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Abstract

This project will aim to unveil the subcellular localization of PP4-2 and two of its believed regulators; PP4R2L and PP4R3L/PSY2L. To achieve this, two fusion-proteins were prepared from each of the genes to be examined. This was done by using molecular cloning. One fusion-protein was designed to carry the EYFP tag on N-terminus and the other fusion-protein carried the EYFP tag on the C-terminus. Molecular cloning was not successful for the PSY2L gene, possibly due to its large size. The two PP4-2 fusion-proteins; PP4-2-EYFP and EYFP-PP4-2, appear to be cytosolic with clusters of protein aggregation and the two PP4R2L fusion-proteins also displayed cytosolic localization. No aggregation was observed for the PP4R2L fusion proteins.

This thesis also wishes to examine the effect of artificial micro RNA (amiRNA) on PP4-2. Two different amiRNAs were used each with two different vectors; the inducible pER10 vector and the constitutive pBA002 vector. The goal was to do expression studies, and observe the phenotypes of the different mutant plants. Expression studies were not done due to a lack of time. No significant difference in phenotype was observed for the different mutant plants. They did however display slower growth rate than that of the wild type.

Finally, one more study was performed to observe the effect of disrupting the PSY2L gene and the PP4R2L gene. This was done by studying plants with a T-DNA insert at specific locations in regards to the gene (see table 1), then observing the resulting phenotype and studying expression of the disrupted gene. For the PSY2L gene, mutants with T-DNA inserts at two different locations were used (one with the insert in exon 3, and one with the insert downstream of the gene) and T-DNA insert at only one location was performed for the PP4R2L gene (insert in exon 7). Expression study was to be preformed on homozygous individuals. At the time of the expression study, homozygous plants were found solely for the PSY2L Salk 125872 mutant. The expression study was performed on a homozygous PSY2L Salk 125872 mutant as well as one heterozygous PSY2L Salk 048064 mutant. The homozygous PSY2L Salk 125872 mutant displayed reduced expression when compared to the wild type. The heterozygous PSY2L Salk 048064 mutant displayed an expression level close to that of the wild type. At a later time, after the expression study had taken place, homozygous individuals for the PP4R2L Salk 093041 mutants were also found. No expression analysis was done on the PP4R2L Salk 093041 mutants, due to time restrictions.

All mutant plants (T-DNA mutants and amiRNA mutants) displayed a reduced growth rate, as well as being shorter and bearing fewer stems than what is commonly observed for the wild type. The time taken for them to produce seeds was also about one month longer than what is observed for the wild type.

List of abbreviations.

amiRNA	artificial micro RNA
Amp	Ampicillin
Bp	base pairs
DNA	Deoxyribonucleic acid
EYFP	Enhanced yellow fluorescent protein
GFP	Green fluorescent protein
Kan	Kanamycin
KAP1	KRAB associated protein 1
KRAB	Krüppel associated box
LB	Luria-Bertani
MS	Murashige and Skoog
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PP2A	Protein phosphatase 2 A
PP2AC	Catalytic subunit of PP2A
PP4/PPX/ppp4	Phosphoprotein phosphatase 4
PP4C	Catalytic subunit of PP4
PP4R2L	PP4 regulator 2-like
PPM	Metal-ion-dependent
PPP	phosphoprotein phosphatse
PSY2L/PP4R3L	PP4 Regulator 3-like
RNA	Ribonucleic acid
RPA2	Replication protein A2
T-DNA	Transfer DNA
YFP	Yellow fluorescent protein

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1 INTRODUCTION

Protein phosphatases are responsible for a myriad of cellular processes. Studying these processes can give useful information about the workings of a biological system. Not much is known about the phosphoprotein phosphatase 4 (PP4 also referred to as PPX or ppp4) in plants. This thesis will try to shed some light on the workings of PP4-2 and two of its putative regulators, namely PP4R2L and PSY2L in *Arabidopsis thaliana*.

When studying a protein of which not much is known, finding the localization of said protein can be a good place to start. Knowing where in the cell the protein is present gives a good indication as to where it acts and what processes it can be involved in. Finding a protein's subcellular localization can be achieved using microscopy. Proteins are usually not visible in a microscope, so the protein to be studied can be visualized by tagging it with a fluorescent tag, often in the form of a protein tag such as green fluorescent protein (GFP) or yellow fluorescent protein (YFP). The protein to be studied is tagged using enhanced yellow fluorescent protein (EYFP) with a technique called molecular cloning, followed by visualization of the fusion-protein using confocal microscopy. Molecular cloning is a technique that aims to introduce recombinant DNA to a host organism, to produce large amounts of the recombinant DNA. *E. coli* is often used as a host organism. The recombinant DNA is produced by amplification of the gene to be studied, followed by digestion of both the gene and the vector to be used by corresponding restriction enzymes. Ligation of the gene into the vector is then preformed, and the host cells are transformed using the vector with the gene in place. Large amounts of the recombinant DNA can then be procured by cultivation of the successfully transformed bacterial colonies. To make sure the colonies are in possession of the recombinant DNA, screening is done with a corresponding screening agent. Most vectors are in possession of a gene for resistance to a certain antibiotic. Spreading the bacterial colonies out on plates containing a growth medium with the antibiotic will result in survival of only transformed bacterial cells. After successful molecular cloning, the gene product can be used for further studies, such as microscopy to check the subcellular localization of the protein.

MicroRNA's are non coding RNA segments acting as post transcriptional regulators of gene expression. This is achieved by gene silencing either by the suppression of mRNA translation or by degrading of the mRNA molecule. MicroRNA's are short, single stranded molecules and their target site is located at the 3´ UTR of the target mRNA. In theory, the production of artificial microRNA's (amiRNA) designed to act upon PP4-2 will give a basis for further study of the role of PP4-2 in plants, by giving information about what happens to the plant in the protein's absence. To achieve this, amiRNA is introduced to the plants by use of a plasmid. For this experiment two different plasmids were used, one constitutive plasmid (continuous production of the amiRNA) and one inducible plasmid (amiRNA production induced by estradiol). The constitutive plasmid; pBA002 and the inducible plasmid; pER10 were introduced to the plants by agrobacterium. Screening of the transformed plants was achieved by utilizing the plasmids resistance to antibiotics/herbicides. The pER10 vector contains a segment encoding kanamycin resistance whereas the pBA002 vector has resistance to the herbicide, BASTA. By sowing seeds on plates with nutrient medium containing the antibiotic or herbicide, only plants containing the vector will survive. These plants can then be used for genotyping and expression studies of the PP4-2 protein. Due to the presence of plants with an inducible amiRNA production, any differences in the phenotype with varying degrees of amiRNA production can be studied.

