

**T.C.
AKDENİZ UNIVERSITY**



**CLONING TWO TOMATO PATHOGENESIS-RELATED-1 (*SIPR*-1) GENES
AND GENERATING VECTORS FOR THEIR HETEROLOGOUS
EXPRESSION**

Kübra YILDIZ

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY

MASTER THESIS

APRIL 2024

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This thesis was unanimously accepted by the jury on 01/04/2024.

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ÖZET

İKİ ADET DOMATES PATOJEN İLİŞKİLİ-1 (*SIPR-1*) GENİN KLONLANMASI VE HETEROLOG İFADELERİNİN BELİRLENMESİ İÇİN FARKLI VEKTÖRLERİN OLUŞTURULMASI

Kübra YILDIZ

Yüksek Lisans Tezi, Tarımsal Biyoteknoloji Anabilim Dalı

Danışman: Doç. Dr. M. Aydın AKBUDAK

Nisan 2024; 60 sayfa

Dünya nüfusunun hızlı bir şekilde artması ve meydana gelen iklimsel değişiklikler, tarımsal ürün kalitesini ve verimini düşürmektedir. Bunun neden olduğu beslenme bozuklukları veya yetersiz beslenme gibi sorunlar, bitkilerin maruz kaldığı stres koşulları karşısında geliştirdikleri mekanizmaların detaylı olarak araştırılmasını elzem kılmıştır. Bu mekanizmalarla birlikte stres yanıtında görev alan genlerin tanımlanması ve karakterize edilmesi büyük önem taşımaktadır. Bitki patojen ilişkili proteinler, bitki savunma mekanizmasının temel bileşenlerinden biridir. Biyotik stres yanıtlarına ek olarak abiyotik stres koşulları altında da ifadelerinin arttığı yapılan çalışmalar ile ortaya konmuştur. Ancak, abiyotik stres unsurlarına karşı oynadıkları rollerin henüz belirlenememiş olması literatürde büyük bir eksikliğe neden olmaktadır. Bundan yola çıkarak bu tez çalışmasında, kuraklık stresi altında ifadelerinin arttığı rapor edilen *SIPR-1.1* ve *SIPR-1.2* genlerinin heterolog ifadelerinin belirlenmesine zemin hazırlamak için farklı vektörler hazırlanmıştır. Genlerin ayrı ayrı pIPKb004 bitki ifade vektörüne klonlanması Gateway klonlama yöntemi ile yürütülürken; iki genin birlikte pGFPGUSPlus bitki ifade vektörüne klonlanması geleneksel klonlama yöntemi ile gerçekleştirilmiştir. Genlerin belirlenen vektörler içindeki varlığı PCR ve kesim reaksiyonları ile doğrulanmıştır. Elde edilen bitki ifade vektörleri, belirlenen iki genin ayrı ayrı ve birlikte ifadelenmesi sonucu özellikle abiyotik stresler karşısında oluşturacakları stres yanıtlarının tespit edilmesine olanak sağlayacaktır.

ANAHTAR KELİMELER: Abiyotik Stres, Biyotik Stres, Gateway Klonlama, Geleneksel Klonlama, Gen Klonlama, Kuraklık, Patojen İlişkili Genler, Vektör

JÜRİ: Doç. Dr. M. Aydın AKBUDAK

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ABSTRACT

CLONING TWO TOMATO PATHOGENESIS-RELATED-1 (*SIPR-1*) GENES AND GENERATING VECTORS FOR THEIR HETEROLOGOUS EXPRESSION

Kübra YILDIZ

MSc Thesis in Department Of Agricultural Biotechnology

Supervisor: Assoc. Prof. Dr. M. Aydın AKBUDAK

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The rapid increase in the world population and the occurrence of climate changes are diminishing the quality and productivity of agricultural products. Issues such as nutritional disorders or inadequate nutrition arising from these factors make it imperative to thoroughly investigate the mechanisms developed by plants in response to stress conditions. The identification and characterization of genes involved in stress response, along with these mechanisms, are of great importance. Pathogenesis-related proteins are fundamental components of plant defense mechanisms. Studies have revealed that these proteins not only respond to biotic stress but also exhibit increased expression under abiotic stress conditions. However, the roles they play in response to abiotic stress factors have not yet been fully determined, leading to a significant gap in the literature. Based on this, in this thesis study, different vectors have been prepared to facilitate the determination of the heterologous expressions of *SIPR-1.1* and *SIPR-1.2* genes, reported to be upregulated under drought stress. While the cloning of genes into the pIPKb004 plant expression vector was conducted using the Gateway cloning method individually, the cloning of the two genes into the pGFPUSPlus plant expression vector was carried out using traditional cloning methods. The presence of genes within the designated vectors was confirmed through PCR and restriction digestion reactions. The obtained plant expression vectors will enable the detection of stress responses, especially under abiotic stresses, resulting from the separate and combined expression of the two genes.

KEYWORDS: Abiotic Stress, Biotic Stress, Drought, Gateway Cloning, Gene Cloning, Pathogenesis-Related Genes, Traditional Cloning, Vector

COMMITTEE: Assoc. Prof. Dr. M. Aydın AKBUDAK

Assoc. Prof. Dr. Aysun ÖZÇELİK

Assoc. Prof. Dr. Sertan ÇEVİK

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AKADEMİK BEYAN

Yüksek Lisans Tezi olarak sunduğum “Cloning Two Tomato Pathogenesis-Related-1 (*SlPR-1*) Genes And Generating Vectors For Their Heterologous Expression” adlı bu çalışmanın, akademik kurallar ve etik değerlere uygun olarak yazıldığını belirtir, bu tez çalışmasında bana ait olmayan tüm bilgilerin kaynağını gösterdiğimi beyan ederim.

01/04/2024

Kübra YILDIZ



LIST OF SYMBOLS AND ABBREVIATIONS

Symbols

% : percent

µg : micro gram

µl : micro liter

bp : base pair

g : gram

mg : milligram

min : minutes

ml : milliliter

nm : nanometer

s : second

V : Volt

dH₂O : distilled water

ng : nanogram

rpm : revolutions per minute

°C : centigrade degree

. : decimal point

Abbreviations

DNA : Deoxyribonucleic acid

RNA : Ribonucleic acid

cDNA : Complementary DNA

gDNA : Genomic DNA

PR : Pathogenesis-related

PCR : Polymerase chain reaction

MS : Murashige & Skoog

CRISP : Cysteine-rich secretory protein

CAP : Cysteine-rich secretory protein, antigen 5, and pathogenesis-related 1

SCP : Sperm coating protein

CTAB : Cetyltrimethyl Ammonium Bromide

LB : Luria-Bertani

S.O.C : Super Optimal broth with Catabolite repression

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1. INTRODUCTION

Nowadays, there are several factors that exert pressure on agricultural activities. The substantial increase in the global population, coupled with the continual decrease in cultivable agricultural land and the occurrence of climate changes, constitutes the major reasons affecting agricultural production (Myers et al., 2017). According to recent data, approximately 720-811 million people suffer from inadequate nutrition, and around 2.3 billion people are reported to be affected by nutritional disorders (World Health, 2021). Therefore, it is crucial to increase studies focusing on environmental stress conditions affecting plant growth and development. Simultaneously, investigating and comprehending the mechanisms that plants develop in response to these stresses are essential topics of concern.

Plants employ various mechanisms to combat environmental threats. Developing mechanisms to sustain vital activities under adverse conditions or adopting specific growth habits to avoid stress conditions are among the various strategies that plants have evolved (Singhal et al., 2016). Additionally, hundreds of genes and their products respond to biotic and abiotic stresses at transcriptional and translational levels (Cushman & Bohnert, 2000). Understanding these mechanisms or strategies devised by plants is of great importance to protect significant agricultural outputs such as yield and quality. Today, molecular biology studies, along with morphological, physiological, and biochemical methods, facilitate the comprehensive analysis of various stresses (Roca Paixão et al., 2019; Liu et al., 2019). Moreover, significant progress has been made through functional genetics and modern genetic applications, identifying and characterizing genes that play roles under various stress conditions (Wani et al., 2017; Khan et al., 2019; Prihatna et al., 2018). As the number of identified and characterized genes increases, understanding plant stress responses and developing strategies against crop losses becomes more feasible.

Plant pathogenesis-related (PR) proteins encompass various proteins that are induced and accumulate in defense responses against microbial pathogens or insects in the host organism. Seventeen PR protein families have been characterized in both dicot and monocot plant species (van Loon et al., 2006; Van Loon & Van Strien, 1999; Sels et al., 2008). Most PR proteins are components with low molecular weight, soluble in acid, and partially resistant to endogenous plant and commercial proteases (Van Loon et al., 1994). These proteins can be produced not only in infected tissues but also in unaffected distant tissues (Ahuja et al., 2012; Ali et al., 2017). The main components inducing the synthesis of PR proteins include polyacrylic acid, acetyl-salicylic acid, and salicylic acid (Gianinazzi, 1984).

PR-1 proteins are the first discovered and most abundantly produced proteins in plant defense responses among the PR proteins (Alexander et al., 1993; Van Loon & Van Kammen, 1970). The regional and systematic accumulation of PR-1 suggests its involvement in a form of plant immunity known as systemic acquired resistance (SAR) (van Loon et al., 2006). Recent studies have also shown that PR-1 genes respond to abiotic stress factors, in addition to biotic stress (Akbulak et al., 2020; Almeida-Silva & Venancio, 2022). The involvement of PR-1 proteins in both biotic and abiotic stress defense mechanisms has increased their popularity in molecular biology studies. Besides

their role in stress response, there is evidence that PR-1 proteins actively participate in plant growth and development (Lotan et al., 1989).

This study aims to lay the foundation for future research on combating stress factors by cloning the tomato Solyc09g007010.1 (*SIPR-1.1*) and Solyc08g068990.1 (*SIPR-1.2*) genes, which have been identified for their role in drought stress. In a study conducted by Akbudak et al., (2020), expression profiles of 13 pathogenesis-related gene-1 (PR-1) genes in the tomato genome were revealed under drought stress. Among these genes, it was found that the expression levels of two of them (*SIPR-1.1* and *SIPR-1.2*) increased significantly, up to 45 and 50 times, under drought stress conditions (Akbudak et al., 2020).

Upon reviewing the literature, no studies were found regarding the roles of tomato *SIPR-1.1* and *SIPR-1.2* genes under abiotic and biotic stresses. Only the Solyc09g007010.1 (*SIPR-1.1*) gene has been reported to show increased expression depend on the hypersensitive response (Xue et al., 2021) under both biotic (Buoso et al., 2019; Zhao et al., 2021) and abiotic stresses (Martínez-Andújar et al., 2021). However, there have been no studies investigating the potential roles of this gene in these conditions, and the reported expression increases were emphasized to be potentially associated with hypersensitive response and the salicylic acid signaling pathway in these studies.

To address this gap in the literature and lay the foundation for studies determining the roles of *SIPR-1.1* and *SIPR-1.2* genes, these genes were individually cloned into pIPKb004 and cloned together into pGFPGUSPlus. Cloning into the pIPKb004 plant expression vector was performed using the gateway cloning method, while cloning together into the pGFPGUSPlus plant expression vector was carried out using traditional cloning methods. The genetic material for cloning the *SIPR-1.1* and *SIPR-1.2* genes, reported to have increased expression under drought stress, was obtained from tomato plants subjected to drought stress. According to the PCR results obtained from the studies carried out with different primer sets, the PR products for cloning were obtained from cDNA for the *SIPR-1.1* gene and genomic DNA for the *SIPR-1.2* gene. PCR products were cloned into the pENTR™/D-TOPO® vector using the pENTR™/D-TOPO® Cloning Kit (Thermo Fisher, USA). The cloning of genes separately into pIPKb004 was successfully accomplished using the Gateway™ LR Clonase™ II Enzyme mix kit (Thermo Fisher, USA). Cloning of *SIPR-1.1* and *SIPR-1.2* genes together into pGFPGUSPlus was achieved through sticky ends created by specific restriction enzymes and the T4 DNA Ligase kit (NEB). The presence of genes in the designated plasmids was confirmed through PCR and restriction enzyme analysis.

The separate and combined cloning of *SIPR-1.1* and *SIPR-1.2* genes into plant expression vectors is expected to greatly facilitate the manipulation of plants using these vectors to determine the roles of genes under stress conditions. This contributes to closing the information gap in the literature regarding these identified genes, which is one of the main objectives of the thesis.

2. LITERATURE REVIEW

Plant pathogenesis-related proteins are fundamental components that constitute the plant defense mechanism. Although they are generally found structurally, they are activated in response to various pathogen attacks that plants are exposed to. In such situations, plants defend themselves by synchronously operating their natural barriers and inducible mechanisms activated against various pathogen attacks, regarded as natural resistance to pathogens. Two different active plant defense responses extensively studied to date against pathogen attacks are: (i) induced systemic resistance (ISR) and (ii) systemic acquired resistance (SAR) (Keen, 1990; McGovern, 2015).

ISR is dependent on ethylene and jasmonate (jasmonic acid) phytohormones. It is induced through exposure to specific rhizobacteria strains. ISR is not associated with the accumulation and transcription of PR proteins. On the other hand, SAR is dependent on salicylate (salicylic acid) phytohormone. It is induced by exposure of plant roots and foliar organs to biotic or abiotic stress factors. It is associated with the accumulation of PR proteins (Vallad & Goodman, 2004).

PR proteins have antimicrobial properties and can break down the pathogen's cell wall. Additionally, they act as messengers of signals generated during pathogen attacks, thereby serving as an excellent molecular marker in plant defense (van Loon et al., 2006).

PR proteins can be produced in infected tissues as well as in unaffected distant tissues. Although they can be acidic or basic based on their isoelectric points, they generally have similar functions. While acidic PR proteins are mostly found in intercellular regions, basic PR proteins are located in vacuoles (Takeda et al., 1991).

Initially classified into five families based on molecular mass, isoelectric points, and biological activities, PR proteins are now categorized into 17 families based on sequence similarities, enzymatic activities, structural features, and functions (Bol et al., 1990; Sels et al., 2008; van Loon et al., 2006). PR-1 is the first gene family identified among PR proteins and was discovered in tobacco leaves infected with Tobacco Mosaic Virus (Alexander et al., 1993). According to Alexander et al. (1993), 2% of the accumulated proteins in infected tobacco leaves are reported to be PR-1 proteins, supporting the idea that PR proteins play a prominent role in plant defense.

PR-1 proteins can be found in every organ of the plant, and they have been identified in numerous plants to date (Kothari et al., 2016; Fraser, 1981). While some PR-1 proteins accumulate in vacuoles, they are mostly secreted into the apoplast, facilitated by N-terminal secretion peptides (Sessa et al., 1995). PR-1 is a member of the CAP [cysteine-rich secretory protein (CRISP), antigen 5, and pathogenesis-related 1] family, also known as the SCP (sperm coating protein) family. CAP proteins share a domain consisting of 150 amino acids (Gibbs et al., 2008; Cantacessi et al., 2009) and adopt a specific α - β - α sandwich fold structure to create a large central cavity (Fernández et al., 1997; Asojo et al., 2005). All PR-1 proteins contain the CAP domain (Breen et al., 2017). Proteins containing CAP have been observed in over 2500 species, including plants, animals, bacteria, and fungi, exhibiting different functions (Schneider & Di Pietro, 2013). These unique features are responsible for their biological functions, primarily associated

with the formation of defense against stress conditions (Kothari et al., 2016; Seo et al., 2008).

Interestingly, different plant species can contain multiple PR-1-like proteins. PR1a was the first discovered PR-1 protein, reported as an acidic protein in tobacco leaves infected by the Tobacco Mosaic Virus (Van Loon & Van Kammen, 1970). On the other hand, in Arabidopsis, a PR1 gene predicted to encode 22 homologs of tobacco PR1a proteins has been identified (van Loon et al., 2006). Among these 22 genes, it has been reported that only one gene (At2g14610) encoding a basic protein is involved in responding to pathogens, while the others do not play an active role against bacteria or fungi (van Loon et al., 2006). Accordingly, it is believed that only one of the Arabidopsis PR1 genes is involved in responding to pathogen attacks, and the other genes may have different functions. Based on this, Mitsuhashi et al., (2008) selected 12 rice *PR-1* genes in their study and examined the responses of these genes to pathogen infection. While all genes were reported to be upregulated in the rice-blast fungus interaction, in the rice-bacterial blight interaction, 4 genes were upregulated, 6 genes were not affected, and one gene was downregulated (Mitsuhashi et al., 2008). These studies conducted with Arabidopsis, a model organism for dicots, and rice (*Oryza sativa* L.), a model organism for monocots, have served as examples for subsequent research.

As many studies have demonstrated, *PR-1* genes also respond to abiotic stress, suggesting their significant role in abiotic stress responses. Akbudak et al., (2020) conducted a study examining the expression profiles of tomato *PR-1* genes under drought stress, revealing for the first time the responses of PR1 genes to abiotic stress. They analyzed 13 different tomato *PR-1* genes in *Fusarium oxysporum f.sp. radicle-lycopersici*-sensitive (Var: Anit) and tolerant (Var: Kizilkaya) tomato varieties. It was observed that the expression of all *SlPR-1* genes increased under drought stress in both Fusarium-sensitive and Fusarium-tolerant varieties. Specifically, the expression of some *SlPR-1* genes, such as Solyc09g007010.1 and Solyc08g068990.1, increased up to 50 times under drought stress. This study demonstrates that *SlPR-1* genes play a crucial role not only in responding to biotic stresses such as Fusarium but also in responding to abiotic stresses like drought (Akbudak et al., 2020). Similarly, Almeida-Silva & Venancio, (2022) characterized soybean PR-1 (*GmPR-1*) genes based on sequence, structure, and transcription levels in various organs and stress conditions. They identified 24 *GmPR-1* genes branching into two separate phylogenetic groups. They highlighted features such as strong purifying selection for all *GmPR-1* genes, especially in tandem-derived paralogs, and their susceptibility to tissue-specific expression. Conducting an extensive transcriptional analysis under a total of 26 different abiotic and biotic stress conditions, including alkalinity, salt, cadmium, drought, temperature, herbicides, high ozone levels, iron deficiency, and phosphorus deficiency, they carried out the most comprehensive investigation to date into the transcriptional stress response of *GmPR-1* genes (Almeida-Silva & Venancio, 2022).

Moreover, there is evidence suggesting that PR-1 proteins play a role in plant growth and development independently of stress responses. For instance, it has been shown that PR-1 proteins accumulate strongly during leaf maturation in adult flowering plants. In a subsequent study, Lotan et al., (1989) demonstrated the accumulation of PR-1 proteins in developing leaf sepals, indicating a potential role in plant flowering (Lotan et al., 1989).

One of the most rational ways to enhance plant tolerance to environmental stresses is to develop cultivars resistant to these stresses (Liang et al., 2006; Krishna et al., 2019). The suitability of genetically modified plants for this purpose has been demonstrated in various ways today (Raina et al., 2018). Developing resilient varieties by combining the advantages of *PR-1* genes in responding to both abiotic and biotic stresses with modern breeding methods is of great importance for genetic engineering, and agricultural activities. Reviewing the literature reveals that transgenic plants overexpressing the PR-1 gene exhibit increased resistance to biotic and abiotic stresses (Shin et al., 2014; Kiba et al., 2007; Sarowar et al., 2005).



3. MATERIALS AND METHODS

3.1. Application of Drought Stress Treatment to Tomato Plants

The genetic material to be used for the cloning study of *SIPR-1.1* and *SIPR-1.2* genes, whose expression is reported to increase under drought stress, was obtained from tomato plants subjected to drought stress. In this context, six tomato plants obtained in the seedling stage were planted in a peat and perlite mixture (2:1) and grown for approximately one week in a climate chamber with a 16-hour day/8-hour night photoperiod, at a temperature of 23 °C and 60% humidity. Throughout the acclimatization process, the plants were watered every two days and grown normally for one week. After one week, three of the plants were subjected to drought stress by withholding watering, while the other three were continued to be irrigated normally for use as a control. After 10 days of drought stress application, the plants subjected to drought stress and those used as controls were harvested, rapidly frozen in liquid nitrogen, and preserved at -80 °C for subsequent analyses.

3.2. Obtaining Expression Vectors for *SIPR-1.1* and *SIPR-1.2*

The cloning experiments in this study were conducted using the pENTR™/D-TOPO® Cloning Kit (Thermo Fisher, USA) and the Gateway™ LR Clonase™ II Enzyme mix kit (Thermo Fisher, USA). The pENTR™/D-TOPO® Cloning Kit facilitates the transition to the Gateway™ cloning system by allowing direct cloning of blunt-ended PCR products into the vector. The Gateway™ LR Clonase™ II Enzyme mix catalyzes in vitro recombination between the entry vector containing the target gene and the expression vector. In this study, the expression vector used was pIPKb004 (Figure 3.1).

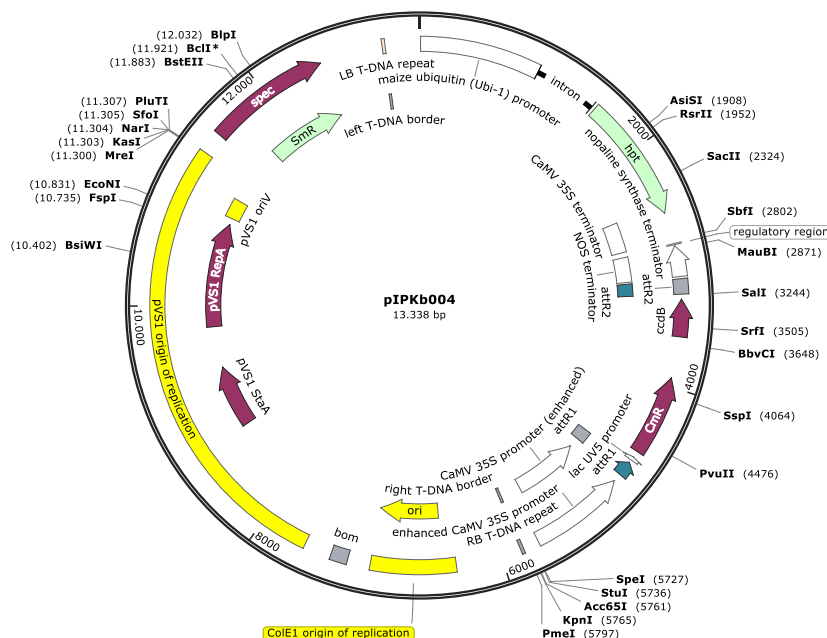


Figure 3.1. The Map of the pIPKb004 Plasmid (Novoprolabs, V005409#)

3.2.1. Cloning of the *SIPR-1.1* Gene

To obtain the cDNA of the *SIPR-1.1* gene, tomato plants subjected to drought stress were utilized. Initially, total RNA was isolated from leaf tissues using the RNA Plant Mini Kit (Qiagen, Cat No: 74904) according to the manufacturer's instructions. Subsequently, 1 µg of the obtained samples was treated with TURBO™ DNase kit (Thermo Fisher, USA) to eliminate any potential DNA contamination, and agarose gel electrophoresis was used to check for DNA contamination and assess RNA quality. The RNA quantity was measured using Qubit (Invitrogen, USA), and cDNA synthesis was carried out using the ABM OneScript® Plus cDNA Synthesis Kit with 1 µg of the sample and a gene-specific reverse primer. Primers used for cloning were designed according to the instructions of the pENTR™/D-TOPO® Cloning Kit, and a 4-base sequence (CACC) was added to the 5' end of the forward primer. Due to issues such as incorrect band sizes or no bands obtained with commercially obtained primers, several primers were designed (Table 3.1). The primers were tested on both genomic DNA and cDNA. Gradient PCRs at different temperature values were set up with both EcoTaq and Phusion to find the optimal T_m temperature for the primers. Once the suitable primer set was selected, the experiments were continued using this primer set. The blunt-ended PCR product required for cloning was obtained using heat-resistant and proofreading-capable Phusion polymerase (Thermo Fisher, USA) with the reaction and PCR cycles shown in Table 3.2. and Table 3.3. To confirm the correct amplification of the region by the primers, the PCR product was digested with *Eco*NI (Thermo Fisher, USA). The PCR-amplified region was verified by Sanger sequencing.

