6
396	2,027	777,5	6,4	100	50	43,6
397	2,208	1115	4,5	100	50	45,5
398	1,995	1601	3,1	100	50	46,9
399	2,157	742	6,7	100	50	43,3
400	1,851	3212	1,6	100	50	48,4
401	2,120	731,5	6,8	100	50	43,2
402	1,445	3035	1,6	100	50	48,4
403	2,020	1185	4,2	100	50	45,8
404	1,285	3414	1,5	100	50	48,5
405	1,036	3432	1,5	100	50	48,5
406	1,413	3398	1,5	100	50	48,5
407	1,880	2797	1,8	100	50	48,2
408	2,079	1896	2,6	100	50	47,4
409	1,981	1279	3,9	100	50	46,1
410	1,352	3396	1,5	100	50	48,5
411	2,095	1475	3,4	100	50	46,6
412	1,845	3228	1,5	100	50	48,5
413	2,072	1656	3,0	100	50	47,0
414	2,013	978,6	5,1	100	50	44,9
415	2,027	2690	1,9	100	50	48,1
416	1,859	3110	1,6	100	50	48,4
417	1,855	3220	1,6	100	50	48,4
418	2,060	2445	2,0	100	50	48,0

APPENDIX 1 (Continued)

Sample No	gDNA Purity	gDNA Con. (ng)	Amount of gDNA to be taken (µl)	Last Con. (ng/µl)	Final Volume (µl)	Amount of NFW to be taken (µl)

419	1,494	3332	1,5	100	50	48,5
420	2,030	1733	2,9	100	50	47,1
421	1,876	2891	1,7	100	50	48,3
422	1,842	3066	1,6	100	50	48,4
423	2,012	2202	2,3	100	50	47,7
424	2,128	1292	3,9	100	50	46,1
425	1,987	2356	2,1	100	50	47,9
426	1,414	3397	1,5	100	50	48,5
427	2,085	1446	3,5	100	50	46,5
428	1,324	3373	1,5	100	50	48,5
429	2,029	2728	1,8	100	50	48,2
430	1,825	3212	1,6	100	50	48,4
431	1,342	3425	1,5	100	50	48,5
432	1,864	3046	1,6	100	50	48,4
433	2,082	2226	2,2	100	50	47,8
434	1,619	3363	1,5	100	50	48,5
435	1,855	3100	1,6	100	50	48,4
436	1,900	3088	1,6	100	50	48,4
437	2,143	1037	4,8	100	50	45,2
438	2,092	730,1	6,8	100	50	43,2
439	2,079	2339	2,1	100	50	47,9
440	2,100	1807	2,8	100	50	47,2
441	1,883	3192	1,6	100	50	48,4
442	2,099	2258	2,2	100	50	47,8
443	1,798	1091	4,6	100	50	45,4
444	2,044	800	6,3	100	50	43,8
445	2,172	726,5	6,9	100	50	43,1
446	2,084	1893	2,6	100	50	47,4
447	1,998	103,6	29,0	100	30	1,0
448	2,201	441,7	11,3	100	50	38,7
449	2,159	707,5	7,1	100	50	42,9
450	1,903	318,3	15,7	100	50	34,3
451	2,149	724,4	6,9	100	50	43,1
452	2,087	1873	2,7	100	50	47,3
453	2,114	2564	2,0	100	50	48,0
454	2,037	1081	4,6	100	50	45,4
455	1,365	403,36	12,4	100	50	37,6
456	2,192	357,5	14,0	100	50	36,0
457	2,171	1067	4,7	100	50	45,3
458	2,107	2156	2,3	100	50	47,7
459	2,181	820	6,1	100	50	43,9
460	2,137	500	10,0	100	50	40,0

APPENDIX 1 (Continued)

Sample No	gDNA Purity	gDNA Con. (ng)	Amount of gDNA to be taken (µl)	Last Con. (ng/µl)	Final Volume (µl)	Amount of NFW to be taken (µl)
461	2,128	354,5	14,1	100	50	35,9

462	2,026	1001	5,0	100	50	45,0
463	1,923	68	73,5	100	50	-23,5
464	1,818	1289	3,9	100	50	46,1
465	2,132	1396	3,6	100	50	46,4
466	2,285	378,5	13,2	100	50	36,8
467	2,025	679	7,4	100	50	42,6
468	1,801	2566	1,9	100	50	48,1
469	1,837	3357	1,5	100	50	48,5
470	1,965	1490	3,4	100	50	46,6
471	2,169	577,5	8,7	100	50	41,3
472	2,205	865,5	5,8	100	50	44,2
473	1,968	2708	1,8	100	50	48,2
474	2,151	894,8	5,6	100	50	44,4
475	2,035	1465	3,4	100	50	46,6
476	2,083	1343	3,7	100	50	46,3
477	2,081	1352	3,7	100	50	46,3
478	2,100	1035	4,8	100	50	45,2
479	2,126	1764	2,8	100	50	47,2
480	2,103	168,2	29,7	100	50	20,3
481	1,913	1890	2,6	100	50	47,4
482	2,132	1019	4,9	100	50	45,1
483	2,137	1693	3,0	100	50	47,0
484	2,155	541,2	9,2	100	50	40,8
485	2,202	900,4	5,6	100	50	44,4
486	2,061	1275	3,9	100	50	46,1
487	2,206	1239	4,0	100	50	46,0
488	1,979	3134	1,6	100	50	48,4
489	2,084	1249	4,0	100	50	46,0
490	2,150	755,7	6,6	100	50	43,4
491	1,970	807,3	6,2	100	50	43,8
492	1,988	2906	1,7	100	50	48,3
493	2,060	825,7	6,1	100	50	43,9
494	2,043	2596	1,9	100	50	48,1
495	2,100	834,2	6,0	100	50	44,0
496	2,149	292,2	17,1	100	50	32,9
497	1,953	2904	1,7	100	50	48,3
498	2,130	528,2	9,5	100	50	40,5
499	1,925	1339	3,7	100	50	46,3
500	1,904	1978	2,5	100	50	47,5
501	2,088	534,2	9,4	100	50	40,6
502	2,025	516,2	9,7	100	50	40,3

APPENDIX 1 (Continued)

Sample No	gDNA Purity	gDNA Con. (ng)	Amount of gDNA to be taken (µl)	Last Con. (ng/µl)	Final Volume (µl)	Amount of NFW to be taken (µl)
503	1,929	643,5	7,8	100	50	42,2

504	2,186	831,5	6,0	100	50	44,0
505	1,802	203,7	24,5	100	50	25,5
506	2,050	380,9	13,1	100	50	36,9
507	1,970	558,1	9,0	100	50	41,0
508	2,076	1164	4,3	100	50	45,7
509	1,874	804,2	6,2	100	50	43,8
510	2,094	313,5	15,9	100	50	34,1
511	1,886	451,3	11,1	100	50	38,9
512	1,843	1997	2,5	100	50	47,5
513	2,122	595,7	8,4	100	50	41,6
514	2,235	905,7	5,5	100	50	44,5
515	1,872	569,2	8,8	100	50	41,2
516	2,211	685,5	7,3	100	50	42,7