When studying a protein whose role is not yet fully understood, studying what it takes to disrupt the protein in question can give useful information about the workings of the protein. This can be achieved by studying the genetic expression and phenotype of mutant plants whose DNA has been altered to contain a non-coding T-DNA insert at specific locations. The gene to be studied can thus be disrupted by the T-DNA if it is placed at locations that interfere with the complete expression of the gene. To be able to say something about the expression and importance of the gene to be studied, homozygous plants should be procured for further studies. Finding both homozygous and heterozygous plants is ideal, to compare the two and check for any noticeable differences. For heterozygous plants with only one allele for the disrupted gene, the "healthy" allele might make up for the disrupted one, leading to a phenotype and expression level close to that observed for the wild type. Genotyping the plants will lead to mapping out which individuals are homozygous and which are not. This can be followed by observations on phenotype and studying the genetic expression of the gene in question. The T-DNA is as mentioned a non-coding sequence, inserted into the sequence for the genes to be studied or in close proximity to it. This is done to be able to draw any conclusions as to the importance of the sequence being intact for the gene being studied.

1.1 Protein Phosphatases

Protein phosphatases are enzymes responsible for the dephosphorylation of a peptide substrate. Kinases are a group of enzymes that have the antagonistic effect of the phosphatases, namely phosphorylation. In general it is believed that the kinases acting upon a protein will turn said protein on, in essence, altering the proteins activity and making it more active. Protein phosphatases, having the opposite effect, can decrease the activity of a protein. This way a cell can carefully control the activity of its proteins and regulate its cellular processes to a high degree. Protein phosphatases and kinases are regulators of protein activity that act on a post-translational level. The protein phosphatases are grouped together based on what substrate they dephosphorylate. The protein phosphatases can be grouped as follows; Tyrosine-specific phosphatases (Zhang 2002). Serine/threonine –specific phosphatases (Mumby and Walter 1993), Dual specificity phosphatases (Camps, Nichols et al. 2000) and Histidine phosphatases (Kowluru, Klumpp et al. 2011).

1.1.1 Ser/Trh phosphatases

The serine/threonine phosphatases in plants will act upon serine and threonine residues and the family can further be divided into two groups; the Plant Ser/Thr phosphoprotein phosphatases (plant PPP family) (Farkas, Dombradi et al. 2007) and the Metal-ion-dependent protein phosphatases (PPM family) (Barford, Das et al. 1998)
PP4 belongs to the PPP family. Proteins from both the PPP and PPM family will dehposhporylate serine or threonine amino acid residues.
It is within the plant PPP family that the major plant phosphatases are found. The proteins of this family are reported to play a part in regulation of the target rapamycin pathway, the auxin and brassinosteroid signalling, in phototropism as well as the cell stress response activity. Understanding the roles of these proteins is laborious work, and only recently has the focus moved over from the widely studied kinases to the phosphatases. The function of the PPP phosphatases is now described as being equally regulated and varied as their kinase counterparts. (Uhrig, Labandera et al. 2013)

The PPP family enzymes in humans are involved in cellular processes connected to conditions such as diabetes, cancer and Alzheimer's disease. These cellular processes include: DNA replication, apoptosis, gene expression, glycogen metabolism, synaptic transmission and morphogenesis, and they are reported to be regulated by enzymes from the PPP family (Pereira, Vasconcelos et al. 2011).

1.1.2 PP4/PPX

PP4 is sometimes also referred to as PPX or ppp4. Not much is known about the function or localization of PP4 in plants, most research available on PP4 has been done on mammalian cells. It has been reported that in mammalian cells, PP4 is localized at the centrosomes during mitosis, the nucleus and somewhat in the cytoplasm (Sumiyoshi, Sugimoto et al. 2002; Hastie, Carnegie et al. 2000; Helps, Brewis et al. 1998). It is indicated that the phosphatase might play a role in microtubule organization. The mammalian PP4 is reported to display 65% identity to PP2A (Brewis, Street et al. 1993).
PP4 has been reported to be involved in processes that facilitate DNA repair by dephosphorylation of RPA2 in eukaryotic cells (Lee, Pan et al. 2010). The enzyme has also been reported to act upon γH2AX, a histone and KAP-1, a regulator of chromatin structure, both of which are involved in repair of DNA (Nakada, Chen et al. 2008; Lee, Goodarzi et al. 2012; Iyengar and Farnham 2011). It is indicated that PP4 along with PP2A could have a regulatory effect upon hedgehog signalling, a signalling pathway associated with the development of embryos and with maintenance of tissue (Jia, Liu et al. 2009).
Overexpression of the catalytic subunit of PP4; PP4C, has been found samples taken from patients suffering from Pancreatic ductal adenocarcinoma (PDAC) in amounts that are regarded to be higher than that of healthy individuals (Weng, Wang et al. 2012).
There is also reported to be overexpression of the catalytic subunit of PP4 (PP4C) in breast and lung tumor tissue taken from humans (Wang, Zhao et al. 2008).

1.1.3 PP4 regulators

PP4 is active in many processes, and regulation of PP4 is needed in the cell to maintain control of its activity. Two main putative regulators of PP4-2 has been identified in *Arabidopsis thaliana* by in silico analysis; PP4R2L and PP4R3L/PSY2L.
PP4 in mammals has been shown to be inhibited by the antitumor drus by the name of fostriecin and cantharidin. Fostriecin has also been shown to inhibit the catalytic subunit of PP2A (Hastie and Cohen 1998). Okadaic acid as well as microcystin also has inhibitory effects on PP4. This effect on PP4 is not the same as PP2AC, although the effect is similar (Brewis, Street et al. 1993).

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Material

Arabidopsis thaliana is a commonly used model organism for laboratory work with plants. For this study, two types of plant mutants were used; t-DNA plants and amiRNA plants. All work was done on plants of the species *Arabidopsis thaliana*. Plants sown on soil were watered using 1x Hoagland solution (Tables 31 and 32).

2.1.1.1 T-DNA plants

Mature plant material (*Arabidopsis thaliana*) used for this study was received from a former employee Zek Ginbot. The genes to be examined in this study were the PSY2L gene and the PP4R2L gene. Two Salk lines were used for the PSY2L gene, and one for the PP4R2L gene (Table 1).
Mature plants already available in the lab were to be tested to find homozygous individuals. Identification of homozygous individuals was achieved by PCR followed by agarose electrophoresis.

Table 1. Overview of T-DNA insert in the plant material

Name	Location	Salk line	T-DNA location
PSY2L	At3g06670	Salk 048064	Insert in exon 3 of 25, segregating
PSY2L	At3g06670	Salk 125872	Flank-tagged downstream of translation, segregating
PP4R2	At5g17070	Salk 093041	Insert in exon 7 of 8, segregating

A more detailed overview is made available in the appendix (A1 and A2).

2.1.1.2 MicroRNA plants

Mutant plants (*Arabidopsis thaliana)* transformed using either the inducible pER10 vector or the constitutive pBA002 vector carrying artificial micro RNA (amiRNA) were already available in the lab when this study started**.** These plants had been treated using agrobacterium by former employee Zek Ginbot**.** Two different amiRNAs were introduced to the plants with the following sequence:
amiRNA1: TAATGAGAGTTATACGGTCTA
amiRNA2: TTAAAAGACGTAACAACGCTG
Both microRNAs are designed to target PP4-2 as well as PP4-1.
Two plants were used for each amiRNA, giving rise to the following classification: RNA 1-1 being amiRNA 1-plant 1, Rna 1-2 being amiRNA 1-plant 2, RNA 2-1 being amiRNA 2-plant 1, and RNA 2-2 being amiRNA 2-plant 2. Plants transformed with only the vector with no amiRNA present were also available for this study (PBA002 only and pER10 only).