Table 3.1. The Primers Designed for the *SIPR-1.1* Gene

Forward Primers (5' → 3')	Reverse Primers (5' → 3')
Forward 1: CACCCTCTCCACTAACCTAAAGAAAA	Reverse 1: AGCAACATGTCAGAAATAGACGA
Forward 2: CACCCTCACGTTTATAATCACAAT	
Forward 3: CACCATGGGGTTGTTCAACATC	Reverse 2: TTAATAAGGACGTTCTCCAACCC

Table 3.2. The Phusion PCR Reaction Mix

5X HF buffer	10 µl
10 mM dNTPs	1 µl
Forward primer	2.5 µl
Reverse primer	2.5 µl
DMSO	1.5 µl
Phusion	0.5 µl
dH ₂ O	26 µl
cDNA	6 µl
Total	50 µl

Table 3.3 The Phusion PCR Cycle

Initial Denaturation	98 °C	30 s	1X
Denaturation	98 °C	10 s	35X
Annealing	63 °C	30 s	
Extension	72 °C	1 min	
Final Extension	72 °C	10 min	1X

The yield and quality of the PCR product were checked by agarose gel electrophoresis at 75V for 1 hour. Once a correctly sized, single, and high-quality band was obtained, a cloning reaction was set up with the pENTR™/D-TOPO® vector (Figure 3.2) following the manufacturer's instructions, as outlined in Table 3.4.

Table 3.4. The Cloning Reaction of *SIPR-1.1* Gene into the pENTR™/D-TOPO® Vector

PCR product	1 µl
Salt solution	1 µl
pENTR™/D-TOPO® vector	1 µl
Sterile dH ₂ O	3 µl
Total	6 µl

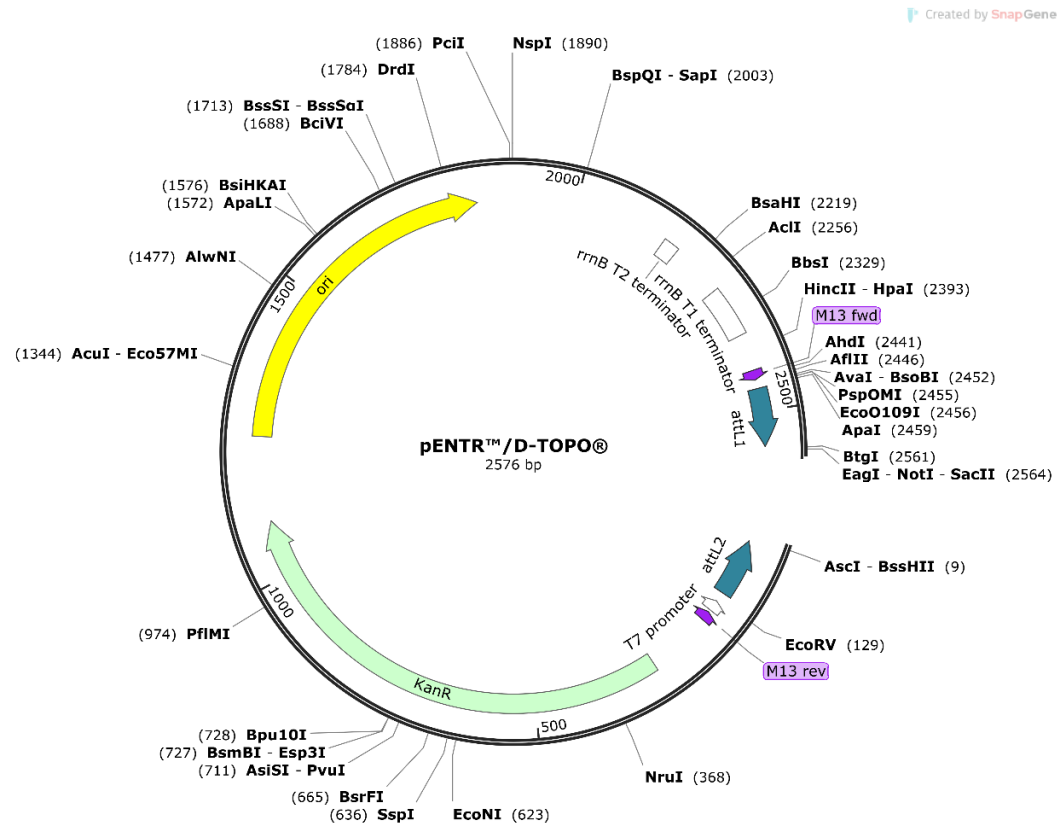


Figure 3.2. The Map of the pENTR™/D-TOPO® Vector (Thermo Fisher, USA)

After the preparation of the reaction, it was gently mixed and incubated at room temperature (22-23 °C) for 5 minutes, then immediately placed on ice. The resulting mixture after cloning was transferred into *Escherichia coli* (DH5α) using the HeatShock method as described below:

1. 2 µL from the TOPO® Cloning reaction was added to a 1.5 ml Eppendorf tube containing 50 µl *Escherichia coli* DH5α cells, and gently mixed.
2. The tubes were incubated on ice for 30 minutes.
3. HeatShock was applied to the cells at 42°C for 30 seconds, followed by immediate transfer of the tubes onto ice.
4. 250 µL of S.O.C. medium at room temperature was added.
5. The tubes were incubated by shaking at 37°C for 1 hour.
6. 50–200 µL of the bacterial culture was spread on agar plates containing 50 mg/L kanamycin and incubated overnight at 37 °C.

The surviving colonies on the selection medium were verified by colony PCR using EcoTaq (EcoTech Biotechnology) PCR (Table 3.5. and Table 3.6.). Colony PCR were conducted using the gene specific primer set and M13 primers located on the pENTR™/D-TOPO® vector (F: 5'-GTAAAACGACGGCCAG-3' R: 5'-CAGGAAACAGCTATGAC-3'). The details of colony PCR are as follows: 1 µl was taken from cells grown in 500 µl LB Broth at 37 °C for 1 hour and used as a template.

The PCR reaction was set up as shown in Table 3.5. According to results of PCR reaction, plasmids expected to be confirmed by digestion were isolated from *E. coli* cells using the GeneJET Plasmid Miniprep Kit (Thermo Fisher, USA). They were separately subjected to a 1-hour digestion reaction at 37 °C with the enzyme pairs *AlwNI* – *AscI* and *NotI* – *EcoRV*.

Table 3.5. The EcoTaq PCR Reaction Mix

EcoTaq 2x PCR Master Mix	8 µl
Forward primer	1 µl
Reverse primer	1 µl
Template DNA	1 µl
dH ₂ O	9 µl
Total	20 µl

Table 3.6. The EcoTaq PCR Cycle

Initial Denaturation	98 °C	30 s	1X
Denaturation	94 °C	10 s	40X
Annealing	59 °C	15 s	
Extension	72 °C	1 min	
Final Extension	72 °C	10 min	1X

After confirming the presence of the *SIPR-1.1* gene in the cloning vector, an LR reaction was set up using the Gateway™ LR Clonase™ II Enzyme mix kit (Thermo Fisher, USA) following the manufacturer's instructions (Table 3.7). The overnight reaction at 25 °C was ended by incubating at 37 °C for 10 minutes after adding 1 µl Proteinase K. The resulting mix after the reaction was transferred into *E. coli* DH5α cells using the HeatShock method and spread onto media containing 100 mg/L spectinomycin. Positive colonies on the selection medium were checked by colony PCR using the gene specific primers. Subsequently, the plasmids were rechecked with the 35S forward primer (5'-GGTAATATCCGGAACCTCCTC-3') on the plasmid and the gene specific reverse primer. After colony PCR, pIPKb004 and the cloned plasmid named as pIPKb004-PR1.1 were simultaneously digested with *EcoNI* – *EcoRV* enzyme pair and *NcoI* separately.

Table 3.7. The LR reaction was set up with the pENTR™/D-TOPO®-PR1.1 and pIPKb004

pENTR™/D-TOPO®-PR1.1 (50-150 ng/reaction)	1.5 µl
pIPKb004 (150 ng/µl)	1 µl
TE buffer, pH: 8	5.5 µl
LR clonase	2 µl
Total	10 µl

3.2.2. Cloning of the *SIPR-1.2* Gene

The cloning of the *SIPR-1.2* gene initially involved the design of primer set containing the CACC sequence at the 5' end of the forward primer (Table 3.8). The primers were obtained through service procurement and were tested by gradient PCR using both genomic DNA and cDNA. Genomic DNA isolation was performed according to the protocol outlined below:

1. Leaf samples were crushed in 150 µl CTAB using a pestle. An additional 450 µl CTAB was added to bring the total volume to 600 µl.
2. The tubes were incubated at 65 °C for 60 minutes, with vortexing for 10-15 seconds every 10 minutes.
3. 600 µl of chloroform:isoamyl alcohol (24:1) was added, and the mixture was thoroughly mixed by vortexing or inversion.
4. Centrifugation was performed at 13,000 rpm for 15 minutes, and the resulting supernatant (approximately 600 µl) was transferred to new tubes.
5. An equal volume of cold isopropanol was added to achieve a 1:1 ratio.
6. After inverting the samples, they were incubated at -20 °C for 60 minutes.
7. Centrifugation was performed at 13,000 rpm for 15 minutes, and the supernatant was discarded. 500 µl of 70% ethanol was added to the pellet.
8. The mixture was inverted for approximately 15 minutes and then centrifuged at 10,000 rpm for 10 minutes.
9. After discarding the upper phase, the pellet was dried and dissolved in 50 µl of dH₂O.

The blunt-ended PCR product required for cloning was obtained from genomic DNA using Phusion polymerase (Thermo Fisher, USA). After experimenting with different PCR protocols and cycles to enhance PCR quality, the reaction and PCR cycles shown in Table 3.9 and Table 3.10 were followed. Phusion PCR was set up with the primer pair selected for cloning. The PCR product was tested for the amplification of the correct region by digestion with *Spe*I at 37 °C for 1 hour.

Table 3.8. The Primers Designed for the *SIPR-1.2* Gene

Forward Primers (5' – 3')	Reverse Primers (5' – 3')
Forward 1: CACC CACTTACCACAAA ACTCCCCAA	Reverse 1: TCTCAACACAAACCCAACAATCA
Forward 2: CACC ATGGCATATTTGCAACCA	Reverse 2: TTAGAAGGGCTTTTCTCC

Table 3.9. The Optimized Phusion PCR Reaction Mix

5X HF buffer	10 μ l
10 mM dNTPs	0.5 μ l
Forward primer	1 μ l
Reverse primer	1 μ l
Phusion	0.7 μ l
dH ₂ O	35.8 μ l
gDNA	1 μ l
Total	50 μ l

Table 3.10. The Optimized Phusion PCR Cycle

Initial Denaturation	94 °C	2 min	1X
Denaturation	94 °C	1 min	25X
Annealing	67 °C	1 min	
Extension	72 °C	2 min	
Final Extension	72 °C	7 min	1X

The 10 μ l PCR product was checked by agarose gel electrophoreses at 75V for 1 hour to examine whether a single band with correct size was present. The cloning reaction was set up using the pENTR™/D-TOPO® vector (Figure 3.2), as per the manufacturer's instructions for the pENTR™/D-TOPO® Cloning Kit, as shown in Table 3.4. A 2 μ l aliquot was taken from the reaction incubated at room temperature (22-23 °C) for 5 minutes and transferred to *E. coli* (DH5 α) cells using the HeatShock method. Bacterial cultures of 50-200 μ L were spread on media containing 50 mg/L kanamycin and incubated overnight at 37°C. After antibiotic selection, colony PCR was performed (Table 3.5; 3.6). In addition to PCR studies conducted with gene-specific primers and M13 primers combinations, the accuracy of the plasmid was also verified by restriction digestion.

E. coli cells that tested positive in colony PCR were used to isolate plasmids with the GeneJET Plasmid Miniprep Kit (Thermo Fisher, USA). The obtained plasmids were subjected to a 1-hour digestion reaction with the *EcoRV-SpeI* enzyme pair at 37 °C. In parallel with the cloning reactions, another reaction was set up using the DNA template and primers obtained from the pENTR™/D-TOPO® Cloning Kit (Table 3.9; 3.10) and was used as a positive control. In HeatShock experiments, transformation efficiency and cell viability were tested using pUC19 plasmids, and *E. coli* competent cells without plasmids were subjected to HeatShock as a negative control.

Once the presence of the *SIPR-1.2* gene in the cloning vector was confirmed, an LR reaction was set up using the Gateway™ LR Clonase™ II Enzyme mix kit (Thermo Fisher, USA) following the manufacturer's instructions. pENTR™/D-TOPO®-PR1.2 (50-150 ng/reaction), pIPKb004 (150 ng/μl), TE buffer (pH: 8), and LR clonase were mixed in the ratios shown in Table 3.7 and incubated overnight at 25 °C. After adding 1 μl Proteinase K, the reaction was ended by incubating at 37 °C for 10 minutes. 2 μl aliquot of the resulting product was transferred to *E. coli* DH5α cells using the HeatShock method and spread on media containing 100 mg/L spectinomycin. Six colonies were randomly selected and subjected to colony PCR using the gene specific primer set and the 35S forward primer (5'-GGTAATATCCGGAAACCTCCTC-3'). The isolated pIPKb004-PR1.2 and pIPKb004 plasmids were subjected to a restriction digestion reaction with *SpeI* at 37 °C for 1 hour after colony PCR.

3.2.3. Cloning of the *SIPR-1.1* and *SIPR-1.2* Genes into pGFPGUSplus

The aim is to clone the *SIPR-1.1* and *SIPR-1.2* genes into the expression vector pGFPGUSplus. To achieve this, the EGFP region in the vector is excised by digesting with *XbaI* and *SacI* enzymes. The excised region, with sticky ends (*XbaI-SacI*), is then replaced with the *SIPR-1.1* gene. Simultaneously, the GUSplus region is removed along with the 35S promoter using *SbfI* and *BstEII* enzymes, and the excised region is replaced with the *SIPR-1.2* gene from the pIPKb004 vector, along with its 35S promoter. Due to the absence of a suitable restriction enzyme in the region between the 35S promoter and the start codon of the GUSplus gene in the pGFPGUSplus vector, it is decided to remove the 35S promoter and clone the *SIPR-1.2* gene with its 35S promoter (Figure 3.3). Inserts for cloning will be obtained through PCR from the pIPKb004-PR1.1 and pIPKb004-PR1.2 vectors. This approach ensures the expression of *SIPR-1.1* and *SIPR-1.2* genes on the same vector.

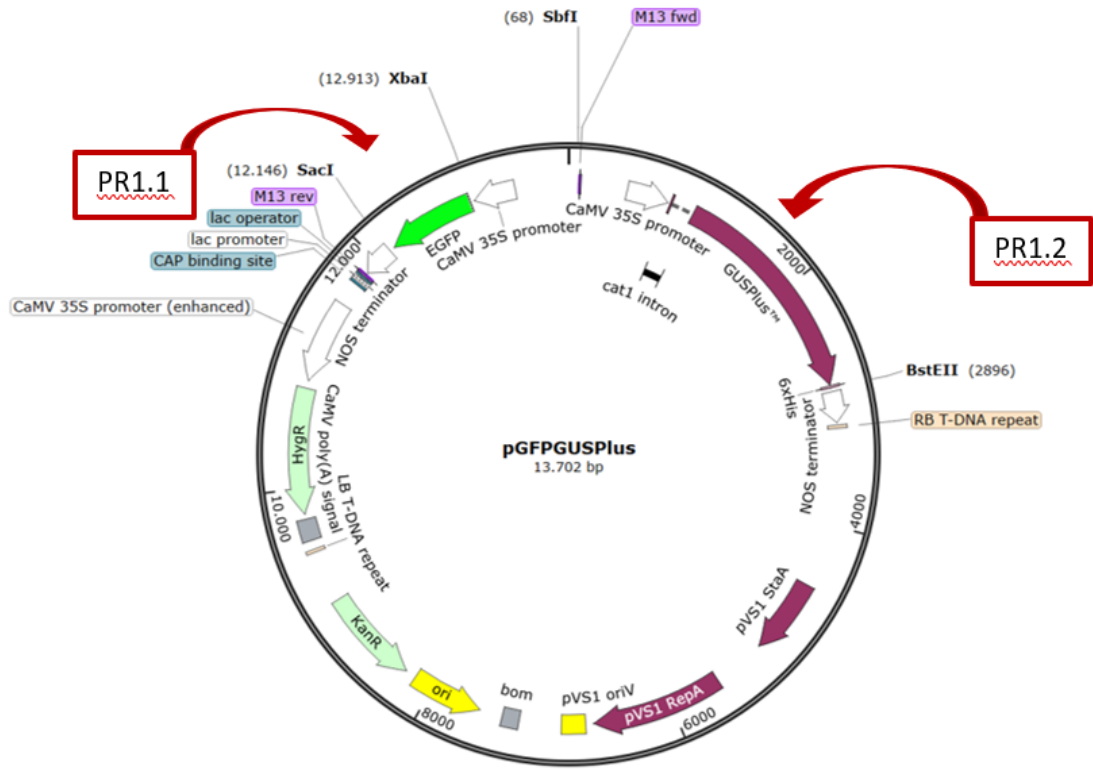


Figure 3.3. The Map of the pGFPGUSPlus Vector (Addgene plasmid #64401)

While ensuring that the restriction enzymes do not exist within the *SIPR-1.1* and *SIPR-1.2* genes during the cloning process, priority was given to *SIPR-1.1* since the *XbaI* restriction enzyme cuts *SIPR-1.2* within the insert. Consequently, primers containing *XbaI* and *SacI* restriction enzyme cutting sites at the ends were designed for the cloning of the *SIPR-1.1* gene. The primers, synthesized through service procurement, are as follows:

*XbaI*_F: GCTCTAGAGCATGGGGTTGTTCAA

*SacI*_R: CGAGCTCGTTAATAAGGACGTTCT

*SacI*_R2: TTGAGCTCTTAATAAGGACGTTCTCCA

Similarly, for the cloning of the *SIPR-1.2* gene, primers containing *SbfI* and *BstEII* restriction enzyme cutting sites at the ends were designed as follows:

*SbfI*_F: TATCCTGCAGGTATATCTGGCCCTTA

*BstEII*_R: GAGGTGACCTGTCTCAACACAAACCC

Firstly, for the cloning of the *SIPR-1.1* gene into the pGFPGUSPlus plasmid, the previously designed pIPKb004-PR1.1 plasmid and primers containing *XbaI* - *SacI* cutting sites were used to set up a Phusion PCR (NEB, Phusion® High-Fidelity DNA Polymerase). Modifications were made to the Phusion PCR protocol listed in Table 3.2; 3.3, following the manufacturer's instructions for the NEB Phusion kit. 1 μ L pIPKb004-

PR1.1 was used as template. The extension time and the annealing temperature was set as the primers.

The PCR product was purified using the NEB, Monarch® PCR & DNA Cleanup kit following the manufacturer's instructions. The purified product, presumed to contain *Xba*I and *Sac*I restriction sites, and the pGFPGUSPlus plasmid were subjected to a digestion reaction using *Xba*I and *Sac*I enzymes (Table 3.11).

Table 3.11. The restriction digestion reaction of pGFPGUSPlus plasmid and the purified *SIPR-1.1* PCR product, amplified using Phusion PCR, with *Xba*I and *Sac*I enzymes

pGFPGUSPlus	<i>SIPR-1.1</i>
5 µL plasmid	15 µL PCR product
5 µL CutSmart Buffer	5 µL CutSmart Buffer
1 µL <i>Sac</i> I	1 µL <i>Sac</i> I
1 µL <i>Xba</i> I	1 µL <i>Xba</i> I
38 µL dH ₂ O	28 µL dH ₂ O

The established restriction digestion reaction was incubated overnight at 37 °C. Before the completion of the incubation period, 2.5 µL of CIP was added to the restriction digestion reaction of the pGFPGUSPlus plasmid. The mixture was further incubated at 37 °C for 1 additional hour for CIP treatment. Prior to completion of the incubation period, 3 µL of 0.5 M EDTA was added to the pGFPGUSPlus cutting reaction after CIP treatment. The reaction was then incubated at 75 °C for 10 minutes for heat inactivation, and the reaction was ended. Heat inactivation for PR1.1 was similarly carried out at 75 °C for 10 minutes. For gel electrophoresis, 5 µL of the *SIPR-1.1* cutting product and 25 µL each of two wells loaded with the pGFPGUSPlus cutting product were run at 60 V for 90 minutes. While performing the clean-up on the *SIPR-1.1* cutting product according to the NEB, Monarch® PCR & DNA Cleanup Kit (NEB) instructions, the background gel resulting from the removal of the EGFP region from the pGFPGUSPlus plasmid was purified using the NEB, Monarch® DNA Gel Extraction Kit (NEB). After measuring the concentrations of the cleaned cutting products using Nanodrop, ligation reactions were set up with DNA T4 Ligase kit according to Table 3.12.

