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Transformation of *Arabidopsis* by Flag tagged PP2A

by
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Master's thesis for partial fulfillment of the Master degree in
Biological Chemistry

at the
Faculty of Science and Technology,
University of Stavanger

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“Success is going from failure to failure without losing enthusiasm.”

Winston Churchill

Abstract

Eukaryotic protein kinases transfer a phosphate group from ATP to the hydroxyl group of Ser, Thr and Tyr residues, whereas protein phosphatases hydrolyze the phosphoester bond to give free phosphate and dephosphorylated protein. Protein phosphorylation and dephosphorylation are essential for the regulation of metabolism, cell division, development, growth, and stress response in all organisms. Protein phosphatase type 2A is a serine and threonine specific phosphatase. The enzyme is characterized by its multi-subunit structure that allow it to participate in a wide range of signaling cascades that regulate plant growth, defense and development. Protein phosphatase type 2A is made up of three subunits, a catalytic (C), scaffold (A) and regulatory (B) subunit. The regulatory subunit is divided into three groups called B, B' and B''. A particular subgroup of B' called *B'φ* (*B'phi*) appears to be crucial for the interaction between plants and microorganisms, especially mycorrhiza. *B'φ* has not been studied much and can also be important for regulating other processes. The model plant *Arabidopsis thaliana* (*Arabidopsis*) does not have the subunit *B'φ*, and the aim of this project is to i) clone *B'φ* from *Solanum lycopersicum* (tomato plant) ii) link the tomato gene with a FLAG tag and transform it into an *Arabidopsis*. *Arabidopsis* plants will be selected on BASTA for further cultivation and the presence of *B'φ* will be confirmed with PCR. Further experiments can provide information about the location of the protein in the cell, and interaction partners to compare the interacting proteins identified in *Arabidopsis* and tomato.

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Stavanger, June 2019

Lene Skår

Abbreviations

PPs	Protein phosphatases
ATP	Adenosine triphosphate
OH	Hydroxyl group
PPP	Phosphoprotein phosphatase
PPM	Metallo-dependent protein phosphatase
PTP	Protein-tyrosine phosphatase
pSer/pThr	Phosphoserine/phosphotreonine
pTyr	Phosphotyrosine
DSP	Dual specificity classes
PP2A	Protein phosphatase 2A
VIGS	Virus-induced gene silencing
HR	Hypersensitivity response
BAK1	Receptor BRI1-associated kinase 1
FLS2	Flagellin sensing receptor 2
CPKs	Calcium-dependent protein kinases
ROS	Reactive oxygen species
TF	Transcription factor
AOX	Alternative oxidases
RLK	Receptor-like kinases
RLP	Receptor-like proteins
Bp	Base pairs
Kb	Kilobases
dsDNA	Double-stranded DNA
E. coli	*Escherichia coli*
ORI	Replication origin
NCBI	National Center for Biotechnology Information
MS	Murashige and Skoog medium
Spec	Spectinomycin
Kan	Kanamycin
Amp	Ampicillin

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1 INTRODUCTION

1.1 Regulatory enzymes

A number of enzymes catalyze the transfer of phosphates to proteins in the cell. The variation is large between the enzymes, yet these enzymes share certain properties and fall into a class of proteins, called protein kinases. Their similarities stem from the group's ability to take phosphate from the chemical energy-bearing molecule ATP and place it on an amino acid side chain of a protein. The hydroxyl groups (-OH) of the serine, threonine, tyrosine or histidine amino acid side chains are the most common targets. A second class of enzymes is responsible for the reverse reaction, in which phosphates are removed from a protein. These are called protein phosphatases (Lillo et al. 2014; Ardito et al. 2017).

The use of phosphorylation/dephosphorylation of a protein as a regulatory mechanism has many advantages: it is rapid, taking as little as a few seconds. It does not require new proteins to be made or degraded. It is easily reversible (DeLong et al. 2006; Brautigan et al. 2013; Ardito et al. 2017).

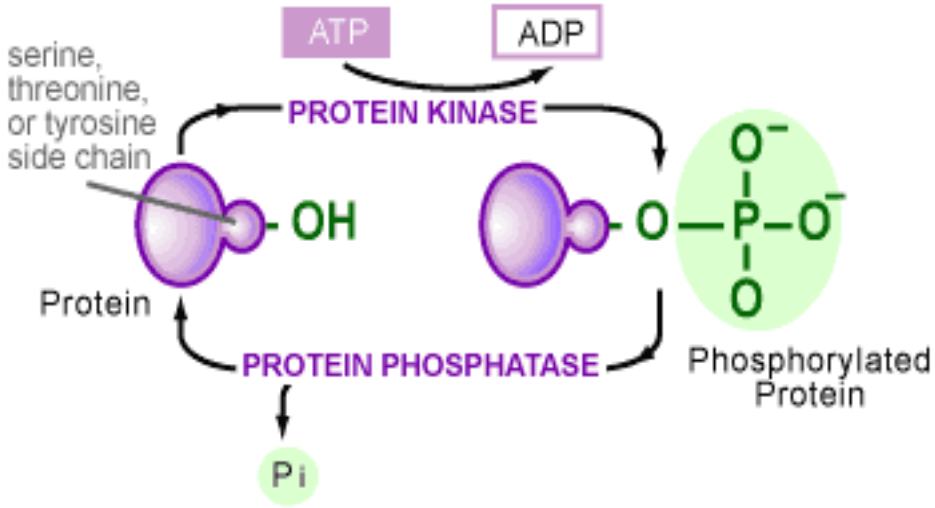


Figure 1.1. Protein kinases catalyze the transfer of a phosphate group to a specific amino acid chain of a protein (phosphorylation). Protein phosphatases dephosphorylate the target protein by removing the phosphate group. The figure is obtained from Ardito et al. 2017.

1.1.1 Protein phosphatases

Eukaryotic phosphatases are classified into three families: the phosphoprotein phosphatase (PPP) family, metallo-dependent protein phosphatase (PPM) family and protein-tyrosine phosphatase (PTP) family (Kerk et al. 2008; Uhrig & Moorhead et al. 2011a,b). The protein phosphatase families are classified according to their substrate specificity (DeLong et al. 2006; Brautigan et al. 2013; Uhrig et al. 2013b). The PPPs are for example serine/threonine (Ser/Thr) specific phosphatases that include PP1, PP2A, PP2B, PP4-7, and protein phosphatases with kelch repeat domains (Uhrig et al. 2013a,b; Maselli et al. 2014). PPMs include Ser/Thr-spesific ion dependent protein phosphatases, also known as PP2Cs.

Members of all subfamilies (PP1, PP2A, PP4-7 and PP2Cs) except PP2B are identified in plants, where PP1 and PP2A are responsible for the majority (80%) of total cellular phosphatase activity (Janssens & Goris et al. 2001; Uhrig et al. 2013b). The *Arabidopsis thaliana* (*Arabidopsis*) genome encodes 26 PPP catalytic subunits related to type PP1 and PP2A and so-called novel phosphatases, including four plant-specific enzymes carrying large N-terminal kelch-domains have been identified, but no apperent homologue of the PP2B family (Farkas et al. 2007). In this project, the focus has been on the Ser/Thr-spesific protein phosphatase 2A (PP2A).

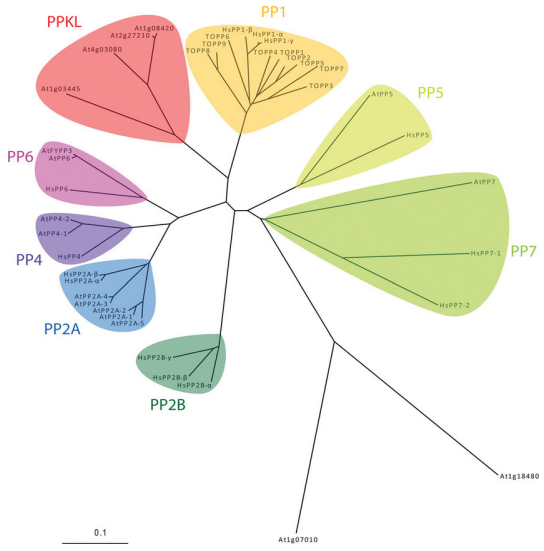


Figure 1.2. The phylogenetic tree includes PPP-enzymes from the organisms *Homo sapiens* and *Arabidopsis* (Cohen et al. 2011). The figure is obtained from Moorhead et al. 2009.

1.1.2 Protein phosphatases 2A

PP2A is an enzyme capable of removing a phosphate modification from Ser/Thr residues on protein substrates to help regulate cellular activity in plants and other organisms (Lillo et al. 2014; Ardito et al. 2017). Recent studies have indicated that PP2A is a crucial component that controls pathogenesis responses in various plant species. Genetic, proteomic and metabolomic approaches have underscored the versatile nature of PP2A, which contributes to the regulation of receptor signaling, organellar signaling, gene expression, metabolic pathways, and cell death, all of which essentially impact plant immunity (Durian et al. 2016).

1.1.3 PP2A structure

PP2A holoenzyme consists of a 36 kDa catalytic subunit that occurs either in association with a 65 kDa regulatory A subunit or together with a third variable B subunit. The B subunits, which determine the substrate specificity and subcellular localization of PP2As, are classified into 55 kDa B, 54-74 kDa B′ and 72-130 kDa B″subunit families. Genes encoding PP2A subunits have been characterized in several plant species (Pais et al. 2009).

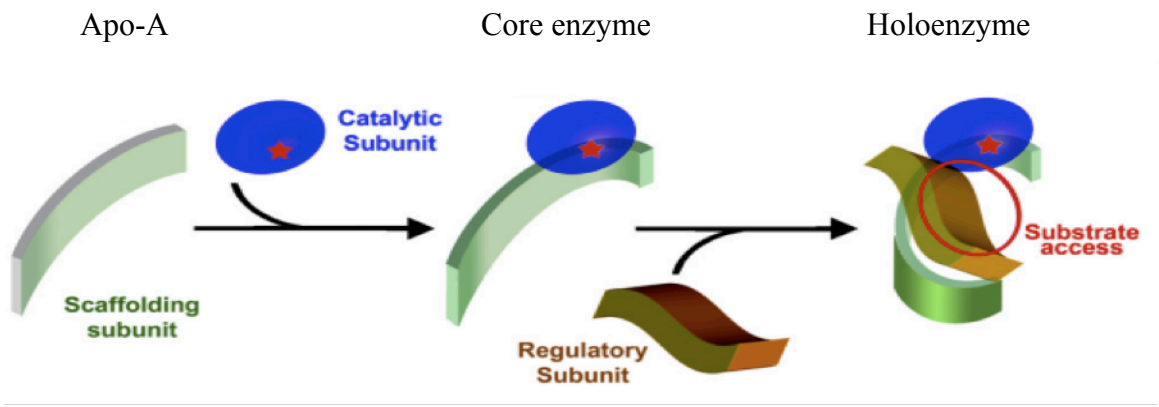


Figure 1.3. A model of the PP2A holoenzyme. The first step in holoenzyme assembly is the association between the free scaffolding subunit and the catalytic subunit to form the core enzyme. This association results in significant conformational changes in the scaffolding subunit. The PP2A core enzyme interacts with a variable regulatory subunit to form a holoenzyme, leading to a more drastic conformational change in the scaffold composition. The regulatory B subunits facilitate substrate access by using their acidic concave trace (red ring). The red star of catalytic subunit denotes the active site (Xu et al. 2006).

In *Arabidopsis*, the C subunits are encoded by five genes (*$PP2A_C$-1, 2, 3, 4 and 5*); A subunits are encoded by three genes (*RCN1, PP2AA2, PP2AA3*); B subunits are encoded by two related genes (*α and β*), B´subunits are encoded by nine related genes (*α, β, γ, δ, ε, ζ, η, θ and κ*); B´´subunits are encoded by six related genes (*α, β, γ, δ, ε and TON2*) and TAP46 is an unrelated regulatory subunit (Janssens et al. 2001). See the figure below.

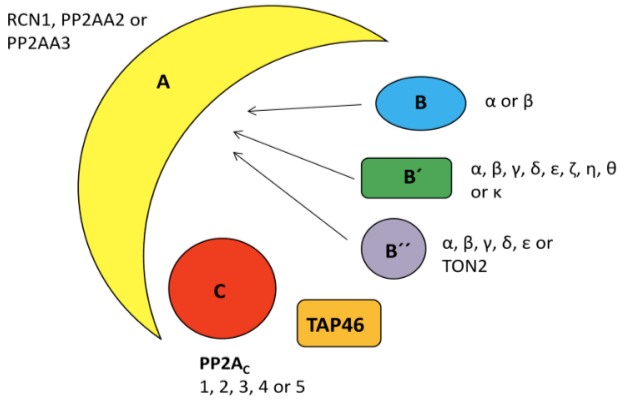


Figure 1.4. PP2A is composed of three hetero-dimers, the scaffold subunit, the catalytic subunit, and the regulatory subunit. There are three families of regulatory subunits, each having multiple isoforms as shown in the figure (Tran et al. 2012). The figure is obtained from Janssens et al. 2001.

1.1.4 Catalytic subunits

The catalytic subunits are extremely conserved throughout eukaryotes, and the five C subunits in *Arabidopsis* show 79% amino acid sequence identity overall (DeLong et al. 2006). The genes encoding the C subunits are grouped into two subfamilies:

- Subfamily 1: $PP2A_{C1}$, $PP2A_{C2}$ and $PP2A_{C5}$ and
- Subfamily 2: $PP2A_{C3}$ and $PP2A_{C4}$ (He et al. 2004).

An earlier research project has shown that silencing members of subfamily 1 in *Nicotiana benthamiana* (a close relative of tobacco) results in increased expression of pathogenesis-related (PR) genes (proteins produced in plants in the event of a pathogen attack) and localized cell death in stems and leaves. The PP2Ac - silenced plants became more resistant to a virulent stain of *Pseudomonas syringae pv.tabaci* (*P.syringae*) and developed an accelerated

hypersensitivity response (HR) to effector proteins from both *P.syringae* and the fungal pathogen, *Cladosporium fulvum*, indicating that these catalytic subunits act as negative regulators of plant defense responses (He et al. 2004).

Generally, plants will recognize pathogens from the external environment with receptor-like kinases (RLK) or receptor-like proteins (RLPs). These receptors are structured in the cell membrane and can transmit signals from the extracellular space to the nucleus to promote defense against pathogens. PP2A is a contributor to this defense mechanism, where PP2A consisting of catalytic subunit C4 (subfamily 2), scaffold subunit A1 and regulatory B subunits B' or B 'ζ can negatively regulate receptor BRI1-associated kinase 1 (BAK1), which is a coreceptor for the flagellin sensing receptor 2 (FLS2) (Durian et al. 2016). See figure 1.5.

1.1.5 Scaffolding subunits

PP2A substrate specificity and localization depends on the binding between the A and B subunits. Regulatory A subunits consist of 15 imperfect repeats of α-helical HEAT forming a horseshoe-like structure (figure 1.4). The catalytic subunit bind to the carboxyl end of the A subunit, and the B subunits bind to the amino end. Both binding interactions use a hydrophobic bonding interface formed by short and variable loops located in the middle of each HEAT repetition. Three important control functions are performed by the A subunit. First, the kinetic property of the C subunit is changed. Second, the binding of A and C subunits causes the C subunit to interact with the B subunit. Third, recent work has indicated that A subunit binding is required for the fully activated C subunit conformation (Blakeslee et al. 2008).

1.1.6 Regulatory subunits

PP2A-B´subfamily members B´ *θ* and B´γ act as negative regulators of plant immunity. B´γ controls a feedback loop where increased abundance of alternative oxidases AOX result in reduced eactive oxygen species (ROS) production. B´γ is active in controlling salicylic acid (SA) -dependent pathogenesis responses and cell death triggered by intracellular ROS signals. Another important regulator is from the PP2A regulatory protein TAP46, which interacts with a catalytic subunit that negatively regulates autophagy and the associated program death (Lillo et al. 2014; Durian et al. 2016). See figure below.

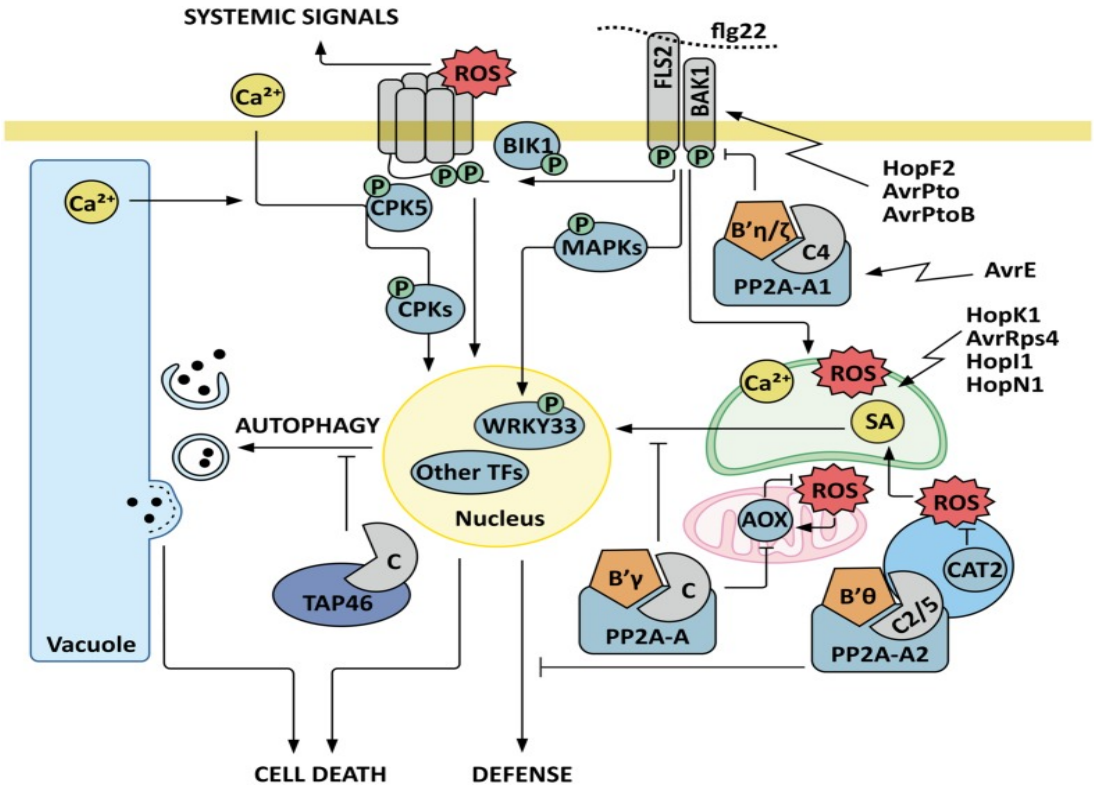


Figure 1.5. When FLS2 senses the bacterial effectors HopF2, AvrPto and AvrPtoB, a downstream signaling effect is trigger: increases cytosolic calcium concentration, activation of membrane NADPH oxidases, increase of reactive oxygen species (ROS) in apoplast and activation of phosphorelay cascades using mitogen-activated protein kinases (MAPKs) or calcium-dependent protein kinases (CPKs). These regulatory actions trigger the first line of transcriptional reprogramming in the nucleus. PP2A is active in defense cascades, where transcription factor (TF) WRK33 is phosphorylated by MPKs. CPK5 mediates ROS signals to distal tissues by accelerating the production of NADPH oxidase RBOHD. Signals from FLS2 are transmitted to nearby chloroplasts, where calcium-dependent signaling interactions trigger chloroplast retrograde signals which further increase plant immunity. The figure is obtained from Durian et al. 2016.

1.1.7 Stress-related processes regulated by PP2A

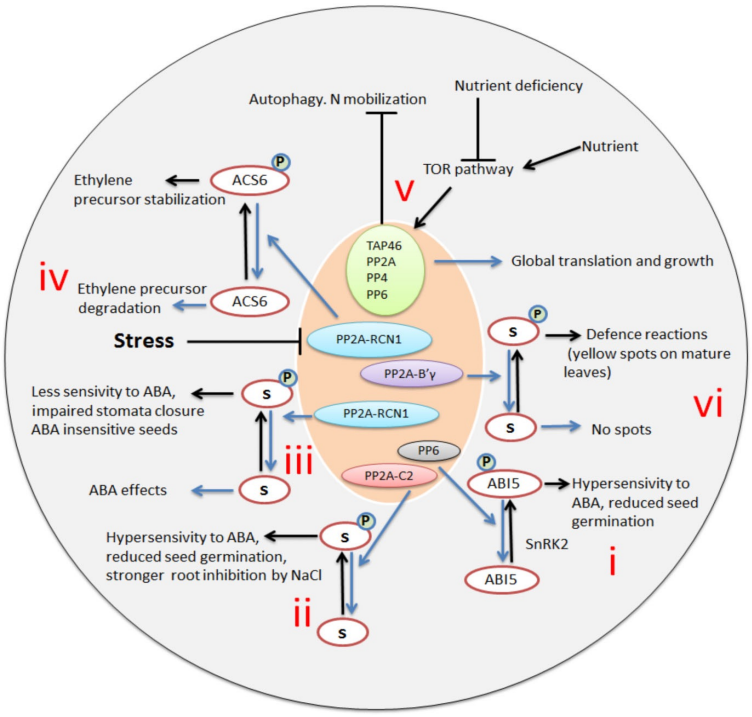


Figure 1.6. (ii) PP2A-C2 knockdown resulted in hypersensitivity to ABA, reduced seed germination and reduced root growth with the presence of NaCl (Pernas et al. 2007). (iii) Knockdown of A1/RCN1 resulted in reduced sensitivity to ABA, impaired stoma closure and ABA insensitive seed germination (Kwak et al. 2002) (iv) Knockdown of A1/RCN1 leds to higher ethylene levels due to lack of a PP2A complex that would otherwise dephosphorylate the ethylene precursor ACS6 (Skottke et al. 2011). (v) Knockdown of TAP46 leads to induction of autophagy and mobilization (Skottke et al. 2011). B´γ knockdown led to yellow spots in leaves (Trotta et al. 2011). Positive effects are indicated by default arrows while inhibition are indicated by blunt end arrows. Unknown substrates are named S, and P indicates that the protein is phosphorylated. The figure is obtained from Lillo et al. 2014.