2.1.2 Vectors

The following vectors were used for molecular cloning: pCAT-EYFP, pCAT-DECR-EYFP and pWEN25. Both pCAT-EYFP and pCAT-DECR-EYFP vectors share the same nucleotide sequence with the exception that pCAT-EYFP will ensure that the resulting fusion-protein will carry the EYFP tag on the N–Terminus, whereas the pCAT-DECR-EYFP will give the resulting fusion-protein an EYFP tag at the C–terminus (see figure 1 for a vector map of the pCAT-EYFP vector).

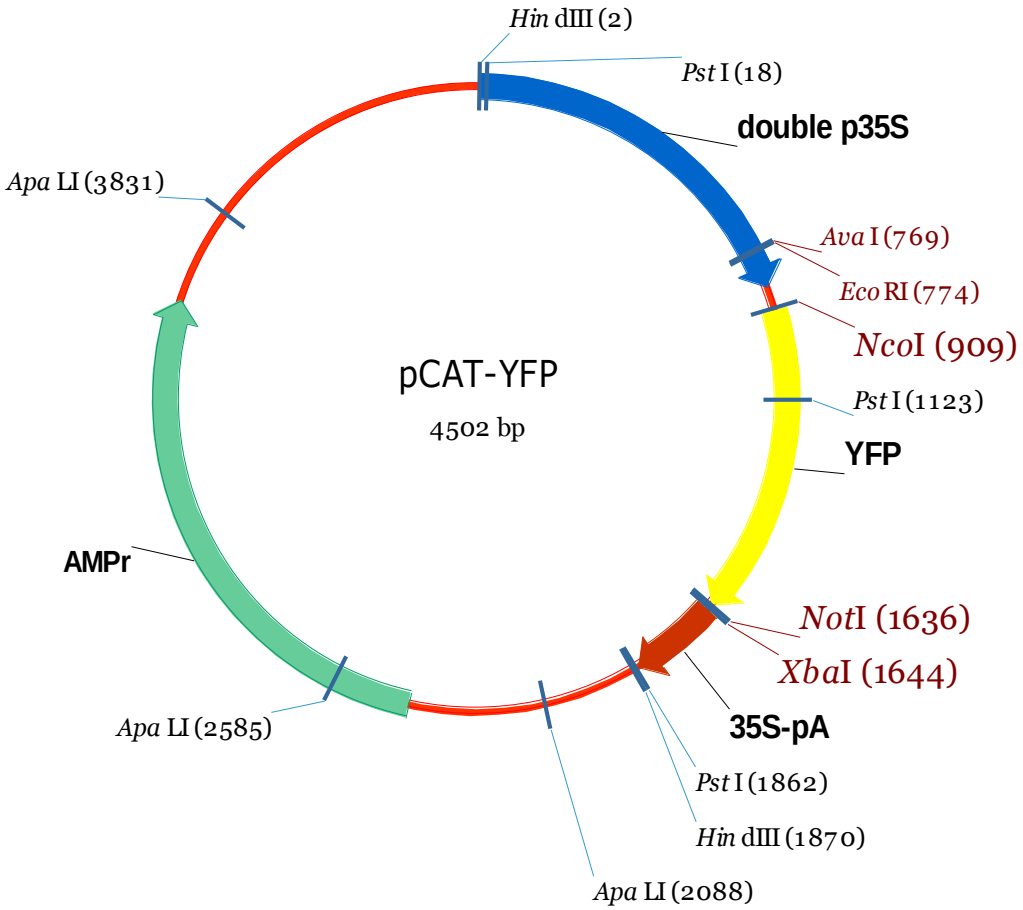

Figure 1. Vector map of pCAT-EYFP. The vector was used for molecular cloning and the vector map was designed by Dr. Amr Kataya (Ma, Haslbeck et al.2006).

A vecotor map of the pWEN25 vector is shown in figure 2.

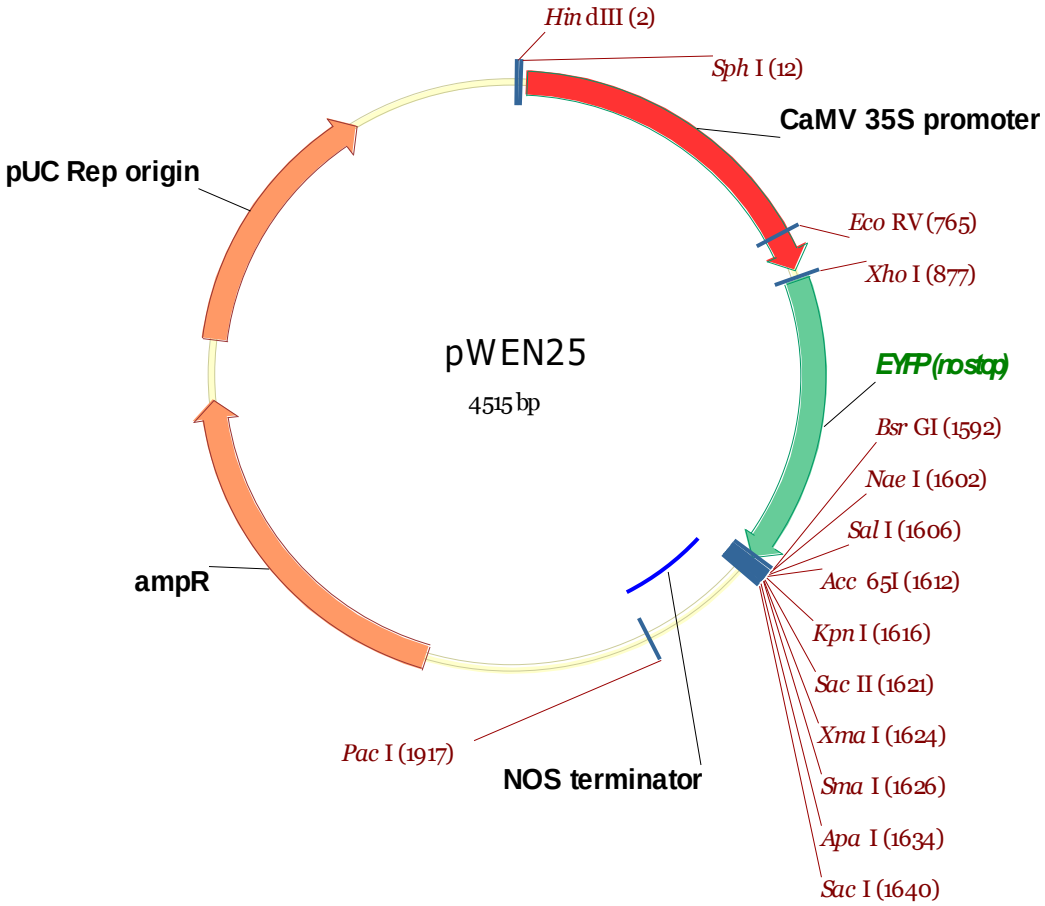

Figure 2. Vector map of the pWEN25 vector used for molecular cloning. The vector map for pWEN25 was designed by Dr. Amr Kataya (Matre, Meyer et al. 2009).