Table 3.12. Ligation reaction set up with T4 DNA Ligase for pGFPGUSPlus plasmid and *SIPR-1.1* insert

	Sample	Negative Control
Buffer	2 µL	2 µL
T4 DNA ligase	1 µL	1 µL
pGFPGUS+ (100 ng)	4.5 µL	1 µL
Insert (300 ng)	7 µL	-
dH ₂ O	5.5 µL	16 µL

The reaction was incubated at 16 °C overnight (~14 hours). Following incubation, the reaction was ended by heat inactivation at 65 °C for 10 minutes. The ligation product was transferred to DH10b cells using the electroporation method as outlined below:

1. DH10b *E. coli* competent cells were thawed on ice.
2. 3 µL of the ligation product was added to 30 µL of DH10b competent cells and gently mixed.
3. The mixture of competent cells and ligation product was transferred into a previously chilled 0.2 cm-gap sterile BIO-RAD Gene Pulser/MicroPulser Electroporation Cuvette, ensuring an even distribution on the cuvette bottom.
4. Electroporation was performed by applying a 2000 V current to the cuvettes placed in the electroporation device.
5. After electroporation, 1 mL of LB liquid culture was added to the cells, and the mixture was transferred to 15 mL Falcon tube.
6. The cells were grown at 37 °C with shaking at 225 rpm for 1 hour. Subsequently, the cells were spread onto LB Agar + Kan50 plates.
7. Petri dishes were incubated overnight at 37 °C.

The same procedures were repeated for the negative control.

Colonies that survived in the selection medium were subjected to colony PCR, and the PCR products were checked by running at 80 V for about an hour using gel electrophoresis. Initially, cells added in 500 µl LB Broth were incubated at 37 °C for 1 hour, and 1 µL from this culture was used as a template. Colony PCR was set up using the primer pairs 35S forward and SacI reverse, as well as the gene-specific designed primer pairs XbaI forward and SacI reverse. PCR details are as shown in Table 3.5; 3.6, with an annealing temperature set at 61 °C. Subsequently, plasmid isolation was performed, and the isolated plasmid was subjected to a restriction digestion reaction at 37 °C for 1 hour using the *Eco*NI enzyme. After heat inactivation, the cutting reaction was loaded onto a 0.8% gel as 7 µL and run at 80 V for 1 hour.

Following the cloning of the *SIPR-1.1* gene into the pGFPGUSPlus plasmid, efforts were directed toward cloning the *SIPR-1.2* gene into the same vector. For the amplification of the *SIPR-1.2* gene, a Phusion PCR was set up with the pIPKb004-PR1.2 plasmid and primers containing *Sbf*I - *Bst*EII cutting sites, as shown in Table 3.2; 3.3. The annealing temperature was set at 67 °C, and the extension time was 1 minute 15 seconds. The PCR product was purified; the PCR product, presumed to contain *Sbf*I and *Bst*EII restriction sites, and the pGFPGUSPlus-PR1.1 plasmid were subjected to a cutting reaction with *Sbf*I and *Bst*EII enzymes (Table 3.13).

Table 3.13. Restriction reaction with *SbfI* and *BstEII* enzymes for the pGFPGUSPlus-PR1.1 plasmid and the purified *SIPR-1.2* PCR product amplified with Phusion PCR

pGFPGUSPlus-PR1.1	<i>SIPR-1.2</i>
5 µL plasmid	15 µL PCR product
5 µL CutSmart Buffer	5 µL CutSmart Buffer
1 µL <i>SbfI</i> -HF	1 µL <i>SbfI</i> -HF
1 µL <i>BstEII</i> -HF	1 µL <i>BstEII</i> -HF
38 µL dH ₂ O	28 µL dH ₂ O

The established restriction digestion reaction was incubated overnight at 37 °C. Before the completion of the incubation period, CIP treatment was applied to the cutting reaction of the pGFPGUSPlus-PR1.1 plasmid, as mentioned earlier. After the inactivation, 5 µL of the inactivated *SIPR-1.2* digested product and 25 µL each from two wells loaded with the pGFPGUSPlus-PR1.1 digested product were run on a gel at 60 V for 90 minutes. While performing the clean-up on the *SIPR-1.2* digested product according to the NEB PCR Clean-up kit instructions, the background gel resulting from the removal of the 35S+GUSPlus region from the pGFPGUSPlus-PR1.1 plasmid was purified using the NEB Gel Purification kit. After measuring the concentrations of the purified digestion products using Nanodrop, ligation reactions were set up with the T4 DNA Ligase kit as shown in Table 3.14.

Table 3.14. Ligation reaction set up with T4 DNA Ligase for pGFPGUSPlus-PR1.1 plasmid and *SIPR-1.2* insert

	Sample	Negative Control
Buffer	2 µL	2 µL
T4 ligase	1 µL	1 µL
pGFPGUSPlus-PR1.1 (100 ng)	4 µL	1 µL
Insert (300 ng)	5 µL	-
dH ₂ O	8 µL	16 µL

The reaction was incubated at 16 °C overnight (~14 hours). Following incubation, the reaction was ended by heat inactivation at 65 °C for 10 minutes. The ligation product was transferred to DH5α cells using the heat shock method, as described earlier.

The same procedures were repeated for the negative control.

From *E. coli* colonies that tested positive in the selection medium, colony PCR was performed using the primer pairs 35S forward and *BstEII* reverse, as well as the gene-specific designed primer pairs *SbfI* forward and *BstEII* reverse. PCR details are as shown in Table 3.5; 3.6, with annealing temperature and extension time adjusted according to the primers. Subsequently, plasmid isolation was carried out, followed by separate restriction digestion reactions of the pGFPGUSPlus-PR1.1PR1.2 plasmid with *NcoI* - *SphI* and *XbaI* - *SphI* enzyme pairs. After incubation at 37 °C for 1 hour, the restriction digestion reactions were heat-inactivated, and the products were run on a 1% agarose gel at 80 V for 1 hour for verification.

4. FINDINGS

4.1. Application of Drought Stress Treatment to Tomato Plants

Tomato plants were subjected to stress treatment to exploit the increased expressions of *SIPR-1.1* and *SIPR-1.2* genes under drought stress conditions. Following drought stress, changes in color in the plant roots and weak growth were observed, while the leaves exhibited fading and wrinkling. In this study, cDNA and gDNA obtained from leaf tissues of the plants treated as drought group and control group grown under normal conditions (Figure 4.1).



Figure 4.1. Images of tomato plants after stress application. A) Control plants without stress application, and B) Plants subjected to drought stress

4.2. Obtaining Expression Vectors for *SIPR-1.1* and *SIPR-1.2*

4.2.1. Cloning of the *SIPR-1.1* Gene

The initial step in the conducted studies, which emerged as the first phase, was the selection of appropriate primers, constituting the most time-consuming part of the process. While the first primer pair designed for the *SIPR-1.1* gene was forward 1 and reverse 1, the expected band was not obtained in the PCR established from gDNA using EcoTaq (Figure 4.2). Instead of the anticipated 534 bp band, an approximately ~300 bp band was obtained. Consequently, the forward primer was modified, and thus, forward 2 was designed.

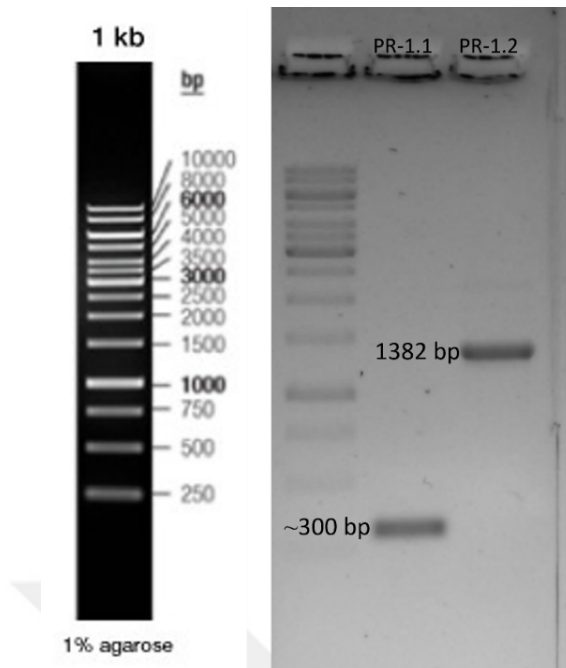


Figure 4.2. The PCR results obtained from gDNA using EcoTaq with PR-1.1 forward 1 and reverse 1, as well as PR-1.2 forward 1 and reverse 1 primers

Using the forward 2 and reverse 1 primers designed for the *SIPR-1.1* gene, a new PCR was set up in a similar manner. According to the PCR results, a wrong sized band of approximately ~350 bp was obtained, similar to the first primer (Figure 4.3).

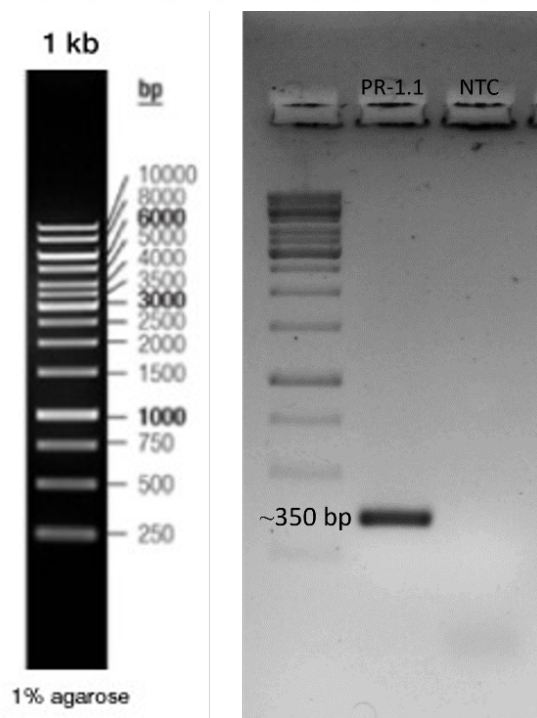


Figure 4.3. The PCR results obtained from gDNA using EcoTaq with PR-1.1 forward 2 and reverse 1 primers

Due to the inability to obtain the desired results from genomic DNA with the available primers, they were also tested by setting up PCR with cDNA. The success of cDNA synthesis was verified by performing PCR with tomato Actin primers as a control. Upon examining the PCR results using PR-1.1 forward 1 - reverse 1 and forward 2 - reverse 1 primer pairs, two bands of 534 bp and 300 bp were obtained from the forward 1 - reverse 1 primer pair, while no band was obtained from the forward 2 - reverse 1 primer pair (Figure 4.4). Due to issues such as the low quality of the 534 bp band obtained from the PR-1.1 forward 1 - reverse 1 primer pair and non-specific binding of primers, forward 3 and reverse 2 primers were designed. The observation of bands in no template control samples used in PCR experiments repeated with the same primer pair, is also one of the reasons for changing the primer (Figure 4.5).

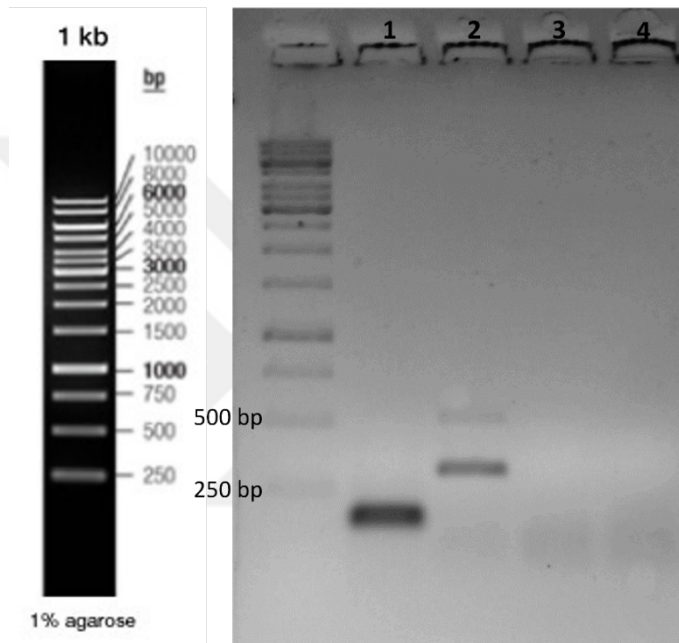


Figure 4.4. The PCR results obtained from tomato cDNA using EcoTaq with the 1) Actin; 2) PR-1.1 Forward 1 and reverse 1; 3) PR-1.1 Forward 2 and reverse 1; and 4) PR-1.2 forward 1 and reverse 1 primers

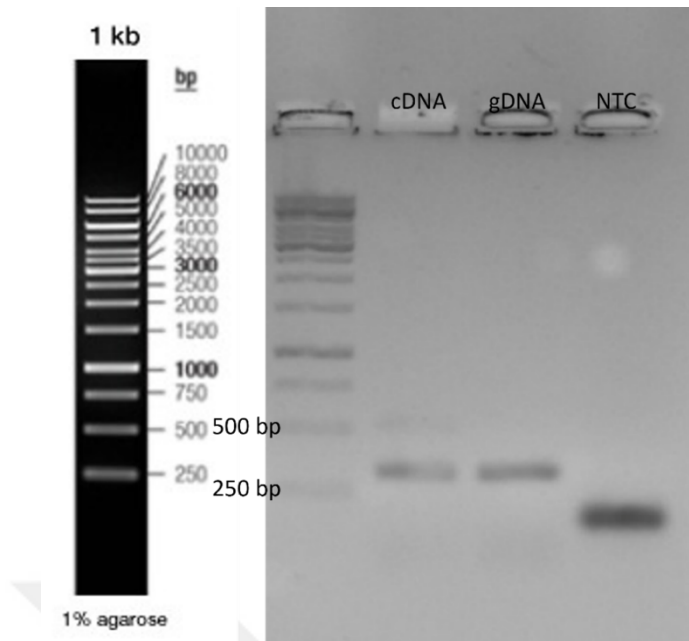


Figure 4.5. The PCR results obtained from tomato cDNA and gDNA using PR-1.1 forward 1 and reverse 1 primers

After obtaining forward 3 and reverse 2 primers through outsourcing, cDNA was synthesized with reverse 2 primer following the instructions of the ABM OneScript® Plus cDNA Synthesis Kit. Subsequently, PCR was set up to confirm the synthesis, resulting in the expected 484 bp band (Figure 4.6). To verify that the primers amplified the correct region, an *Eco*NI restriction digestion reaction was established, and the expected bands of 238 bp and 242 bp were observed (Figure 4.7).

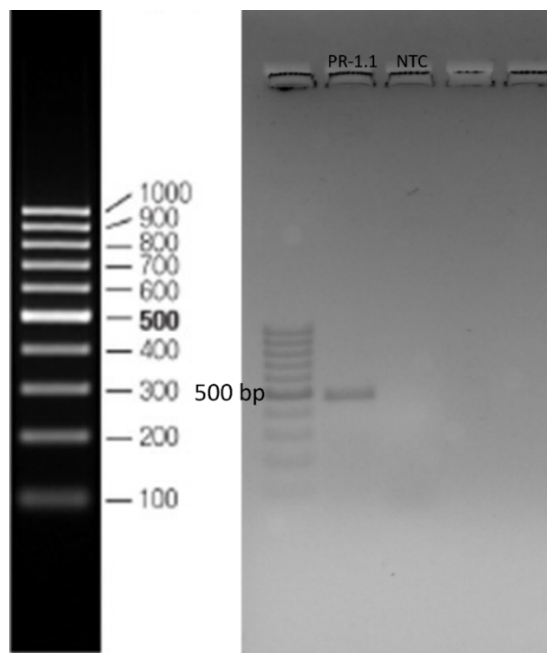


Figure 4.6. The PCR results obtained from cDNA using PR-1.1 forward 3 and reverse 2 primers

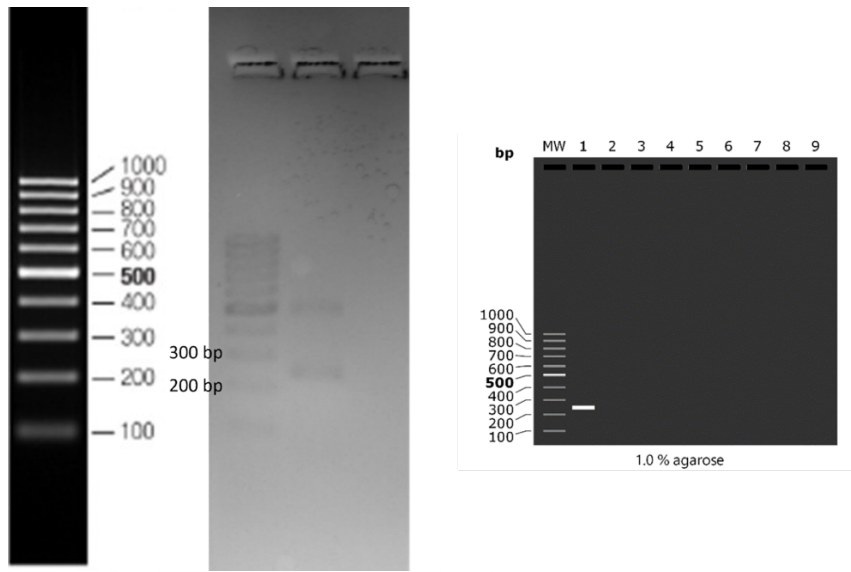


Figure 4.7. The result of *EcoNI* enzyme digestion of the *SIPR-1.1* gene amplified from cDNA using forward 3 and reverse 2 primers

After obtaining the expected results through PCR and restriction digestion, the *SIPR-1.1* gene, which was subsequently validated through Sanger sequencing (as depicted in Figure 4.8), is now prepared for cloning studies.

<i>S.lycopersicum</i> 1	ATGGGGTTGTTCAACATCTCATTGTTACTCACTTGTCTCATGGTATTAGCCATATT--C -----AATATTTC *****	58 10
<i>S.lycopersicum</i> 1	ACTCTTGT-GAGGCCCAAATTACCCCAAGACTATCTTGCGGTTCAACGATGCCCGT CCTCTTGTGGAGGCCCAAATTACCC-CAGACTATCTTGCGGTTCAACGATGCCCGT *****	117 69
<i>S.lycopersicum</i> 1	GCCCAAGTCGGAGTCGGGCAATGTCTTGGGATGCCAACTTGGCATCCCGAGCACAAAAC GCCCAAGTCGGAGTCGGGCAATGTCTTGGGATGCCAACTTGGCATCCCGAGCACAAAAC *****	177 129
<i>S.lycopersicum</i> 1	TATGCCAACTCAAGAGCGGGTGATTGTAATTTGATTCACTCTGGTGCTGGGGAGAACCTT TATGCCAACTCAAGAGCGGGTGATTGTAATTTGATTCACTCTGGTGCTGGGGAGAACCTT *****	237 189
<i>S.lycopersicum</i> 1	GCCAAGGGTGGTGGTGACTTCACGGGAGGGCAGCCGTGCAATTGTGGGTGTCGAGAGG GCCAAGGGTGGTGGTGACTTCACGGGAGGGCAGCCGTGCAATTGTGGGTGTCGAGAGG *****	297 249
<i>S.lycopersicum</i> 1	CCAGACTATAACTACGTACCAACCAATGTGTTGGTGGAAAAATGTGTGGACATTATACT CCAACTATAACTACGTACCAACCAATGTGTTGGTGGAAAAATGTGTGGACATTATACT ***	357 309
<i>S.lycopersicum</i> 1	CAAGTAGTCTGGCGCAACTCAGTCCGACTAGGTTGTGGTCGGGCTCGTTGCAACAATGGG CAAGTAGTCTGGCGCAACTCAGTCCGACTAGGTTGTGGTCGGGCTCGTTGCAACAATGGG *****	417 369
<i>S.lycopersicum</i> 1	TGGTGGTTCATTTCTTGCAACTACGATCCTGTAGGCAACTGGGTTGGAGAACGTCCTTAT TGGTGGTTCATTTCTTGCAACTACGATCCTGTAGGCAACTGGGTTGGAGAACGTCCTTAT *****	477 429
<i>S.lycopersicum</i> 1	TAA--- TTAAAA *:*	480 436

Figure 4.8. Sanger sequencing result of the *SIPR-1.1* gene amplified with forward 3 and reverse 2 primers

After identifying suitable primers, the *SIPR-1.1* gene was amplified using Phusion PCR and subsequently cloned into the pENTR™/D-TOPO® vector following the instructions of the pENTR™/D-TOPO® Cloning Kit. Concurrently, the positive control template included in the kit was subjected to Phusion PCR using the kit's primers, and the resulting PCR product was also cloned into the pENTR™/D-TOPO® vector. Following cloning studies, 2 µl of the reaction was taken and transformed into DH5α cells using the HeatShock method. In parallel, transformation with pUC19 was performed to assess the success and efficiency of the transformation, and the cells were spread onto LB agar plates containing Ampicillin. As a negative control, competent cells subjected to HeatShock without the addition of any plasmid were used and spread onto media containing Kanamycin. As expected, cells grew densely in antibiotic-free LB media, while in media containing antibiotics, only colonies believed to be positive survived. A total of 4 colonies were obtained in the cloning of the *SIPR-1.1* gene (Figure 4.9).

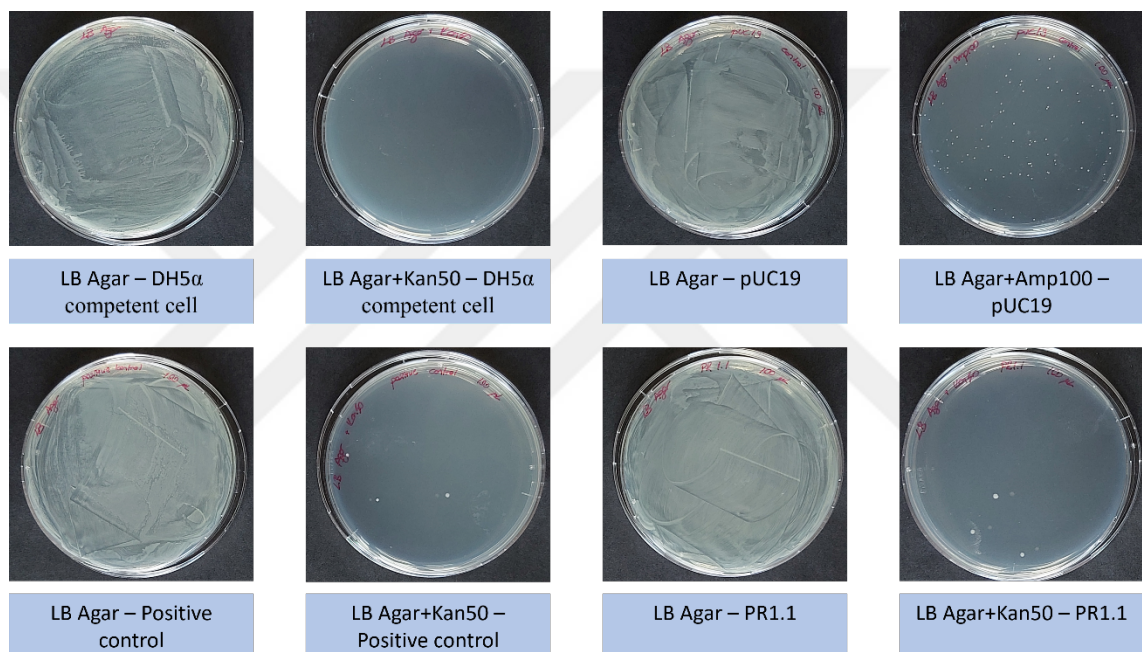


Figure 4.9. The petri dish images obtained as a result of cloning the *SIPR-1.1* gene into the pENTR™/D-TOPO® vector

Colonies that survived from the antibiotic selection were subjected to colony PCR using forward 3 - reverse 2 primers and M13 primers. Based on the PCR results, the bands of 484 bp and 805 bp was observed correctly for colony 2 (Figure 4.10).