1.2 Cloning

Molecular cloning is a set of methods, which are used to insert recombinant DNA into a vector - a carrier of DNA molecules that will replicate recombinant DNA fragments in host organisms (Lessard et al. 2013).

1.2.1 Molecular cloning

One of the challenges of molecular cloning has been to isolate a smaller sequence of the DNA molecule, such as a gene. For example, the human genome contains 23 pairs of chromosomes, which contain approximately 6 × 109 base pairs (bp). Cleavage of human DNA with restriction enzymes that cut for every 3000 base pairs would yieled about 2 million fragments, far too many to separate from each other directly. This challenge for obtaining pure DNA samples has been overcome with recombinant DNA technology. The recombinant DNA technology has made it possible for almost any gene to be purified, sequenced and reintroduced into cells or into whole organisms (Harvei et al. 2000).

The essence of recombinant DNA technology is to produce a large number of identical DNA molecules, then the DNA molecules are engineered into a vector with standard 3 '→ 5' phosphodiester bonds. Vectors are molecules that can replicate themselves when introduced into a host cell. The inserted gene is replicated together with the vector, thus producing a large number of recombinant DNA molecules. *Escherichia coli* (*E. coli*) plasmid vectors and bacteriophage λ vectors are the most commonly vectors. Plasmid vectors replicate along with their host cells, while λ vectors replicate as lytic viruses, killing the host cell and packaging the DNA into virions (Harvei et al. 2000).

1.2.2 Plasmid vectors

Plasmids are found naturally in bacteria and consist of double-stranded DNA (dsDNA) circulating freely in the cytosol. The plasmids commonly used in recombinant DNA technology are from the bacterium *E. coli*, and may consist of a few thousand base pairs to more than 100 kilobases (kb). Like the chromosomal DNA, the plasmids are duplicated before each cell division. At least one copy of the plasmid DNA is segregated into each daughter cell that ensures further propagation of the plasmid over generations (Harvei *et al*. 2000). The plasmid vectors contain little more than the essential nucleotide sequences required for their use in DNA cloning: a replication origin, an antibiotic resistant gene, and a region in which exogenous DNA fragments can be inserted (Harvei et al. 2000).

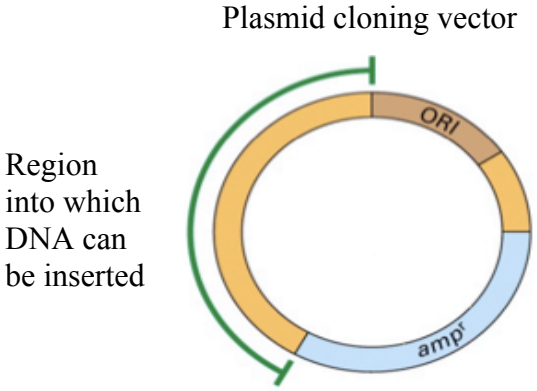


Figure 1.7. Plasmid vectors contain a replication origin (ORI) sequence, a gene that makes the bacterium resistant to a particular antibiotic and the herbicide BASTA. Here the selective gene is amp^r; it encodes the enzyme β-lactamase, which inactivates ampicillin. Exogenous DNA can be inserted into the bracketed region without disturbing the ability of the plasmid to replicate or express the genes. The figure is obtained from Harvei et al. 2000.

1.2.3 Plasmid DNA replication

ORI is a specific sequence of around 50-100 bp and is absolutely essential for replication. Specific enzymes in the host cell bind to this sequence and promote replication of the circular plasmid (see figure below). Then, the enzymes continue throughout the plasmid independently of the plasmid's nucleotide sequence. Any gene inserted into the plasmid is replicated along with the rest of the plasmid DNA, and this property provides the basis for molecular DNA cloning.

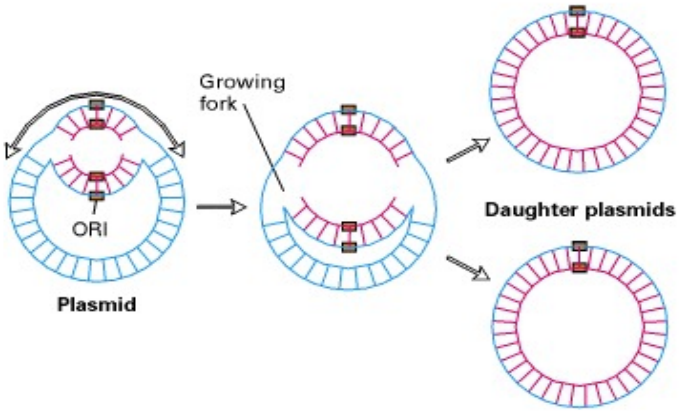


Figure 1.8. Original thread is blue and new synthesized thread is red. Replication starts in the ORI and continues in both directions. The figure is obtained from Harvei et al. 2000.

1.2.4 Selection of transformed cells

For the DNA cloning to be successful, the vector must contain a selected gene that constitutes a resistant to specific antibiotics. As we have seen, the ampicillin-resistance gene (amp^r) encodes β-lactamase, which inactivates the antibiotic ampicillin (see 1.5.2 Plasmid vectors). The bacterial cells must be made competent so that they can take up extra chromosomal DNA. Bacterial cells are made competent by weakening the cell wall and membranes of the bacteria, so the extra chromosomal DNA can enter the cell. This can be achieved by exposing the bacteria to high concentration of, for example $CaCl_2$. The ions will neutralize the charge of the phospholipid head in cell membranes, and phosphate groups on the DNA. This allows for the DNA of interest to easily enter the bacterial cell.

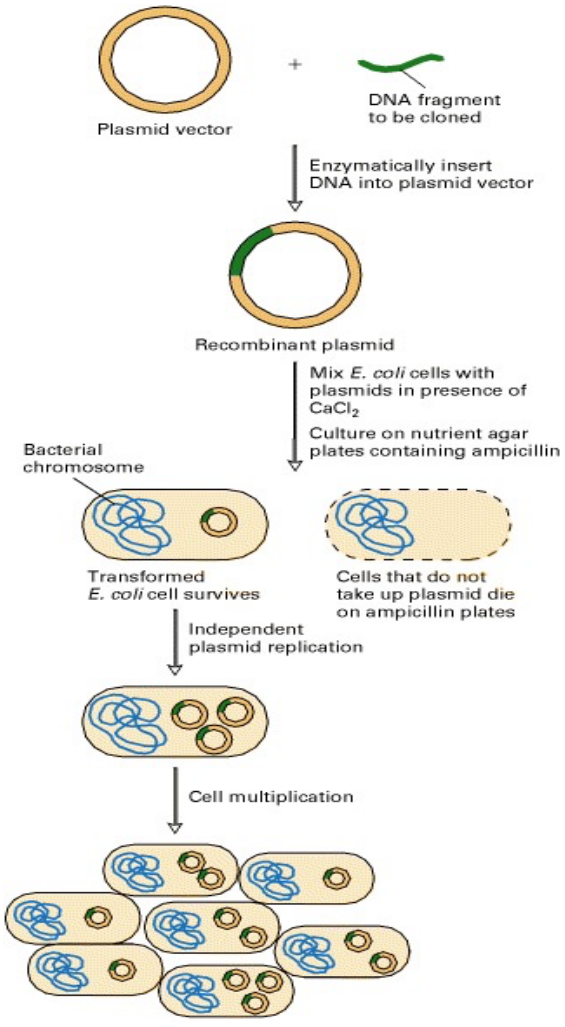


Figure 1.9. Model of *E. coli* produces colonies containing copies of the same recombinant plasmids. The figure is obtained from Harvei et al. 2000.

1.2.5 Restriction enzymes

Restriction enzymes are found naturally in bacteria (provide a defense mechanism against invading viruses) and recognize 4-8 bp sequences in the DNA, called restriction sites. To clone a gene fragment into a vector, both plasmid DNA and gene insert are typically cut with the same restriction enzymes, and then glued together with the assistance of an enzyme known as a DNA ligase. The enzymes recognize the restriction sites and cut the DNA strands at both sites. The enzymes are also called restriction endonucleases to separate them from exonucleases, which digest nucleic acids from end to end (Harvei et al. 2000).

1.2.6 DNA ligase

Ligase is an enzyme used to ligate together fragments of DNA that have complementary overhanging ends or straight ends. During in vivo DNA replication, ligase catalyzes formation of 3′→5′ phosphodiester bonds between the short fragments of the discontinuously synthesized DNA strand at a replication fork. In recombinant DNA technology, purified DNA ligase is used to covalently join the ends of restriction fragments in vitro. When DNA ligase and ATP are added to a solution containing restriction fragments with sticky ends, the restriction fragments are covalently ligated together through the standard 3′→5′ phosphodiester bonds of DNA (Harvei et al. 2000).

1.2.7 Transgenic plants

Transgenic (GM) plants have been genetically modified using recombinant DNA technology. The modified plants will express genes that are not natural in their genome. Specific proteins are synthesized and thus the plants have new properties. One can avail of this technology in several ways, for example, to resist resistance to abiotic stresses, such as drought, extreme temperature or salinity and biotic stresses, such as insects and pathogens that would normally be harmful to plant growth or survival. One can also use technology to improve the plant's nutritional content, a program that can be particularly useful in the developing world. New generation GM crops have now also been developed for the production of recombinant drugs and industrial products, such as monoclonal antibodies, vaccines, plastics and biofuels (Key et al. 2008; Van Lijsebettens et al. 2013).

1.3 Aim of this project

Protein phosphatase type 2A (PP2A) from *Solanum lycopersicum* (tomato plant) is one of the major Ser/Thr specific phosphatases. It is characterized by its multi-subunit structure that allows it to participate in a wide range of signalling cascades that regulate plant growth, defense and development (Janssens et al. 2001). The PP2A protein is made up of three subunits, a catalytic (C), scaffold (A) and regulatory (B) subunit. The regulatory subunit is divided into three groups called B, B' and B" (Booker et al. 2017). A particular subgroup of B' called *B'φ* (*B'phi*) appears to be crucial for the interaction between plants and microorganisms, especially mycorrhiza (Charpentier et al. 2014; Booker and deLong et al. 2016). *B'φ* has not been studied much and can also be important for regulating other processes. The model plant *Arabidopsis* does not have the subunit *B'φ*, and the aim of this project is to i) clone *B'φ* from *Solanum lycopersicum* ii) link the tomato gene with a FLAG tag and transform it into an *Arabidopsis*. For this purpose, primers must be constructed so that *B'φ* with the FLAG tag is amplified (and *Arabidopsis B'Z* as control/comparison). The amplified product will be inserted into pBA002 plasmid (using restriction enzymes and ligases) and *Agrobacterium tumefaciens* (*Agrobacterium)* will be transformed. Then, *Agrobacterium* can be used to transform an *Arabidopsis*. *Arabidopsis* plants will be selected on BASTA and seedlings further grown and presence of the gene will be confirmed by PCR. Further experiments can provide information about the location of the protein in the cell, and interaction partners and subsequent function in the future. Experiments such as immunoblotting and immunoprecipitation experiments can be used for this purpose. The precipitate should contain interacting proteins, and then one can compare the interacting proteins identified in *Arabidopsis* and tomato.

2 MATERIALS AND METHODS

2.1 Outline

Molecular cloning: the different approaches and methods in order to genetically modify an *Arabidopsis thaliana* with the genes *B'phi* and *B'Z* are listed below

Isolation and fragmentation of the source DNA:

- Expand high fidelity PCR system
- Purification of DNA from agarose gel
- Digestion of genes
- Gel electrophoresis
- ChemiDoc™ Imaging Systems
- NanoDrop

Digestion and inserting the DNA fragment into a cloning vector:

- Digestion of plasmids
- Ligation
- *Escherichia* transformation
- Plasmid isolation
- Gel electrophoresis
- UV-light
- NanoDrop

Isolation and introduction of the cloned DNA into a host organism:

- *Agrobacterium* transformation
- Floral dipping
- Gel electrophoresis
- Colony PCR
- ChemiDoc™ Imaging Systems
- UV-light
- NanoDrop

2.2 Plant material

2.2.1 Seeds from *Arabidopsis*

Seeds from *Arabidopsis* with a Colombia background were used in this project. The *Arabidopsis* seeds were seeded in 15 pots, each pot containing 3/4 soil, 1/4 vermiculite and 5 seeds. In addition, Hoaglenad solution (Hoagland & Arnon et al. 1950) was used for watering. The pots were placed in a cold (4°C) and dark room for three days before being placed in a light room.

2.2.2 Hoagland solution

A complete Hoagland solution (Hoagland & Arnon et al. 1950) was used for watering of plants grown on soil. Chemicals for making the nutrient solution are listed in table 1.

Table 2.1. Chemicals used for making Hoagland solution.

Chemicals	**Per 5 L (10 * concentrated)**
1 M KH_2PO_4	50 ml
1 M KNO_3	250 ml
1 M $Ca(NO_3)_2$*$4H_2O$	250 ml
$MgSO_4$*$7H_2O$	100 ml
1% Fe-EDTA	50 ml
Micronutrients	50 ml
Micronutrients per L	
2.86 g H_3BO_3	
1.81 g $MnCl_2$*4 H_2O	
0.089 g $CuSO_4$ *5 H_2O	
0.22 g $ZnSO_4$*7 H_2O	
0.029 g H_2MoO_4* H_2O	

The solution was kept in the dark at room temperature.
For watering, 1* Hoagland solution was used.

2.2.3 ½ MS Medium

Agar containing 1⁄2 MS medium salts (Murashige and Skoog et al. 1962) was used for growth of seedlings in Petri dishes. Chemicals for making MS stock solutions and 1⁄2 strength MS are listed in table 2.

Table 2.2. Chemicals to make MS stock solutions and ½ MS salt solution

Chamicals for making MS-medium, stock solutions		**Chemicals to make 1 liter ½ MS-medium**	
Chemicals	g/L	Chemicals	½ MS
A: KNO_3	95 g	A: KNO_3	10 ml
B: NH_3NO_3	125 g	B: NH_3NO_3	6.5 ml
C: $MgSO_4*7H_2O$	37 g	C: $MgSO_4*7H_2O$	5 ml
D: KH_2PO_4	17 g	D: KH_2PO_4	10 ml
E: $CaCl_2*2\ H_2O$	44 g	E: $CaCl_2*2\ H_2O$	5 ml
Fe/EDTA:		Minor I:	5 ml
Na_2*EDTA	0.373 g	Minor II:	5 ml
$FeSO_4*7\ H_2O$	0.278 g	Fe/EDTA	25 ml
Minor I:		Agar-Agar	7 ml
$ZnSO_4*7\ H_2O$	0.920 g	(Sucrose, 1%)	(10 g)
H_3BO_3	0.620 g	Water	Up to 1 L
$MnSO_4*4\ H_2O$	2.230 g		
Minor II:			
$Na_2MoO_4*2\ H_2O$	0.025 g		
$CuSO_2*5\ H_2O$	0.003 g		
$CoCl_2*6\ H_2O$	0.003 g		
KI	0.083 g		

Chemicals for 1⁄2 strength MS medium were added together and the pH was adjusted to 5.8 with KOH (1M). The solution was autoclaved at 110°C for 30 minutes. After autoclaving, the media was transferred to Petri dishes in the sterile flow hood when the temperature of the media was approximately 40 °C. Dishes were stored at +4°C

2.2.4 Enzymes and kits

The different enzymes and kits that were used in this project are listed in table 3.

Table 2.3. Enzymes and kits

Purpose	Name	Manufacturer
DNA Amplifications	Expand High Fidelity PCR System	Roche Applied Science (Mannheim, Germany)
Colony PCR	DreamTaq Polymerase	Thermo Fisher Scientific (USA)
Ligation	T4 DNA ligase 5U/µl	Invitrogen by Life TechnologiesTM (Carlsbad, California)
Digestion	Restriction endonucleases	New England BioLabs (Ipswich, MA, USA)
Gel extraction	Illustra^{TM}GFX^{TM} PCR DNA and Gel Band Purification Kit	GE Healthcare, Life Sciences (Piscataway, New Jersey, USA)
Plasmid isolation	GenEluteTM Plasmid Miniprep Kit	Sigma Aldrich (St. Louis, MO, USA)

2.2.5 Cloning vectors

The bacteria used for cloning are listed in the table 4.

Table 2.4. Cloning vectors

Vector	Size bp	Antibiotics selection	Description	References
pBA002	10182	Spectinomycin	Constitutive	(Moller et al., 2003)
pGEM-T Easy	3015	Ampicillin	Blue/white screening	(Promega, Madison, Wisconsin, USA)

2.2.6 Bacteria

The bacteria used for cloning/transformation are listed in the table 5.

Table 2.5. Bacterial strains

Bacterium	Strain	Description
Escherichia coli	JM109	Cloning/transformation
Agrobacterium tumefaciens	ABI	*Arabidopsis* transformation

2.2.7 Antibiotics

The different antibiotics and concentrations used for selection of positive transformants are listed in table 6.

Table 2.6. The concentration of antibiotics used in bacterial growth medium

Antibiotic	Stock solution	Final conc.
Ampicillin (Amp)	100 mg/ml	100 μg/ml
Kanamycin (Kan)	50 mg/ml	25 μg/ml
Spectinomycin (Spec)	50 mg/ml	50 μg/ml

2.3 Isolation and fragmentation of the source DNA

2.3.1 Expand high fidelity PCR system

In molecular biology, the polymerase chain reaction (PCR) method is used to make multiple copies of a specific DNA segment. Using PCR, a single copy (or more) of a DNA sequence is exponentially amplified to generate thousands to millions of copies of that particular DNA segment (Kary et al. 1983).

When a segment of DNA is amplified using PCR, the sample is first heated so the DNA denatures, or separated into two pieces of single-stranded DNA. Next, an enzyme called "Taq polymerase" synthesizes - builds - two new strands of DNA, using the original strands as templates. This process results in the duplication of the original DNA, with each of the new molecules containing one old and one new strand of DNA. Then, each of the new strands can

be used to make two new copies, and so on, and so on. The cycle of denaturation and synthesis of new DNA repeats around 30 to 40 times, leading to more than one million exact copies of the original DNA segment (Kary et al. 1983).

The entire cycling process of PCR is automated and can be completed in just a few hours. The process of cycling is performed by a machine called thermocycler and is programmed to alter the temperature of the reaction every few minutes to allow DNA denaturing and synthesis (Kary et al. 1983).

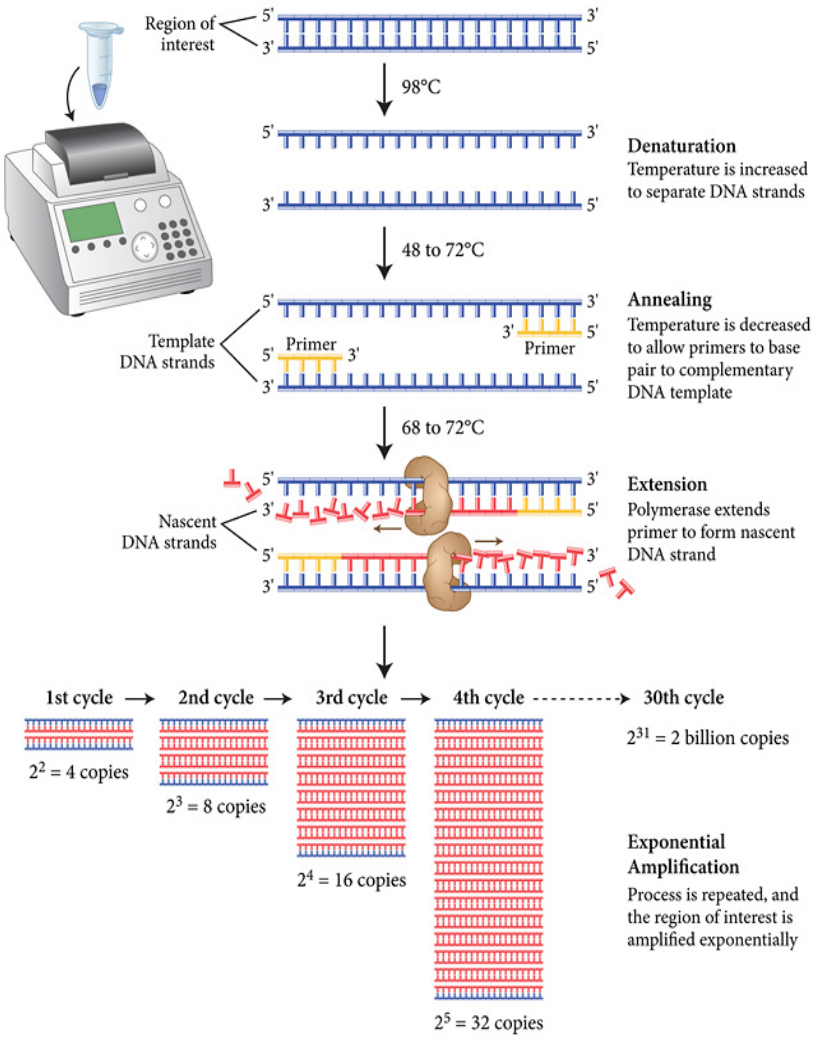


Figure 2.1. Polymerase chain reaction (PCR) is a method widely used in molecular biology to make many copies of a specific DNA segment. The figure is obtained from BioLabs, 2019.