When studying the effect of amiRNA on plants, the following vectors were used to introduce the amiRNA to the plants: pER10 and pBA002. Figures 3 and 4 shows vector maps of pER10 and pBA002, respectively.

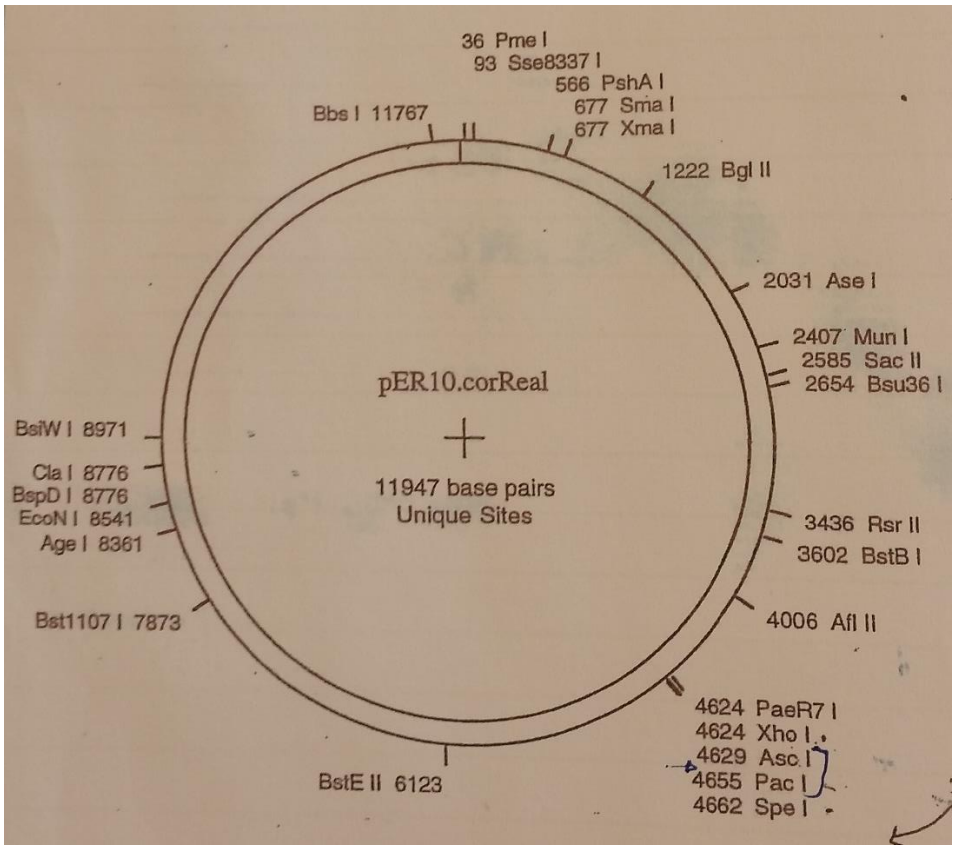

Figure 3. Vector map of pER10 vector (Moller and Chua 2002). The pER10 vector contains resistance against the antibiotic, Kanamycin. It is an inducible vector for the production of amiRNA.

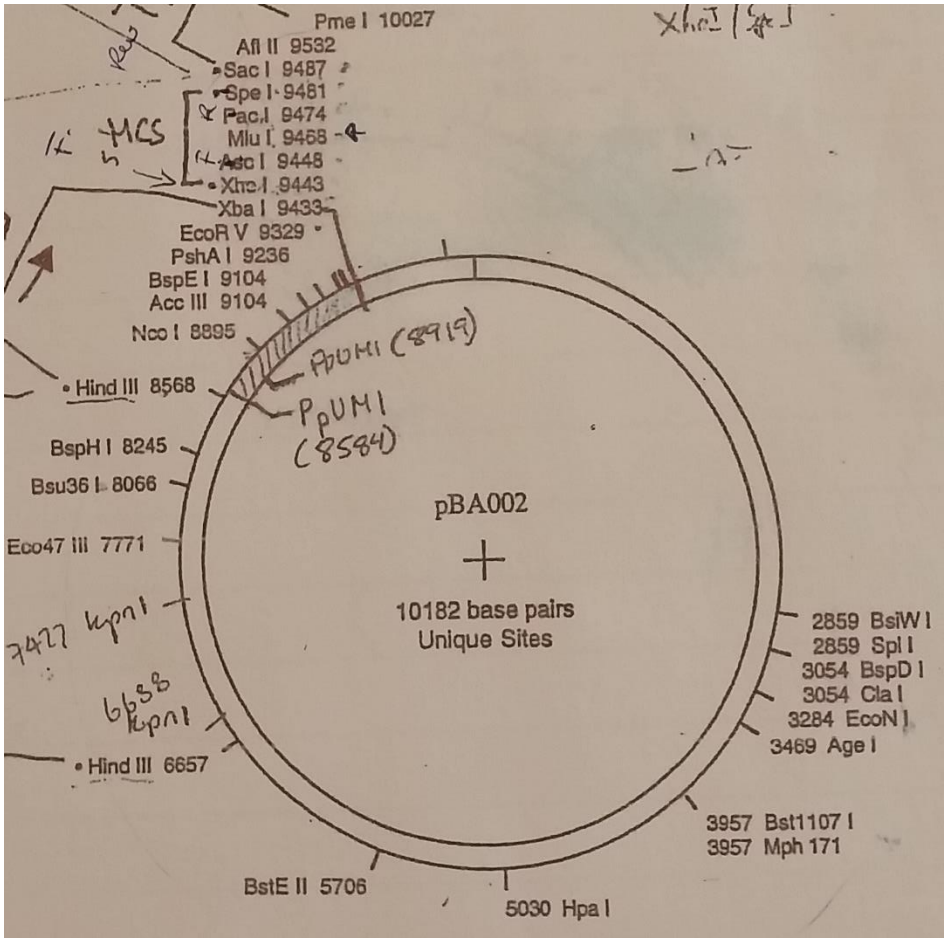

Figure 4. Vector map of pBA002 (Moller, Kim et al. 2003). The pBA002 vector contains resistance against the herbicide BASTA. It is a constitutive vector for the amiRNA.

2.1.3 Bacterial cells

For the molecular cloning, bacterial cells were used and transformed. The bacterial cells used for this study were competent *Escherichia coli JM109* from Promega.

2.1.4 Kits

Kits are made available for easy and rapid reactions and processes. For this study, PCR cleanup kit was used to isolate DNA after PCR and after digestion. This was done to remove any reagents from the PCR/digestion mixture. When isolating amplified DNA from a PCR reaction where the PCR product was run on gel (high fidelity PCR), a kit for gel extraction was used. This kit ensured that all gel was removed from the sample so that the purified DNA could be used for cloning. Plasmid isolation performed to isolate plasmids from transformed bacterial cells was also done using a kit, namely plasmid miniprep kit. Genotyping of plants was performed using the Phire ® Plant Direct PCR Kit from New England Biolabs.
The RNeasy kit from QIAGEN was used when isolating RNA to be used for the expression study for T-DNA plants.