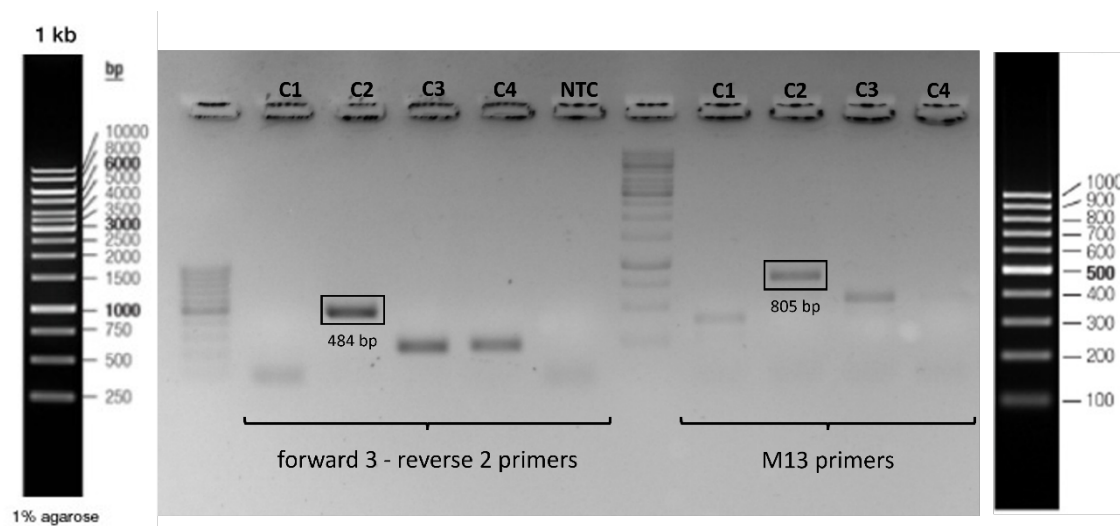


Figure 4.10. The colony PCR image of pENTR™/D-TOPO®-PR1.1 plasmids that survived from the antibiotic selection (C: Colony)

Before proceeding to the restriction digestion experiments, to ensure the insertion of the target gene into the pENTR™/D-TOPO® vector, a PCR was set up using the M13 forward primer on the vector and the reverse 2 primer specific to the *SIPR-1.1* gene. Cells streaked from the glycerol stock of Colony 2 were plated on LB agar + Kan50 medium, and two colonies were randomly selected for PCR analysis. The expected band size with the primer pair used was 611 bp, and the expected outcome was obtained from the selected two colonies (Figure 4.11).

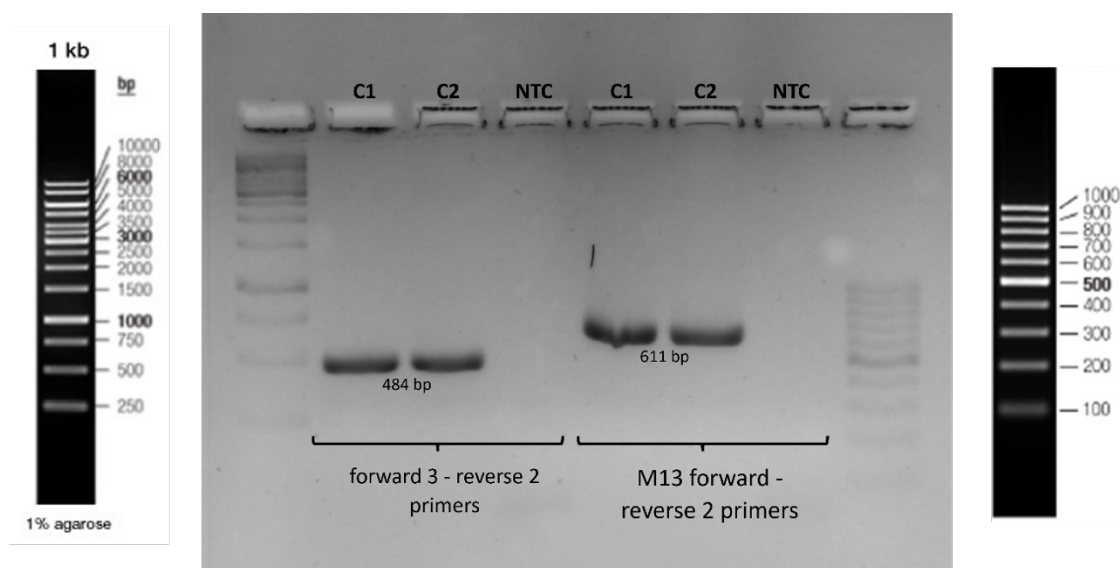


Figure 4.11. The PCR results obtained using M13 forward and PR-1.1 reverse 2 primers

The restriction digestion reactions of the colonies confirmed with the established PCRs were performed using *AlwNI* - *AscI* and *NotI* - *EcoRV* enzymes. The expected band sizes from *NotI* - *EcoRV* digestion were 625 bp and 2435 bp, while the expected band sizes from *AscI* - *AlwNI* digestion were 1468 bp and 1592 bp. Bands of the expected sizes

were obtained from the digestion reactions, confirming the integrity of the pENTR™/D-TOPO®-PR1.1 vector (Figure 4.12).

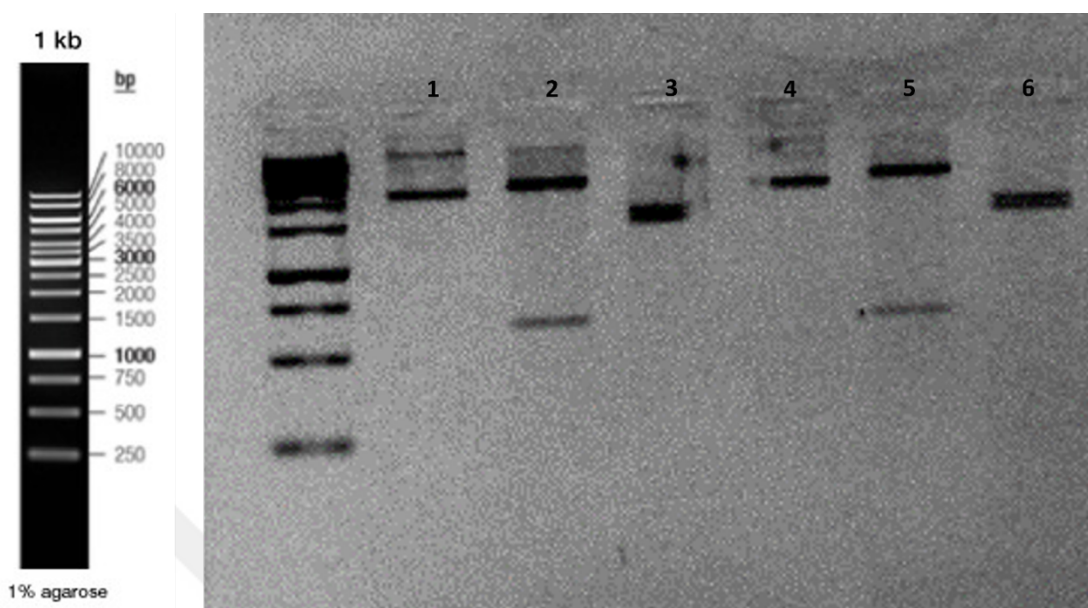


Figure 4.12. The restriction digestion results of the pENTR™/D-TOPO®-PR1.1 vector using the *NotI* - *EcoRV* and *AlwNI* - *AscI* enzyme pairs 1) colony 1 uncut; 2) *NotI* – *EcoRV* digestion of colony 1; 3) *AlwNI* – *AscI* digestion of colony 1; 4) colony 2 uncut; 5) *NotI* – *EcoRV* digestion of colony 2; 6) *AlwNI* – *AscI* digestion of colony 2

After cloning the *SIPR-1.1* gene into the pENTR™/D-TOPO® vector and confirming it through various analyses, the LR reaction was initiated. Simultaneously with the reaction established with pIPKb004 following the instructions of the Gateway™ LR Clonase™ II Enzyme Mix kit (Thermo Fisher, USA), a reaction was set up with the positive control vector included in the kit. 2 µl of the reaction obtained after LR was taken, subjected to HeatShock in 100 µl competent DH5α cells, and spread onto media containing 100 mg/L Spectinomycin. HeatShock experiments were also conducted as controls using pUC19 and without the addition of any plasmid. According to the transformation results, it was observed that all cells survived in antibiotic-free LB agar medium, but in the antibiotic-containing medium, only colonies believed to be positive were present. Cloning of the *SIPR-1.1* gene into pIPKb004 resulted in the acquisition of hundreds of colonies (Figure 4.13).

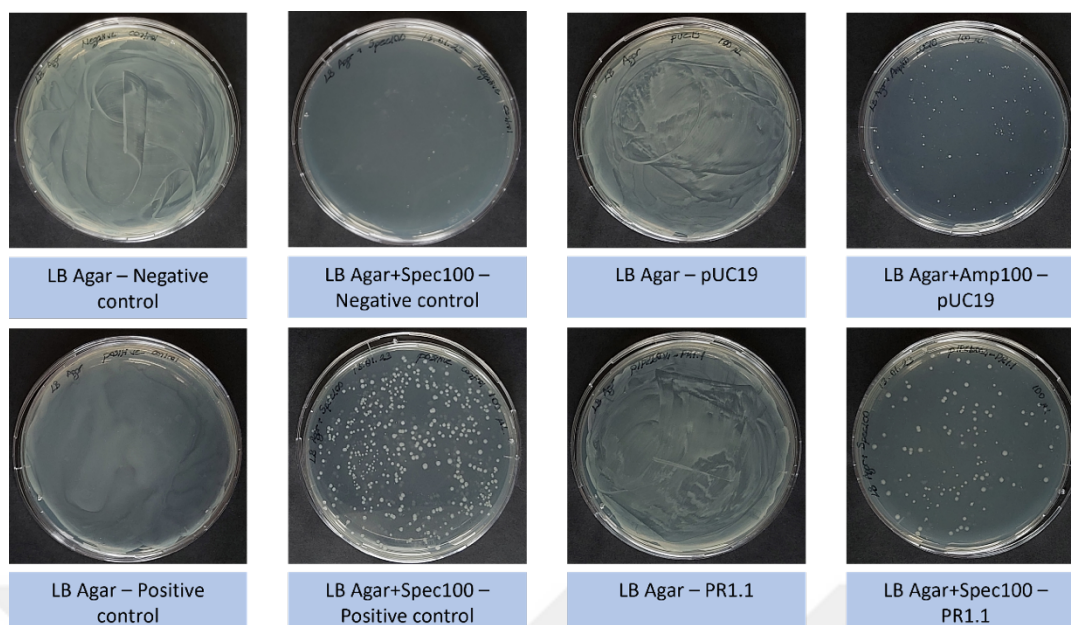


Figure 4.13. The petri dish images of the LR reaction conducted with the pENTR™/D-TOPO®-PR1.1 and pIPKb004

Colony PCR was performed using forward 3 - reverse 2 primers on colonies that survived from the antibiotic selection. The positivity of all colonies was confirmed with the obtained 484 bp bands (Figure 4.14).

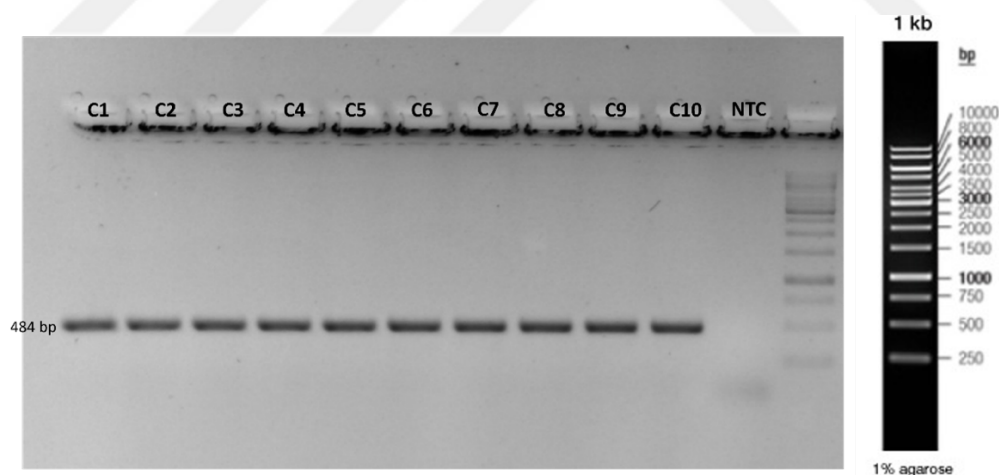


Figure 4.14. The colony PCR results of pIPKb004-PR1.1 plasmids that survived from the antibiotic selection

Colony 7, identified as positive, was selected, and plasmid isolation was performed using the GeneJET Plasmid Miniprep Kit (Thermo Fisher, USA). Prior to verification through restriction digestion, a PCR was set up using the 35S F and PR-1.1 reverse 2 primers on the isolated plasmid from Colony 7 to ensure the insertion of the target gene into pIPKb004. The expected band sizes with the primer pair used were 1289 bp and 962 bp. Due to the presence of two 35S promoters, two distinct bands were observed in the PCR results (Figure 4.15).

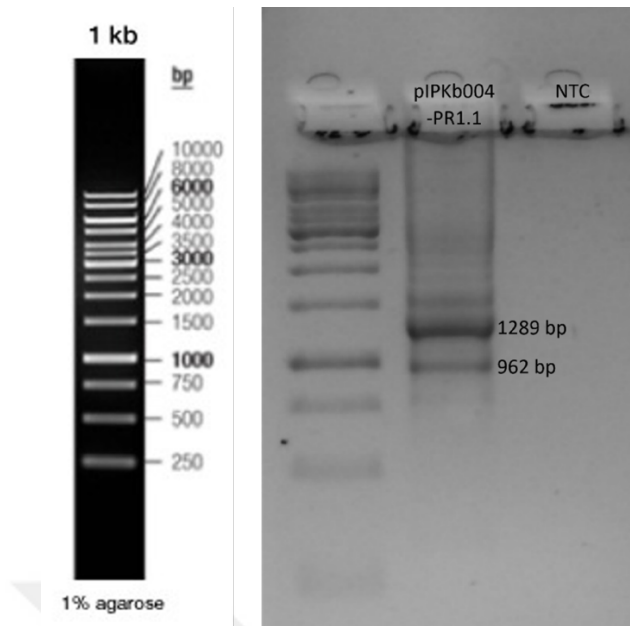


Figure 4.15. The PCR results of pIPKb004-PR1.1 amplified with the 35S_F and PR-1.1 reverse 2 primers

After confirming the plasmid from Colony 7 through PCR, restriction digestion reactions with the *Eco*NI - *Eco*RV enzyme pair and *Nco*I were set up for both pIPKb004 and pIPKb004-PR1.1 plasmids. For *Eco*NI - *Eco*RV digestion, the expected band sizes for pIPKb004 were 7532 bp, 4522 bp, and 1284 bp, while for pIPKb004-PR1.1, the expected sizes were 5891 bp, 4523 bp, 1284 bp, and 485 bp. The expected bands were successfully obtained according to the digestion results (Figure 4.16). For the *Nco*I digestion, the expected band sizes for pIPKb004 were 10323 bp, 1243 bp, 932 bp, and 840 bp, while for pIPKb004-PR1.1, they were 9619 bp, 932 bp, 840 bp, and 792 bp. All expected bands were successfully obtained (Figure 4.17).

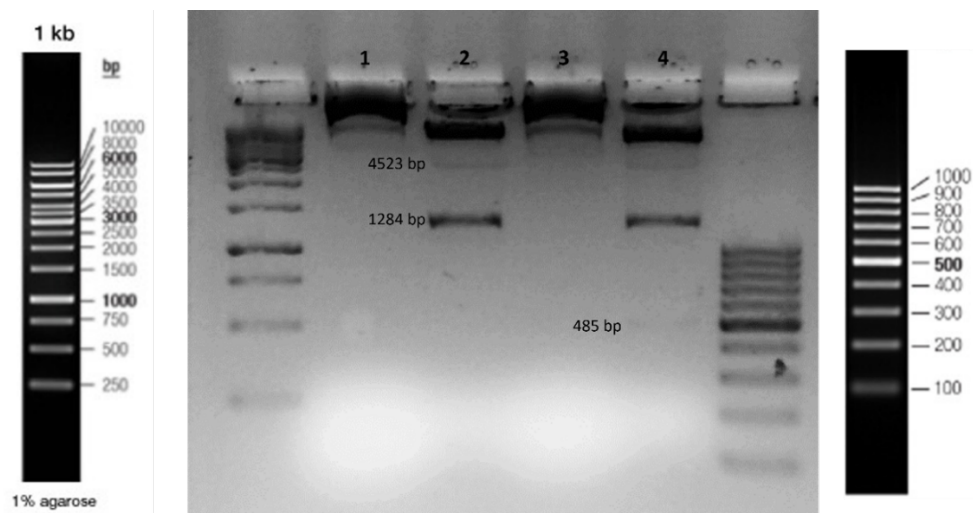


Figure 4.16. The restriction digestion results of the pIPKb004 and pIPKb004-PR1.1 plasmids with the *Eco*NI - *Eco*RV enzyme pair 1) pIPKb004 uncut; 2) pIPKb004 cut; 3) pIPKb004-PR1.1 uncut; 4) pIPKb004-PR1.1 cut

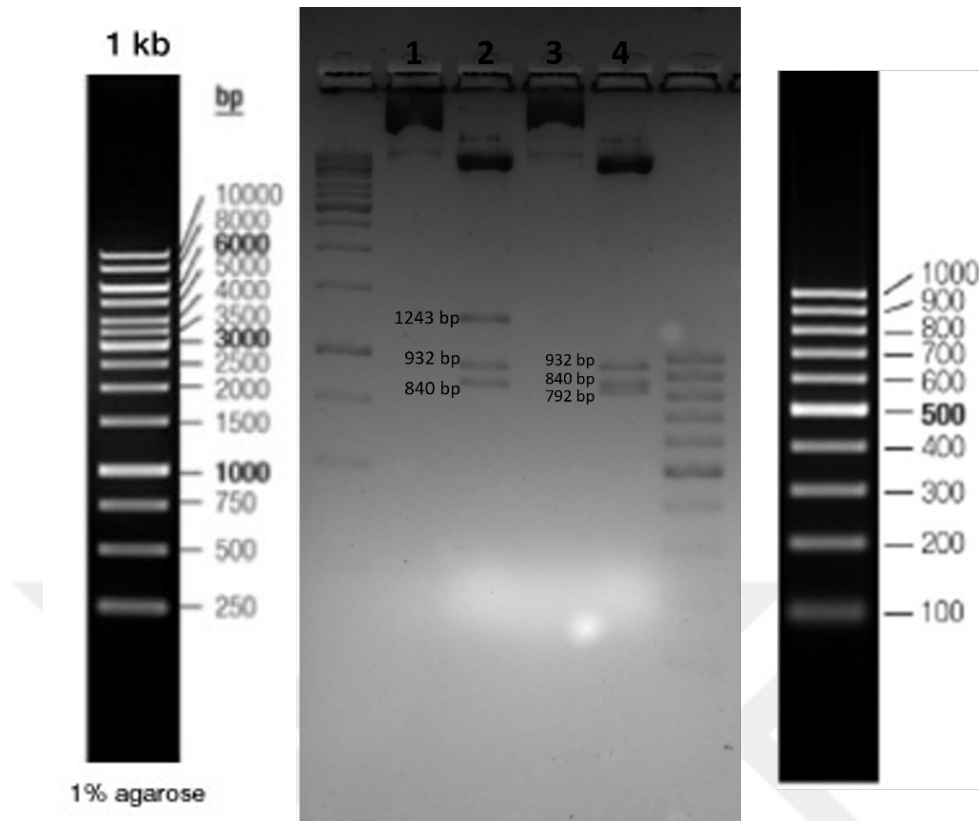


Figure 4.17. The restriction digestion results of the pIPKb004 and pIPKb004-PR1.1 plasmids with the *NcoI* enzyme 1) pIPKb004 uncut; 2) pIPKb004 cut; 3) pIPKb004-PR1.1 uncut; 4) pIPKb004-PR1.1 cut

Thus, the cloning of the *SIPR-1.1* gene into the pIPKb004 vector has been successfully completed.

4.2.2. Cloning of the *SIPR-1.2* Gene

The primary issue encountered in the *SIPR-1.1* gene is also evident in the *SIPR-1.2* gene. While the first primer pair designed for the *SIPR-1.2* gene consists of forward 1 and reverse 1, PCR conducted with EcoTaq and gDNA yielded the expected 1382 bp band size accurately (Figure 4.2). Upon obtaining results from gDNA, the PCR product was validated by digestion with the *SpeI* enzyme. As a result of the digestion, the anticipated 883 bp and 499 bp bands were successfully obtained, as shown in Figure 4.18. However, the same primer did not yield consistent results with cDNA (Figure 4.4).

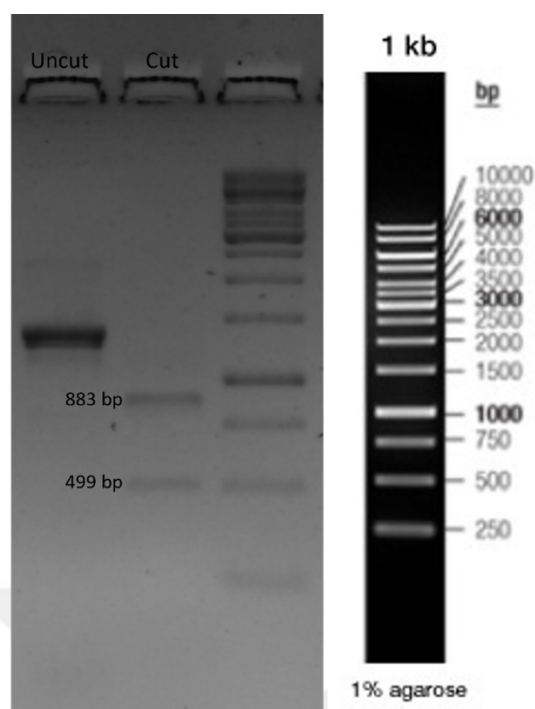


Figure 4.18. The *SpeI* digestion result of the EcoTaq PCR product, amplified with PR-1.2 forward 1 and reverse 1 from gDNA

Due to the inability to obtain bands from cDNA using PR-1.2 forward 1 and reverse 1 primers, new primers (PR-1.2 forward 2 and reverse 2) were designed. The primer pair was tested by performing PCR with EcoTaq on both gDNA and cDNA. In the PCR results, where 1312 bp was expected from gDNA and 550 bp from cDNA, additional bands were observed in gDNA while obtaining the expected band from cDNA (Figure 4.19).