In this experiment, expand high fidelity PCR system kit was used. The PCR system is designed to amplify DNA fragments up to 5 kb. The system is composed of a special enzyme mix containing thermostable Taq DNA polymerase and Tgo DNA polymerase, a thermostable DNA polymerase with proofreading activity. This mixture is designed to generate PCR products of high yield, high fidelity and high specificity from all types of DNA (Refers to protocol Expand High Fidelity PCR System, Roche Applied Science, 2011). The PCR system was used to amplify two genes, *B'φ* from *Solanum lycopersicum* and *B'Z* from *Arabidopsis thaliana*.

Table 2.7. Sample material

Target genes	**Annotation**	**Description**
B'φ	XM_010317091.2	PREDICTED: *Solanum lycopersicum* PP2A 57 kDa regulatory subunit B′iota isoform-like, mRNA 1212 bp. The gene encodes the serine/threonine protein phosphatase PP2A.
B'Z	AT3G21650.1	*Arabidopsis* PP2A B′ regulatory subunit. The gene encodes the protein phosphatase PP2A *B`zeta* subunit.

It was recommended to prepare two reaction mixes for a large number of reactions. This circumvents the need of "Hot Start" and avoids that the 3`-5`exonuclease activity of the proofreading polymerase partially degrades primers and template during the reaction set-up (Expand High Fidelity PCR System kit, 2011).

Table 2.8. Mix 1

Reagent	**Volume**	**Final conc.**
Sterile double-dist. water	16 µl	
Deoxynucleotide mix, 10 mM of each dNTP	4 µl	200 µM of each dNTP
Upstream primer	2 µl	300 nM
Downstream primer	2 µl	300 nM
Template DNA	1 µl	0.1 – 250 ng[a]
Final volume	**25 µl**	

Table 2.9. Mix 2

Reagent	**Volume**	**Final conc.**
Sterile double-dist. water	19.25 µl	
Expand High Fidelity buffer, 10*conc. with 15 mM $MgCl_2$	5 µl	1 * (1,5 mM $MgCl_2$)
Expand High Fidelity enzyme mix	0.75 µl	2.6 U/reaction
Final volume	**25 µl**	

Designed primers were used to attach a FLAG tag sequence to the N- and C-terminals of the target genes. Primers are short, synthetic fragments of DNA used in many molecular techniques from PCR to DNA sequencing. These primers are used to induce DNA synthesis in the PCR system.

Primer pair to attach the FLAG tag sequence to the N-terminal coding region of the target gene *B'φ*

Forward primer

5' GCCCTCGAGATGGACTACAAAGACGATGACGACAAGATG**ACAAATTTTCTTGATTCTG** 3'

XhoI Start FLAG-tag Start *B'φ*

Translated into 5'3' Frame 1: ALEMDYKDDDDKMTNFLDSE

Reverse primer

5' CGCACTAGTTCA**CATTGCTGCATTTTCAATTTT** 3'

SpeI Stop *B'φ*

Translated into into 3'5' Frame 1: KIENAAM-TSA

The reverse primer (3'end) includes a restriction enzyme site, SpeI a stop codon, and the DNA sequence for the carboxy-terminus of the target protein.

Primer pair to attach the FLAG tag sequence to the C-terminal coding region of the target gene *B'φ*

Forward primer

5' GCCCTCGAGATG**ACAAATTTTCTTGATTCTGAG** 3'

XhoI Start *B'φ*

Translated into into 5'3' Frame 1: ALEMTNFLDSE

Reverse primer

5' CGCACTAGTTCA**CTTGTCGTCATCGTCTTTGTAGTC**CATTGCTGCATTTTCAATTTT 3'

SpeI Stop FLAG-tag *B'φ*

Translated into 3'5' Frame 1: KIENAAMDYKDDDDK-TSA

The reverse primer (3'end) includes a restriction enzyme site, SpeI, a stop codon, the epitope tag sequence, and the DNA sequence for the carboxy-terminus of the target protein.

Primer pair to attach the FLAG tag sequence to the N-terminal coding region of the target gene *B'Z*

Forward primer

5' GCCCTCGAGATGGACTACAAAGACGATGACGACAAGATG**ATCAAACAGATATTTGGG** 3'

XhoI Start FLAG-Tag Start *B'Z*

Translated into 5′3′ Frame 1 ALEMDYKDDDDKMIKQIFG

Reverse primer

5' TATTAATTAACTA**CGACCCTGTGGACTCAGA** 3'

Pac1 Stop *B'Z*

Translated into into 3′5′ Frame 1 SESTGS-LIN

The reverse primer (3´end) includes a restriction enzyme site, PacI a stop codon, and the DNA sequence for the carboxy-terminus of the target protein.

Primer pair to attach the FLAG tag sequence to the C-terminal coding region of the target gene *B'Z*

Forward primer

5'GCCCTCGAGATG**ATCAAACAGATATTTGGG**3'

XhoI Start *B'Z*

Translated into 5′3′ Frame 1 ALEMIKQIFG

Reverse primer

5'TATTAATTAATCACTTGTCGTCATCGTCTTTGTAGTC**CGACCCTGTGGACTCAGAGCT**3'

Pac1 Stop FLAG-tag *B'Z*

Translated into into 3′5′ Frame 1 1SSESTGSDYKDDDDK-LIN

The reverse primer (3´end) includes a restriction enzyme site, PacI, a stop codon, the epitope tag sequence, and the DNA sequence for the carboxy-terminus of the target protein.

Four reaction samples were made with specific content to perform the expand high fidelity PCR system. Each sample was made with specific content of DNA template, PCR Mix and pre-made primers:

Table 2.10. Four reaction samples

Flag-B'φ	***B'φ-Flag***	***Flag-B'Z***	***B'Z-Flag***
DNA template from *Solanum lycopersicum*	DNA template from *Solanum lycopersicum*	DNA template from *Arabidopsis thaliana*	DNA template from *Arabidopsis thaliana*
Mix 1 and 2 from Expand High Fidelity PCR System kit	Mix 1 and 2 from Expand High Fidelity PCR System kit	Mix 1 and 2 from Expand High Fidelity PCR System kit	Mix 1 and 2 from Expand High Fidelity PCR System kit
Primer pair to attach the FLAG tag sequence to the N-terminal coding region of the target gene (*B'φ*)	Primer pair to attach the FLAG tag sequence to the C-terminal coding region of the target gene (*B'φ*)	Primer pair for attaching the FLAG-tag sequence to the N-terminal coding region of the target gene (*B' Z*)	Primer pair for attaching the FLAG-tag sequence to the C-terminal coding region of the target gene (*B' Z*)

The samples were placed in the thermal block cycler with specific settings for three hours:

Table 2.11. Thermal cycling

Process	**Temperature**	**Time**	**Cycles**
Initial Denaturation	94°C	2 min	35*
Denaturation	94°C	30 sec	
Annealing	60°C	30 sec	
Elongation	72°C	2.30 min	
Final Elongation	72°C	7 min	
Cooling	4°C	∞	

2.3.2 Gel electrophoreses

The samples were run on gel electrophoresis. Gel electrophoresis is a method for separating a mixture of macromolecules such as DNA or proteins in a matrix of agarose, one of the two main components of agar (Lee et al. 2012). The agarose powder was added to 1 * TAE buffer (2.12) and heated in a microwave oven. Then, the mixture was cooled to about 50 ° C and added to the electrophoresis tray. The tray contains a comb for making wells for application of the samples. The comb was removed after the gel had cooled and solidified. Prior to mounting the samples, the tray was placed in the electrophoresis apparatus containing enough 1x TAE buffer to cover all wells. A HyperLadder was used in the range of 200 to 10000 bp as the reference marker (figure 2.3). All samples were added to Gel Red ™ and loading buffer before being placed in the wells. Gel Red^{TM} is a fluorescent dye that enables visualization of DNA bands under UV light. The buffer causes the samples to be inserted into the wells and not diffuse out into the buffer. Power of 80 V was applied and the run was set for about 40 minutes. For analytical analysis, 1-3 µl of DNA was loaded onto the gel. For preparative electrophoresis, 15 µl was added to the gel.

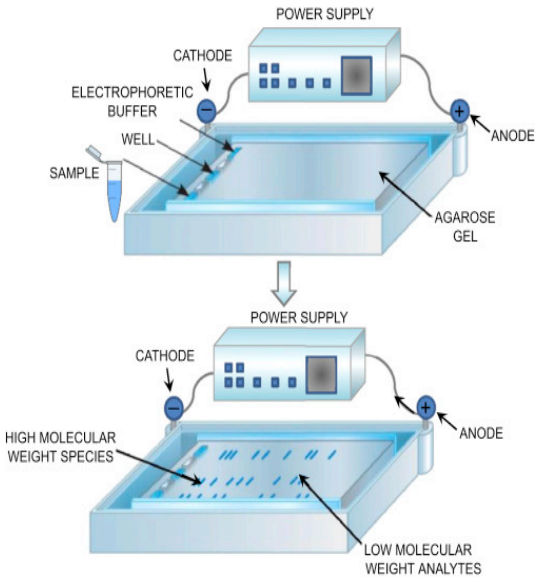


Figure 2.2. The fragments are separated from each other either by charge and/or size by applying an electrical field to move the charged molecules through an agarose matrix. The smallest DNA fragments will migrate faster down the gel relative to the larger molecules. The molecules can then be observed with UV light (Lee et al. 2012).

Table 2.12. Agarose gel electrophoresis buffer

50* TAE Buffer	**Per L**
2 M Tris-Base	242 g
Aceric acid (glacial)	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
H_2O	Up ti 1 L

To get a 1* TAE concentartion, 20 ml of the stock solution was added per liter distilled water

A HyperLadder was added to the gel electrophoresis system, which is a ready-to-use molecular weight marker, especially designed for easy size determination of DNA fragments, plasmids, and other downstream techniques. The molecular weight marker is made for easy size determination of linear double-stranded DNA fragments on 1% to 2% Tris-acetate-EDTA (TAE) agarose.

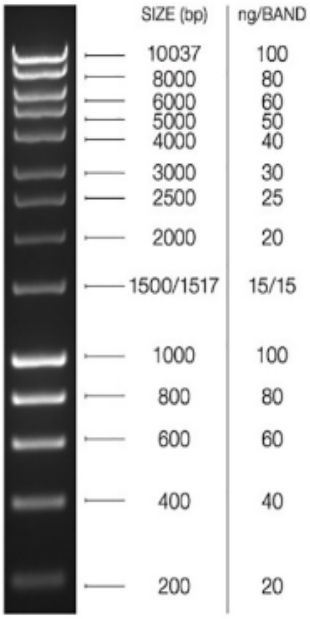


Figure 2.3. HyperLadder 1 (Bioline). Used as the reference marker in agarose gel electrophoresis

2.3.3 Purification of DNA from TAE agarose gel

The samples were purified from the agarose gel (Refer to the protocol Illustra^{TM}GFX^{TM} PCR DNA and Gel Band Purification Kit, GE Healthcare, Life Sciences). Sample capture: four empty DNase 1,5 ml microcentrifuge tube were weighed and noted. A clean scalpel was used to cut the agarose gel band containing the samples of interest. Then, the samples were placed in each of the four empty DNase 1,5 ml microcentrifuges. Each 1,5 ml microcentrifuge tubes plus the agarose band were weighed, and the weight of the agarose slice was calculated. 1 µl Capture buffer type 3 was added for each 1 mg of gel slice. For example, 300 µl buffer to each 300 mg gel slice. Then the samples were incubated for 60 °C until the agarose was completely dissolved. Sample binding: four GFX MicroSpin^{TM} columns were placed in four collection tubes. 600 µl dissolved sample mix from each microcentrifuge tubes was added to each column. The samples were incubated 60 second in room temperature and spun 30 second with 1600 g (1600 times Earth's gravitational force). Wash and dry: 500 µl Wash buffer type 1 was added the GFX MicroSpin columns. The samples were spun 30 second with 1600 g. After the spin, the columns were placed in a fresh DNase-free 1,5 ml microcentrifuge and spun 30 second with 1600 g. Elution: 50 µl distilled water was added to the samples, and the samples were incubated 60 second in room temperature, and spun 60 second with 1600 g. The samples were stored at -20 °C.

2.3.4 NanoDrop

The samples were measured with the instrument NanoDrop. NanoDrop allows to quickly and easily quantify and assess purity of samples such as proteins and nucleic acids. With this instrument, the concentrations were measured as ng/µl.

2.3.5 Restriction enzyme digestion

The samples were added restriction enzymes. Restriction digestion is the process of cutting DNA molecules into smaller pieces with special enzymes called restriction endonucleases (sometimes just called restriction enzymes or RE's). These special enzymes recognize specific sequences in the DNA molecule (for example GATATC) wherever that sequence occurs in the DNA. Restriction digestion is usually used to prepare a DNA fragment for molecular cloning. The samples were incubated for four hours allowing the enzymes to recognize and bind specific DNA sequences and cleave specific nucleotides within the recognition sequence of the DNA.

Table 2.13. Restriction enzymes and their cutting sites

Restriction enzymes	Recognition site
Xho1	C/TCGAG
	GAGCT/C
Spe1	A/CTAGT
	TGATC/A
Pac 1	TTA/ATTAA
	AATTA/ATT

Table 2.14. Digestion set up for the genes

Reagent	*FLAG-B′φ*	*B′φ-FLAB*	*FLAG-B′Z*	*B′Z-FLAG*
Destiled water	9.4	9.4	9.4	9.4
100*BSA	2.0 µl	2.0 µl	2.0 µl	2.0 µl
10*Neb Buffer (Cut smart)	2.0 µl	2.0 µl	2.0 µl	2.0 µl
Template	5.0 µl	5.0 µl	5.0 µl	5.0 µl
Enzyme 1	0.8 µl	0.8 µl	0.8 µl	0.8 µl
	Xhol + Spe1	Xhol + Spe1	Xhol + Pac1	Xhol + Pac1
Enzyme 2	0.8 µl	0.8 µl	0.8 µl	0.8 µl
	Xhol + Spe1	Xhol + Spe1	Xhol + Pac1	Xhol + Pac1
Final volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**

Table 2.15. Digestion set up for the plasmids

Reagent	**PBA002**	**PBA002**	**PBA002**	**PBA002**
Destiled water	9.4	9.4	9.4	9.4
100*BSA	2.0 µl	2.0 µl	2.0 µl	2.0 µl
10*Neb Buffer (Cut smart)	2.0 µl	2.0 µl	2.0 µl	2.0 µl
Template	5.0 µl	5.0 µl	5.0 µl	5.0 µl
Enzyme 1	0.8 µl	0.8 µl	0.8 µl	0.8 µl
	Xhol + Spe1	Xhol + Spe1	Xhol + Pac1	Xhol + Pac1
Enzyme 2	0.8 µl	0.8 µl	0.8 µl	0.8 µl
	Xhol + Spe1	Xhol + Spe1	Xhol + Pac1	Xhol + Pac1
Final volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**

2.4 Inserting the DNA fragment into a cloning vector

2.4.1 Transformation to obtain more plasmid

Competent cells of *E. coli* (JM109) were thawed on ice. 3µl plasmid solution was added. The mixture was incubated on ice for 30 minutes. Furthermore, the cells were heat-shocked for 50 seconds at 42 °C using a water bath. Then the cells were incubated on ice for 5 minutes. 0.5 ml of LB-Broth agar was added to the cells, and the cells were incubated for 1-2 hour at 37 °C on a shaker (225 rpm). The cells were spread on LB agar containing the appropriate antibiotics (Spectinomycin 50 µl/ml). The cells were incubated over night at 37 °C. The next day, colonies were picked and transferred to 5 ml LB-Broth. 5 µl spectinomycin was added to the mix. Then, the cells were incubated over night at 37 °C

2.4.2 Vector pBA002

In this project, binary vector pBA002 was used. pBA002 vector has plant selectable marker glufosinate ammonium (BASTA), 35S promoter of the cauliflower mosaic virus (CaMV) and bacterial selectable marker (spectinomycin) to enable selection of transformed bacterial colonies.

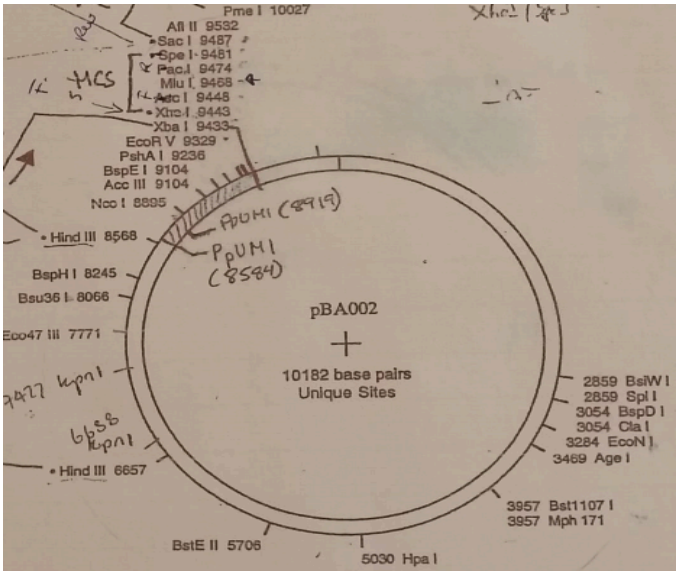


Figure 2.4. Vector map of pBA002 (Moller, Kim et al. 2003). The pBA002 vector contains resistance against the herbicide BASTA.

2.4.3 Ligation

A protocol for ligation was used to glue the target gene together with the plasmid pBA002. Ligation is the joining of two nucleic acid fragments through the action of an enzyme. It is an essential procedure in the molecular cloning of DNA whereby DNA fragments are joined together to create recombinant DNA molecules, such as when a foreign DNA fragment is inserted into a plasmid. The ends of DNA fragments are joined together by the formation of phosphodiester bonds between the 3'-hydroxyl of one DNA terminus with the 5'-phosphoryl of another (Gaastra et al. 1985).

pBA002 Xho1 + Spe1 = 5.9 ng/µl (refer to table 3.4 of the result section)

pBA002 Xho1 + Pac1 = 3.8 ng/µl (refer to table 3.4 of the result section)

FLAG-B′φ

((5.9 * 4 µl vector) * 1.5 bp insert) * 8 / 12.2 bp vector = 27.76 ng

27.76 ng / 5.7 = 4.87 µl FLAG-B′φ (refer to table 3.2 of the result section)

27.76 ng / 7.9 = 3.51 µl B′φ-FLAB(refer to table 3.2 of the result section)

FLAG-B′Z

((3.8 * 4 µl vector) * 1.7 bp insert) * 8 / 10.2 bp vector = 20.27 ng

20.27 ng / 4.2 = 4.80 µl FLAG-B′Z (refer to table 3.2 of the result section)

20.27 ng / 7.0 = 2.89 µl B′Z-FLAG (refer to table 3.2 of the result section)

Table 2.16 Ligation set up for the genes and plasmids

Reagent	**PBA002+ *FLAG-B′***	**PBA002+ *B′φ-FLAB***	**PBA002+ *FLAG-B′Z***	**PBA002+ *B′Z-FLAG***
Vector	4 µl	4 µl	4 µl	4 µl
Insert	4 µl	4 µl	4 µl	4 µl
Buffer	1 µl	1 µl	1 µl	1 µl
Ligase	1 µl	1 µl	1 µl	1 µl
Final volume	**10 µl**	**10 µl**	**10 µl**	**10 µl**

2.4.4 *Escherichia* transformation

The bacterium *E. coli* was selected to make multiple copies of the recombinant plasmid pBA002. The recombinant pBA002 was inserted into competent cells of *E. coli*-JM109. Bacteria can take up foreign DNA in a process called transformation. Transformation is a key step in DNA cloning. It occurs after restriction digest and ligation and transfers newly made plasmids to bacteria. After transformation, bacteria are selected on antibiotic plates. Bacteria with a plasmid are antibiotic-resistant, and each one will form a colony. Colonies with the right plasmid can be grown to make large cultures of identical bacteria (see figure 2.3). Competent cells of *E. coli* (JM109) were thawed on ice. Next, 3 µl of the recombinant pBA002 was added. The mixture was incubated on ice for 30 minutes. Furthermore, the cells were heat-shocked for 50 seconds at 42 °C using a water bath. Then the cells were incubated on ice for 5 minutes. 0.5 ml of LB-Broth agar was added to the cells, and the cells were incubated for 1-2 hour at 37 °C on a shaker (225 rpm). The cells were spread on LB agar containing the appropriate antibiotics (Spectinomycin 50 µl/ml). Next, the cells were incubated over night at 37 °C. The next day (after PCR), colonies were picked and transferred to 5 ml LB-Broth. 5µl spectinomycin was added to the mix. Furthermore, the cells were incubated over night at
37 °C.

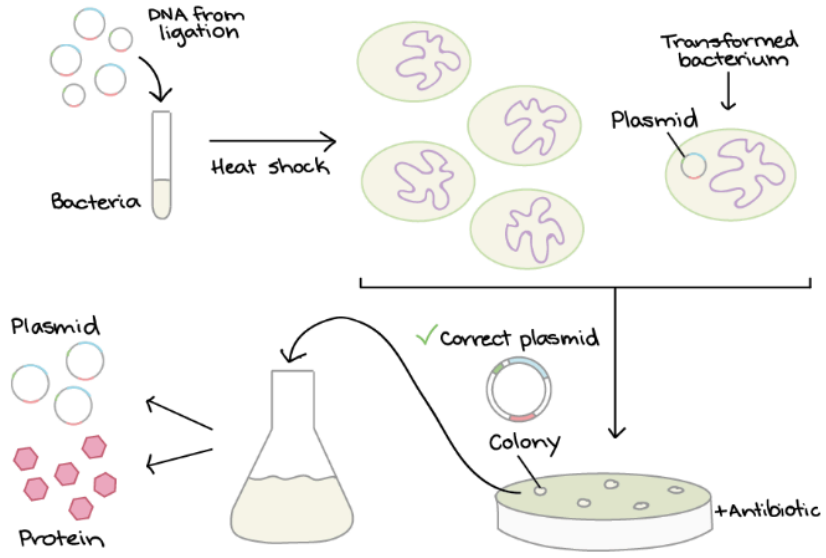


Figure 2.5. Bacteria without the recombinant plasmid die. Each bacterium *with* the recombinant plasmid gives rise to a cluster of identical, plasmid-containing bacteria called a colony. Several colonies are checked to identify one with the right plasmid.