2.1.5 Chemical list

Table 2 gives an overview of the enzymes and buffers used during this thesis.

Table 2. Overview of all chemicals, enzymes and buffers used

Reagent	Classification	Supplier
BSA, Purified BSA 100x (10 mg/ml)	Protein	New England Biolabs
NcoI	Restriction enzyme	New England Biolabs
NotI	Restriction enzyme	New England Biolabs
SacII	Restriction enzyme	New England Biolabs
KpnI	Restriction enzyme	New England Biolabs
SalI	Restriction enzyme	New England Biolabs
Taq polymerase(Aug-Dec)	Enzyme	Invitrogen
Taq polymerase(Jan-Apr)	Enzyme	SIGMA-ALDRICH
High fidelity polymerase	Enzyme	ROCHE
T4 DNA Ligase	Enzyme	Promega
rSap	Enzyme	New England Biolabs
NeBuffer1	Buffer	New England Biolabs
NeBuffer2	Buffer	New England Biolabs
NeBuffer3	Buffer	New England Biolabs
PCR buffer, 10X PCR rxn buffer -$MgCl_2$	Buffer	Invitrogen
High fidelity PCR buffer, Expand high fidelity ^{Plus}PCR system Reaction Buffer (5x) with $MgCl_2$	Buffer	ROCHE
T4 DNA Ligase 10X buffer	Buffer	Promega
rSap buffer	Buffer	New England Biolabs
dNTP's, 2.5 mM of each NTP, total 10 mM	Chemical	Bioline

2.2 Methods

2.2.1 PCR

The PCR is performed to amplify DNA based on a template strand. A thermocycler is utilized to make sure the sample will go through the correct amplification step at the correct time. For this study, PCR was used when genotyping T-DNA plants to find homozygous individuals, it was used to amplify genes used for molecular cloning (high fidelity PCR), and the technique was additionally used to check if any transformed bacterial colonies from the molecular cloning contained the insert in question (colony PCR).

2.2.1.1 Genotyping of individual plants to identify homozygous mutants

Genotyping was performed on individual plants of *Arabidopsis thaliana* T-DNA plants for the following Salk lines: PSY2L Salk 125872, PSY2L Salk 048064 and PP4R2L Salk 093041, T-DNA plants.
Two types of mastermix was prepared, one designed using primers that would amplify only T-DNA, the other using primers that would amplify the wild type DNA. Finding bands in both series means the plant in question is heterozygous; it being in possession of both TDNA and that of the wild type. Finding a band only in one of the two, means the plant is homozygous for that particular gene. Here, the samples using primers to detect TDNA was denoted series A, whereas the wild type detection samples was denoted series B. Plants exhibiting a band only for series A were confirmed to be homozygous for the T-DNA.
Any homozygous plants found were genotyped at least twice.

2.2.1.1.1 Primer working solution

A ten-fold dilution of the primer stock solution was used as the primer working solution, 10 µl primer stock solution was added to 90 µl dH_2O.

2.2.1.1.2 Primers used for genotyping

Table 3 gives an overview of all the primers used when genotyping the T-DNA plants.

Table 3. Overview of primers used for genotyping of T-DNA plants

Type	Salk line	Nucleotide sequence
TDNA LB LBb1.3	Used on all lines	ATTTTGCCGATTTCGGAAC
PSY2L RP	125872	AAARGAATATGGCTTTTGGGG
PSY2L LP	125872	AAGCCTCTGAGGATGAGGAAG
PSY2L RP	048064	TGTTGAATTGAGATGGAAGGG
PSY2L LP	048064	ATGTTTCGCCTGTTCAATCAC
PP4R2 RP	093041	TGTTCAACAGATCCTTTTGGC
PP4R2 LP	093041	CAACATATTTGGCATTTTGGC

2.2.1.1.3 PCR mix and PCR program used when genotyping

The PCR mix was prepared following the below table, table 4, and the PCR program used is shown in table 5.

Table 4. PCR mix used for genotyping of T-DNA plants

Reagent	**Amount (µl)**	**Final concentration**
dH_2O	3.3	-
Buffer	5	1x
Primer 1	0.5	0.5 µM
Primer 2	0.5	0.5 µM
Enzyme	0.2	-
Plant material	0.5	-
Total volume	10	NA

Table 5. PCR program used for genotyping of T-DNA plants

Step	**Temperature**	**Time**
1, Initial denaturation	98˚C	5 min
2, Denaturation*	98˚C	5 sec
3, Annealing*	60˚C	10 sec
4, Extension*	70˚C	40 sec
5, Final extension	72˚C	1 min
6, Hold	12˚C	∞

Steps 2, 3 and 4 were repeated 40 times in order.

2.2.1.2 High fidelity PCR, Amplification of constructs to be used for molecular cloning

High fidelity PCR was performed to ensure that the amplified constructs were correct in regards to the original sequence**.** The following genes were amplified using high fidelity PCR: PP4-2, PSY2L and PP4R2L.

2.2.1.2.1 Primers used for High fidelity PCR

The following primers were used when amplifying PP4-2, PP4R2L and PSY2L to be used for molecular cloning (table 6. For the nucleotide sequence of each primer, see table 7).

Table 6. Overview of primers used, High fidelity PCR for cloning

Gene	**Destination vector**	**Forward Primer**	**Revers Primer**
PP4-2	pCAT-DECR-EYFP	AK77F	AK75R
PSY2L	pCAT-EYFP	EYFPPSY2Lf	EYFPPSY2Lr
PSY2L	pCAT-DECR-EYFP	PSY2LEYFPf	PSY2LEYFPr
PP4R2L	pCAT-EYFP	EYFPPP4R2Lf	EYFPPP4R2Lr
PP4R2L	pCAT-DECR-EYFP	PP4R2LEYFPf	PP4R2LEYFPr
NA-Vector specific	pCAT-EYFP	c.term.EYFPf	AK92r
NA-Vector specific	pCAT-DECR-EYFP	AK93f	AK94r
NA-Vector specific	pWEN25	c.term.EYFPf	NOS

Table 7. Nucleotide sequence of primers used, High fidelity PCR for cloning

Primer	Nucelotide sequence
AK77F	ATCCATGGGTATGTCAGACCTAGACAAGCA
AK75R	ATGCGGCCGCTATGTCAGACCTAGACAAGCAA
EYFPPSY2Lf	AAAGCGGCCGCTTATGGGCGCTCCGGAAAAGTCT
EYFPPSY2Lr	ATTGCGGCCGCTCAGGATCCATTTACAGCCAT
PSY2LEYFPf	ATCCATGGGTATGGGCGCTCCGGAAAAGTCT
PSY2LEYFPr	ATTGCGGCCGCTCAGGATCCATTTACAGCCAT
PSY2L kpnI	AAGGTACCTCAGGATCCATTTACAGCCAT
PSY2L SalI	AAAGTCGACCCATGGGCGCTCCGGAAAAGTCT
EYFPPP4R2Lf	AAAGCGGCCGCTATGGAGAATCCGTCATCATCG
EYFPPP4R2Lr	ATTCCGCGGCTAGGCACACGTTGTAGGCAA
PP4R2LEYFPf	ATCCATGGGTATGGAGAATCCGTCATCATCG
PP4R2LEYFPr	AAGCGGCCGCGGCACACGTTGTAGGCAACCG
C.Term.EYFPf	ACTACCTGAGCTACCAGTCC
AK92r	CCTTATCTGGGAACTACTCAC
AK93f	GCATTCTACTTCTATTGCAGC
AK94r	AACTTCAGGGTCAGCTTGCCGT
NOS	GATAATCATCGCAAGACCGGCAACAGGA

2.2.1.2.2 PCR mix and PCR program used for high fidelity PCR

Table 8 shows the amount of reagents used when performing high fidelity PRC to amplify constructs used for molecular cloning.