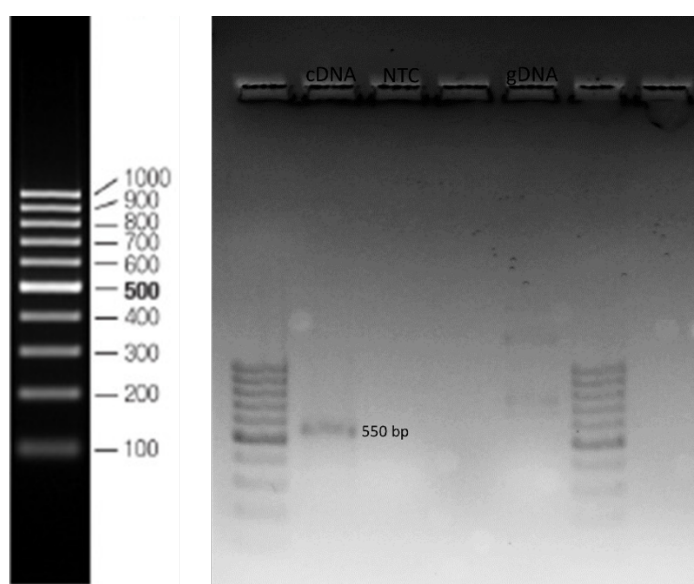


Figure 4.19. The EcoTaq PCR results obtained from cDNA and gDNA using the PR-1.2 forward 2 and reverse 2 primer pair

Since the desired band size was obtained from cDNA, the PCR product was subjected to a digestion reaction with the *Xba*I enzyme. Unfortunately, the expected 443 bp and 107 bp bands could not be obtained as shown in Figure 4.20.

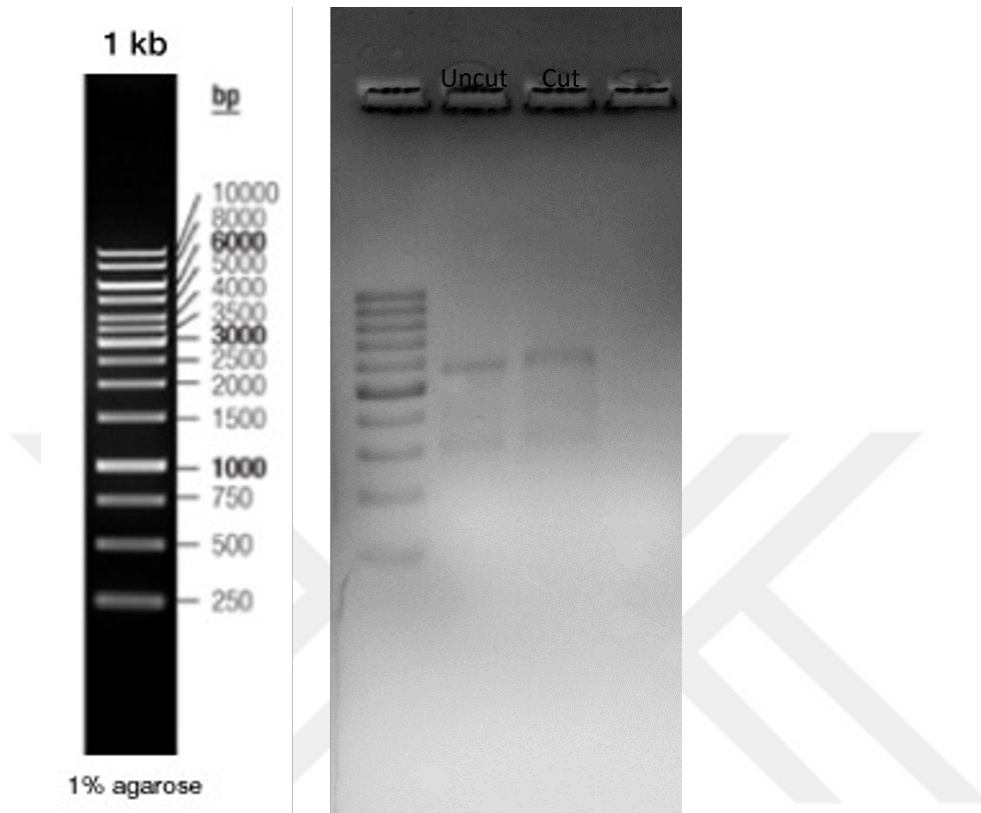


Figure 4.20. The *Xba*I digestion of the PCR product obtained from cDNA using the PR-1.2 forward 2 and reverse 2 primer pair

Despite the incorrect results from the digestion reaction, the PCR product was submitted for Sanger sequencing. However, the obtained sequence did not match the sequence of the *SLPR-1.2* gene, as illustrated in Figure 4.21.

```

PR1.2      ATGGCATATTTGCAACCAAGACTCTTAGGAAAACCTTAGTATTTTCCTTCTCCTTTGCATA 60
1          -----TT 2
                                     *:

PR1.2      GTCCAAACTTCATTCTCTTCTTCTCCCATCAAC--ATTTGTCCTCTAGACACCTTTTGA 118
1          TTACAATAT-----TCATCAGCAGGTTTGCCCACTGCTCACCTCTCGG 45
          *,***:,*          *****,* ,*** **:*, :***** *,

PR1.2      CA-----ACTACCCCAAGCAACATAA-----TCAAAC--AATACCTTAACCCC 159
1          AAAGTAATCGGCC--AAAAAACAGAATTGGAGAAAGACTCAAATAATTTTCTTATACCC 103
          ,*          :*,** **,**** **          ***** :*: *****:***

PR1.2      CACAACAAAGCTAGAGCAAAACTTGGACTTCCACCACTCAAATGGAGCAATAAATTAGCA 219
1          -A--AAAAAGCGTTG-----AAATTACACCT--AT-----TTTCCT 136
          * *,***** :*,          ,*,**,* **,          *:          **: *:

PR1.2      AATTACGCA-----A-----GCTGGTGGGGACACCAACGA 249
1          TATTACGAATTTGTGCCCTATCGAAAAGGTTTCTAAGGGCTACCTGGTTTCCAGCAG 196
          :*****,*          *          ***,          **: :***,*,

PR1.2      CAG-----G---TG---AGTTGTGAG-TTGGTTCATTCTCGT-----GCGACT 286
1          CTTTGTTAAGGTAGATGGAATGAAGATTGTATGGCACATCCCCCTGTTCCCTTATCTCCG 256
          *:          *          ** :*:*: :* :*** :*** * * **          * ,*

PR1.2      ACGGGGAGAACCTCTTTTGGGGGAGCGGAAAGGATTGGAAGCCAAGCGATGCTGTGACGA 346
1          TCTGGAT---T-----CGTGTTCGAAACGGAAG-----281
          :* **, :          * *: **,*,***:

PR1.2      TGTGGGCGAAAGAAAGACCTTATTATGATTATAAGAGCAATTCTTGCAAGAAAAATGAGC 406
1          -----AGC-----284
                                     ***

PR1.2      AATGCTTGCATTATACACAAATTATTTGGAACAAAGCACAGAGTTGGGTGTGCAAAAG 466
1          -----284

PR1.2      TTCTATGCAAAACAGGAGATACTTTGATTAGTTGCAACTATGATCCTCATGGTAATGTAG 526
1          -----284

PR1.2      AAGGAGAAAAGCCCTTCTAA 546
1          -----284

```

Figure 4.21. The Sanger sequencing result of the *SIPR-1.2* gene amplified with the PR-1.2 forward 2 and reverse 2 primer pair

Due to the unexpected results from cDNA, it was decided to use the PR-1.2 forward 1 and reverse 1 primers, which yield the expected results from gDNA. The PR-1.2 gene was amplified using the designated primer pair through Phusion PCR and cloned into the pENTR™/D-TOPO® vector following the pENTR™/D-TOPO® Cloning Kit instructions. Simultaneously, a positive control reaction provided in the kit was performed. Two microliters of the resulting reaction after cloning were transformed into DH5α cells using the HeatShock method. Additionally, for the transformation success control, transformation was carried out with pUC19, and as a negative control, competent cells were used without adding any plasmid. As expected, cells grew densely in LB agar media without antibiotics, while only colonies believed to be positive survived in media containing antibiotics. In the cloning of the *SIPR-1.2* gene, a total of 5 colonies were obtained from cells spread on two separate Petri dishes (Figure 4.22).

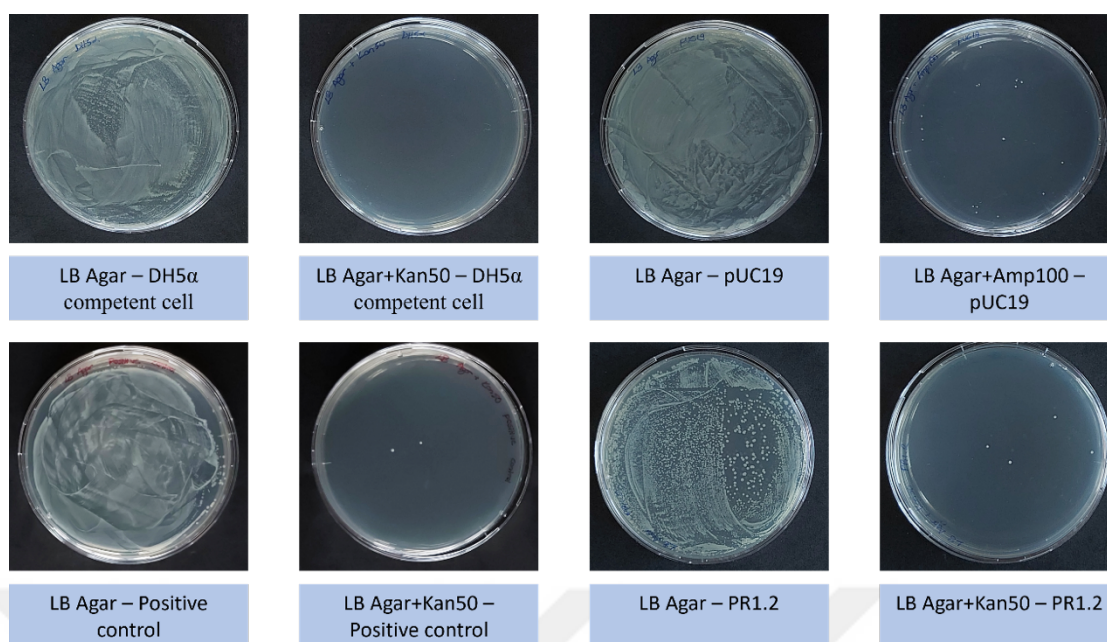


Figure 4.22. The petri dish images of cloning of the *SIPR-1.2* gene into the pENTR™/D-TOPO® vector

Colony PCR was performed using the forward 1 - reverse 1 primers and M13 F – PR-1.2 reverse 1 primers on surviving colonies in the antibiotic selection. According to the PCR results, bands of 1382 bp and 1530 bp were obtained and confirmed sequentially from colony 1 (Figure 4.23).

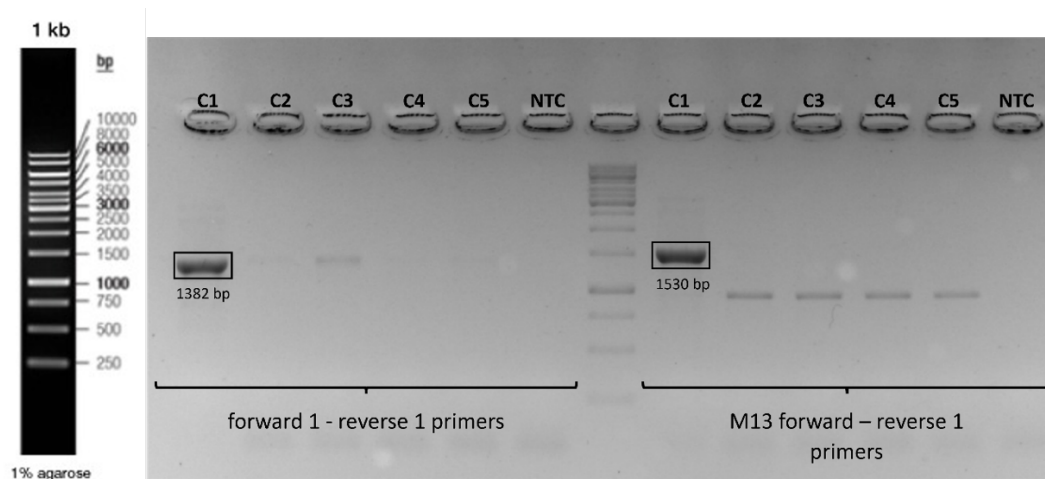


Figure 4.23. The colony PCR results of pENTR™/D-TOPO®-PR1.2 plasmids that survived from antibiotic selection

Plasmid isolation was carried out from colony 1, which was confirmed by the established PCRs, and a cutting reaction was performed with *EcoRV-SpeI* enzymes. The expected bands from the digestion were 2946 bp and 1012 bp. The bands of the expected sizes were obtained from the cutting reaction, confirming the pENTR™/D-TOPO®-PR1.2 vector (Figure 4.24).

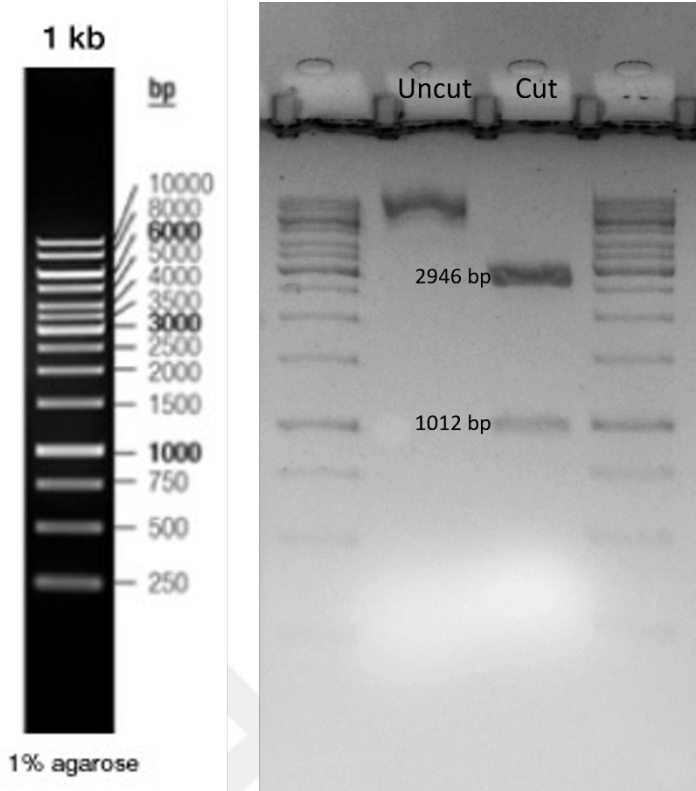


Figure 4.24. The results of the *EcoRV* – *SpeI* enzyme pair digestion of the pENTR™/D-TOPO®-PR1.2 plasmid

After the successful cloning of the *SIPR-1.2* gene into the pENTR™/D-TOPO® vector, which was confirmed by various analyses, the LR reaction was initiated. Simultaneously with the reaction set up with pIPKb004 following the instructions of the Gateway™ LR Clonase™ II Enzyme mix kit (Thermo Fisher, USA), a negative control reaction without LR clonase was also established. 2 µl of the resulting reaction after LR were transformed into 100 µl competent DH5α cells using the heat-shock method and spread onto media containing 100 mg/L Spectinomycin. HeatShock experiments carried out without the addition of pUC19 and plasmids were used as controls. According to the transformation results, it was observed that all cells survived in media without antibiotics, but only colonies believed to be positive survived in media with antibiotics. Hundreds of colonies were obtained as a result of cloning the *SIPR-1.2* gene into pIPKb004 (Figure 2.25).

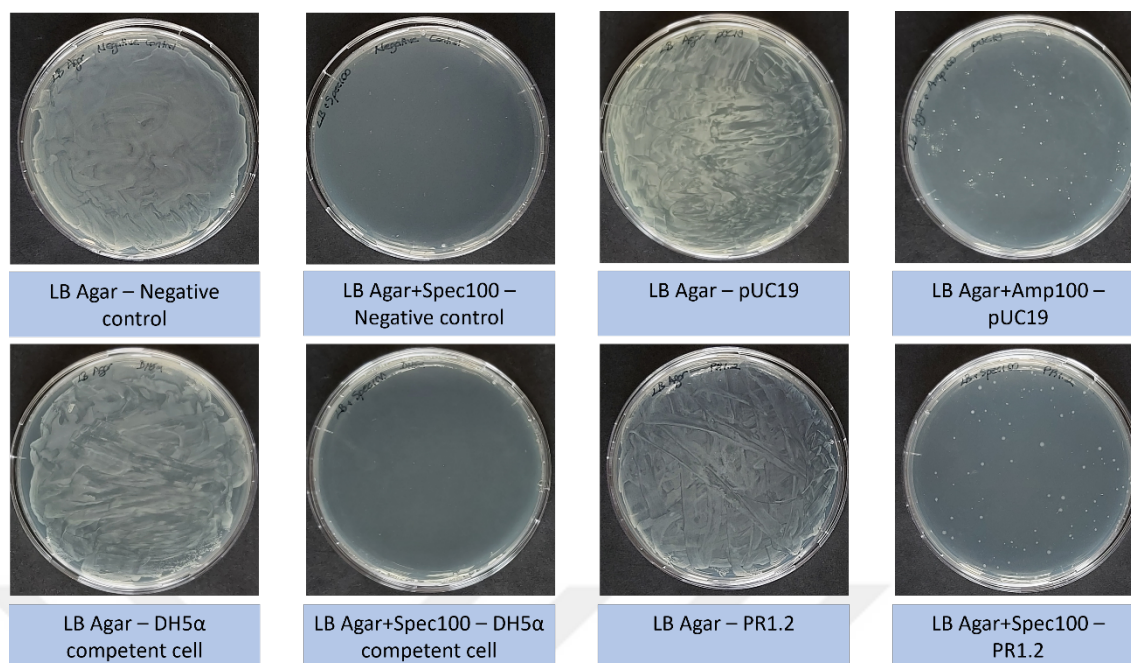


Figure 4.25. The petri dish images of the LR reaction conducted with the pENTR™/D-TOPO®-PR1.2 and pIPKb004

Six randomly selected colonies that survived from the antibiotic selection were subjected to colony PCR using the forward 1 - reverse 1 and 35S F – reverse 1 primer pairs. The expected band sizes from the PCR were 1382 bp for the forward 1 – reverse 1 primer pair, and 2187 bp and 1860 bp for the 35S F – reverse 1 primer pair. The expected bands were obtained with both primer pairs, and all selected colonies were confirmed to be positive (Figure 4.26).

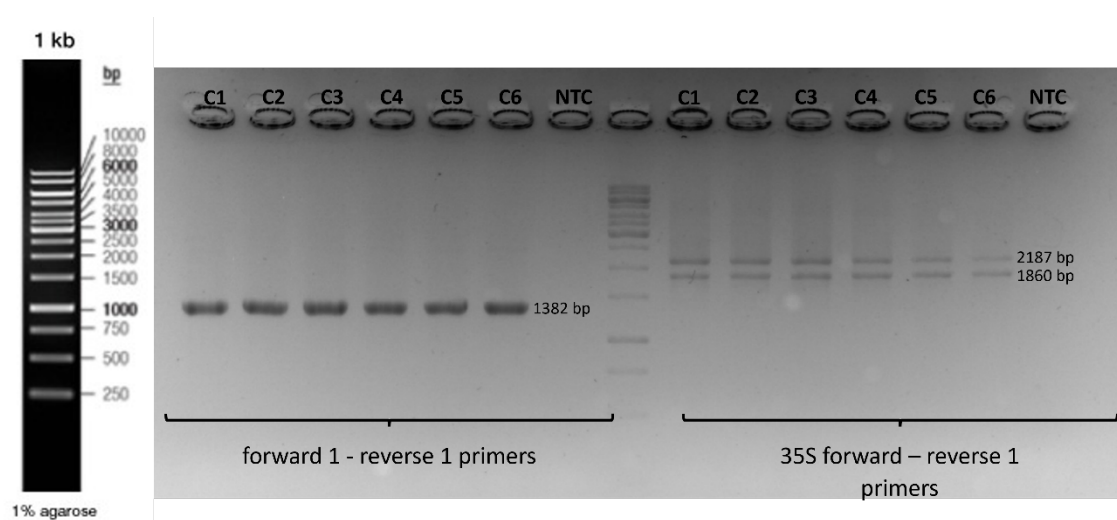


Figure 4.26. The colony PCR results of the pIPKb004-PR1.2 plasmids that survived from the antibiotic selection

Colony 2 was randomly chosen, and plasmid isolation was conducted using the GeneJET Plasmid Miniprep Kit (Thermo Fisher, USA). Simultaneously, restriction digestion reactions were set up for the plasmids obtained from colony 2, namely pIPKb004-PR1.2 and pIPKb004, using the *SpeI* enzyme. Since *SpeI* digest pIPKb004 at a single site, a linear band of 13338 bp is expected. For pIPKb004-PR1.2, the anticipated band sizes are 11633 bp and 1448 bp. The digestion reactions were carried out at 37 °C for 1 hour and were subsequently verified by gel electrophoresis at approximately 75 V for about 1 hour. According to the digestion results, the expected bands were successfully obtained for both plasmids (Figure 4.27).