2.4.5 Colony PCR (DreamTaq)

Colony PCR was performed to identify whether the growing colonies /cells contained the vector construct. PCR was performed using taq DNA polymerase (taqDNA Polymerase, recombinant, Invitrogen ™ by Life Technologies ™, Carlsbad, California).

Table 2.17. Colony PCR set up

Reagent	Volume	Final conc.
10*Dream taq buffer	1.0 µl	1*
dntp´s	0.4 µl	0.1 mM of each
pBA002 F	0.5 µl	0.5 mM
pBA002 R	0.5 µl	0.5 mM
Dream taq polymerase	0.1 µl	0.05 U
Water	7.5 µl	Variable
DNA templat		Variable
Final volume	**10 µl**	

Thermal Cycling

The samples were placed in the thermal block cycler with specific settings for three hours.

Table 2.18. Thermal cycling

Process	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	38*
Denaturation	95°C	30 sec	
Annealing	51°C	30 sec	
Elongation	72°C	2.30 min	
Final Elongation	72°C	5 min	
Cooling	5°C	∞	

2.4.6 Plasmid isolation

The recombinant pBA002 was isolated from *E. coli*-JM109 with the kit GenElute^{TM} Plasmid Minipreo Kit, Sigma Aldrich (St. Louis, MO, USA). Harvest cells: two samples with 5 ml bacterial (*E. coli* + recombinant pBA002) were centrifuged several times at 12,000 g for 1 minute to pellet cells. The supernatant was discarded each time. Resuspend cells: the bacterial pellet was completely resuspended with 200 µl Resuspension Solution. The cells were vortexed until homogeneous mixture. Lyse cells: the resuspended cells were lysed by adding 200 µl of the Lysis Solution. The contents was immediately mixed by gentle inversion (6-8 times) until the mixture became clear andviscous. At this point it was important not to vortex and not allow the lysis reaction to exceed 5 minutes. Neutralize: the cells debris were precipitated adding 350 µl of the Neutralization/Binding Solution. The samples were gently inverted 4-6 times. The cells debris where pellet at 12000 x g or maximum speed for 10 minutes. Prepare Column: GenElute Miniprep Binding Columns were inserted into 1.5 ml microcentrifuge tubes. 500 µl of the Column Preparation Solution was added to each miniprep column and centrifuged 12.000 x g for 30 seconds to 1 minute. The flow-through liquid was discarded. Load cleared lysate: the cleared lysate from step 3 was transferred to the columns prepared in step 4 and centrifuged 12,000 x g for 30 seconds. The flow-through liquid was discarded. Wash column: 750 µl of the diluted wash solution was added to the each column. The samples were centrifuged 12,000 x g for 1 minute. The flow-through liquid was discarded. The samples were centrifuged again at maximum speed for 2 minutes without any additional wash Solution to remove excess ethanol. Elute DNA: The columns were transferred to fresh collection tubes. 50 µl of water was added to each column. The samples were centrifuged at 12000 x g for 1 minute. The samples were stored at -20 ° C.

2.4.7 Blue-White screening

In the blue/white screening experiment, a vector called pGEM®-T Easy (pGEM®-T Easy Vector System I, Promega) was used. Easy vectors are linearized and have thymidine overhangs at the 3 'ends. In the PCR reactions, the enzyme polymerase that binds adenosine to the insert will produce complementary overhangs. The insert is therefore ligated to the vector without having to be digested in advance. The primers T7 and SP6 which are flanking the multiple cloning sites and which bind to the inserted polymerase promoter can be used for sequencing (primer sequences are listed in table 2.19). The cloning region contains genes for β-galactosidase that are controlled by the lac operon. β-galactosidase forms a blue pigment in the presence of X-gal and the lac operon inducer IPTG. If the insert is present in the operon, it will interrupt the gene and the colony will remain white. For this reason, it is possible to detect positive colonies with blue/white screening where positive colonies will appear white.

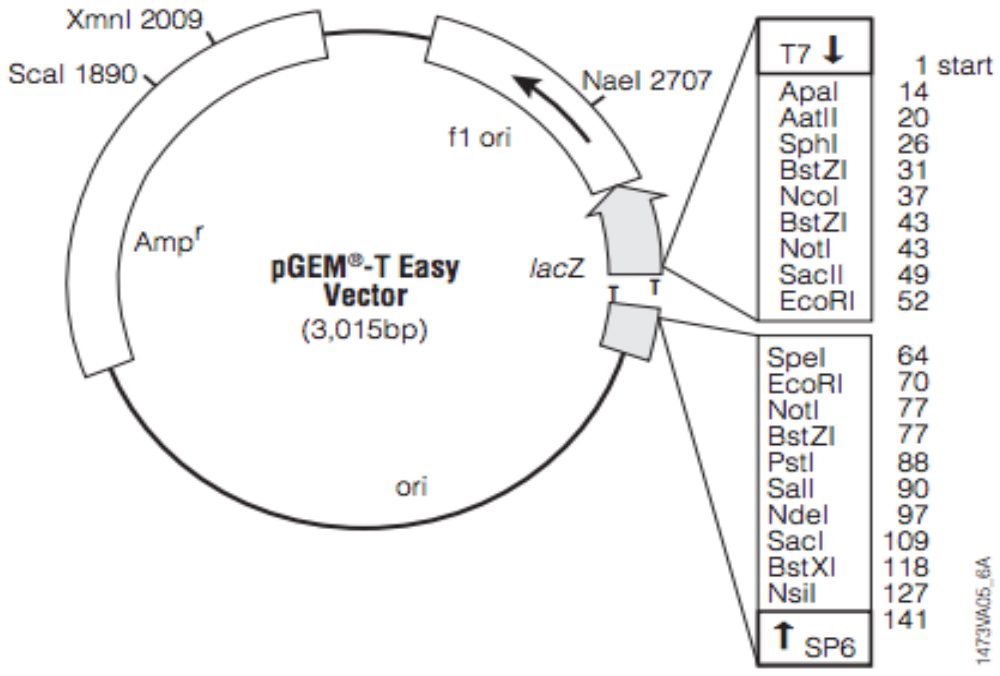


Figure 2.6. The pGEM®-T Easy Vector Map and Sequence Reference Points

The information about the pGEM®-T Easy Vector System 1 is retrieved from from the Promega web site: http://no.promega.com/~/media/Files/Resources/Protocols/Technical%20Manuals/0/pGEM-T%20and%20pGEM-T%20Easy%20Vector%20Systems%20Protocol.pdf.

Table 2.19. Oligonucleotide primers

Name	**Sequence 5'→3'**
T7	TAATACGACTCACTATAGGG
Sp6	ATTTAGGTGACACTATAG

B'φ and *B´Z* with tag were amplified by using the Expand High Fidelity PCR System. Thereafter the samples wee run on gel electrophoresis and purified from the agarose gel. Next, the target genes were ligated into the pGEM (R)-T Easy vector. The vector was transformed into competent cells of *E. coli* (JM109). X-gal (40 μl, 20 mg/ml) and IPTG (4 μl, 1M) were added to the LB-agar containing ampicillin (100 μg/μl) for the activation of β-galactosidase. Then the bacteria were spread and plates. Then, the cells were incubated over night at 37 °C. The samples were analyzed by colony PCR (DreamTaq) and gel electrophoresis. The plasmids were isolated from the overnight cultures of positive transformants. A portion of the plasmids were digested by restriction enzymes and analyzed by agarose gel electrophoresis for verifying the correct size. The plasmids were sent for sequencing.

2.4.8 Colony PCR (Blue/White- DreamTaq)

Several colonies with *E. coli* were checked after the transformation. In this experiment, gene-specific genes were used to search for positive transformants of *E. coli*.

Table 2.20. Colony PCR set up for *Flag-B'φ*

Reagent	**Volume**	**Final conc.**
10*Dream taq buffer	1.0 µl	1*
dntp´s	0.4 µl	0.1 mM of each
Flag-B'φ F	0.5 µl	0.5 mM
B'φ R	0.5 µl	0.5 mM
Dream taq polymerase	6,5 µl	0.05 U
Water	6,5 µl	Variable
DNA Template	1.0 µl	Variable
Final volume	**10 µl**	

Table 2.21. Colony PCR set up for *Flag-B'Z*

Reagent	**Volume**	**Final conc.**
10*Dream taq buffer	1.0 µl	1*
dntp´s	0.4 µl	0.1 mM of each
Flag-B'φ F	0.5 µl	0.5 mM
B'φ R	0.5 µl	0.5 mM
Dream taq polymerase	6,5 µl	0.05 U
Water	6,5 µl	Variable
DNA Template	1.0 µl	Variable
Final volume	**10 µl**	

The samples were placed in the thermal block cycler with specific settings for three hours.

Table 2.22. The cycling process of the PCR system

Process	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	38*
Denaturation	95°C	30 sec	
Annealing	51°C	30 sec	
Elongation	72°C	2.30 min	
Final Elongation	72°C	5 min	
Cooling	5°C	∞	

2.5 Introduction of the cloned DNA in host organism

2.5.1 *Agrobacterium* transformation

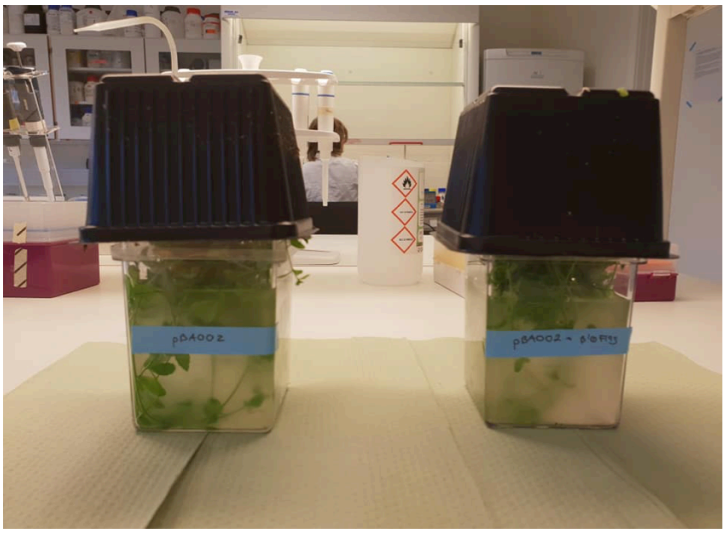
Competent cells of ABI strain of *Agrobacterium tumefaciens* were thawed on ice for 10 minutes. DNA (1 μl) was added to the cells (each tube). The mixtures were kept on ice for 10 minutes. After this, the tubes were submerged into liquid nitrogen to freeze. The frozen cells were transfered directly into a 37ºC water-bath for 15 minutes. After the heat shock, 500 μl LB-brot was added to each tube. The tubes were incubated for 3 hours at 28ºC on a shaker (200 rpm). The cells were spread on LB-agar containing the appropriate antibiotics, spectinomycin (50 μg/ml) and kanamycin (25 μg/ml). Next, the cells were incubated for 48 hours at 28ºC. Next, 150 μl overnight culture was added to Eppendorf tubes. Then the tubes were centrifuged for 1 minute at 14 000 * g and the supernantants were discarded. The pellets were re-suspended in dH_2O (20 μl). Then the suspensions were heated at 96ºC for 10 minutes followed by centrifugation at 14 000 * g for 5 minutes. Posetive colonies were to be used for floral dipping.

2.5.2 Floral dipping

Wild type of *Arabidopsis thaliana* plants was grown at a 12 h light regiemn. The plants were watered regularly with Hoagland solution (Hoagland & Arnon, 1950), and the first flowering bolts were cut to generate more flowers. 100 µl of overnight culture from the transformed *Agrobacterium* was added to LB-broth (150-170 ml) in an autoclaved beaker together with kanamycin (25 µg/ml) and spectinomycin (50 µg/ml). The solution was incubated at 28 ºC overnight on a shaker (250 rpm). After incubation, the bacterial culture was centrifuged at 4000 rpm for 15 minutes, and the supernantant were discarded. The pellets were re-suspended with 2 volumes of the buffer (see table below). An *Arabidopsis* plant was emersed in the buffer along with the vector with the insert. Another *Arabidopsis* plant was immersed in the buffer without the insert.

Table 2.23. Buffer for *Agrobacterium* floral dipping

Components	Per liter water
Sucrose	50 g
MgC12-6-hydrate	2.03 g
Silwet L-77	50 µl



The re-suspended bacteria solution was transfered to a megneta box. The flowers were submerged into the bacterial solution for 20 minutes. After this, the plants were covered with a plastic and they were laid sideways in a plant tray. Next day, the plastic bags were removed and the plants were transfered to the growth chamber

Figure 2.7. Floral dipping of *Arabidopsis thaliana*

Figure 2.8. The plants were covered with a plastic and they were laid sideways in a plant tray.

2.5.3 Surface steralization of seeds

Ca-hypochlorite (0.25 g) was added to distilled water (35 ml) and Tween (1 drop) in a measuring cylinder. The cylider was covered and shaked well. Then, Ca- hypochlorite was added to the solution 2.5 ml) to ethanol (22.5 ml, 95 %). Seeds were distributed to Eppendorf tubes. Then, the Ca- hypochlorite/ethanol solution (1ml) was added and left for 5 min. The supernantant was discarded and washed twice with ethanol (1ml, 95 %). The tubes were left on a sterile flow hood overnight for the seed to dry. The tubes were closed the following day, and sealed with Para-film

2.5.4 Phire Plant Direct PCR kit

PCR reaction set up using the Phire Plant Direct PCR Kit, Catalog number F130Wh (ThermoFisher Scientific, Waltham, Massachusetts, USA)

Table 2.24. Phire plant PCR setup

Components	**Per 10 µl**
2* Phire Plant PCR Buffer containning	5 µl
dNTP´s and $MgCl_2$	0.1 nM of each
Primer pBA002 F	0.5 µl
Priner pBA002 R	0.5 µl
Phire Hot Start II DNA Polymerase	0.2 µl
Sample	1 µl

Table 2.25. Phire plant PCR setup

Components	**Per 10 µl**
2* Phire Plant PCR Buffer containning	5 µl
dNTP´s and $MgCl_2$	0.1 nM of each
Primer gene spesific, F	0.5 µl
Priner gene spesific, R	0.5 µl
Phire Hot Start II DNA Polymerase	0.2 µl
Sample	1 µl

Table 2.26. Thermal cycling

Process	Temperature	Time	Cycles
Initial Denaturation	98°C	5:00 min	40*
Denaturation	98°C	5.0 sec	
Annealing	60°C	10 sec	
Elongation	72°C	40 sec	
Final Elongation	72°C	1 min	
Cooling	5°C	∞	

2.5.5 Sequencing

Sequencing was done to verify that the insert contained the correct nucleotide sequence and that it was ligated into the correct vector. The sequencing was performed at Seqlab-Sequence Laboratories in Göttingen, Germany. A PCR reaction tube was added the sample (0.6-0.7 μg), the primer specific for the sequence of interest (20 pmol) and dH_2O up to 7 μl. The sequencing PCR process reads nucleotides up to 1000 of length so samples exceeding this length were sequenced from both sides to ensure complete coverage.

3 RESULTS

3.1 Isolation and fragmentation of *B'φ* and *B´Z*

3.1.1 Amplified *B'φ* and *B´Z* with a FLAG tag

B'φ and *B´Z* with a FLAG tag were amplified with an expand high fidelity PCR System (refer to point 2.3.1 of the method section). The PCR products were run on an agarose gel with a gel electrophoresis system, and the products were separated by size (refer to point 2.3.2 of the method section). The gel was transferred to a ChemiDoc^{MP} Imaging System. ChemiDoc^{MP} is a full-feature instrument for imaging and analyzing gels. Using this instrument, four bands were observed in the range of about 1500 base pairs. According to National Center for Biotechnology Information (NCBI), the *B'φ* gene from the organism *S. lycopersicum* consists of 1212 base pairs, and the control gene *B´Z* from *Arabidopsis* consists of 1486 base pairs. The cycle of denaturation and synthesis of new DNA from the PCR systen is repeated around 30 to 40 times, leading to more than one million exact copies of the original DNA fragment. The large number of the synthesized genes together with a fluorescent substance (GelRed) creates thick bands in the gel that result in them being visible with either UV light or ChemiDoc^{MP}.

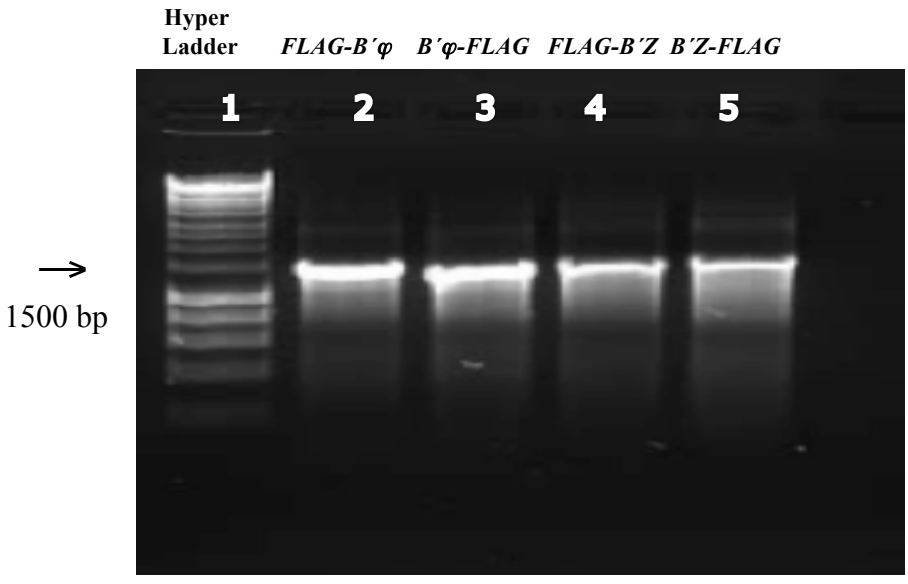


Figure 3.1. With the ChemiDoc^{MP} Imaging System, four bands were observed in the range of about 1500 base pairs. Lane 1. HyperLadder 1 (Bioline). Lane 2. *FLAG-B´φ*. Lane 3. *B´φ-FLAG*. Lane 4. *FLAG-B´Z*. Lane 5. *B´Z-FLAG*.

3.1.2 Purified *B′φ* and *B′Z* with a FLAG tag

B′φ and *B′Z* with a FLAG tag were purified from the agarose gel. The DNA fragments were cut out of the gel with a scalpel, and the gel was dissolved with chemicals (refer to point 2.3.3 of the method section). The purified products were run on an agarose gel with a gel electrophoresis system, and the products were separated by size (refer to point 2.3.2 of the method section). The gel was transferred to ChemiDoc^{MP} so that the gel could be examined for bands. With the ChemiDoc^{MP} Imaging System, four bands were observed in the range of about 1500 base pairs. The concentrations were measured with Nanodrop. 1 µl of each sample was transferred to the NanoDrop pedestal and the concentration was analyzed by the spectrophotometer (refer to point 2.3.4 of the method section).

Table 3.1. Nanodrop concentrations of purified *B′φ* and *B′Z* with a FLAG tag

Sample	Genes	Concentration
1	*FLAG-B′φ*	67.3 ng/µl
2	*B′φ-FLAG*	68.8 ng/µl
3	*FLAG-B′Z*	63.1 ng/µl
4	*B′Z-FLAG*	66.0 ng/µl

3.1.3 Digested *B'φ* and *B´Z* with a FLAG tag

B'φ with the FLAG tag was digested with the restriction enzymes Xho1 and Spe1. *B'Z* with the FLAG tag was digested with the enzymes Xho1 and Pac 1 (refer to point 2.3.5 of the method section). The digested products were run on an agarose gel with a gel electrophoresis system, and the products were separated by size (refer to point 2.3.2 of the method section). Two DNA ladders in the electrophoresis system were used in two separate regions of the gel. In this experiment, low intensity UV light was used to observe the bands. Low intensity UV light was used to minimize the destruction of the DNA fragments. Three bands were observed in the range of about 1500 base pairs in the upper part of the gel, and one band in the range of about 1500 base pairs was observed in the lower part of the gel. This picture was taken was taken after the products were cut out of the gel.

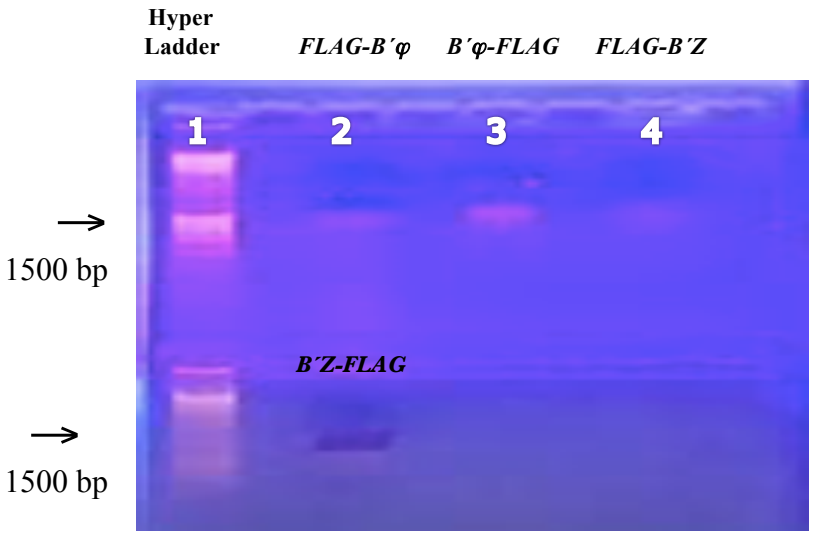


Figure 3.2. Using low intensit UV light, four bands were observed in the range of about 1500 base pairs. Upper part of the gel: Lane 1. HyperLadder 1 (Bioline). Lane 2. *FLAG-B´φ* digested with the restriction enzymes Xho1 and Spe1. Lane 3. *B´φ- FLAG* digested with the enzymes Xho1 and Spe1. Lane 4. *FLAG-B´Z* digested with the enzymes Xho1 and Pac1. Lower part of the gel: Lane 1. HyperLadder 1 (Bioline). Lane 2. *B´Z-FLAG* digested with the enzymes Xho1 and Pac1.