Table 8. PCR mix used, High fidelity PCR for cloning

Reagent	Amount (µl)	Final conc.
dH_2O	30.5	-
Buffer (5x Exp.Hifi. buffer)	10	1x
dNTP	4	0.8 mM
Primer 1	2	0.16 µM
Primer 2	2	0.16 µM
Enzyme	0.5	2.5 U
Template DNA	1	5-500 ng (genomic DNA) 100 pg-10 ng (plasmid DNA)
Total volume	50	-

The above table (table 8) shows the amount of reagents used for one PCR reaction.
The concentration of template DNA was altered to ensure that 1 µl template solution was within the range of 100 pg-10 ng plasmid concentration. The PCR program used follows in table 9.

Table 9. High fidelity PCR program, cloning

Step	Temperature	Time
1, Initial denaturation	98˚C	5 min
2, Denaturation*	98˚C	5 s
3, Annealing*	60˚C	10 s
4, Extension*	70˚C	40 s
5, Final extension	72˚C	1 min
6, Hold	12˚C	∞

Steps 2, 3 and 4 were repeated 40 times in order. The resulting PCR mixture was run on agarose-gel followed by cleanup of the DNA using the gel extraction kit from SIGMA-ALDRICH.

2.2.1.3 Colony PCR for molecular cloning

Colony PCR was performed on transformed bacterial colonies using both vector-specific primers and gene-specific primers. It was discovered that using one vector-specific primer and one gene specific primer gave the best results in regards to the visualization of the PCR product. Using vector-specific primers gave rise to two clear bands, one band being the empty vector or the vector with insert, and the other band believed to be primer-dimer product.

2.2.1.3.1 Primers used for colony PCR

When screening colonies for the correct insert, both the vector specific and gene-specific primers were used. The gene-specific primers are listed in table 6, and the vector-specific primers are found below, in table 10.

Table 10. Overview of primers used to determine the presence of transformed bacterial cells

Vector specific primers	
Name	Vector
AK93f	pCAT-DECR-EYFP
AK94r	pCAT-DECR-EYFP
c.term.EYFPf	pCAT-EYFP
AK92r	pCAT-EYFP
NOS	pWEN 25
c.term.EYFPf	pWEN 25

Due to ineffective molecular cloning, colony PCR was performed throughout the entirety of this project. Acquisition of a new Taq polymerase gave rise to two different protocols being used; one protocol was followed from August through December and a new protocol was followed from January throughout April.

2.2.1.3.2 Colony PCR performed on bacterial colonies (August-December 2014)

Colony PCR performed from August through December was done with a taq polymerase from Invitrogen, following the procedure described below.

2.2.1.3.2.1 PCR mix and PCR program used for colony PCR (August-December 2014)

Tables 11 and 12 gives an overview of the PCR mix and PCR program used for colony PCR performed from August to December 2014.

Table 11. Colony PCR mastermix (August-December 2014)

Reagent	**Amount (µl)**
PCR buffer (-$MgCl_2$)	2
$MgCl_2$ (50mM)	0.6
dNTP mix	0.4
Primer 1	1
Primer 2	1
Taq polymerase	0.1
dH_2O	14.9

Masttermix was prepared using the above table and template DNA was added by transferring a tiny amount of bacteria from one bacterial colony using the tip of a pipette/ toothpick. The pipette tip/toothpick was placed tip down in the PCR tube then stirred around a bit before removing it from the PCR tube. Whether a pipette tip or a toothpick was used did not influence the PCR results.

Table 12. Colony PCR program (August-December 2014)

Step	**Temperature**	**Time**
1, Initial denaturation	94˚C	3.00 min
2, Denaturation*	94 ˚C	45 s
3, Annealing*	60 ˚C	30 s
4, Extension*	72 ˚C	2 min 30 s
5, Final extension	72 ˚C	10.00 min
6, Hold	4 ˚C	∞

*Steps 2 through 4 were repeated 35/32 times.

2.2.1.3.3 Colony PCR performed on bacterial colonies (January- April 2015)

Due to the acquisition of a new Taq polymerase from a new supplier, SIGMA-ALDRICH, the procedure for colony PCR had to be altered for any colony PCR performed after December 2014. Some optimization of the PCR program was needed before finding the preferred program.

2.2.1.3.3.1 PCR mix and PCR program used for colony PCR (January- April 2015)

Below follows table 13; an overview of the PCR mix and the optimal PCR program used after the acquisition of the new Taq polymerase.

Table 13. Colony PCR mastermix (January- April 2015)

Reagent	Amount (µl)	Final concentration
10x rxn buffer	2	1x
DNA template	(bacterial colonies)	200 pg
dNTP mix	1.6	800 µM
Primer 1	0.8	0.1 µM
Primer 2	0.8	0.1 µM
Taq polymerase	0.2	0.05 U
dH_2O	14.6	-

Mastermix was prepared using the above table and bacterial colonies were transferred using the tip of a pipette/toothpick. Table 14 gives an overview of the PCR program used.

Table 14. Colony PCR program (January- April 2015)

Step	Temperature	Time, minutes
1, Initial denaturation	94˚C	4.00
2, Denaturation*	94 ˚C	1.00
3, Annealing*	60 ˚C	1.00
4, Extension*	72 ˚C	4.00
5, Final extension	72 ˚C	5.00
6, Hold	4 ˚C	∞

*Steps 2 through 4 were repeated 27 times.

2.2.1.4 cDNA synthesis, Expression study for T-DNA plants

The following plant material was used for expression studies; PSY2L Salk 048064 and PSY2L Salk 125872.
Plants were first genotyped to find homozygous individuals.

The seeds harvested from the homozygous plants were sown directly on soil and placed in darkness at 4˚C for 72 h. The plants were then moved to a growth incubator and genotyped when large enough to withstand the removal of leaves. Leaves were harvested from two of the plants believed to be homozygous, one PSY2L Salk 048064 mutant, (later confirmed to be heterozygous) and one PSY2L 125872 mutant (confirmed to be homozygous at a later time).