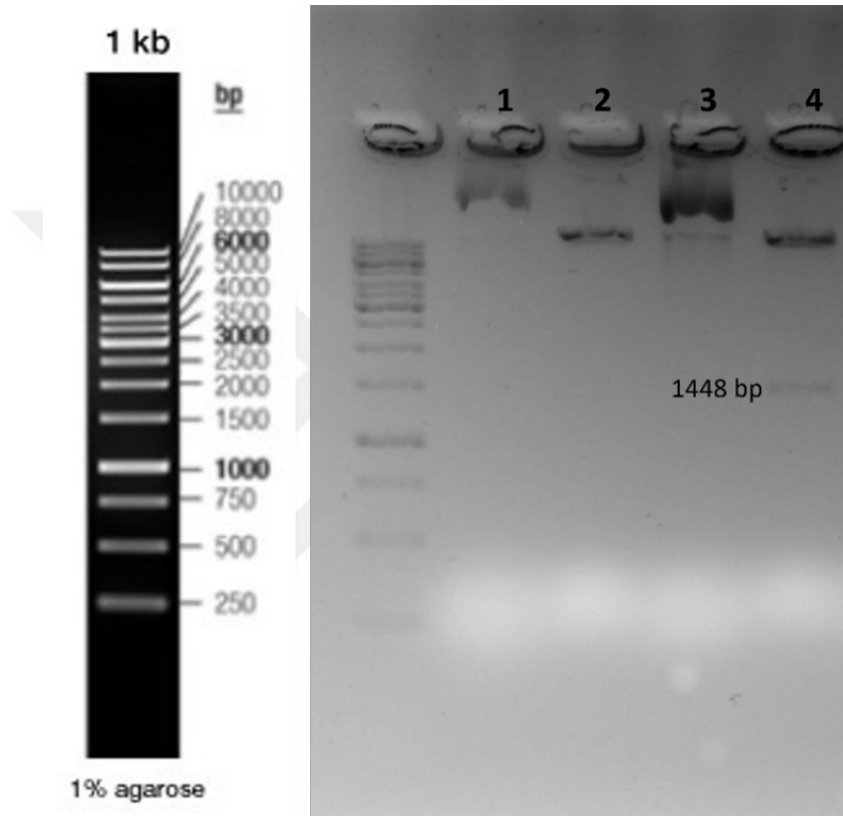


Figure 4.27. The results of the *SpeI* enzyme digestion of the pIPKb004 and pIPKb004-PR1.2 1) pIPKb004 uncut; 2) pIPKb004 cut; 3) pIPKb004-PR1.2 uncut; 4) pIPKb004-PR1.2 cut

Thus, the cloning of the *SIPR-1.2* gene into the pIPKb004 vector has been successfully completed.

4.2.3. Cloning of the *SIPR-1.1* and *SIPR-1.2* Genes into pGFPGUSPlus

The Phusion PCR, established with the pIPKb004-PR1.1 template using XbaI_F and SacI_R primers, was validated through gel electrophoresis. The anticipated 498 bp band was successfully obtained, as expected (Figure 4.28).

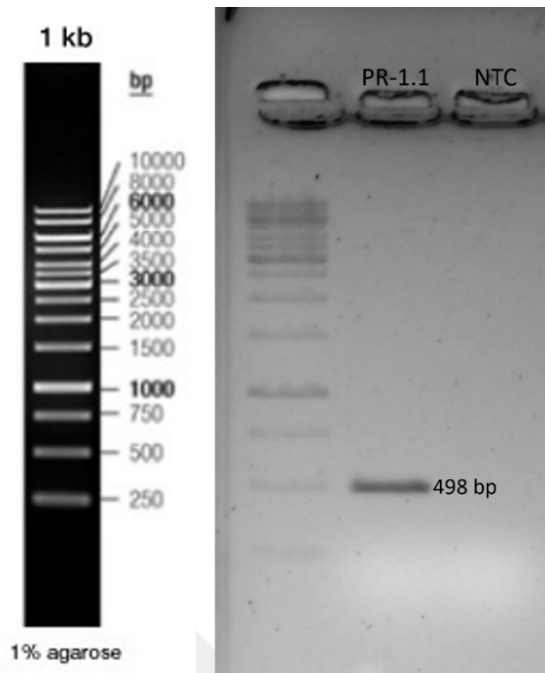


Figure 4.28. Phusion PCR results conducted with the pIPKb004-PR1.1 plasmid using *Xba*I forward and *Sac*I reverse primers

The obtained PCR product and pGFPGUSPlus were digested with *Xba*I and *Sac*I restriction enzymes aiming to create sticky ends. The expected bands of 12935 bp and 767 bp were successfully obtained from the digestion reaction with pGFPGUSPlus, as illustrated in Figure 4.29.

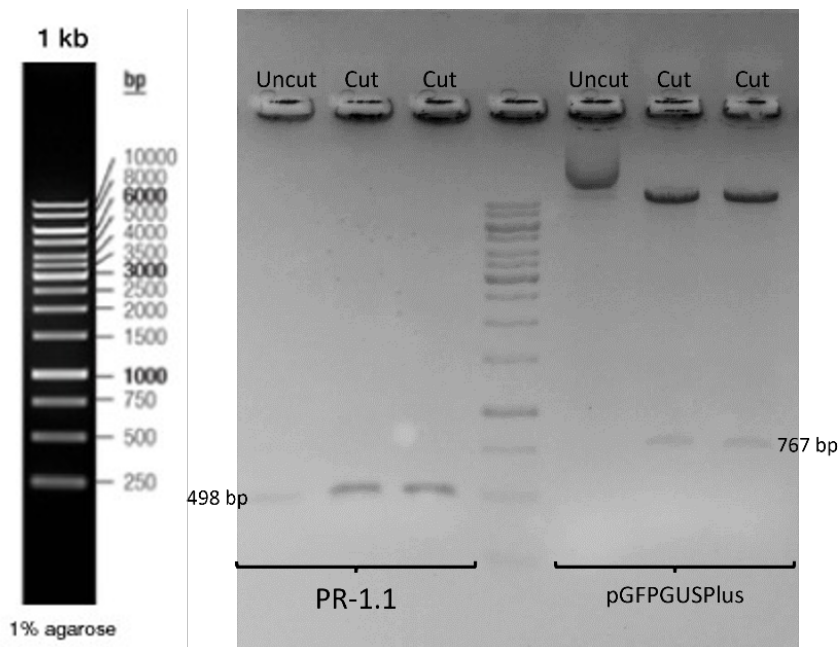


Figure 4.29. The result of the *Xba*I and *Sac*I enzyme digestion of the *SlPR-1.1* PCR product and pGFPGUSPlus plasmid

To minimize product loss during gel purification, the entire digestion products were loaded into two separate wells. Following the excision of the EGFP region, the remaining 12935 bp band and the *SIPR-1.1* digestion product were purified from the gel. The quantification after gel purification resulted in 35 ng for pGFPGUSplus and 11 ng for *SIPR-1.1*. To integrate the *SIPR-1.1* gene into pGFPGUSplusΔGFP, a ligation reaction was set up, and the ligation product was transferred to *Escherichia coli* DH5α cells using the HeatShock method. From the transformed cells spread on media containing kanamycin, a single colony was obtained. The transformed cells exhibited robust growth on LB agar without antibiotics, indicating high transformation efficiency likely due to cells resulting from pUC19 transformation. In the reaction evaluated as a negative control without *SIPR-1.1*, no colonies were observed on antibiotic-containing media (Figure 4.30).

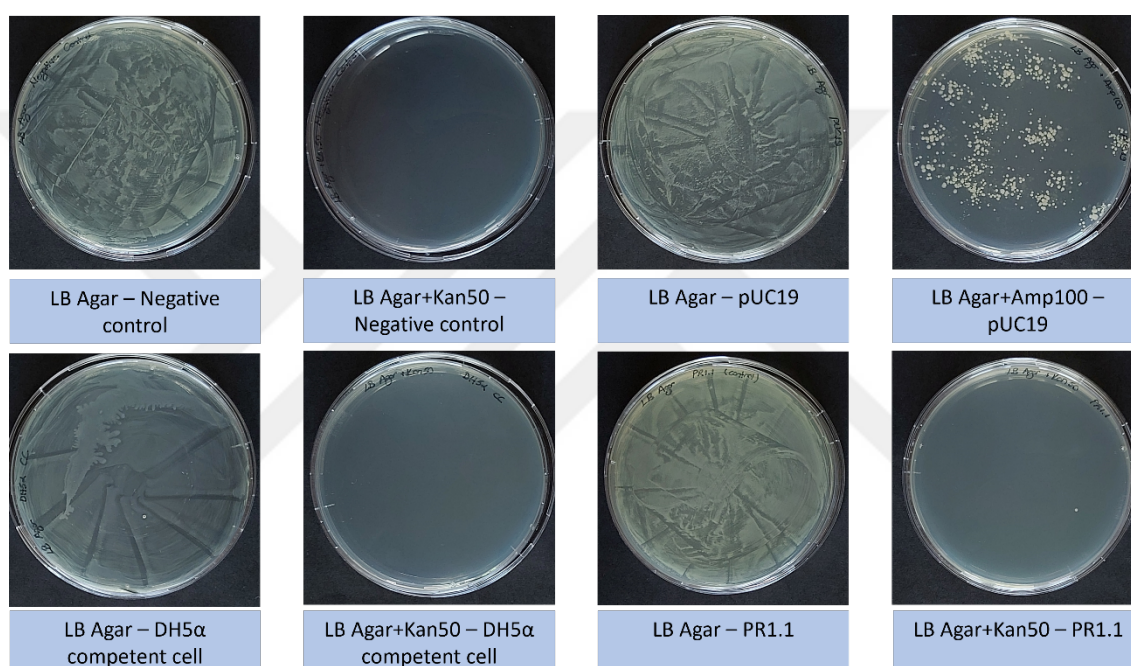


Figure 4.30. Images of the petri dishes after the transfer of the ligation product of the *SIPR-1.1* gene into pGFPGUSplusΔGFP to DH5α cells via heat shock

Following ligation, a colony PCR was conducted using the XbaI_F/SacI_R and 35S_F/SacI_R primer pairs to verify whether the insert integrated into the plasmid. However, no bands were obtained in the PCR results (Figure 4.31).

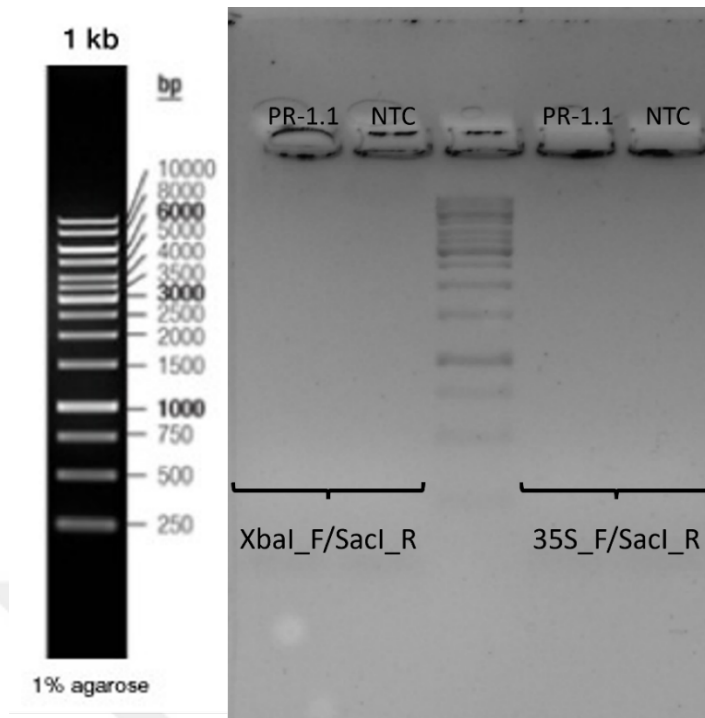


Figure 4.31. Colony PCR results using the XbaI_F/SacI_R and 35S_F/SacI_R primer pairs from the single colony obtained after ligation

Upon the initial unsuccessful ligation attempt, adjustments were made to the insert:pGFPGUSplusΔGFP ratios, the duration of the restriction reaction was extended, and modifications were applied to the method of transferring the ligation product to *E. coli*. Additionally, instead of purifying the *SIPR-1.1* digestion product from the gel, purification was performed using a PCR cleanup kit. Despite obtaining colonies in subsequent ligation attempts, negative results were consistently observed in colony PCR. To enhance the transformation efficiency of the ligation product into *E. coli* cells, the electroporation method was employed, and DH10b competent cells were utilized. Despite the formation of 9 colonies after electroporation, the expected bands were not obtained in colony PCR (Figures 4.32; 4.33).

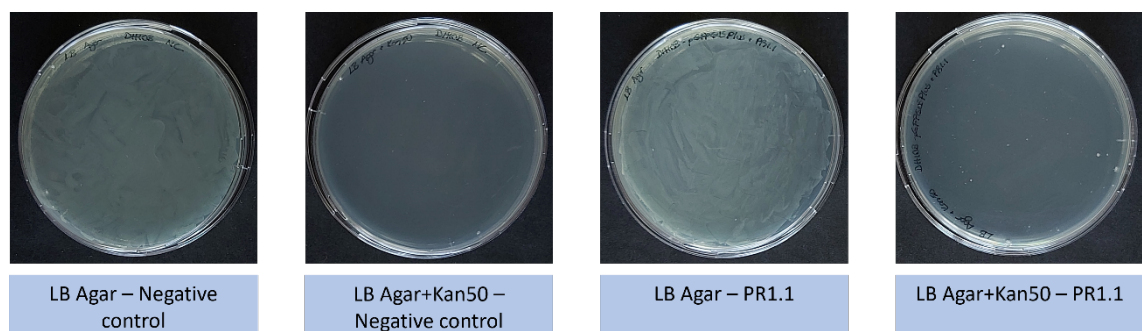


Figure 4.32. Images of petri dishes after the transfer of the ligation product of the *SIPR-1.1* gene into pGFPGUSplusΔGFP to DH10b via electroporation

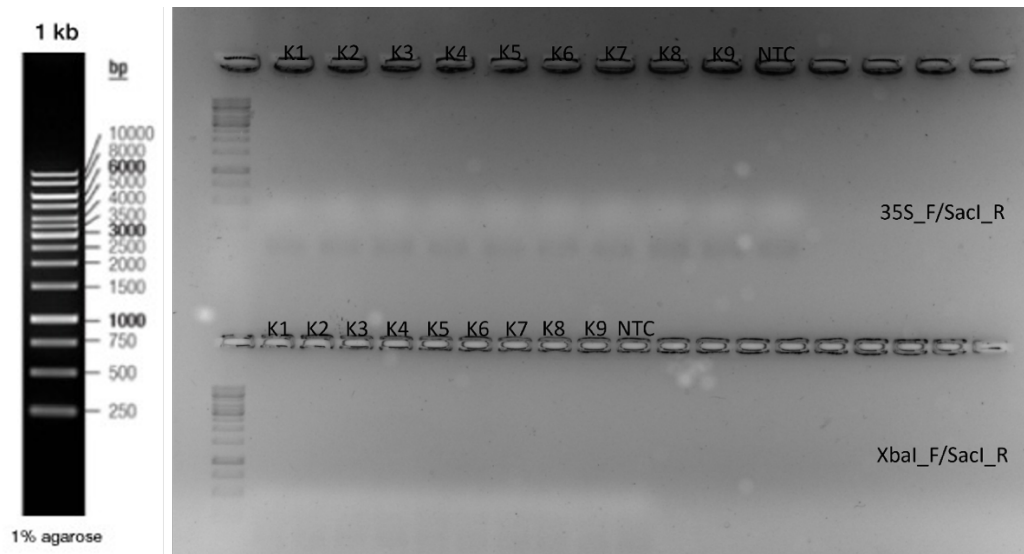


Figure 4.33. After ligation, colony PCR was conducted using primer pairs XbaI_F/SacI_R and 35S_F/SacI_R

Upon the inability to obtain results from ligation experiments, a second reverse primer (SacI_R2) incorporating the *SacI* cleavage site was designed. Following the gel electrophoresis observation of the expected 486 bp band obtained from PCR with pIPKb004-PR1.1 and XbaI_F/SacI_R2 primers, ligation experiments were initiated (Figure 4.34).

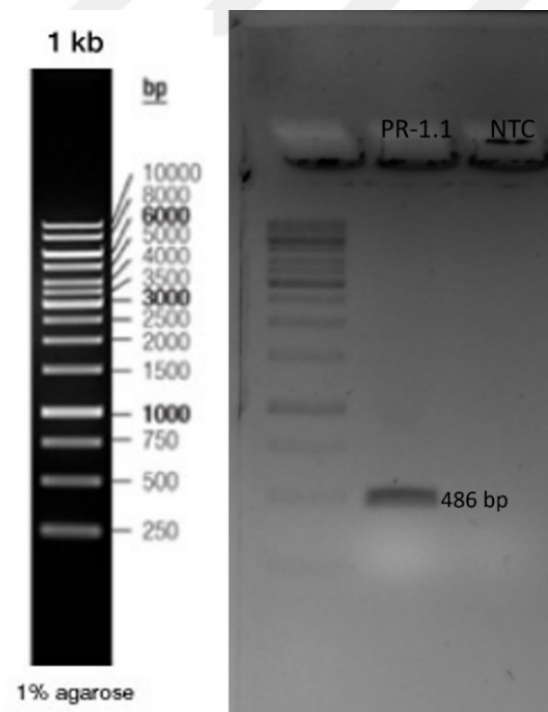


Figure 4.34. The gel image of Phusion PCR of the pIPKb004-PR1.1 plasmid with XbaI_F and SacI_R2 primers

The amplification of the *SIPR-1.1* gene was carried out by performing two 50 μ L reactions using pIPKb004-PR1.1, XbaI_F, and SacI_R2 primers. After loading 3 μ L of the obtained PCR products onto a 1% gel for verification (Figure 4.35), the products were purified using a PCR cleanup kit.

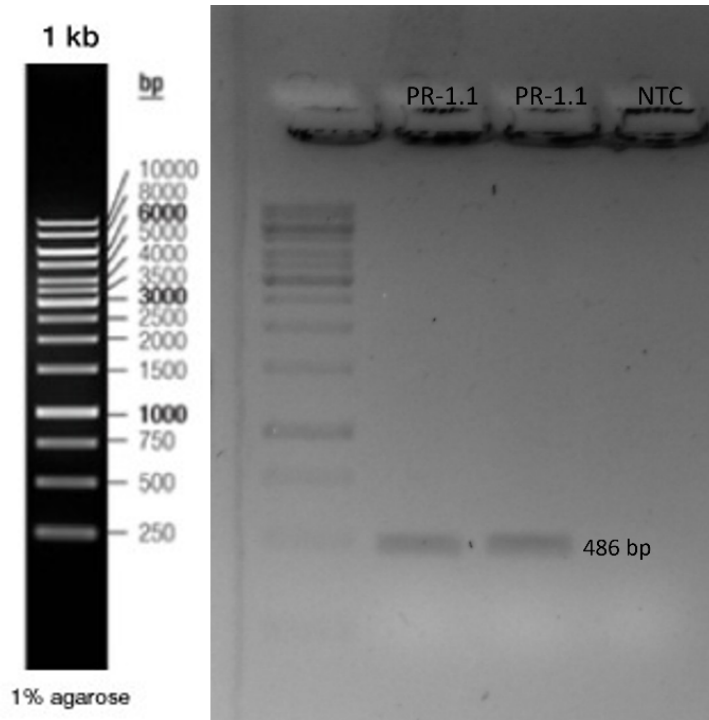


Figure 4.35. The PCR result of the pIPKb004-PR1.1 plasmid with XbaI_F and SacI_R2 primers obtained prior to the restriction digestion and purification process

The purified *SIPR-1.1* PCR product, obtained after clean-up, and the pGFPGUSPlus plasmid were subjected to a restriction digestion with *Xba*I and *Sac*I to generate sticky ends. To eliminate the possibility of self-ligation, a CIP treatment was applied to the pGFPGUSPlus digestion reaction before its completion, regardless of the digestion with different enzymes. Following the termination of digestion and CIP treatments, the resulting fragments were electrophoresed on a 1% gel at 60 V for approximately 90 minutes for verification purposes (Figure 4.36).

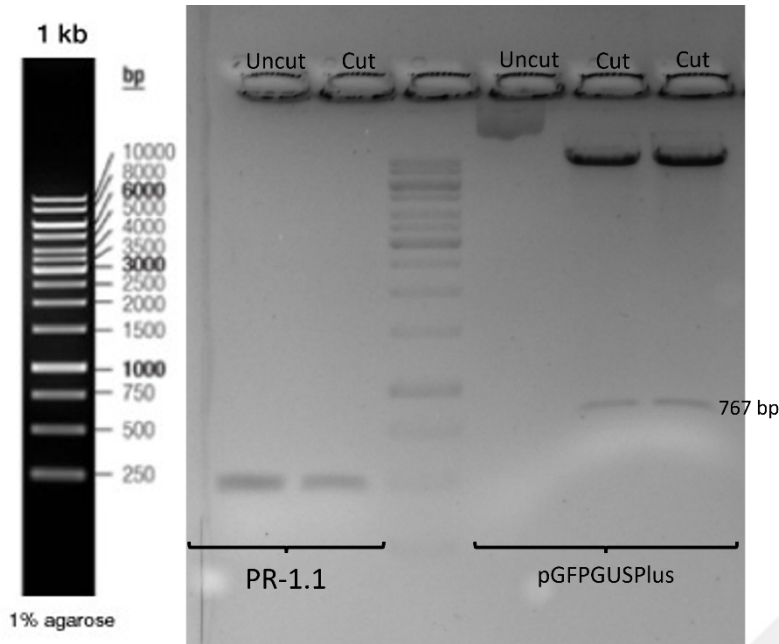


Figure 4.36. The gel image of the restriction digestion results of *SIPR-1.1* PCR product and pGFPGUSPlus plasmid using *Xba*I and *Sac*I enzymes

After the digested product of *SIPR-1.1* was purified by PCR clean-up, pGFPGUSplusΔGFP was extracted from the gel. The quantities of the obtained products after the digestion and purification processes were measured using a spectrophotometer, revealing a concentration of 42 ng/μL for *SIPR-1.1* (insert) and 15 ng/μL for pGFPGUSplusΔGFP. The ligation reaction was performed with an insert:pGFPGUSplusΔGFP ratio of 3:1 and transformed into DH5α cells using the HeatShock method. Despite spreading the transformed cells on a selection medium containing 50 mg/mL Kanamycin, no colonies were obtained. Subsequently, the same ligation product was transferred to DH10b cells using the electroporation method, resulting in the obtaining of 4 colonies after antibiotic selection. As expected, abundant growth was observed on Kanamycin-free petri dishes, confirming the viability of the cells. Simultaneously, the negative control, which was carried alongside the ligation reaction and lacked the insert, did not yield any colonies, as anticipated (Figure 4.37).