3.1.4 Purification of digested *B′φ* and *B′Z*

The digested products were purified from the gel (refer to point 2.3.3 of the method section). The concentrations of the digested and purified products were measured with Nanodrop. 1 µl of each sample was transferred to the NanoDrop pedestal and the concentration was analyzed by the spectrophotometer (refer to point 2.3.4 of the method section). The concentrations were significantly reduced after digestion and purification.

Table 3.2. Nanodrop concentrations of digested *B′φ* and *B′Z* with a FLAG tag

Sample	Gene	Concentration
1	*FLAG-B′φ*	5.7 ng/µl
2	*B′φ-FLAG*	7.9 ng/µl
3	*FLAG-B′Z*	4.2 ng/µl
4	*B′Z-FLAG*	7.0 ng/µl

3.2 Inserting of *B′φ* and *B′Z* into a cloning vector

3.2.1 Transformation to obtain more plasmid

Transformation of *E. coli* (JM109) was performed to obtain more plasmid. After transformation, the bacteria were selected on antibiotic plates. The bacteria with the vector construct became resistant to the antibiotic spectinomycin, which resulted in a cluster of identical plasmid-containing bacteria called a colony. Furthermore, overnight cultures were made with two posetive colonies (refer to point 2.4.1 of the method section). The next day, the plasmids were isolated from *E. coli* (refer to point 2.4.5 of the method section), for further work in relation to restriction digestion and ligation (refer to point 2.3.5 and 2.4.2 of the method section). Isolated plasmid concentrations were measured with Nanodrop. 1 µl of each sample was transferred to the NanoDrop pedestal and the concentration was analyzed by the spectrophotometer (refer to point 2.3.4 of the method section).

Table 3.3. Nanodrop concentrations of pBA002

Sample	Plasmid	Concentration
1	pBA002	113.2 ng/µl
2	pBA002	101.7 ng/µl

3.2.2 Digestion of pBA002

The plasmids (refer to table 3.3 of the result section) were digested with restriction enzymes. Two samples with plasmids were digested with the restriction enzymes Xho1 and Spe1, and two samples with Xho1 and Pac1 (refer to point 2.3.5 of the method section). The digested products were run on an agarose gel with a gel electrophoresis system, and the products were separated by size (refer to point 2.3.2 of the method section). Two DNA ladders in the electrophoresis system were used in two separate regions of the gel. The gel was continuous but the image presentation is divided into two. In this experiment, low intensity UV light was used to observe the bands. Low intensity UV light was used to minimize the destruction of the DNA fragments. Four bands were observed in the range of about 10 000 base pairs in the upper part of the gel, and six bands in the range of about 10 000 base was observed in the lower part of the gel.

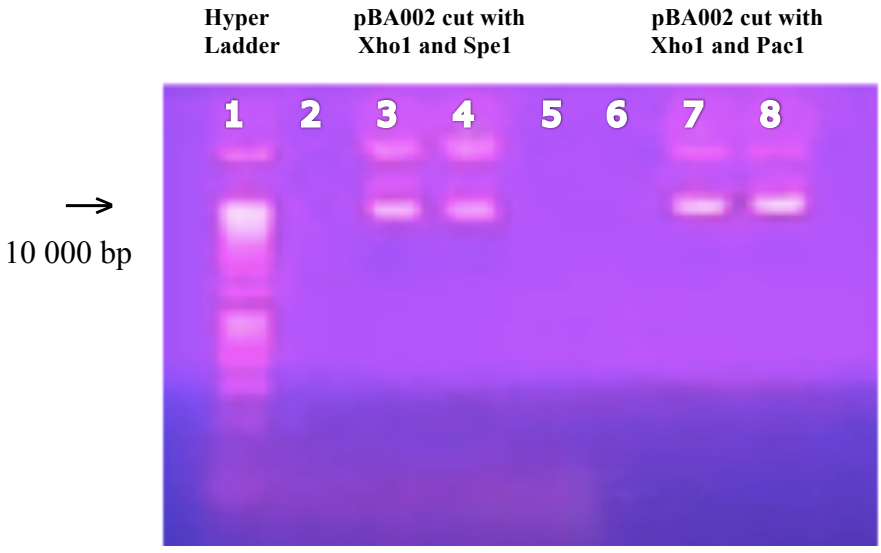


Figure 3.3. Using low intensit UV light, four bands were observed in the range of about 10 000 base pairs. Upper part of the gel: Lane 1. HyperLadder 1 (Bioline). Lane 3 and 4.

pBA002 digested with the restriction enzymes Xho1 and Spe1. Lane 7 and 8. pBA002 digested with the enzymes Xho1 and Pac1.

Six bands were observed in the lower part of the gel. In columns 2 and 3, two bands were observed in the range of about 8 000 base pairs. These bands represent plasmids that were not digested with restriction enzymes. In columns 4 and 5, two bands were observed in the range of about 10 000 base pairs. These bands represent plasmids digested with the enzymes Xho1 and Spe1. In columns 6 and 7, two bands were observed in the range of about 10 000 base pairs. These bands represent plasmids digested with the enzymes Xho1 and Pac1 (refer to point 2.3.5 of the method section).

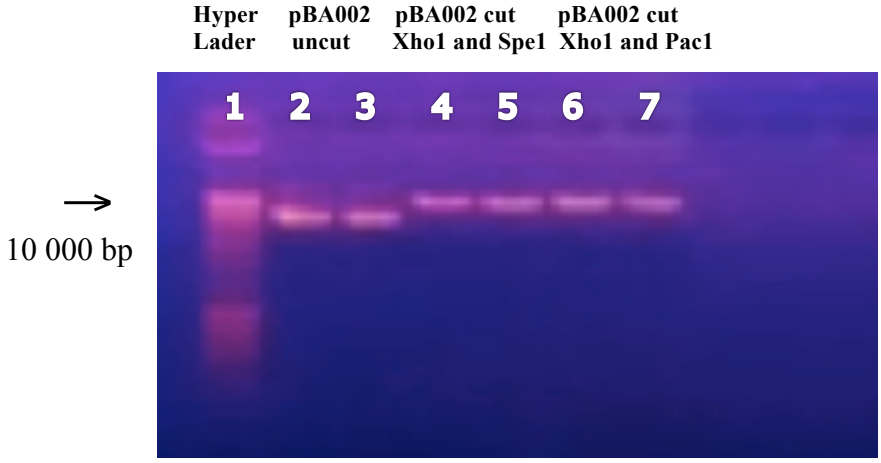


Figure 3.4. Using low intensit UV light, two bands in the range of about 8 000 base pairs and four bands in the range of about 10 000 base pairs were observed in the gel. Upper part of the gel: Lane 1. HyperLadder 1 (Bioline). Lane 2 and 3. pBA002 not digested with restriction enzymes. Lane 4 and 5. pBA002 digested with the restriction enzymes Xho1 and Spe 1. Lane 6 and 7. pBA002 digested with the enzymes Xho1 and Pac1.

The digested products were purified from the gel (refer to point 2.3.3 of the method section). Plasmid concentrations were measured with Nanodrop. 1 µl of each sample was transferred to the NanoDrop pedestal and the concentration was analyzed by the spectrophotometer (refer to point 2.3.4 of the method section). The NanoDrop concentrations of the plasmids were significantly reduced after digestion with restriction enzymes and purification (refer to table 3.3 of the result section).

Table 3.4. Nanodrop concentrations of digested pBA002

Sample	Plasmid	Concentration
1	pBA002	5.9 ng/µl
2	pBA002	3.8 ng/µl

3.2.3 Ligation and transformation

B'φ with a FLAG tag digested with the enzymes Xho1 and Spe1 was ligated with pBA002 digested with the same restriction enzymes. *B'Z* with a FLAG tag digested with the enzymes Xho1 and Pac1 was ligated with pBA002 digested with the same restriction enzymes. Ligation is the result of ends of DNA fragments being joined together by the formation of phosphodiester bonds between the 3'-hydroxyl of one DNA terminus with the 5'-p hosphoryl of another (refer to point 2.4.3 of the method section). *E. coli* (JM109) transformation was performed with the vector construct pBA002 + gene (refer to point 2.4.4 of the method section). After transformation, the bacteria were selected on antibiotic plates. Bacteria with the vector construct pBA002 + gene became resistant to the antibiotic spectinomycin, which resulted in a cluster of identical plasmid-containing bacteria called a colony. Overnight cultures were made with posetive colonies (refer to point 2.4.4 of the method section).

3.2.4 Colony PCR

Colony PCR with the overnight cultures was performed to find positive transformants of the *E. coli* (refer to point 2.4.5 of the method section). The PCR products were run on an agarose gel with a gel electrophoresis system, and the products were separated by size (refer to point 2.3.2 of the method section). The gel was transferred to ChemiDoc^{MP} so that the gel could be examined for bands. In column 10, one band were observed in the range of about 2000 base pairs representing *B'φ-FLAG* and some additional sequence from the vector contruct. When *B'φ-FLAG* is amplified by PCR, a part of the plasmid is synthesized before and after the gene resulting in a longer band than the band representing only the *B'φ-FLAG*.

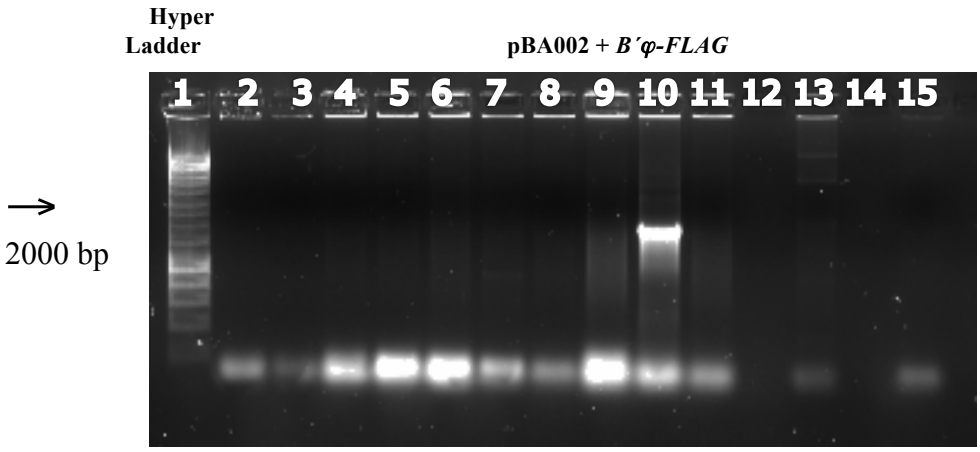


Figure 3.5. With the ChemiDoc^{MP} Imaging System, one band were observed in the range of about 2000 base pairs representing *B'φ-FLAG* and some additional sequence from the vector contruct. Lane 1. HyperLadder 1 (Bioline). Lane 10. *FLAG-B´φ* + some of the plasmid sequence.

An overnight culture was made with the posetive colony with pBA002 + *B'φFlag*. Colony PCR was performed (refer to point 2.4.4 of the method section) and the PCR products were run on an agarose gel with a gel electrophoresis system, and the products were separated by size (refer to point 2.3.2 of the method section). The gel was transferred to ChemiDoc^{MP} so that the gel could be examined for bands. A band was observed in the range of about 2000 base pairs. Furthermore, the sample was sent for sequencing to SecLab in Germany.

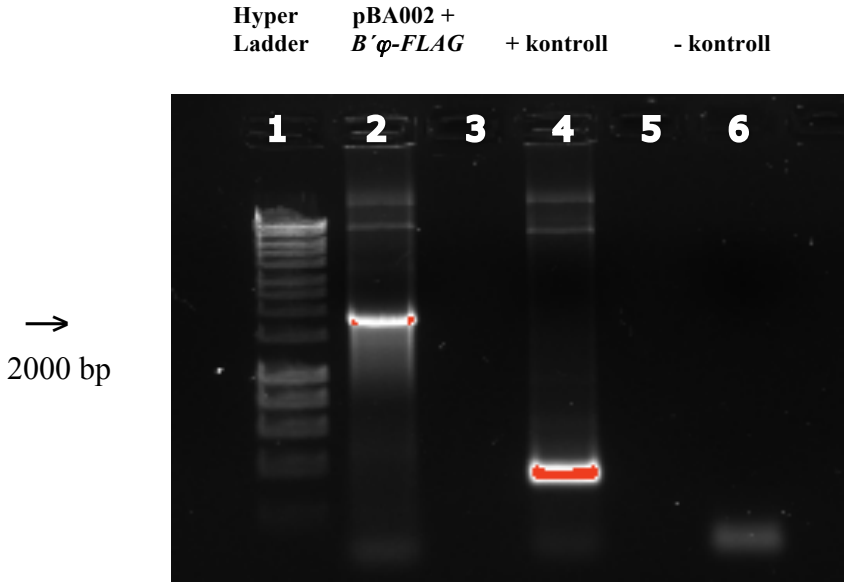


Figure 3.6. With the ChemiDoc^{MP} Imaging System, one band were observed in the range of about 2000 base pairs. Lane 1. HyperLadder 1 (Bioline). Lane 2. *FLAG-B´φ*. Lane 4. Posetive comtrol. Lane 4. Posetive control with pBA002. Lane 6. Negative control.

The vector construct pBA002 + *B'φFlag* was purified from the agarose gel (refer to point 2.3.3 of the method section). The vector construct concentration was measured with Nanodrop. 1 µl of each sample was transferred to the NanoDrop pedestal and the concentration was analyzed by the spectrophotometer (refer to point 2.3.4 of the method section).

Table 3.5. Nanodrop concentrations of pBA002 + *B'φFlag*

Sample	Recombinant plasmid	Concentration
1	pBA002 + *B'φFlag*	160 ng/µl

PCR was performed with plasmids of different sizes (refer to point 2.4.5 of the method section). The plasmids that were amplified were pBA000 not digested with restriction enzyme, the vector construct pBA002 + *B'φFlag* and pBA002 digested with Xho1 and Spe1 amplified (refer to point 2.7 of the method section). The products were run on an agarose gel with a gel electrophoresis system, and the products were separated by size (refer to point 2.3.2 of the method section). The gel was transferred to ChemiDoc^{MP} so that the gel could be examined for bands. Three bands of different sizes were observed in the gel.

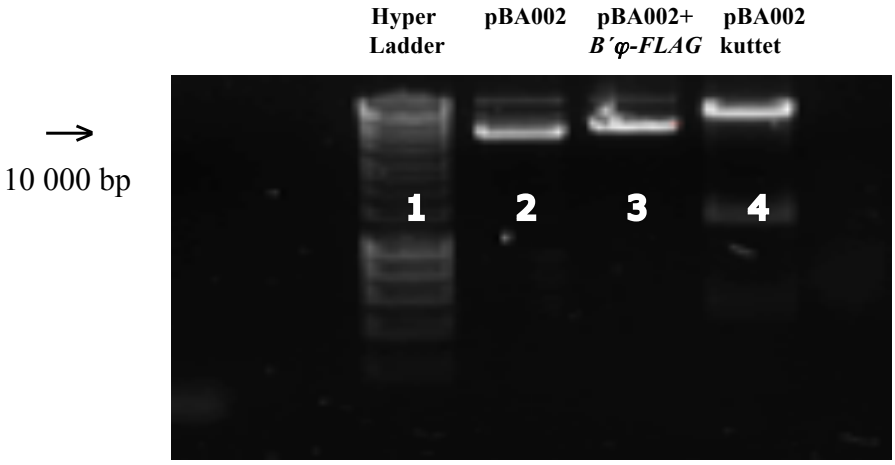


Figure 3.7. With the ChemiDoc^{MP} Imaging System, three bands of different sizes were observed in the gel. HyperLadder 1 (Bioline). Lane 2. pBA002 not digested with restriction enzymes. Lane 3. vectror construct pBA002 + *B'φFlag* and Lane 4. pBA002 digeste with the restriction enzymes Xho1 and Spe1.

3.2.5 Blue white screening

B'φ and *B´Z* with a FLAG tag were amplified by using the Expand High Fidelity PCR System (refer to point 2.3.1 of the method section). The PCR products were run on an agarose gel with a gel electrophoresis system, and the products were separated by size (refer to point 2.3.2 of the method section). Furthermore, the products were purified from the gel (refer to point 2.3.3 of the method section) and ligated (refer to point 2.4.3 of the method section) into the pGEM (R)-T Easy vector. Competent cells of *E. coli* (JM109) were transformed with the vector construct (refer to point 2.4.4 of the method section). Several white colonies were observed in three of the petri dishes, dish A) Easy vector + *FLAG-B'ϕ*, dish B) Easy vector + *B'ϕ-FLAG* and dish C) Easy vector + *FLAG-B´Z*. Colonies were not observed in the Petri dish with the Easy vector + *B´Z- FLAG*. The cloning region contains genes for β-galactosidase that are controlled by the lac operon. β-galactosidase forms a blue pigment in the presence of X-gal and the lac operon inducer IPTG. If the insert is present in the operon, it will interrupt the gene and the colony will remain white. For this reason, it is possible to detect positive colonies with blue/white screening where positive colonies will appear white (refer to point 2.4.7 of the method section).

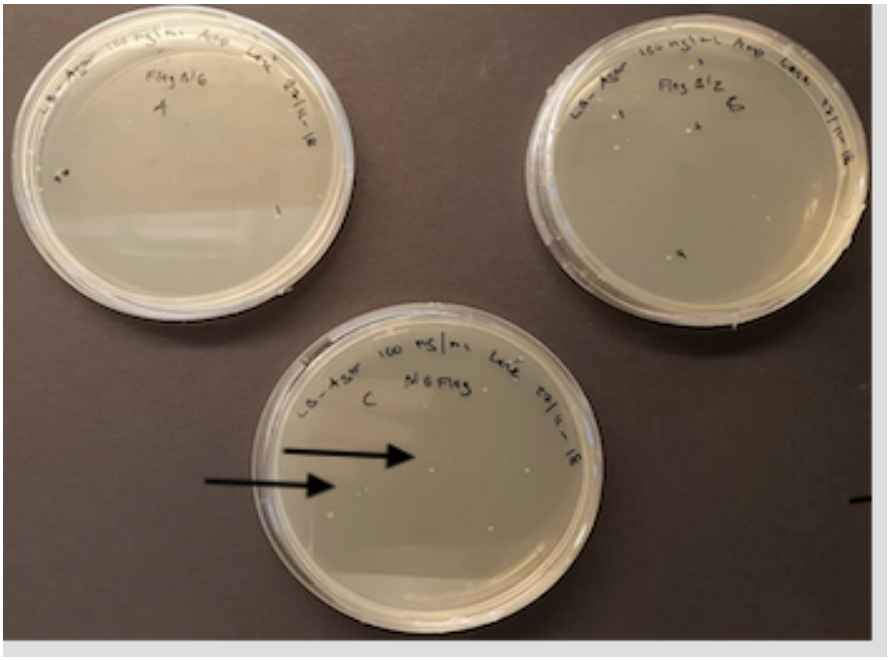


Figure 3.7. White colonies were observsed in three petri dishes. The top arrow in the picture shows a white colony and the bottom arrow shows a blue colony. White colonies were observed in three petri dishes: A) Easy vector + *FLAG-B'ϕ*, B) Easy vector + *FLAG-B´Z* and C) Easy vector + *B'ϕ-FLAG*.

White colonies of *E. coli* were picked from the petri dishes (refer to figure 3.7 A and C) and checked with colony PCR. Gene-specific primers were used in this experiment (refer to point 2.4.8 of the method section). The PCR products were run on an agarose gel with a gel electrophoresis system, and the products were separated by size (refer to point 2.3.2 of the method section). The gel was transferred to ChemiDoc^{MP} so that the gel could be examined for bands. Eight bands were observed in the range of about 1500 base pairs. The bands represent the vector construct Easy vector + *FLAG-B'ϕ*.