The plant material harvested from the two believed homozygous individuals (PSY2L Salk 048064 and PSY2L Salk 125872) was used to isolate RNA to be used for cDNA production. To isolate RNA from the plant material, the RNeasy kit from QIAGEN was used. The protocol given with the product was followed.

2.2.1.4.1 Protocol for production of cDNA, T-DNA plants

The RNA samples obtained using the RNeasy kit were diluted to give a final concentration of 20ng/µl.
All chemicals to be used were vortexed and centrifuged briefly before use. Two chemical mixes were prepared in two Eppendorf tubes, RNA mix (table 15) and Revers transcriptase mix (table 16).

The samples containing RNA mix were heated in a PCR machine to 70˚C for 5 min., then placed on ice for >5 min. They were then centrifuged briefly (5000prm, <1 min.) to make sure any condensation was removed from the inner surface of the lids. The reverse transcriptase mix was added (4,25µl pr. sample) before they were placed back into the PCR machine. The full program for the PCR machine is found in table 17.

Table 15. RNA mix for cDNA production, T-DNA plants PSY2L Salk 048064 and Salk 125872

Reagent	Amount, µl	Final concentration
Experimental RNA	4.75	95 ng/µl
Primer, RT_PSY2L_RP	1	10 µM
Total volume	5.75	NA

Table 16. Reverse transcriptase mix for cDNA production, T-DNA plants

Reagent	Amount, µl
Goscript 5x reaction buffer	201
$MgCl_2$	1.25
dNTP	0.5
Reverse transcriptase	0.5
Total volume	4.25

Table 17. PCR program for cDNA production, T-DNA plants

Temperature	Time
70˚C	15 min.
25˚C	1 min.
42˚C	1 h.
70˚C	15 min.
12˚C	∞

The samples were kept on ice until the expression analysis was started.

To analyse the expression, the amount of cDNA was measured using PCR followed by agarose gel electrophoresis. The protocol follows.

2.2.1.4.2 Protocol for expression analysis, T-DNA plants

The DNA content of the cDNA samples was amplified using the PCR protocol for colony PCR with some alterations.
To find out the optimal amount of template DNA as well as the optimal amount of cycles, four mixes for each sample was prepared; 1µl/25 cycles, 3µl/25 cycles, 1µl/30 cycles, 3µl/30 cycles. The amount of reagents used is given in table 18, and the PCR program for cDNA analysis is given in table 19.

Table 18. Overview of PCR for cDNA analysis, T-DNA plants

Reagent	Amount for 1 µl cDNA samples, µl	Amount for 3 µl cDNA samples, µl
Buffer	2	2
$MgCl_2$	0.6	0.6
dNTP	0.4	0.4
Primer 1	1	1
Primer 2	1	1
cDNA	1	3
Taq polymerase	0.1	0.1
dH_2O	13.9	10.9

Table 19. PCR program for cDNA analysis, T-DNA plants

Step	Temperature	Time
1, Initial denaturation	94˚C	3.00 min
2, Denaturation*	94 ˚C	45 s
3, Annealing*	60 ˚C	30 s
4, Extension*	72 ˚C	2.00 min
5, Final extension	72 ˚C	10.00 min
6, Hold	4 ˚C	∞

*Steps 2 through 4 were repeated 25/30 times.

2.2.2 Agarose gel electrophoresis protocol

1g agarose was used for every 100 ml 1xTAE buffer.

The agarose-buffer mixture was heated until all agarose was solved, then poured into the cast to solidify. To visualize the DNA bands, gel-red (1:50 Gel-red:water ratio) was used. Loading buffer was added to ensure that the sample maintained its position in the correct well. Hyperladder I (4 µl) mixed with Gel-red (2 µl) was used to ascertain the size of the PCR product**.** Loading buffer (2 µl) and Gel-red (2 µl, 1:50) was added to each sample before running it on agarose gel. The gel was run for 40 minutes at 80V, and then analyzed using UV light to visualize the DNA bands.

2.2.3 Molecular cloning of fusion-proteins

The following fusion-proteins were attempted to produce; PP4-2-EYFP, EYFP-PSY2L, PSY2L-EYFP, PP4R2L-EYFP and EYFP-PP4R2L.
Cloning was performed on constructs to be used in localization studies. Three genes were to be examined, and each of the three genes were cloned into two different vectors; pCAT-EYFP and pCAT-DECR-EYFP. Cloning into pCAT-EYFP resulted in the protein being tagged on the N-terminus, and cloning into pCAT-DECR-EYFP resulted in the protein being tagged on the C-terminus. For the PSY2L gene, a third vector was also used. See table 20 for a full overview of the genes and vectors used.

Table 20. Overview of genes to be cloned

Gene	Template plasmid	Destination Vector	Restriction site	EYFP tag at terminus	Expected size including primer nucleotides
PP4-2	U83558	pCAT-EYFP	NotI/SacII	N	932
PP4-2	U83558	pCAT-DECR-EYFP	NotI/NcoI	C	929
PSY2L	U21916	pCAT-EYFP	NotI	N	2621
PSY2L	U21916	pWEN 25	KpnI/SalI	N	2621
PSY2L	U21916	pCAT-DECR-EYFP	NotI/NcoI	C	2619
PP4R2L	U24915	pCAT-EYFP	NotI/SacII	N	857
PP4R2L	U24915	pCAT-DECR-EYFP	NotI/NcoI	C	855

The one marked in red was already provided for transformation into protoplasts, all work done on them by Dr. Amr Kataya. The one marked in blue proved to be difficult due to the large size of the insert combined with the fact that only one restriction enzyme could be used. After multiple failed attempts, it was decided to proceed with the cloning but using a different vector, namely pWEN25, marked green.

2.2.3.1 Molecular cloning overview

Below follows an overview of the entire molecular cloning procedure.

Overview of the entire molecular cloning procedure

- Constructs were amplified using high fidelity PCR.
- They were then analysed using agarose gel electrophoresis.
- The PCR product was cleaned using gel extraction kit from sigma Aldrich, and concentration was measured using Nanodrop.
- A small amount of sample was then run on gel to check that the gel extraction worked well.
- The vectors and genes were then digested using the corresponding restriction enzymes, before the samples were cleaned using PCR cleanup kit from sigma Aldrich.
- A small amount of the samples was run on gel to ensure that the PCR cleanup worked as intended, and the concentration was measured using Nanodrop.
- The genes were ligated into the vectors.
- Competent cells were transformed, using the ligation mix, and incubated overnight at 37˚C.

- Any surviving colonies were checked by colony PCR, then analysed on agarose.
- Overnight cultures were made from any positive colonies, and plasmids were isolated using plasmid isolation kit from sigma Aldrich.
- A small portion of the plasmid sample were digested using the corresponding restriction enzymes to check that both the insert and vector were present in the sample.
- The digested portion of the sample was analysed on agarose.
- Any positive samples were then sent to sequencing.

2.2.3.2 Digestion, molecular cloning

The following tables give an overview of the reagents and restriction enzymes used for the vectors and inserts (tables 21, 22, 23, 24and 25).