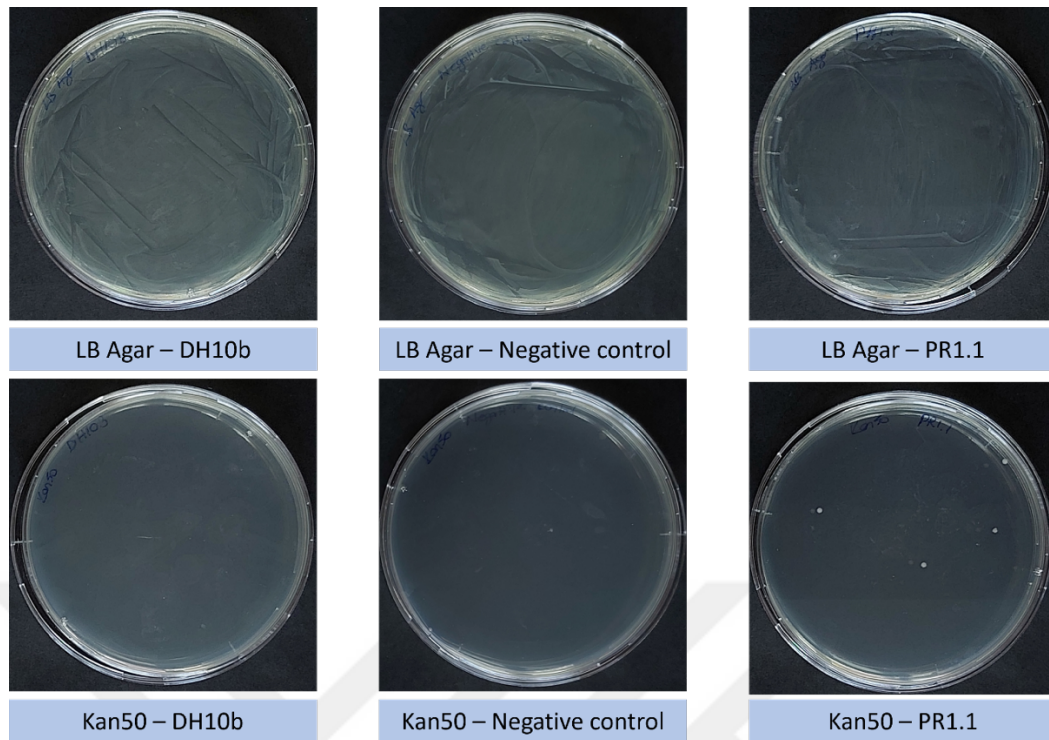


Figure 4.37. The petri dish images obtained after the ligation of pGFPGUSPlus Δ GFP and *SIPR-1.1*

After ligation, four colonies were subjected to colony PCR using the primer pairs 35S_F and gene-specific SacI_R2, as well as gene-specific XbaI_F/SacI_R2, for testing. Gel electrophoresis of the PCR products revealed the expected bands of 828 bp and 486 bp, respectively. This observation indicates the successful completion of the ligation process (Figure 4.38).

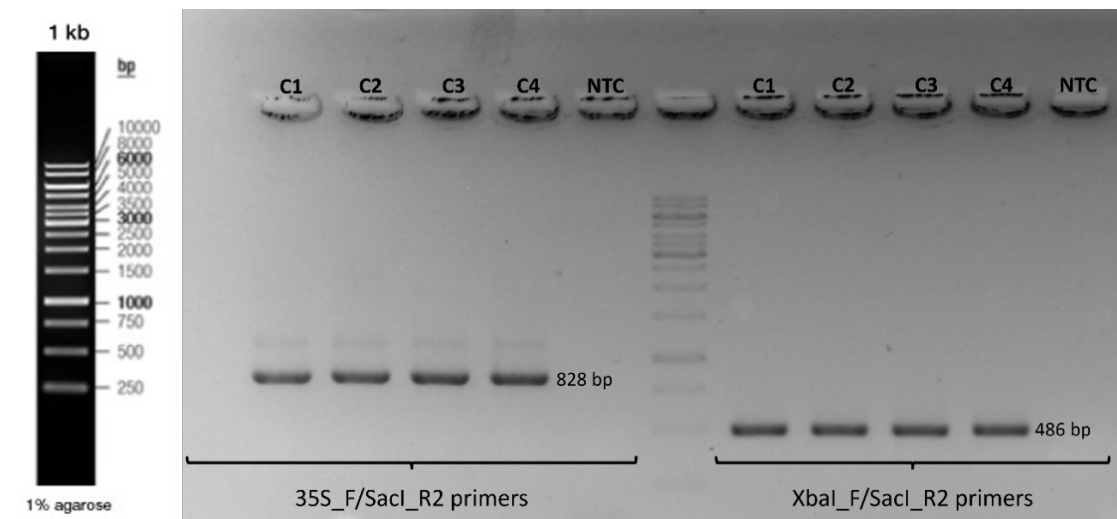


Figure 4.38. The colony PCR results obtained with the primer pairs 35S_F/SacI_R2 and XbaI_F/SacI_R2 for the four colonies after the ligation of *SIPR-1.1* and pGFPGUSPlus Δ GFP

A colony, verified through colony PCR, was selected (colony #2), grown in LB Broth, and subjected to plasmid isolation. The cloned plasmid, believed to carry the *SIPR-1.1* gene, and the control plasmid pGFPGUSPlus were both cut with the *Eco*NI restriction enzyme to confirm their authenticity. After digestion, it was expected that the plasmid believed to contain the insert would produce bands of 7684 bp and 5769 bp, while the control pGFPGUSPlus plasmid, being cut at a single site, was expected to yield a single band of 13702 bp. The desired bands were obtained from both digestions, confirming the successful cloning of *SIPR-1.1* into the pGFPGUSPlus plasmid (Figure 4.39).

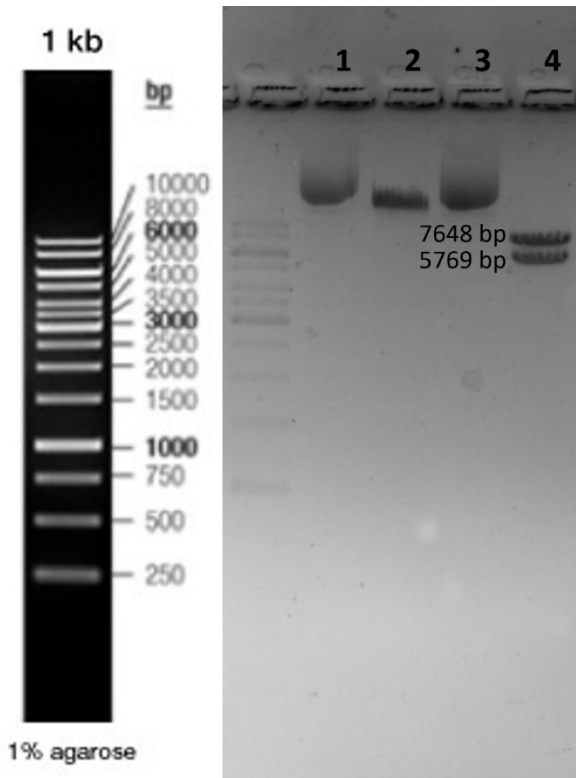


Figure 4.39. The gel image depicting the results of *Eco*NI digestion of the plasmids pGFPGUSPlus-PR1.1 and pGFPGUSPlus 1) pGFPGUSPlus uncut; 2) pGFPGUSPlus cut; 3) pGFPGUSPlus-PR1.1 uncut; 4) pGFPGUSPlus-PR1.1 cut

After confirmation of pGFPGUSPlus-PR1.1 through PCR, and digestion, efforts have commenced towards the cloning of *SIPR-1.2* into the pGFPGUSPlus-PR1.1. Initially, the pIPKb004-PR1.2 plasmid was subjected to Phusion PCR using the *Sbf*I_F/*Bst*EII_R primer pair, and the efficacy of the primers was assessed (Figure 4.40).

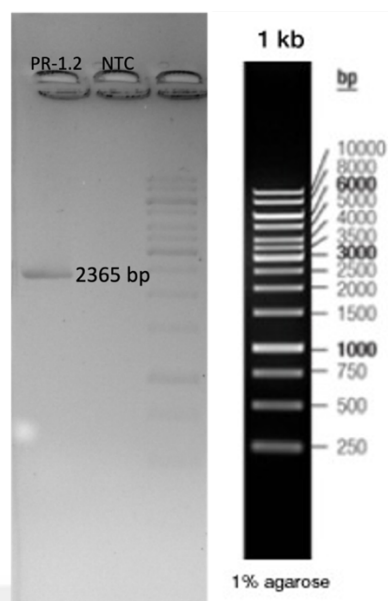


Figure 4.40. The gel image resulting from the amplification of the pIPKb004-PR1.2 plasmid using the SbfI_F/BstEII_R primer pair

Following the amplification of the *SIPR-1.2* gene using the SbfI_F and BstEII_R primers, resulting in the expected 2365 bp band, the gene was amplified in two separate 50 μ L Phusion reactions for cloning purposes. After loading 3 μ L of the PCR products onto a 1% agarose gel for verification, PCR cleanup was performed to purify the products (Figure 4.41).

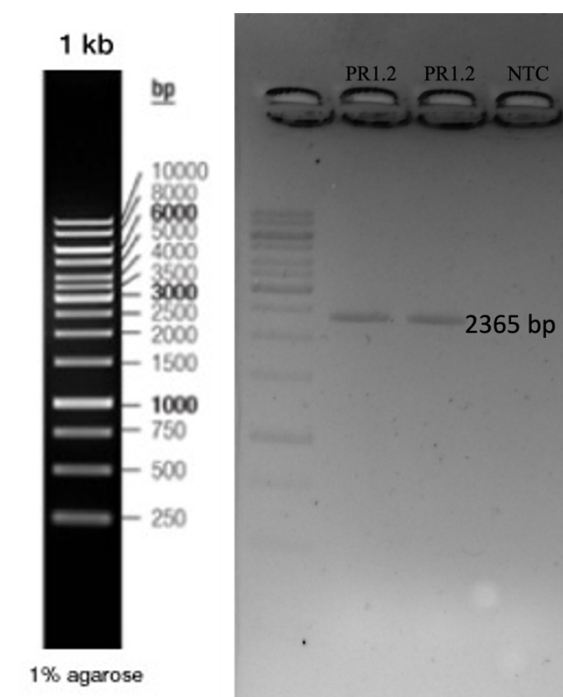


Figure 4.41. The PCR result of the pIPKb004-PR1.2 with SbfI_F and BstEII_R primers obtained prior to the restriction digestion and purification process

pGFPGUSPlus-PR1.1 and the purified *SIPR-1.2* PCR product were digested with *SbfI* and *BstEII* restriction enzymes to obtain sticky-ended products. Before terminating the digestion reaction, a CIP treatment was applied to the pGFPGUSPlus-PR1.1 digestion reaction to prevent the possibility of self-ligation of the sticky ends. The digestion and CIP treatment were then terminated by inactivation, and the digestion products were run on a 1% agarose gel at 60 V for approximately 90 minutes to verify the results. As expected, the 35S+GUSPlus region of 2828 bp aimed to be excised from the pGFPGUSPlus-PR1.1 plasmid and the backbone of 10589 bp were observed on the gel (Figure 4.42).

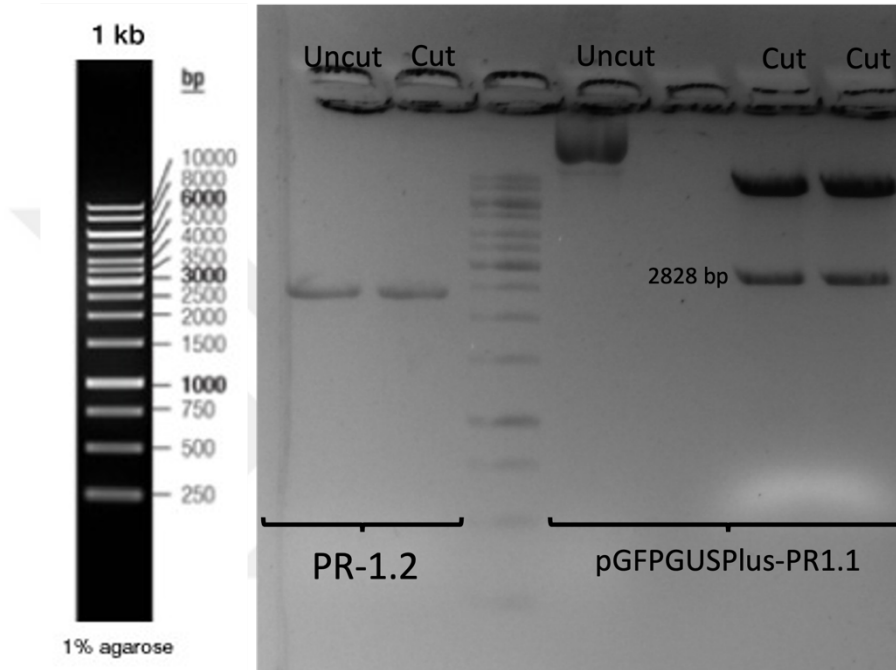


Figure 4.42. The gel image showing the results of the digestion reactions of both the *SIPR-1.2* PCR product and the pGFPGUSPlus-PR1.1 plasmid with *SbfI* and *BstEII* restriction enzymes

Following the digestion, the *SIPR-1.2* digested product underwent PCR cleanup for purification, while the pGFPGUSplus-PR1.1ΔGUSPlus was purified from the gel. Spectrophotometric measurements conducted after digestion and purification procedures indicated a concentration of 60 ng/μL for *SIPR-1.2* (insert) and 32 ng/μL for pGFPGUSplus-PR1.1ΔGUSPlus. A ligation reaction was performed at a ratio of 3:1 insert to pGFPGUSplus-PR1.1ΔGUSPlus, followed by transformation into DH10b cells via electroporation. After transformation, the cells were spread onto selection medium containing 50 mg/mL Kanamycin, resulting in the observation of 6 colonies. Upon examination of the Petri dishes, dense growth was observed on Kanamycin-free plates as expected, confirming the viability of the cells. Simultaneously, the negative control reaction, which lacked the insert, did not yield any colonies as expected (Figure 4.43).

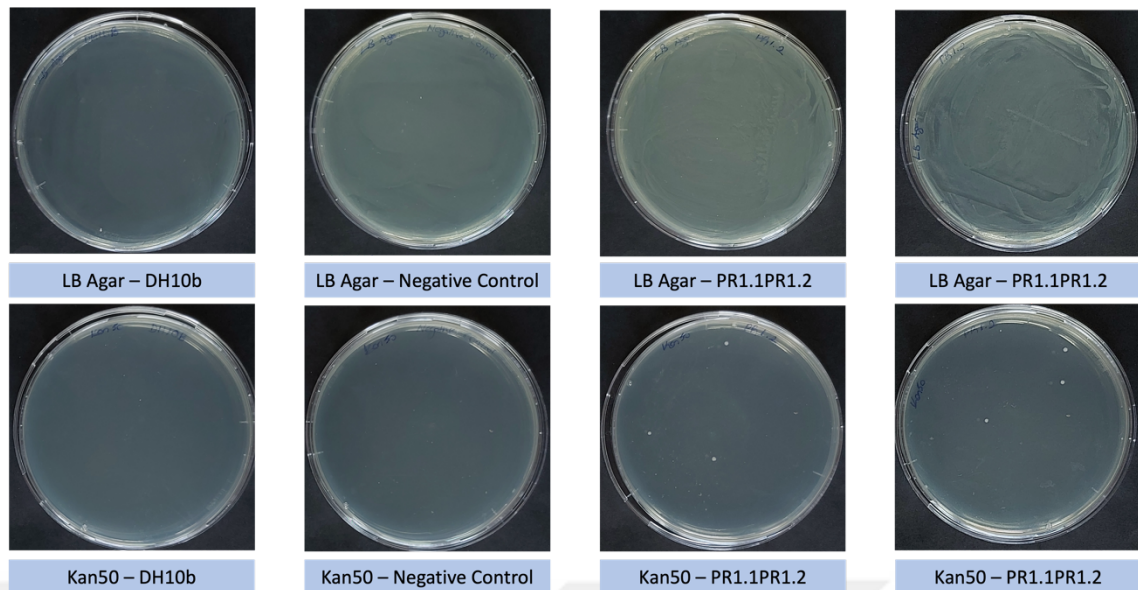


Figure 4.43. The Petri dish images obtained after the ligation of pGFPGUSplus-PR1.1 and *SIPR-1.2*

After cloning, the 6 colonies obtained were confirmed by colony PCR using the 35S_F and gene-specific BstEII_R primers targeting the plasmid. Upon running the PCR products on a 1% agarose gel, bands of the expected sizes, 2198 bp and 1871 bp, were obtained (Figure 4.44).

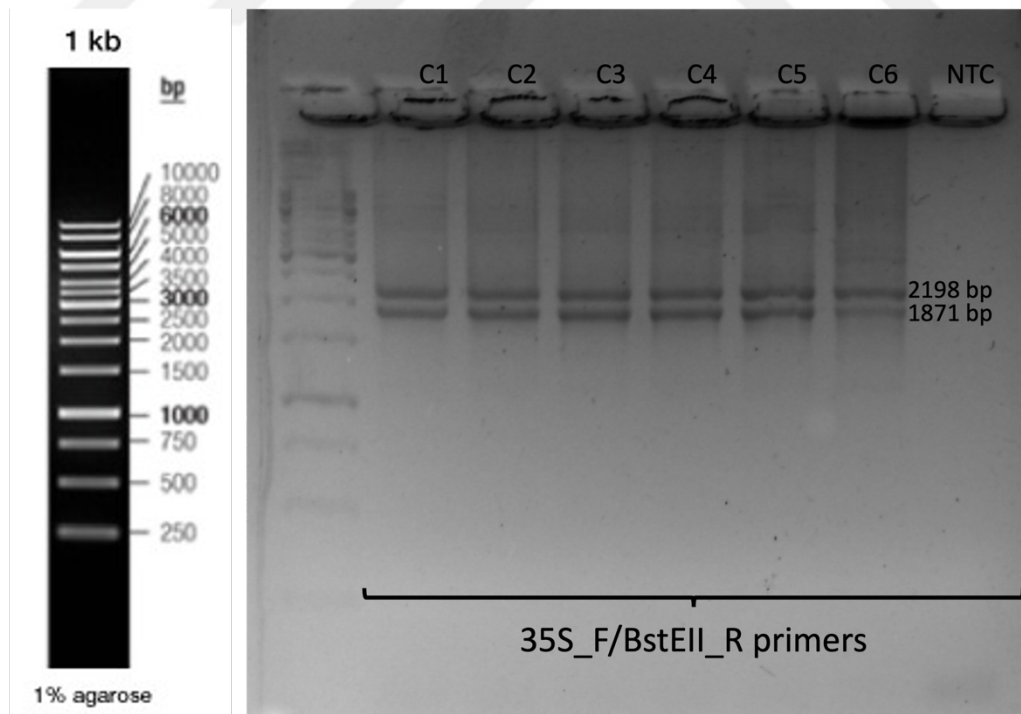


Figure 4.44. The gel image displaying the colony PCR results obtained with the 35S_F/BstEII_R primers for the 6 colonies obtained after the ligation of *SIPR-1.2* and pGFPGUSplus-PR1.1

After colony PCR, colony #1 was selected, and plasmid isolation was performed. The cloned plasmid, believed to carry both *SIPR-1.1* and *SIPR-1.2* genes, was cut with the *XbaI* restriction enzyme for verification purposes. Following digestion, the expected outcome was a single band of 13417 bp for the pGFPGUSPlus-PR1.1 plasmid, resulting from a single cleavage site. For the plasmid presumed to carry the *SIPR-1.2* insert, two bands were expected at 10985 bp and 1958 bp (Figure 4.45).

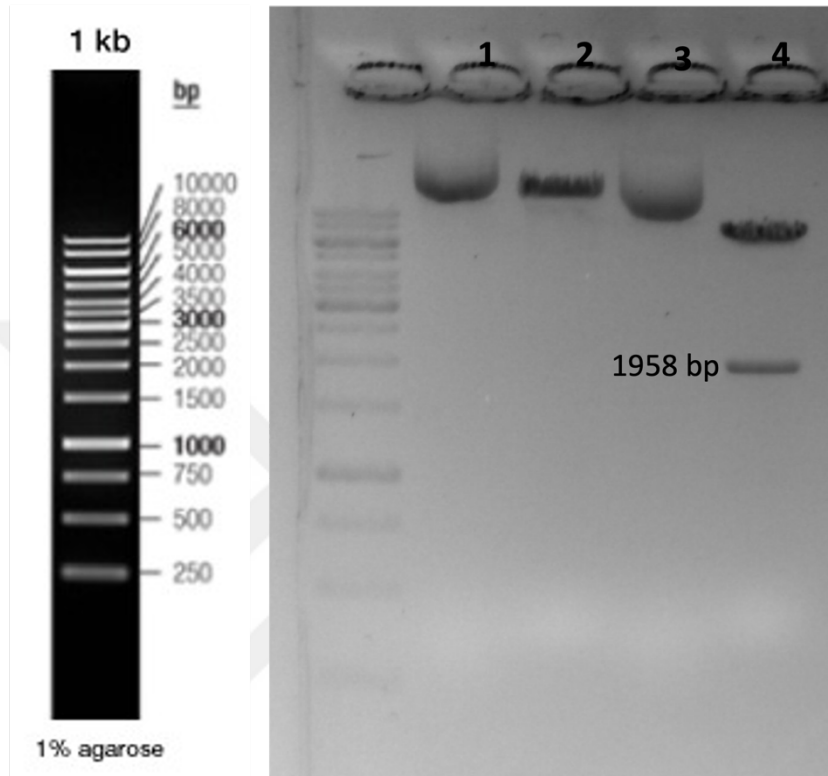


Figure 4.45. The gel image showing the results of digestion with *XbaI* for the plasmids pGFPGUSPlus-PR1.1PR1.2 and pGFPGUSPlus-PR1.1 obtained after cloning 1) pGFPGUSPlus-PR1.1 uncut; 2) pGFPGUSPlus-PR1.1 cut; 3) pGFPGUSPlus-PR1.1PR1.2 uncut; 4) pGFPGUSPlus-PR1.1PR1.2 cut

Although a band of 1958 bp was obtained from the digestion, the band of 10985 bp did not appear at the expected size. Following this, colony #6 from the other colonies obtained after ligation was selected. Plasmid isolation was carried out, and simultaneously with colony #1, it was subjected to digestion reactions using the enzyme pairs *NcoI-SphI* and *XbaI-SphI*. The digestion results, as shown in Figures 4.46 and 4.47, did not yield the expected bands from either colony. Therefore, the ligation was deemed unsuccessful.