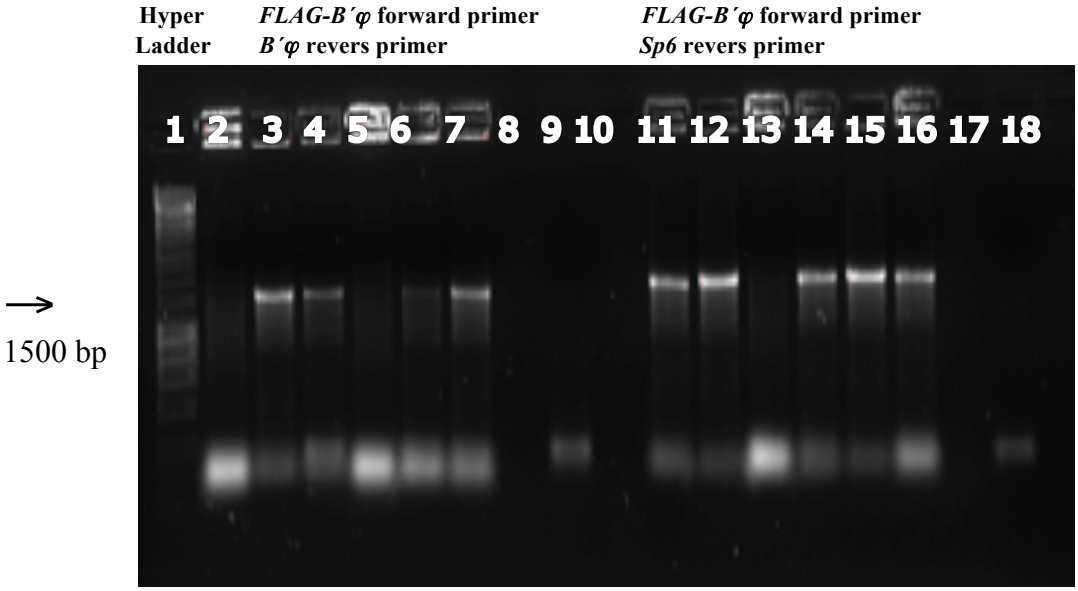


Figure 3.8. With the ChemiDoc^{MP} Imaging System, nine bands were observed in the range of about 1500 base pair. HyperLadder 1 (Bioline). Lane 3, 4 and 5. *FLAG-B´φ* forward primer and *B´φ* revers primer. Lane 11,12 and 14-16. *FLAG-B´φ* forward primer and *Sp6* revers primer.

White colonies of *E. coli* were picked from the petri dishes (refer to figure 3.7 B) and checked with colony PCR. Gene-specific primers were used in this experiment (refer to point 2.4.8 of the method section). The PCR products were run on an agarose gel with a gel electrophoresis system, and the products were separated by size (refer to point 2.3.2 of the method section). The gel was transferred to ChemiDoc^{MP} so that the gel could be examined for bands. Six bands were observed in the range of about 1500 base pairs. The bands represent the vector construct Easy vector + *FLAG-B'Z*

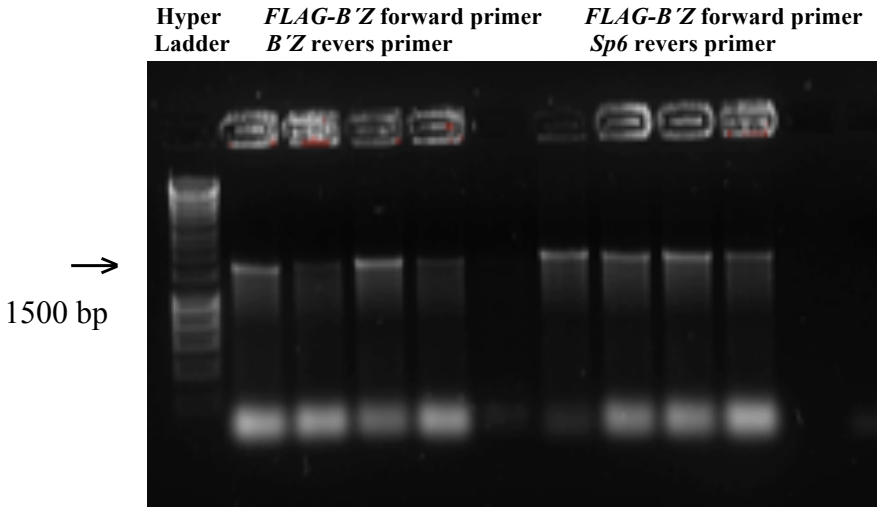


Figure 3.9. With the ChemiDoc^{MP} Imaging System, six bands were observed in the range of about 1500 base pairs. HyperLadder 1 (Bioline). Lane 2 and 3. *FLAG-B´Z* forward primer and *B´φ* revers primer. Lane 6-9. *FLAG-B´φ* forward primer and *Sp6* revers primer.

Overnight cultures were made with the posetive colonies. The products were isolated from *E. coli* the next day (refer to point 2.4.6 of the method section). The concentrations were measured with Nanodrop. 1 µl of each sample was transferred to the NanoDrop pedestal and the concentration was analyzed by the spectrophotometer (refer to point 2.3.4 of the method section).

Table 3.6. Nanodrop concentrations Easy vector + gene

Colony	Easy vector construct	Concentration
2	Vector + *FLAG-B´φ*	249.4 ng/µl
6	Vector + *FLAG-B´φ*	191.9 ng/µl
1	Vector + *B´φ-FLAG*	147.9 ng/µl
1	Vector + *FLAG-B´Z*	166.6 ng/µl
3	Vector + *FLAG-B´Z*	200.6 ng/µl

3.2.6 Easy-vector digested

Easy-vector + *B′φ* with a FLAG tag was digested with the restriction enzymes Xho1 and Spe1. Easy-vector + *B′Z* with a FLAG tag was digested with the restriction enzymes Xho1 and Pac1 (refer to point 2.3.5 of the method section). The products were run on an agarose gel with a gel electrophoresis system, and the products were separated by size (refer to point 2.3.2 of the method section). The gel was transferred to ChemiDoc^{MP} so that the gel could be examined for bands. Five bands were observed in the range of about 1500 and 3000 base pairs. The upper bands represent the Easy vector and the lower bands represent the genes.

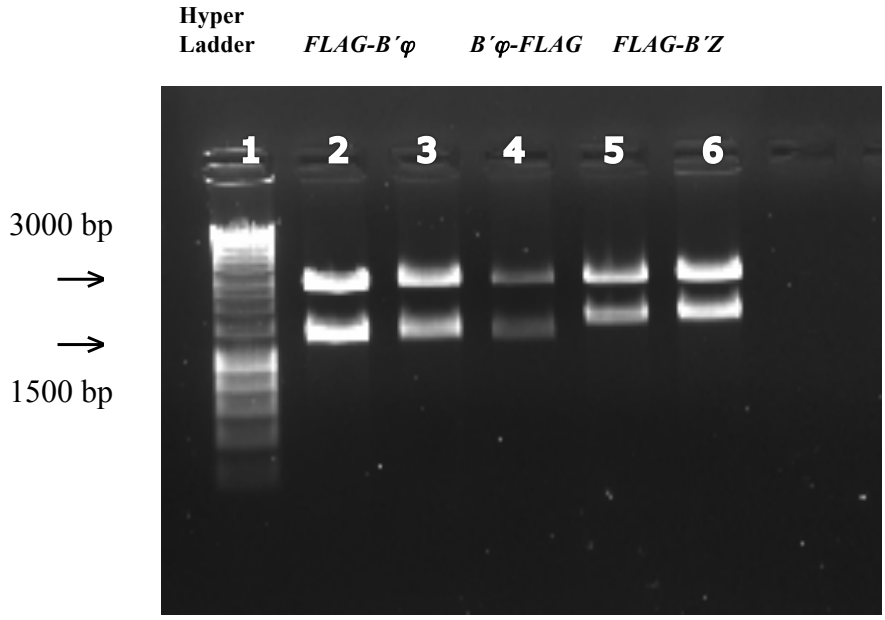


Figure 3.10. With the ChemiDoc^{MP} Imaging System, ten bands were observed in the range of about 1500 and 3000 base pairs. Upper part of the gel: HyperLadder 1 (Bioline). Lane 2-6. pGEM (R)-T Easy vector. Lower part of the gel: HyperLadder 1 (Bioline). Lane 2 and 3. *FLAG-B´φ.* Lane 4. *B´φ-FLAG.* Lane 5 and 6. *FLAG-B´Z*

The DNA fragments were cut out of the gel with a scalpel, and the gel was dissolved with chemicals (refer to figure 3.10 and 2.3.3 of the method section). The concentrations were measured with Nanodrop. 1 µl of each sample was transferred to the NanoDrop pedestal and the concentration was analyzed by the spectrophotometer (refer to point 2.3.4 of the method section).

Table 3.6. Nanodrop concentrations of *B′φ* and *B′Z* with tag

Colony	Primer	Concentration
2	*FLAG-B′φ*	7.3 ng/µl
6	*FLAG-B′φ*	10.1 ng/µl
1	*B′φ-FLAG*	9.2 ng/µl
1	*FLAG-B′Z*	4.4 ng/µl
3	*FLAG-B′Z*	7.9 ng/µl

3.2.7 Colony PCR (blue/white screening)

Purified *B′φ* and *B´Z* with a FLAG tag (refer to table 3.6 of the result section) were ligated into purified pBA002 (refer to point 2.4.3 of the method section). *E. coli* (JM109) transformation was performed with the vector construct pBA002 + gene (refer to point 2.4.4 of the method section). After transformation, the bacteria were selected on antibiotic plates. Colony PCR was performed to find positive transformants of the *E. coli* (refer to point 2.4.8 of the method section). The PCR products were run on an agarose gel with a gel electrophoresis system, and the products were separated by size (refer to point 2.3.2 of the method section). The gel was transferred to ChemiDoc^{MP} so that the gel could be examined for bands. Five bands were observed in the range of about 1500 base pairs representing *FLAG-B′φ* (upper part of the gel). No bands were observed with *B´Z* with a FLAG (lower part of the gel).

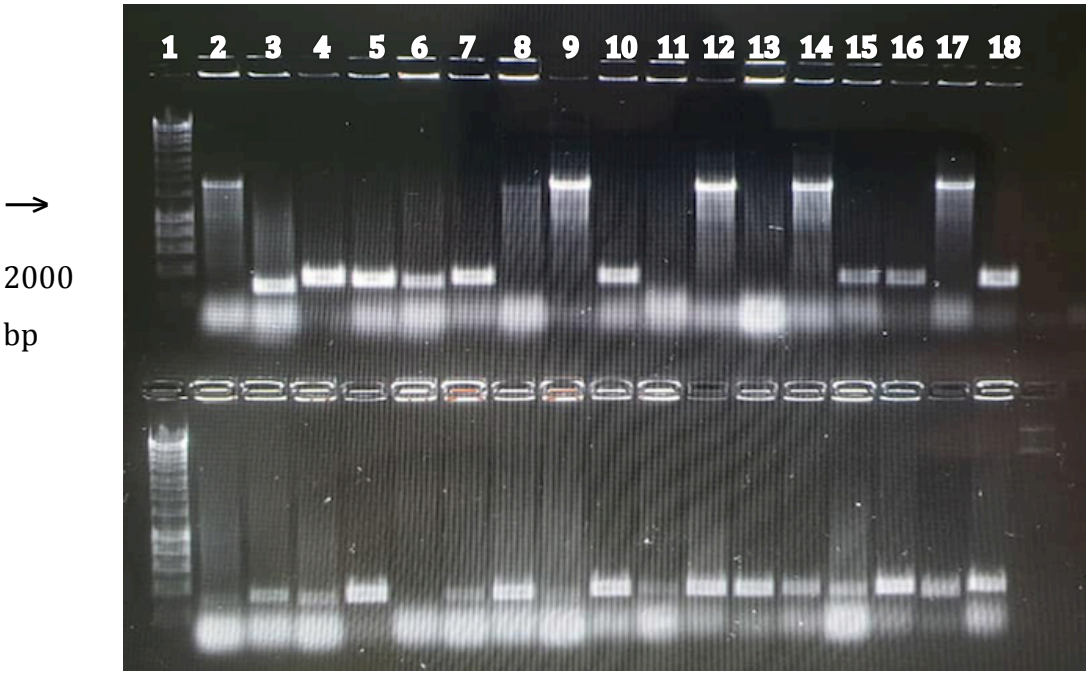


Figure 3.11. With the ChemiDoc^{MP} Imaging System, five bands were observed in the range of about 1500 base pairs. Upper part of the gel: Lane 1. HyperLadder 1 (Bioline). Lane 2. *FLAG-B′φ*. Lane 9. *FLAG-B′φ*. Lane 12. *FLAG-B′φ*. Lane 14. *FLAG-B′φ* and Lane 17. *FLAG-B′φ*

A second colony PCR was performed after overnight cultures with two of the same colonies to confirm the presence of the vector construct in the bacteria. Two new bands were observed in the range of about 2000 base pairs representing *FLAG- B'φ*.

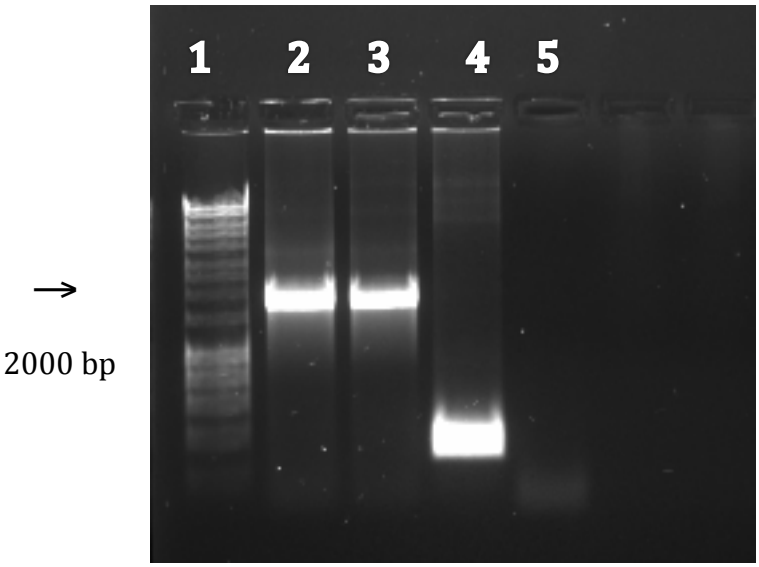


Figure 3.12. With the ChemiDoc^{MP} Imaging System, two bands were observed in the range of about 1500 base pairs. Lane 1. HyperLadder 1 (Bioline). Lane 2. FLAG-*B'φ*. Lane 3. FLAG-*B'φ*. Lane 4. Posetive control with pBA002. Lane 5. Negative control

The vector construct pBA002 + *FLAG-B'φ* was purified from the agarose gel (refer to point 2.3.3 of the method section). The vector construct concentration was measured with Nanodrop. 1 µl of each sample was transferred to the NanoDrop pedestal and the concentration was analyzed by the spectrophotometer (refer to point 2.3.4 of the method section).

Table 3.4. Nanodrop concentrations of pBA002 + *FlagB'φ*

Sample	Recombinant Plasmid	Concentration
1	pBA002+ *FlagB' φ*	63.7 ng/µl
2	pBA002+ *FlagB' φ*	38.7 ng/µl

3.3 Introduction of *B'φ* and *B´Z* in a host organism

3.3.1 *Agrocacterium* transformation

An ABI strain of competent *Agrobacterium* cells were transformed with the construct pBA002 + *B'φ-FLAG* (refer to point 2.5.1 of the method section). Colony PCR was performed the next day to find positive transformants of *Agrocacterium* (refer to point 2.4.5 of the method section). The PCR products were run on an agarose gel with a gel electrophoresis system, and the products were separated by size (refer to point 2.3.2 of the method section). In this experiment, low intensity UV light was used to observe the bands. Low intensity UV light was used to minimize the destruction of the DNA fragments. Three bands were observed in the range of about 2000 base pairs.

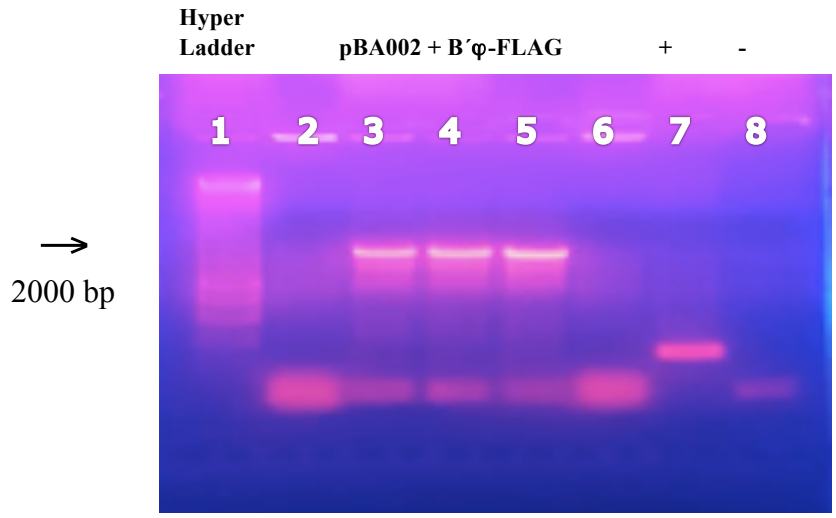


Figure 3.11. Using low intensit UV light, three bands were observed in the range of about 2000 base pairs. Lane 1. Hyperladder 1 (Bioline). Lane 3-4. pBA002 + *B´φ-FLAG.* Lane 6. Posetive control with pBA002. Lane 8. Negative control.

Agrobacterium were spread on three dishes. In the first petri dish, *Agrobacterium* without the pBA002 was spread on LB-agar containing spectinomycin and kanamycin. In the second petri dish was *Agrobacterium* with the plasmid pBA002 (without the insert) spread on LB-agar containing spectinomycin and kanamycin. In the last petri dish was *Agrobacterium* with the construct pBA002 + *B'φ-FLAG* spread on LB-agar containing spectinomycin and kanamycin. Plates with transformed bacterial cells were screened after 48 hours of incubation at 28°C. No colonies were observed in the Petri dish with *Agrobacterium* without the pBA002. Colonies were observed in the dishes where *Agrobacterium* was spread with the plasmid, both with and without the insert.

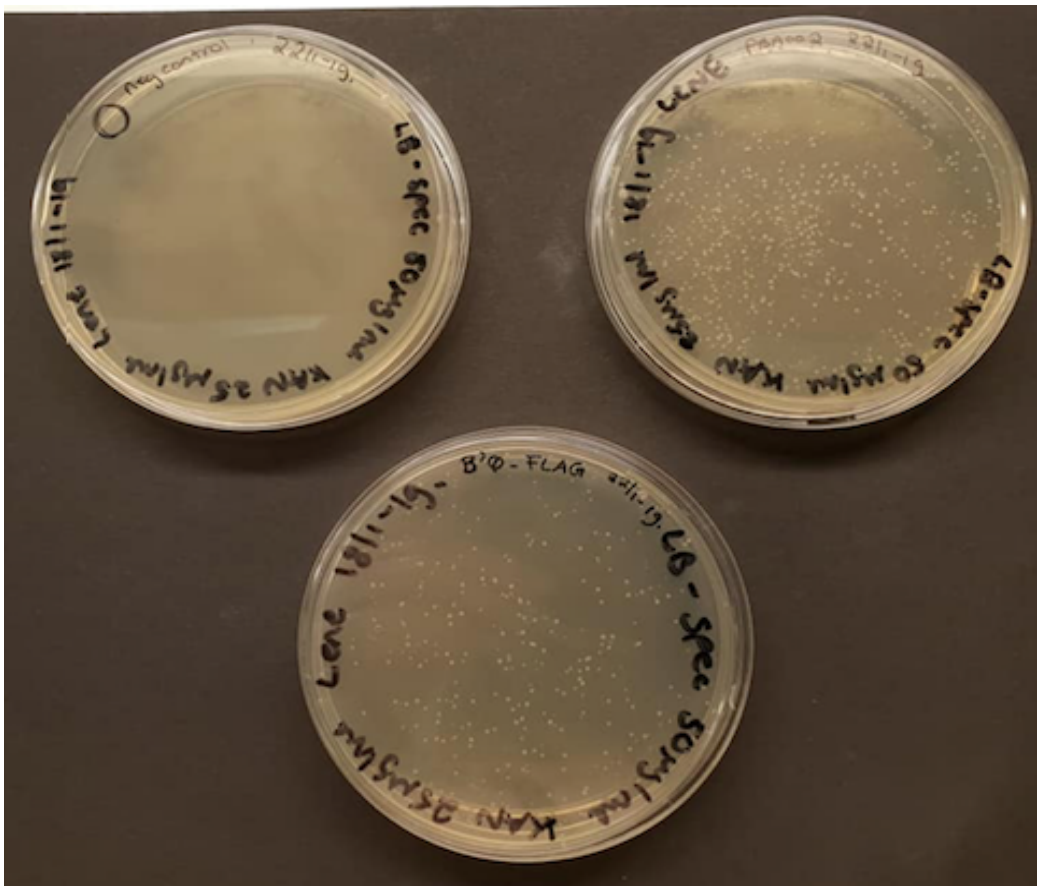


Figure 3.11. The top left dish contained *Agrobacterium* without the plasmid pBA002. The dish up to the right contained *Agrobacterium* with pBA002 without the insert. The middle dish contained *Agrobacterium* with the construct pBA002 + *B'φ-FLAG*.

3.3.2 Colony PCR, *Agrocacterium*

Colony PCR was performed to find positive transformants of *Agrobacterium* (refer to point 2.4.5 of the method section). The PCR products were run on an agarose gel with a gel electrophoresis system, and the products were separated by size (refer to point 2.3.2 of the method section). The gel was transferred to ChemiDoc^{MP} so that the gel could be examined for bands. Five bands were observed in the range of about 2000 base pairs. All the colonies that were checked were posetive for *Agrobacterium* with the construct pBA002 + *B'φ-FLAG*

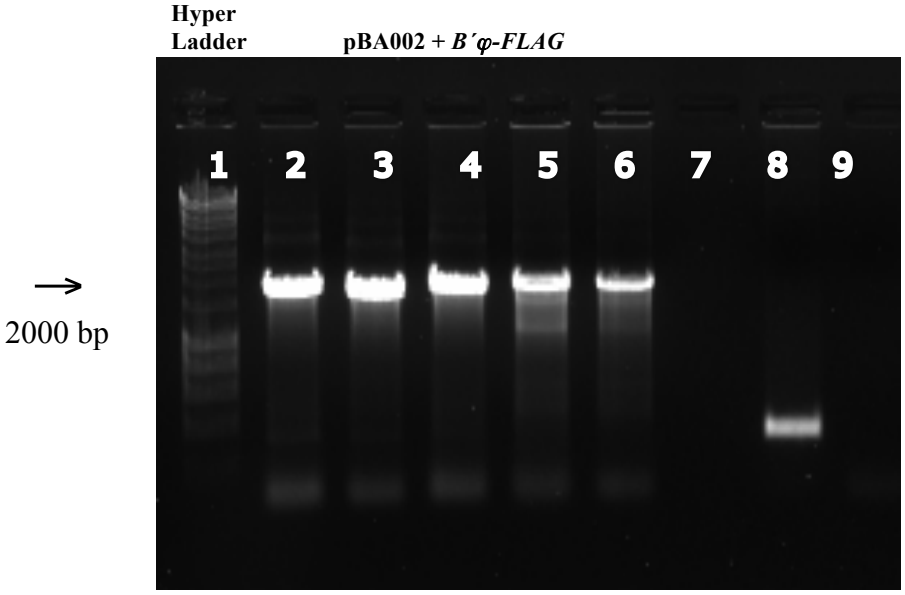


Figure 3.12. With the ChemiDoc^{MP} Imaging System, five bands were observed in the range of about 2000 base pairs. Lane 1. Hyperladder 1 (Bioline). Lane 2-6. pBA002 + *B´φ-FLAG* Lane 8. Posetive control. Lane 9. Negative control.

3.3.3 Floral dipping

Seeds from *Arabidopsis* with a Colombia background were planted in several pots. The pots were placed in a cold (4°C) and dark room for three days before being placed in a light room. The wild type of *Arabidopsis* plants was grown at a 12 h light regiemn. The plants were watered regularly with Hoagland solution (Hoagland & Arnon et al. 1950), and the first flowering bolts were cut to generate more flowers size (refer to point 2.2.1 of the material section).

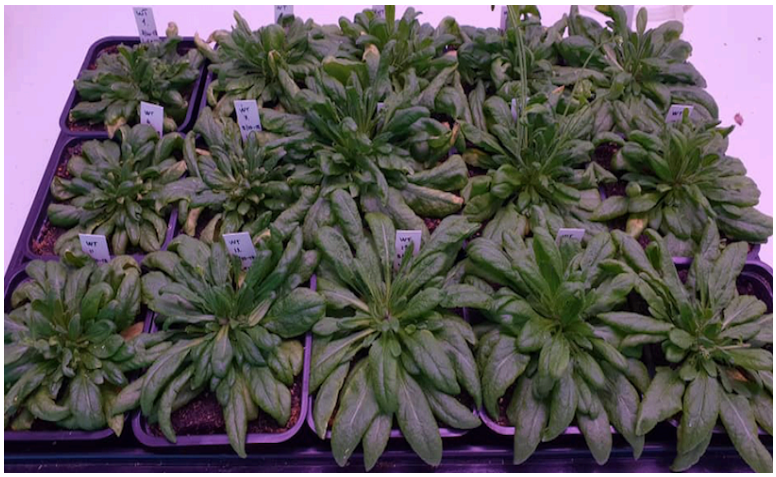


Figure 3.13. Cultured wild-type *Arabidopsis thaliana* plants.

Overnight cultures were made with the posetive transformants of *Agrobacterium* and the plasmid pBA002 (refert to figure 2.5.4 of the result section). The next day the bacteria cultures were pelleted before being resuspended in the buffer used for floral dipping (refer to point 2.5.2 of the method section) (Clough et al. 1998).

Figure 3.14 The bacteria cultures were pelleted before being resuspended in the buffer used for floral dipping (Clough et al. 1998).

An *Arabidopsis* plant was emersed in the buffer along with the vector with the insert. Another *Arabidopsis* plant was immersed in the buffer without the insert. The re-suspended bacteria solution was transfered to a megneta box. The flowers were submerged into the bacterial solution for 20 minutes. After this, the plants were covered with a plastic and they were laid sideways in a plant tray. Next day, the plastic bags were removed and the plants were transfered to the growth chamber (Clough et al. 1998).

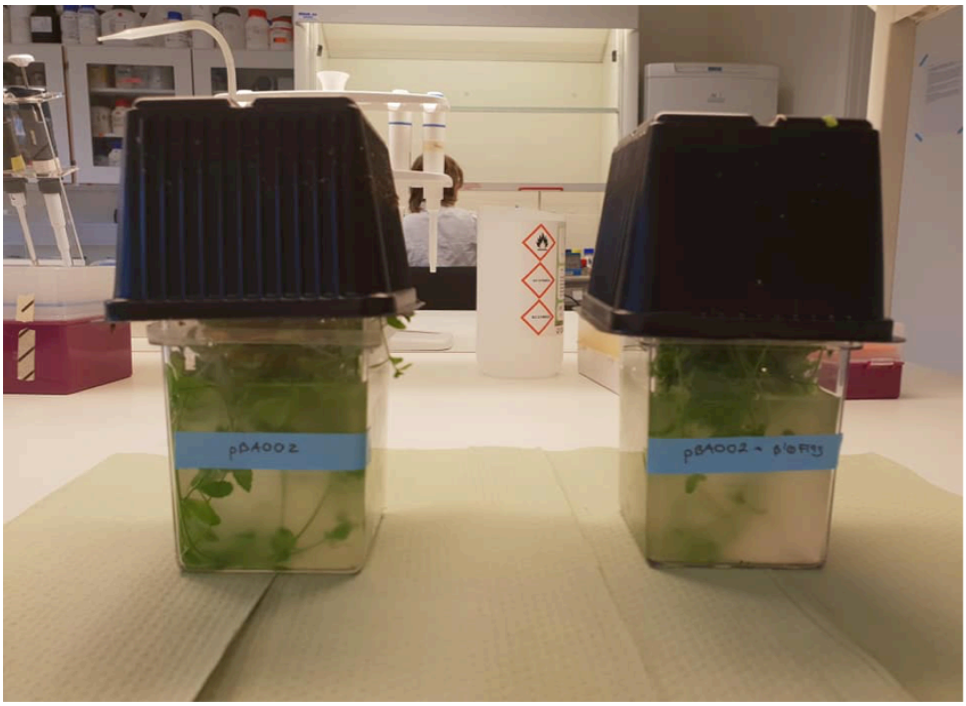


Figure 3.15. Floral dipping. Megneta box to the left, an Arabidopsis was immersed in the buffer solution without the insert. Megneta box to the right Arabidopsis plant was emersed in the buffer solution along with the vector with the insert (Clough et al. 1998).

3.3.4 Screening of *Agrobacterium* transformants

Surface sterilized seeds harvested from the *Agrobacterium* transformed plants were screened on selective media (refer to point 2.5.3 of the method section). This was done on agar plates containing 1/2 MS medium (refer to point 2.2.3. of the material section). For selection of positive *Arabidopsis* transformants Basta was added to the media. The seeds were sprinkled onto the agar plates and were left to stratify for 3 days at 4°C in the dark. Plates were after stratification transferred to 16 hours light. All plates were screened after approximately 10 days.

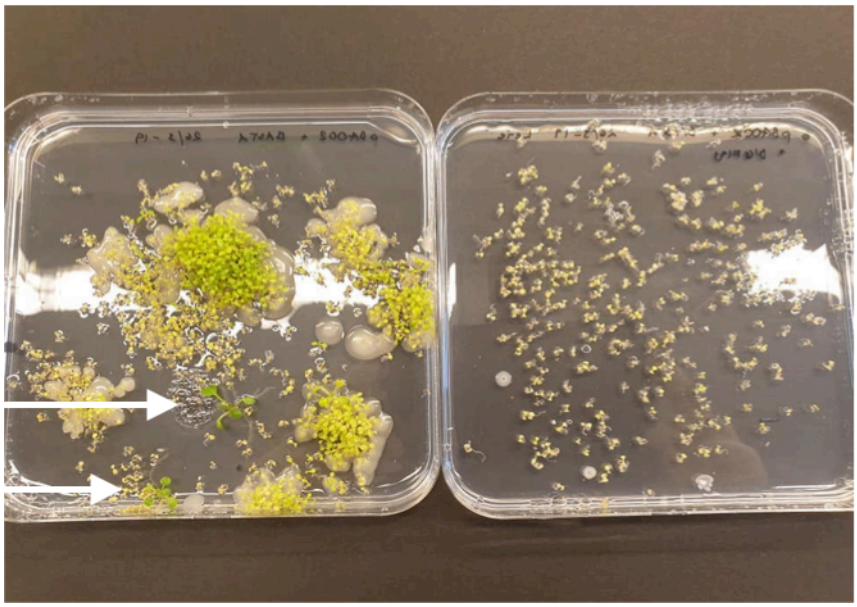


Figure 3.16. Petri dish to the left, transformed *Arabidopsis* with the construct pBA002 + *B'φ-FLAG* on ½ MS meduim with BASTA. The transformants are seen as darker green seedlings having 4-6 leaves including cotyledons. These were transferred to soil and grown at 12 hours light to get seeds. The transformants are seen as darker green seedlings having 4-6 leaves including cotyledons (see white arrows)**.** Petri dish to the right, transformed *Arabidopsis* with pBA002. No plants were observed in this dish.

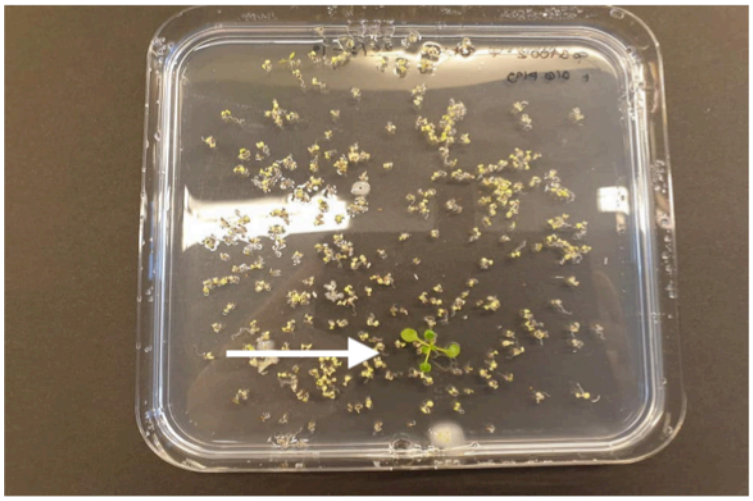


Figure 3.17. Transformed *Agrobacterium* with the construct pBA002 + *B'φ-FLAG* spread on ½ MS mediun with BASTA. The transformed plant is seen as darker green seedling having 4-6 leaves including cotyledons (see white arrow)**.** The plant was transferred to soil and grown at 12 hours light to get seeds.

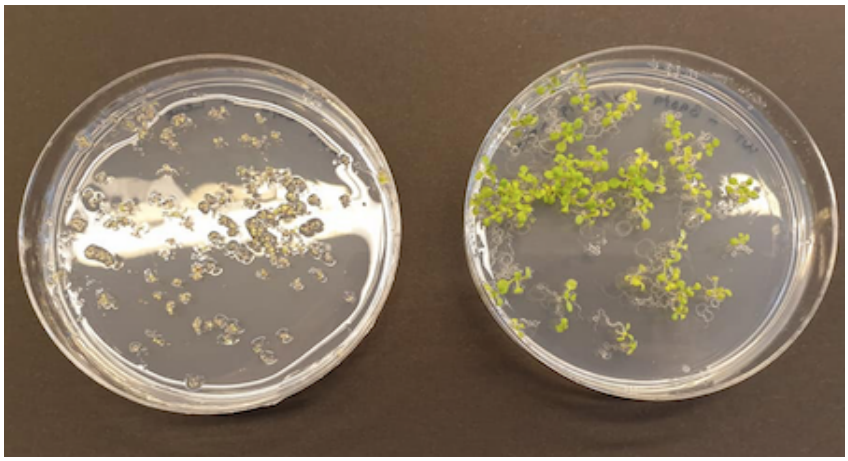


Figure 3.18. Petri dish to the left, wildtype *Arabidopsis* was grown on ½ MS medium with BASTA. No plants were observed in this dish due to lack of resistance to BASTA. Petri dish to the right, wild type *Arabidopsis* was grown in the petri dish without BASTA and clusters with plants were observed.

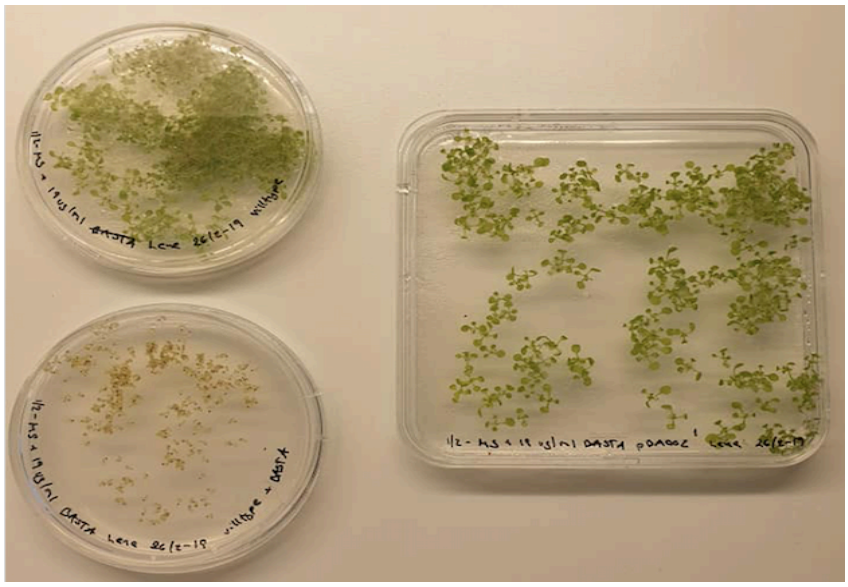


Figure 3.19. In the petri dish up to the left, wildtype *Arabidopsis* was grown without BASTA and and clusters with plants were observed. In the petri dish on the lower left, wildtype *Arabidopsis* was grown with BASTA and no plants survived due to lack of resistance to BASTA. In the Petri dish to the right, *Arabidopsis* with the plasmid pBA002 was grown on BASTA and several plants were observed due to resistance to BASTA.

The transformed *Arabidopsis* plants in figure 3.16 and 3.17 (refer to point 3.13 of the result section) were transferred to soil and grown at 12 hours light to get seeds.

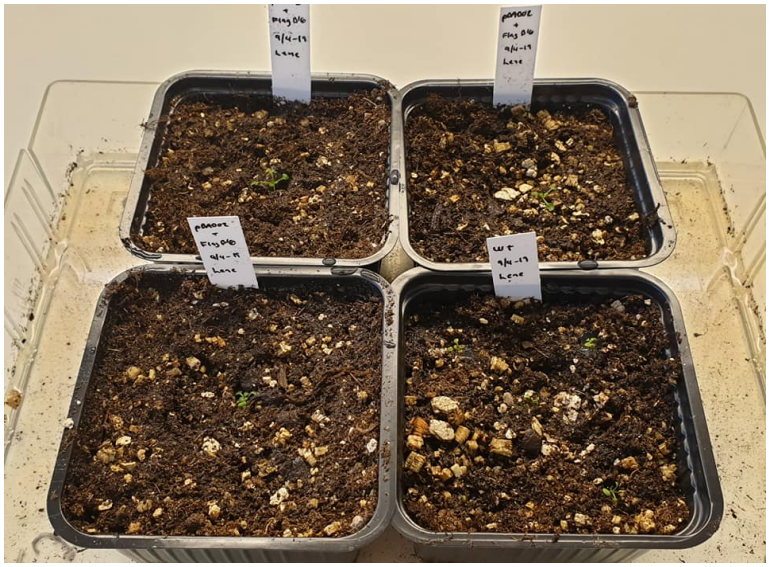


Figure 3.20. Transformed *Arabidopsis* plants for further cultivation. The pot in the lower right contained wildtype *Arabidopsis,* and the other three pots contained trangenic plants.

Figure 3.21. Transgenic *Arabidopsis* plants after some few weeks of cultivation. The pot in the lower right contained wildtype *Arabidopsis,* and the other three pots contained trangenic plants.

The pot in the lower right contained wildtype *Arabidopsis,* and the other three pots contained trangenic plants. Very small plants were observed from the pot with wildtype *Arabidopsis* compared to the pots with transgenic plants. It appeared that the wild-type *Arabidopsis* leaves had been eaten by insects, but not the transgenic plants.

Figure 3.22. Transformed *Arabidopsis* after some few weeks of cultivation

3.3.5 Phire Plant Direct PCR kit

Phire Plant Direct PCR kit was performed to find positive transformants of *Arabidopsis* (refer to point 2.5.4 of the method section). The PCR products were run on an agarose gel with a gel electrophoresis system, and the products were separated by size (refer to point 2.3.2 of the method section). The gel was transferred to ChemiDoc^{MP} so that the gel could be examined for bands. Two bands were observed in the range of about 2000 base pairs. In the upper part of the gene, vector specific primers were used during PCR amplification. In the lower part of the gel, gene specific primers were used during the amplification by PCR.

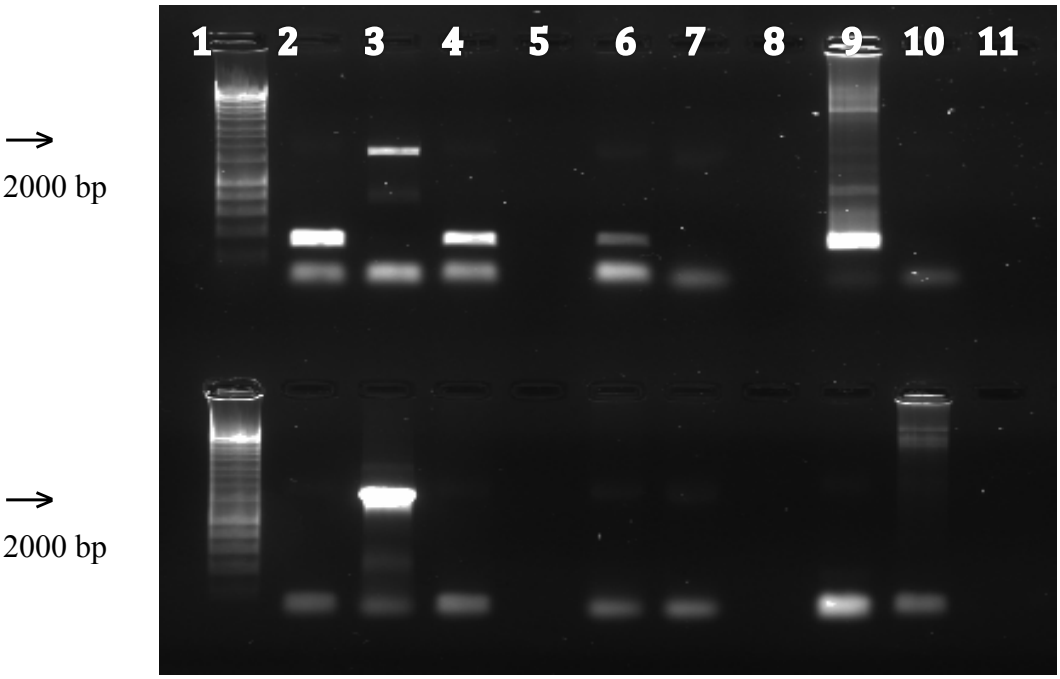


Figure 3.23. With the ChemiDoc^{MP} Imaging System, two bands were observed in the range of about 2000 base pairs. Upper part of the gel. Lane 1. HyperLadder 1 (Bioline). Lane 3. *B′φ-FLAG*. Lane 9. Posetive control. Lane 10. Negative control. Lower part of the gel. Lane 1. HyperLadder 1 (Bioline). Lane 3. *B′φ-FLAG.* Posetive control. Lane 10. Negative control.

4 DISCUSSION AND FINAL REMARKS

4.1 Analysis of isolation and fragmentation of the source DNA

B'φ and B´Z with a FLAG tag were amplified with expand high fidelity PCR system, separated by gel electrophoresis, analyzed by ChemiDoc^{MP}. Four bands in the gel were observed in the range of about 1500 base pairs (refer to figure 3.1 of the result section). According to NCBI, the *B'φ* gene from *Solanum lycopersicum* (tomato plant) consists of 1212 base pairs, and the control gene *B´Z* from *Arabidopsis* consists of 1486 base pairs (refer to table 2.7 of the method section). It was decided to proceed with the PCR products because the bands in the gel matched the sizes reported in the NCBI. It was assumed that the FLAG tag sequences were attached to either the N- or C –terminal of the genes during amplification with the PCR system. The PCR products were purified from the gel and the concentrations were measured with NanoDrop. It was assumed that NanoDrop concentrations were high enough to continue the work (refer to table 3.1 of the result section). The genes were digested with restriction enzymes, separated by gel electrophoresis and analyzed by low intensity UV light. Four bands were observed in the range of about 1500 base pairs (refer to figure 3.2 of the result section). It was decided to proceed with the digested products because the bands in the gel matched the sizes reported in the NCBI. The genes *B'φ and B´Z* with a FLAG tag were purified from the gel and the concentrations were measured with Nanodrop. The NanoDrop concentrations were significantly reduced after digestion and purification, which was thought to be due to dilution of the samples. It was assumed that NanoDrop concentrations were high enough to continue the work (refer to table 3.2 of the result section).

4.2 Analysis of inserting the DNA fragment into a cloning vector

It was necessary to obtain more of the plasmid pBA002. *E. coli* transformation was performed and the bacteria were selected on antibiotic plates. The colonies in the petri dishes were observed and analyzed, and it was assumed that some of the colonies were positive transformants. Plasmids were isolated from the bacteria and the concentrations were measured with Nanodrop. It was assumed that NanoDrop concentrates were high enough to continue the work (refer to table 3.3 of the result section).