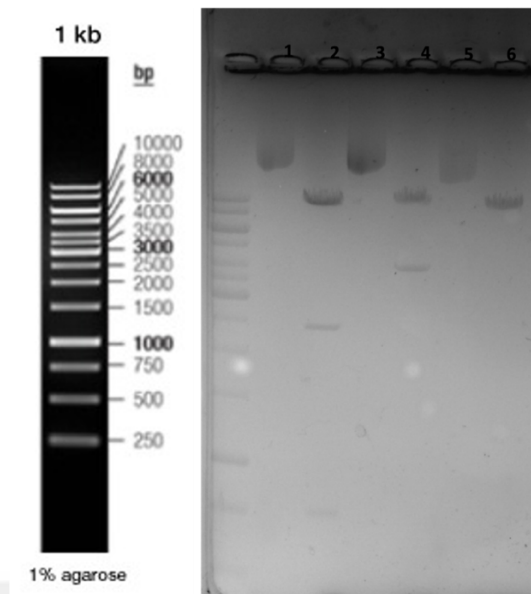


Figure 4.46. The gel image showing the results of digestion with the *NcoI-SphI* enzyme pair for the plasmids pGFPGUSPlus-PR1.1PR1.2 obtained from colonies 1 and 6, as well as for the pGFPGUSPlus-PR1.1 plasmid 1) pGFPGUSPlus-PR1.1 uncut; 2) pGFPGUSPlus-PR1.1 cut; 3) pGFPGUSPlus-PR1.1PR1.2 uncut (colony #6); 4) pGFPGUSPlus-PR1.1PR1.2 cut (colony #6); 5) pGFPGUSPlus-PR1.1PR1.2 uncut (colony #1); 4) pGFPGUSPlus-PR1.1PR1.2 cut (colony #1)

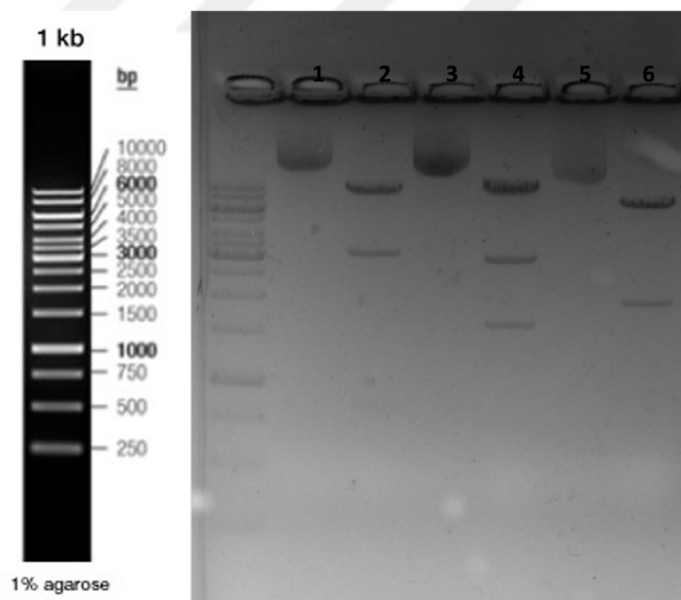


Figure 4.47. The gel image showing the results of digestion with the *XbaI-SphI* enzyme pair for the plasmids pGFPGUSPlus-PR1.1PR1.2 obtained from colonies 1 and 6, as well as for the pGFPGUSPlus-PR1.1 plasmid 1) pGFPGUSPlus-PR1.1 uncut; 2) pGFPGUSPlus-PR1.1 cut; 3) pGFPGUSPlus-PR1.1PR1.2 uncut (colony #6); 4) pGFPGUSPlus-PR1.1PR1.2 cut (colony #6); 5) pGFPGUSPlus-PR1.1PR1.2 uncut (colony #1); 4) pGFPGUSPlus-PR1.1PR1.2 cut (colony #1)

Due to the failure of the cloning experiment as confirmed by the digestion, a new ligation reaction was set up. While previously digested and purified products were used, the transformation method was changed from electroporation to heat shock. The ligation product was transferred to DH5 α cells via heat shock. Following antibiotic selection with Kanamycin, only one colony was observed, and no colonies were observed in the negative control. The viability of the cells was confirmed by the dense growth of cells spread on antibiotic-free media, and the efficiency of transformation was further verified by the growth of pUC19-transferred cells in Ampicillin-containing media (Figure 4.48).

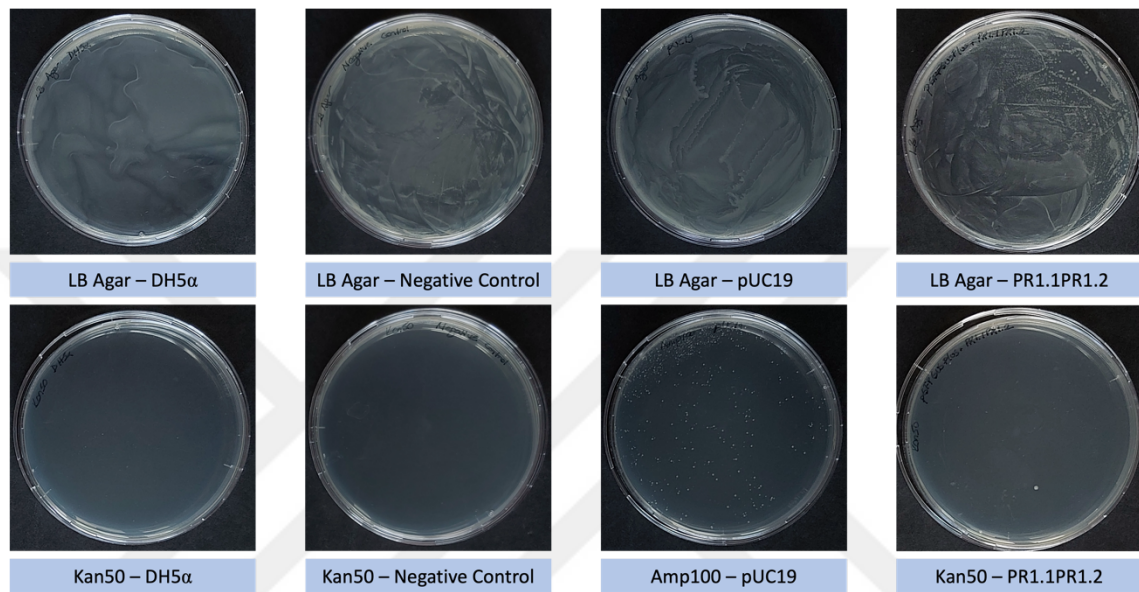


Figure 4.48. The petri dish images obtained after the second ligation of pGFPGUSPlus-PR1.1 and *SIPR-1.2*

Following cloning, the single colony obtained was confirmed by colony PCR using the 35S_F/BstEII_R and SbfI_F/BstEII_R primer pairs. Upon running the PCR products on a 1% agarose gel, bands of the expected sizes were obtained: for the 35S_F/BstEII_R primers, bands at 2198 bp and 1871 bp, and for the SbfI_F/BstEII_R primers, a band at 2365 bp (Figure 4.49).

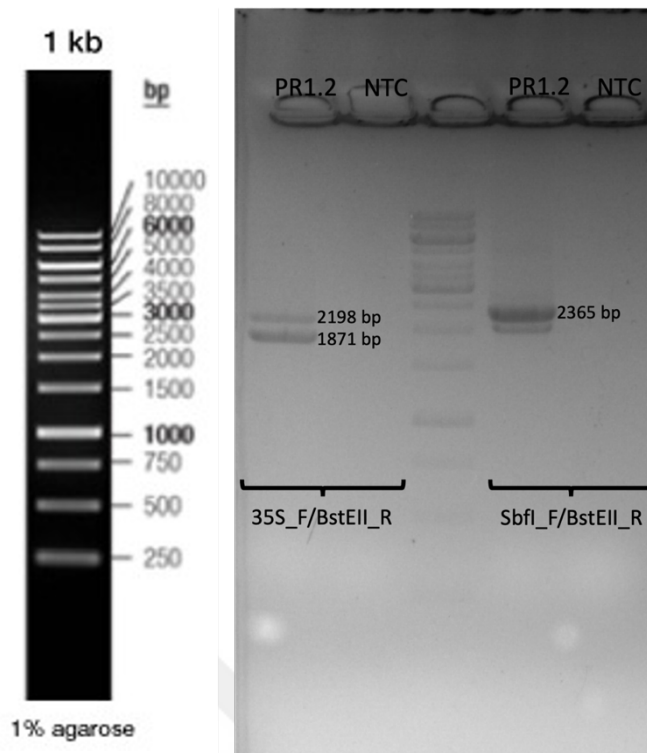


Figure 4.49. The gel image showing the results of colony PCR using the 35S_F/BstEII_R and SbfI_F/BstEII_R primers for the single colony obtained after the ligation of *SIPR-1.2* and pGFPGUSPlus-PR1.1

After plasmid isolation following colony PCR, two separate digestion reactions were performed to verify the success of ligation. The cloned plasmid, suspected to carry the *SIPR-1.1* and *SIPR-1.2* genes, was subjected to separate digestion reactions using the *NcoI-SphI* and *XbaI-SphI* enzyme pairs for confirmation. Unlike the first ligation, this time, bands of the expected sizes were observed correctly, as shown in Figure 4.50.

The plasmid is fully sequenced and confirmed. *SIPR-1.1* and *SIPR-1.2* genes were successfully cloned into the pGFPGUSPlus plasmid, and all the goals of the thesis were achieved.

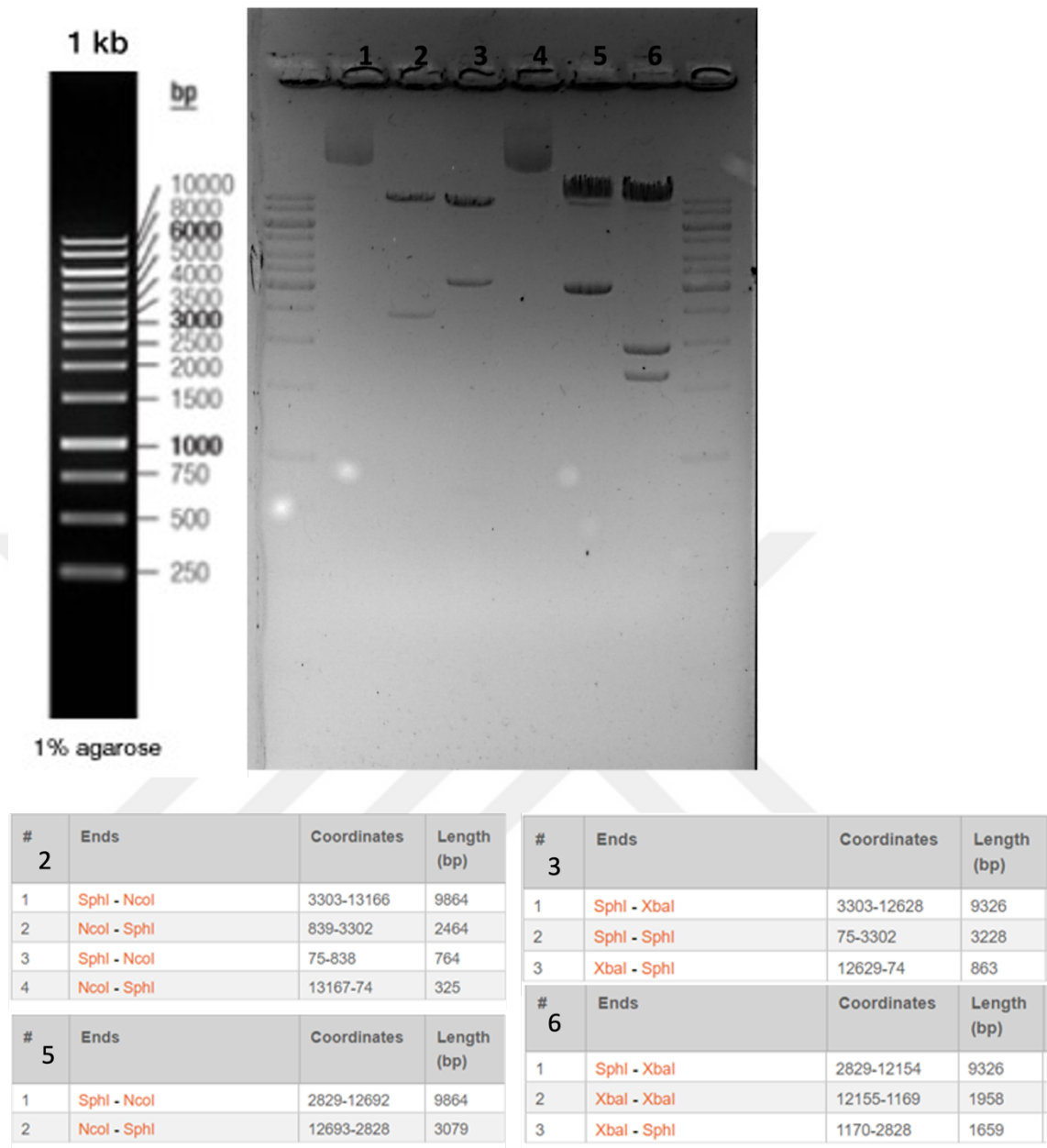


Figure 4.50. The gel image showing the results of digestion with the *NcoI-SphI* and *XbaI-SphI* enzyme pairs for the single colony obtained from ligation of *SLPR-1.2* and the pGFPGUSPlus-PR1.1 plasmid 1) pGFPGUSPlus-PR1.1 uncut; 2) pGFPGUSPlus-PR1.1 cut (*NcoI-SphI*); 3) pGFPGUSPlus-PR1.1 cut (*XbaI-SphI*); 4) pGFPGUSPlus-PR1.1PR1.2 uncut; 5) pGFPGUSPlus-PR1.1PR1.2 cut (*NcoI-SphI*); 6) pGFPGUSPlus-PR1.1PR1.2 cut (*XbaI-SphI*)

*Even though the bands at 863 bp, 764 bp, and 325 bp sizes might not be visible after running the gel for an extended period, a suitable image conforming to the cutting motif was obtained within the first half-hour of running.

5. DISCUSSION

Plants need to cope with various threats in their natural habitats, including abiotic stresses such as drought, salt, cold, and alkalinity, as well as biotic stresses caused by various pathogen infections like fungi, oomycetes, viruses, bacteria, and nematodes (Chen et al., 2020). Particularly, harmful pathogens posing a threat to plants lead to significant economic and production losses in agricultural activities. Globally, it is estimated that yield losses due to plant diseases range from 10% to 40% (Savary et al., 2019).

Plants, being sessile organisms, lack specialized immune cells and a circulatory system. Consequently, they have evolved a complex and versatile immune system to combat environmental threats (Sun et al., 2020). The first line of defense in plants consists of physical barriers formed in response to attacks. Among these barriers are structures like the cell wall and cuticle. Pathogens capable of breaching these physical barriers encounter an evolved multilayered immune response system, involving both innate and acquired stress responses (Jones & Dangl, 2006). Simultaneously, plants have developed a range of molecular components, both constitutive and inducible, as part of their defense mechanisms. These components include cell wall polymers, secondary metabolites, and proteins with antimicrobial activities (Slusarenko et al., 2012; van Loon et al., 2006). Defense responses are triggered upon recognition of invading pathogens. Following pathogen recognition, the production of signal components spreading systemically from the infected region initiates (Durrant & Dong, 2004). As a result, increased resistance against secondary infections is conferred in distant tissues. The spread of this resistance is explained by the term Systemic Acquired Resistance (SAR).

Following the induction of defense in the infection site, the activation of Systemic Acquired Resistance (SAR) is triggered. This activation typically occurs in distant tissues from the infected areas. In this way, non-infected tissues become protected against potential subsequent infections by pathogens (Pieterse et al., 2009). SAR is associated with increased levels of salicylic acid (SA), a plant hormone, in both the infection site and systemic tissues. SA is widely produced in prokaryotes and plants. The accumulation of SA leads to the induction of pathogenesis-related (PR) genes with antimicrobial activity (van Loon et al., 2006).

Systemic Acquired Resistance (SAR) is a plant stress response mechanism activated at the molecular level by the high expression of a group of Pathogenesis-Related (PR) genes. Since the initial identification of PR genes by van Loon in the 1970s, numerous PR genes have been isolated from various plants, and a range of PR proteins has been biochemically characterized. Some PR proteins have been observed to possess *in vitro* antimicrobial activities (Van Loon & Van Strien, 1999; van Loon et al., 2006). Notably, it has been reported in previous studies that PR proteins also respond to abiotic stresses such as drought (Akbudak et al., 2020). Additionally, PR proteins have garnered significant attention in recent research due to their roles in plant growth and development (Lotan et al., 1989). However, transgenic plants with overexpressed PR genes or mutants with disrupted expression of PR genes often do not exhibit a significant phenotype in defense responses (Linthorst et al., 1989; Yoshikawa et al., 1993; van Loon et al., 2006). Consequently, their physiological functions remain incompletely understood from various perspectives. Therefore, in recent years, it has been hypothesized that the

coordinated effects of multiple PR proteins largely contribute to the phenomenon of SAR in defense responses (Durrant & Dong, 2004; van Loon et al., 2006).

In this thesis study, vectors containing tomato PR1.1 and PR1.2 genes, whose expression profiles under drought stress were determined for the first time in a study by Akbudak et al., (2020), were successfully generated individually and in combination. Observing the defense responses in the plant resulting from the separate and combined expression of the identified genes will fill several gaps in the existing literature. Therefore, the construction, a crucial step in obtaining transgenic plants, was successfully completed through this study.

In all created vectors, the *SIPR-1.1* and *SIPR-1.2* genes were driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter. The 35S promoter is one of the most commonly used promoters in plant biotechnology studies. Its prominence in such studies stems from its ability to significantly enhance expression when transferred to plants (Datta & Muthukrishnan, 1999).

The 35S promoter is derived from Cauliflower Mosaic Virus (CaMV) and has been utilized in various studies for over 30 years since its identification (Odell et al., 1985; Somssich, 2019). It is considered a strong constitutive promoter that exhibits high transcriptional activity (Bak & Emerson, 2020; Dutt et al., 2014). The duplication of the CaMV 35S promoter has been shown to increase its transcriptional activity tenfold (Kay et al., 1987), and since then, this promoter construct has been widely used to achieve high-level expression of foreign genes in transgenic plants. In a study conducted by (Kiselev et al., 2021), they systematically and comprehensively investigated the activity of the CaMV 35S promoter in the model plant species *Arabidopsis thaliana* L. They performed extensive analyses of transgenes (*neomycin phosphotransferase* II [*NPTII*] and enhanced green fluorescent protein [EGFP]) driven by the double CaMV 35S promoter, considering organ type, different time periods during the day, plant age, and abiotic stress conditions. According to the results of quantitative reverse transcription PCR (qRT-PCR) analysis, they demonstrated that the transcript levels of *NPTII* and EGFP were significantly higher in cotyledons, young leaves, and roots compared to other plant organs (inflorescences, stems, and adult leaves). The expression of *NPTII* and EGFP varied throughout the day and increased as the plant aged. While drought and cold stress significantly affected transgene expression, temperature, high salinity, and injury did not have a significant impact (Kiselev et al., 2021).

In this study, the pIPKb004 vector was employed for the separate cloning of *SIPR-1.1* and *SIPR-1.2* genes using Gateway cloning. Gateway technology, utilizing a modified bacteriophage λ recombination system, allows for highly efficient, target-specific, and reliable exchange of DNA fragments between plasmids. Similar to other binary destination vectors used in gateway cloning, pIPKb004 includes compatible recombination regions (attR1 and attR2) integrated downstream of the 35S promoter. Between the attR1 and attR2 regions, the negative bacterial selection marker *ccdB* is present. Bacterial selection was applied to obtain the successfully recombined target vector with high efficiency. Spectinomycin was used as the antibiotic selection marker for pIPKb004. The vector also contains *hygromycin phosphotransferase* (*hpt*), widely used as a plant selection marker (Himmelbach et al., 2007).

In their study, Kavas et al., (2016) investigated the response of transgenic tobacco plants to salt stress by overexpressing the *TaSTRG* gene associated with salt tolerance in *Triticum aestivum*. Similar to the thesis study, the *TaSTRG* gene was initially cloned into the pENTR/D-TOPO vector by pENTR Directional TOPO Cloning Kit, allowing the gene to be cloned into the target region with high efficiency, specificity, and reliability, thanks to the overhang added to the 3' end. They used the pIPKb004, a Gateway-based binary plant expression vector, and performed an LR reaction between the entry vector pENTR/D-TOPO with *TaSTRG* and the binary destination vector pIPKb004 using the Gateway LR Clonase II enzyme mix. As a result of the study, it was observed that the overexpression of the *TaSTRG* gene significantly increased salt tolerance in tobacco plants (Kavas et al., 2016).

The cloning of the *SIPR-1.1* and *SIPR-1.2* genes into the pGFPGUSPlus vector was performed using the traditional cloning method. The traditional cloning method, broadly defined as the cut-and-paste approach, remains a widely used technique today. pGFPGUSPlus, carrying enhanced green fluorescent protein (EGFP) (Chalfie et al., 1994) and enhanced β -glucuronidase (GUSPlus) (Broothaerts et al., 2005) expression cassettes, was developed by (Vickers et al., 2007). Each reporter gene is controlled by a constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter. The reporter gene cassettes and a hygromycin resistance gene cassette are located between the T-DNA borders, enabling *Agrobacterium*-mediated transformation. The ability to remove and replace both expression cassettes with different genes of interest is a key feature in the utilization of this system in the study (Vickers et al., 2007).

Musfir Mehaboob et al., (2019) developed an effective and simple *Agrobacterium*-mediated transient transformation method for "ginger," and obtained transgenic plants through the pGFPGUSPlus vector containing the β -glucuronidase (*gus*) reporter gene and *hygromycin phosphotransferase* (*hptII*) selection marker. Histochemical GUS analysis provided confirmation of the temporary expression of the *gus* gene, a finding further validated through PCR analysis using specific primers for the *hptII* and *nptII* genes (Musfir Mehaboob et al., 2019). However, pGFPGUSPlus is commonly utilized to prepare fusion gene constructs containing GFP or GUSPlus reporter genes (Cao et al., 2011; Dai et al., 2020).

6. CONCLUSION

Gene cloning refers to the process of isolating and making copies of a specific gene. Genes associated with pathogenesis are those that play a role in the development of diseases. Biotic stress refers to the adverse effects on plants caused by living organisms, such as pathogens. Abiotic stress, on the other hand, includes environmental factors like drought, salinity, and temperature that affect plant growth and development. Drought is a specific form of abiotic stress characterized by a lack of water.

A vector is a carrier molecule used to introduce foreign genetic material into a host organism, facilitating gene cloning. Gateway cloning is a modern molecular biology technique that allows for the efficient and rapid transfer of DNA fragments between different vectors. Traditional cloning methods involve the manual insertion of DNA fragments into vectors.

This study focuses on cloning pathogenesis-related genes (*SIPR-1.1* and *SIPR-1.2*) that are known to be upregulated under drought stress. The genes are cloned into plant expression vectors, specifically using the pIPKb004 vector through gateway cloning for individual cloning of genes and the pGFPGUSPlus vector through traditional cloning for cloning the genes together. The presence of the genes within the plasmids is confirmed through PCR and restriction reactions. The resulting plant expression vectors will be used to investigate the stress responses, particularly under abiotic stresses, induced by the separate and combined expression of these genes.

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