The plasmids were digested with restriction enzymes and separated by gel electrophoresis. In this experiment, low intensity UV light was used to observe the bands. Four bands were observed in the range of about 10 000 base pairs (refer to figure 3.3 of the result section). It was decided to proceed with the digested products because the bands in the gel matched the vector map of pBA002 (refer to figure 2.5 of the method section). The digested plasmids were purified from the gel and the concentrations were measured with Nanodrop. The NanoDrop concentrations were significantly reduced after digestion and purification, which was thought to be due to dilution of the samples. It was assumed that the NanoDrop concentrations were high enough to continue the work (refer to table 3.4 of the results section). Digested *B′φ* and *B′Z* with a FLAG tag (refer to table 3.2 of the result section) were ligated into digested and purified pBA002. *E. coli* transformation was performed and the bacteria were selected on antibiotic plates. Colony PCR was performed to identify positive transformants of *E. coli.* The PCR products were separated by gel electrophoresis and analyzed by ChemiDoc^{MP}. One band were observed in the range of about 2000 base pairs representing *B′φ-FLAG* and some additional sequence from the vector contruct (refer to figure 3.5 of the result section). A second colony PCR was performed after an overnight culture with the same colony to confirm the presence of the vector construct in the bacteria. A new band was observed in the range of about 2000 base pairs representing *B′φ-FLAG* (refer to figure 3.6 of the result section).
The vector constructs were purified from *E. coli* and the concentrations were measured with Nanodrop (refer to table 3.5 of the result section). The samples were sent for sequencing (refer to point 6.1 of the appendix section). It is important to point out that the gene *B′φ-FLAG* was inserted directly into the pBA002 vector without the help of another vector first.

4.2.1 Blue white screening

B′φ and *B´Z* with a FLAG tag (refer to table 3.2 of the result section) were ligated into the pGEM$^{(R)}$ -T Easy vector. *E. coli* transformation was performed and several blue and white colonies were observed in the petri dishes the next day. It was thought that if the vector construct were present in the bacteria, the colonies would turn white (refer to point 2.4.6 of the method section). White colonies were observed and analyzed in three of the petri dishes: (A) Easy vector + *FLAG-B′ϕ*, (B) Easy vector + *B′ϕ-FLAG* and (C) Easy vector + *FLAG-B´Z* (refer to figure 3.7 of the result section). White colonies were picked from dish A and C and checked with colony PCR to confirm the presence of the vector construct in the bacteria. The PCR products were separated by gel electrophoresis and analyzed by ChemiDoc^{MP}. Eight

bands were observed in the range of about 1500 base pairs, which represented the Easy vector + *FLAG-B′ϕ*. Six bands were observed in the range of about 1500 base pairs, which represented the Easy vector + *FLAG-B′Z* (refer to figure 3.8 and 3.9 of the result section). It was decided to proceed with the PCR products because the bands in the gel matched the sizes reported in the NCBI (refer to table 2.7 of the method section). Overnight cultures were made with the white colonies from the petri dishes A, B and C, and the products were isolated from *E. coli* the next day. The concentrations were measured with Nanodrop and it was assumed that the concentrates were high enough to continue the work (refer to table 3.6 of the result section). The isolated products were digested with restriction enzymes, separated by gel electrophoresis and analyzed by ChemiDoc^{MP}. Five bands were observed in the range of about 1500 base pairs in the upper part og the gel, which represented the genes. Then five bands were observed in the range of about 3000 base pairs in the lower part of the gel, which represented the digested Easy vector (refer to figure 3.10 of the result section). The vector bands matched the size of the pGEM®-T Easy Vector Map (refer to figure 2.6 of the method section). The digested genes were purified from the gel and the concentrations were measured with Nanodrop. It was assumed that NanoDrop concentrates were high enough to continue the work (refer to table 3.6 of the result section). Purified *B′φ* and *B´Z* with a FLAG tag (from the Easy vector) were ligated into digested pBA002 (refer to table 3.4 of the result section). *E. coli* transformation was performed and the bacteria were selected on antibiotic plates. Colony PCR was performed to find positive transformants of *E. coli*. The PCR products were separated by gel electrophoresis and analyzed by ChemiDoc^{MP}. Five bands were observed in the range of about 2000 base pairs (refer to figure 3.11 of the result section). It was decided to proceed with the PCR products because the bands in the gel matched the sizes reported in the NCBI (refer to table 2.7 of the method section). A second colony PCR was performed after an overnight culture with two of the same colonies to confirm the presence of the vector construct in the bacteria. Two new bands were observed in the range of about 1500 base pairs representing *FLAG- B′φ*. The vector constructs were purified from the gel and the concentrations were measured with Nanodrop (refer to table 3.4 of the result section). The samples were sent for sequencing (refer to point 6.2 of the appendix section). It is important to point out that the gene *FLAG-B′φ* was first inserted into the Easy vector and then into the pBA002 vector. At this time, new competent cells of the *E .coli* were made and a new set of ligase and buffer were purchased.

4.3 Analysis of introduction of the cloned DNA in host organism

Agrobacterium transformation was performed with the construct pBA002 + *B'φ-FLAG* (refer to figure 3.6 of the result section). Colony PCR was performed the next day to find positive transformants of *Agrocacterium*. The PCR products were separated by gel electrophoresis, analyzed by ChemiDoc^{MP}. In this experiment, low intensity UV light was used to observe the bands. Three bands were observed in the range of about 2000 base pairs (refer to figure 3.11 of the result section). It was decided to proceed with the transformed *Agrocacterium* because the bands in the gel matched the sizes reported in the NCBI. *Agrobacterium* were spread on three dishes with LB-agar containing spectinomycin and kanamycin. 1) *Agrobacterium* without pBA002. 2) *Agrobacterium* with pBA002 without the insert. 3) *Agrobacterium* with the vector construct pBA002 + *B'φ-FLAG.* Petri dishes with transformed bacterial cells were screened after 48 hours of incubation at 28°C. No colonies were observed in the petri dish with *Agrobacterium* without the pBA002. Colonies were observed in the petri dishes with *Agrobacterium* with the plasmid - insert and *Agrobacterium* with the plasmid + insert.
It was expected that bacteria that survived and grew on the medium had resistance to spectinomycin and kanamycin. Several colonies were picked (*Agrobacterium* + pBA002 + *B'φ-FLAG*) and colony PCR was performed to confirm the presence of the vector construct in the bacteria. The PCR products were separated by gel electrophoresis, analyzed by ChemiDoc^{MP}. Five bands were observed in the range of about 2000 base pairs. All the colonies that were checked were posetive for *Agrobacterium* with the construct pBA002 + *B'φ-FLAG* (refer to figure 3.12 of the result section).

Floral dipping was performed with the transformed *Agrobacterium* and *Arabidopsis.* Two *Arabidopsis* plants were emersed in a buffer solution with *Agrobacterium* with pBA002 + *B'φ-FLAG*. Two other *Arabidopsis* plants were immersed in a buffer with *Agrobacterium* with pBA002. The flowers were submerged into the bacterial solution for 20 minutes. After this, the plants were covered with a plastic bag and they were laid sideways in a plant tray. Next day, the plastic bags were removed and the plants were transfered to a growth chamber (refer to figure 3.15 of the result section). Surface sterilized seeds harvested from the *Arabidopsis* transformed plants were screened on selective media. This was done on agar plates containing 1/2 MS medium. For selection of positive *Arabidopsis* transformants BASTA was added to the media. The seeds were sprinkled onto the agar plates and were left to stratify for 3 days at

4°C in the dark. Plates were after stratification transferred to 16 hours light. All plates were screened after approximately 10 days. It was assumed that if the trangenic *Arabidopsis* would survive on the medium with BASTA, the plants would contain the vector construct with *B'φ-FLAG,* and they would be observed as darker green seedlings with 4-6 leaves, including cotyledons (refer to figure 3.16 of the result section). The transgenic *Arabidopsis* plants were transferred to soil and grown at 12 hours light to get seeds. After a few weeks, a difference in size was observed between the transgenic plants and wildtype *Arabidopsis* (refer to figure 3.18 of the result section). The transgenic plants were significantly larger in size. The leaves of the wildtype *Arabidopsis* had been eaten by insects while the transgenic had not been eaten. In further research it can be investigated whether the transgenic *Arabidopsis* (with *B'φ*) develops better resistance to the external environment compared to wildtype *Arabidopsis*. Phire Plant Direct PCR kit was performed to identify positive transformants of *Arabidopsis*. The PCR products were separated by gel electrophoresis and analyzed by ChemiDoc^{MP}. Two bands were observed in the range of about 2000 base pairs (refer to figure 3.23 of the result section). The bands in the gel matched the sizes reported in NCBI and it was assumed that the transfer of *B'φ-FLAG* from a tomato plant to an *Arabidopsis* was successful. Further experiments can provide information about the location of the protein in the cell, and interaction partners and subsequent function in the future. Experiments such as immunoblotting and immunoprecipitation experiments can be used for this purpose. The precipitate should contain interacting proteins, and then one can compare the interacting proteins identified in *Arabidopsis* and tomato.

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6 Appendix

6.1 Multiple Sequence Alignment of *B'φFLAG*

B'φFLAG

>XM_004231407.1 PREDICTED: Solanum lycopersicum

ATGACAAATTTTCTTGATTCTGAGACAGAGGAAATGCTTTCTGTAATATCTTACTGTTCTTTCACTGATC
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>BphiFLAG

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>BphiFLAG_pba rev

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>BphiFLAG _pba rev_complement

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FORWARD

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CLUSTAL O(1.2.4) multiple sequence alignment


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REVERSE COMPLEMENT

CLUSTAL O(1.2.4) multiple sequence alignment

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BphiFLAGrev_complement      ------------------------------------------------------------ 0

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XM_004231407.1              ----------      1212
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6.2 Multiple Sequence Alignment of *FLAGB'φ*

FLAG-B'φ

>XM_004231407.1 PREDICTED: Solanum lycopersicum

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FORWARD

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CLUSTAL O(1.2.4) multiple sequence alignment


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XM_004231407.1      ------------------------------------------------------------ 0
FLAGBPHI2_F         ATTTCATTTGGAGAGAACACGGGGGACTCTAGAGGATCTCGAGATGGACTACAAAGACGA 120


XM_004231407.1      ----------ATGACAAATTTTCTTGATTCTGAGACAGAGGAAATGCTTTCTGTAATATC 50
FLAGBPHI2_F         TGACGACAAGATGACAAATTTTCTTGATTCTGATACAGAGGAAATGCTTTCTCTAATATC 180
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XM_004231407.1      TTACTGTTCTTTCACTGATCCACAAGAATCCCCTTCACTACAAGATTTAAAGAGGCTAAA 110
FLAGBPHI2_F         TTACTGTTCTTTCACTGATCCACAAGAATCCCCTTCACTACAAGATTTAAAGAGGCTAAA 240
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XM_004231407.1      ACTCATCCAACTCCTTTCCATCATCAAGACTCTCATTAGACCACTTGATGATCAAGTACT 170
FLAGBPHI2_F         ACTCATCCAACTCCTTTCCATCATCAAGACTCTCATTAGACCACTTGATGATCAAGTACT 300
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XM_004231407.1      GTCACCCCTTTTCATAATGTTGTCATCTAATCTTTTTAGGCCTCTCCCTCCACCAATTCA 230
FLAGBPHI2_F         GTCACCCCTTTTCATAATGTTGTCATCTAATCTTTTTAGGCCTCTCCCTCCACCAATTCA 360
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XM_004231407.1      TTCTGCCGTCTCAGTATTACTGGATGACGATGATCTTATCAGCAATCCAACACCCTCCTG 290
FLAGBPHI2_F         TTCTGCCGTCTCAGTATTACTGGATGACGATGATCTTATCAGCAATCCAACACCCTCCTG 420
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XM_004231407.1      GCCACATTTGCAAATAGTTTACGACATTTTCCTCAGGATTGTCAGTAGAACAAGTGTTGA 350
FLAGBPHI2_F         GCCACATTTGCAAATAGTTTACGACATTTTCCTCAGGATTGTCAGTAGAACAAGTGTTGA 480
                    ************************************************************

XM_004231407.1      ATCGCTTCGTATCTACATAGACCATGCTTTCCTCCTTAGTCTCCTCACGTTGTTCCAATC 410
FLAGBPHI2_F         ATCGCTTCGTATCTACATAGACCATGCTTTCCTCCTTAGTCTCCTCACGTTGTTCCAATC 540
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XM_004231407.1      TGAAGATCAAAGAGAACGCGACAACTTAAAGAATGTGTTCCACAGAATCTATTCAAAGTT 470
FLAGBPHI2_F         TGAAGATCAAAGAGAACGCGACAACTTAAAGAATGTGTTCCACAGAATCTATTCAAAGTT 600
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XM_004231407.1      AACATTCTACAGACCATTCATGAGAAAGACTATGCATGATGTATTCTTGCACTATGTTTT 530
FLAGBPHI2_F         AACATTCTACAGACCATTCATGAGAAAGACTATGCATGATGTATTCTTGCACTATGTTTT 660
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XM_004231407.1      CGAGACTGATCAAAGGCACCCTGGAATCGGAGAGCTTCTTGAAATATGGGGCACAATTAT 590
FLAGBPHI2_F         CGAGACTGATCAAAGGCACCCTGGAATCGGAGAGCTTCTTGAAATATGGGGCACAATTAT 720
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XM_004231407.1      AAATGGATTTAGTGTTCCTTTGAAAGAAGAACACAAGTTTTTCTTGAATAGAGTTCTTGT 650
FLAGBPHI2_F         AAATGGATTTAGTGTTCCTTTGAAAGAAGAACACAAGTTTTTCTTGAATAGAGTTCTTGT 780
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XM_004231407.1      CCCTTTGCATAAACCAAAAGGGATGCAAGTTTATCATAGGCAGTTGACTTATTGTGTATC 710
FLAGBPHI2_F         CCCTTTGCATAAACCAAAAGGGATGCAAGTTTATCATAGGCAGTTGACTTATTGTGTATC 840
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XM_004231407.1      TCAGTTTGTGCAAAAAGAGCCTGAGCTTGGTGAGGTTGTTATAAGAGGCATATTGAAGTA 770
FLAGBPHI2_F         TCAGTTTGTGCAAAAAGAGCCTGAGCTTGGTGAGGTTGTTATAAGAGGCATATTGAAGTA 900
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XM_004231407.1      CTGGCCAATTACCAATTGCCAGAAGGAAGTTCTGTTTATCGGTGAATTGGAAGAACTTGT 830
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XM_004231407.1      GGAGACTGTTGATCCACAATTGTACAAGGAACTTGCCCTGCCTTTGTGCACCAAAATTAC 890
FLAGBPHI2_F         GGAGACTGTTGATCCACAATTGTACAAGGAACTTGCCCTGCCTTTGTGCACCAAAATTAC 1020
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XM_004231407.1      CAAGTGTTTAAACAGTTGGAACTCGCAGGTTGCTGAACGCGCATTGTATGTGTGGAACAA 950
FLAGBPHI2_F         CAAGTGTTTAAACAGTTGGAACTCGCAGGTAAATTACTGTTCTTTGGACTTGGCGTACTG 1080
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XM_004231407.1      TGAGCAATTCTGGAAGATGT-----TATCACAAGCAATGGAAGAAGTCTTTCCAGTTCTA 1005
FLAGBPHI2_F         TTCAAGGTTAACCATTAGAAAAATTGTTCATCTTCAATTTTCAATGTTTATTAATAATAT 1140
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XM_004231407.1      GTGGAAGGGATGGAGAAGAACTTGCAAGGACATTGGA-GCAAAAGTGTTAAGGAATTGAC 1064
FLAGBPHI2_F         TTGCAAATGGG-------TGATTTCATGATGATTTTAAGTAGTACGATTGNWTAA----- 1188
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XM_004231407.1      GGAGAATGTGAAGGGAATGCTGGAAGCTCTAGCACCATTTCTCTATTCCAAGTGCCTTCT 1124
FLAGBPHI2_F         ----------CATCCWWTGACGAAGCCATTTACTCCATTGATGAAGRA------------ 1226
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XM_004231407.1      ACAGCTTGAAATCCAAGAAGCCAGTGAACGCATAGAAGAGATGAGAAGGAAAGAAATTTG 1184
FLAGBPHI2_F         ------------------------------------------------------------ 1226


XM_004231407.1      GGAAAAAATTGAAAATGCAGCAATGTGA    1212
FLAGBPHI2_F         ----------------------------    1226
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RERERSE COMPLEMENT

CLUSTAL O(1.2.4) multiple sequence alignment

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XM_004231407.1      ------------------------------------------------------------ 0
FLAGBPHI2_F         ATTTCATTTGGAGAGAACACGGGGGACTCTAGAGGATCTCGAGATGGACTACAAAGACGA 120


XM_004231407.1      ----------ATGACAAATTTTCTTGATTCTGAGACAGAGGAAATGCTTTCTGTAATATC 50
FLAGBPHI2_F         TGACGACAAGATGACAAATTTTCTTGATTCTGATACAGAGGAAATGCTTTCTCTAATATC 180
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XM_004231407.1      TTACTGTTCTTTCACTGATCCACAAGAATCCCCTTCACTACAAGATTTAAAGAGGCTAAA 110
FLAGBPHI2_F         TTACTGTTCTTTCACTGATCCACAAGAATCCCCTTCACTACAAGATTTAAAGAGGCTAAA 240
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XM_004231407.1      ACTCATCCAACTCCTTTCCATCATCAAGACTCTCATTAGACCACTTGATGATCAAGTACT 170
FLAGBPHI2_F         ACTCATCCAACTCCTTTCCATCATCAAGACTCTCATTAGACCACTTGATGATCAAGTACT 300
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XM_004231407.1      GTCACCCCTTTTCATAATGTTGTCATCTAATCTTTTTAGGCCTCTCCCTCCACCAATTCA 230
FLAGBPHI2_F         GTCACCCCTTTTCATAATGTTGTCATCTAATCTTTTTAGGCCTCTCCCTCCACCAATTCA 360
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XM_004231407.1      TTCTGCCGTCTCAGTATTACTGGATGACGATGATCTTATCAGCAATCCAACACCCTCCTG 290
FLAGBPHI2_F         TTCTGCCGTCTCAGTATTACTGGATGACGATGATCTTATCAGCAATCCAACACCCTCCTG 420
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XM_004231407.1      GCCACATTTGCAAATAGTTTACGACATTTTCCTCAGGATTGTCAGTAGAACAAGTGTTGA 350
FLAGBPHI2_F         GCCACATTTGCAAATAGTTTACGACATTTTCCTCAGGATTGTCAGTAGAACAAGTGTTGA 480
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XM_004231407.1      ATCGCTTCGTATCTACATAGACCATGCTTTCCTCCTTAGTCTCCTCACGTTGTTCCAATC 410
FLAGBPHI2_F         ATCGCTTCGTATCTACATAGACCATGCTTTCCTCCTTAGTCTCCTCACGTTGTTCCAATC 540
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XM_004231407.1      TGAAGATCAAAGAGAACGCGACAACTTAAAGAATGTGTTCCACAGAATCTATTCAAAGTT 470
FLAGBPHI2_F         TGAAGATCAAAGAGAACGCGACAACTTAAAGAATGTGTTCCACAGAATCTATTCAAAGTT 600
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XM_004231407.1      AACATTCTACAGACCATTCATGAGAAAGACTATGCATGATGTATTCTTGCACTATGTTTT 530
FLAGBPHI2_F         AACATTCTACAGACCATTCATGAGAAAGACTATGCATGATGTATTCTTGCACTATGTTTT 660
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XM_004231407.1      CGAGACTGATCAAAGGCACCCTGGAATCGGAGAGCTTCTTGAAATATGGGGCACAATTAT 590
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XM_004231407.1      AAATGGATTTAGTGTTCCTTTGAAAGAAGAACACAAGTTTTTCTTGAATAGAGTTCTTGT 650
FLAGBPHI2_F         AAATGGATTTAGTGTTCCTTTGAAAGAAGAACACAAGTTTTTCTTGAATAGAGTTCTTGT 780
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XM_004231407.1      CCCTTTGCATAAACCAAAAGGGATGCAAGTTTATCATAGGCAGTTGACTTATTGTGTATC 710
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XM_004231407.1      TCAGTTTGTGCAAAAAGAGCCTGAGCTTGGTGAGGTTGTTATAAGAGGCATATTGAAGTA 770
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XM_004231407.1      CTGGCCAATTACCAATTGCCAGAAGGAAGTTCTGTTTATCGGTGAATTGGAAGAACTTGT 830
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XM_004231407.1      CAAGTGTTTAAACAGTTGGAACTCGCAGGTTGCTGAACGCGCATTGTATGTGTGGAACAA 950
FLAGBPHI2_F         CAAGTGTTTAAACAGTTGGAACTCGCAGGTAAATTACTGTTCTTTGGACTTGGCGTACTG 1080
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XM_004231407.1      TGAGCAATTCTGGAAGATGT-----TATCACAAGCAATGGAAGAAGTCTTTCCAGTTCTA 1005
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                    *      **    *  *          ***    ****     * ** * *  *

XM_004231407.1      GTGGAAGGGATGGAGAAGAACTTGCAAGGACATTGGA-GCAAAAGTGTTAAGGAATTGAC 1064
FLAGBPHI2_F         TTGCAAATGGG-------TGATTTCATGATGATTTTAAGTAGTACGATTGNWTAA----- 1188
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XM_004231407.1      GGAGAATGTGAAGGGAATGCTGGAAGCTCTAGCACCATTTCTCTATTCCAAGTGCCTTCT 1124
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                               *     **  * *  *  *  * *****  *  *

XM_004231407.1      ACAGCTTGAAATCCAAGAAGCCAGTGAACGCATAGAAGAGATGAGAAGGAAAGAAATTTG 1184
FLAGBPHI2_F         ------------------------------------------------------------ 1226


XM_004231407.1      GGAAAAAATTGAAAATGCAGCAATGTGA    1212
FLAGBPHI2_F         ----------------------------    1226
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