Table 21. Digestion of pCAT-EYFP and PP4R2

Reagent	**Amount (µl)**
BSA	1
Buffer 2	2
NotI	1
SacII	1
Template	15

Table 22. Digestion of pCAT-EYFP and PSY2L

Reagent	**Amount used for pCAT-EYFP (µl)**	**Amout used for PSY2L (µl)**
BSA	2	2
Buffer 3	2	2.5
NotI	1.5	1.5
Template	13	25
H_2O	2	0

Table 23. Digestion of pCAT-DECR-EYFP and PSY2L

Reagent	**Amount used for pCAT-DECR-EYFP (µl)**	**Amount used for PSY2L (µl)**
BSA	1	2
Buffer 3	2	2
NotI	1	1
NcoI	1	1
Template	15	25

Table 24. First digestion of PSY2L to be ligated into pWEN25

Reagent	**Amount used for pWEN25 (µl)**
BSA	2
Buffer 1	4
KpnI	2
Template	35

Table 25. Second digestion of PSY2L to be ligated into pWEN25

Reagent	**Amount used for pWEN25 (µl)**
BSA	2
Buffer 3	4
SalI	2
Template (ligation mix from table 20)	35

2.2.3.3 PCR cleanup, cleaning digested vectors and inserts

PCR cleanup was performed using the PCR cleanup kit from Sigma. The procedure given with the kit was followed with one exception;-being the amount of water used for elution. Elution was done using 38µl water, free of any DNase-, RNase- and protease activity.

2.2.3.4 Dephosphorylation of 5´end of PSY2L for pCAT-EYFP

When cloning PSY2L into vector pCAT-EYFP, only one restriction enzyme could be used. This resulted in no positive colonies found for any bacteria transform with said plasmid. Phosphorylation of the 5´end of PSY2L was thought to help improve the process, eliminating some of the problems arising when ligating the vector and insert, such as the insert being ligated into the vector in tandem, or being ligated in upside down. The shrimp alkaline ligase system was used to attempt to improve the process.
The following mix was prepared (Table 26).

Table 26. Shrimp alkaline ligase mix

Reagent	Amount
DNA	9 µl
rSap buffer	2 µl
rSap	1 µl
dH_2O	8 µl

The mix was incubated at 30˚C for 30 min., followed by heat inactivation of the enzyme at 65˚C for 5 min. The resulting DNA mix was then used for ligation.

2.2.3.5 Ligation, molecular cloning

When ligating the insert into the vector, the amount of insert and vector was calculated following the instruction from the provider (Promega). The following equation was used:

Equation 1. Calculations of the amount of insert (ng) to be used for ligation into vector, 1:1 vector/insert ratio

$$Insert\,(ng) = \frac{Vector\,(ng) \cdot Insert\,(kbp)}{Vector\,(kbp)}$$

This equation gives the amount of insert in ng to be used for a 1:1 Vector/insert ratio. The optimal ratio differs with the size of the insert and vector. For this study a 1:6 ratio was used for most of the genes.

To find the amount of insert in µl for the correct ratio, the following equation was used:

Equation 2. Calculations of the amount of insert (µl) to be used for ligation into vector, Not 1:1 vector/insert ratio

$$Insert\,(\mu l) = \frac{Insert\,(ng) \cdot Ratio(3,6,8)}{Gene\,(ng)}$$

Due to difficulties encountered when cloning the large PSY2L gene, ligation was performed using three different ratios for both vectors; pCAT-DECR-EYFP and pWEN25. The following ratios were used: 1:3, 1:6 and 1:8, shown as ratio (3,6,8) in equation 2. Meaning that the insert amount was multiplied with 3 for 1:3 ratio, 6 for 1:6 ratio and 8 for 1:8 ratio. None of the ratios proved more effective than the others for this study. An overview of the amount of reagents used for ligation follows in table 27.

Table 27. Overview of generic ligation mix

Reagent	**amount**
Vector	2 µl (variable depending on concentration of vector)
Insert	3 µl (variable depending on ratio and concentration)
Buffer	1 µl
Ligase	1 µl
dH_2O	3 µl (variable, used to make final amount 10 µl)
Total	10 µl

Following the above equations and tables, the amount of reagents for each ligation mix was prepared. The ligation mixture was left at room temperature overnight before it was used in transformation of bacterial cells.

2.2.3.6 Transformation of bacterial cells

The heat-shock method was used for transformation of competent bacterial cells, here *Escherichia coli JM109*.

The competent cells were first thawed on ice for 10 min. DNA material was added to the bacterial cells and the resulting mixture was incubated on ice for 30 min. The bacterial cells were then given a heat-shock at 42˚C for 90 min. This was done on a heat block. Thereafter, the bacterial mixture was incubated on ice for five minutes, before LB medium (500 µl) was added. The bacterial suspension was then incubated at 37˚C for 1 h and 30 min (larger plasmids were incubated 3 h). Bacterial colonies were then transferred to LB agar plates containing Ampicillin.

2.2.4 Plasmid isolation, localization studies of proteins; PP4-2, PSY2L and PP4R2L

Plasmid isolation was performed using the plasmid miniprep kit from SIGMA-ALDRICH. The protocol given by the supplier was followed.

2.2.5 Plasmid check, localization studies of proteins; PP4-2, PSY2L and PP4R2L

To ensure that the plasmids obtained from the bacterial cells did indeed contain the gene of interest, the plasmids were first digested using the restriction enzymes corresponding to the restriction sites present, then analyzed on gel to make sure the plasmids displayed two distinct bands. Any samples deemed to be positive were sent to sequencing. The procedure for agarose-gel electrophoresis described earlier was followed.

2.2.6 Sequencing, localization studies of proteins; PP4-2, PSY2L and PP4R2L

The isolated plasmids were sent to Seqlab-Sequence Laboratories Göttingen GmBh, address; Hannah-Vogt-Str.1 37085 Göttingen Postfach 3343 37023 Göttingen Germany. After sequencing, the resulting sequence was used to check its alignment with the CDS from the original sequence for the gene in question (PP4-2, PSY2L or PP4R2L). The alignments were produced using T-Coffee alignment. (appendix A-3, A-4, A-5 and A-6)

2.2.7 Protoplast isolation and DNA-Peg transfection

The method used was devised by Dr. Behzad Heidari Ahootapeh after studying the following articles: “Signal transduction in Maize and Arabidopsis Mesophyll Protoplasts” and “Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis” (Sheen 2001; Yoo, Cho et al. 2007). The PSY2L gene was not successfully cloned and localization studies were only performed for PP4-2 and PP4R2L. Below follows an overview of the fusion-proteins studied (Table 28).

Table 28. Overview of fusion-proteins used in localization studies

Gene	Vector	EYFP tag	Fusion-protein
PP4R2L	pCAT-EYFP	N	EYFPPP4R2L
PP4R2L	pCAT-DECR-EYFP	C	PP4RL2EYFP
PP-4	pCAT-EYFP	N	EYFPPP4-2
PP-4	pCAT-DECR-EYFP	C	PP4-2EYFP

Enzyme solution was prepared following table 29.

Table 29. Enzyme solution for Protoplast isolation

Reagent	Amount for 1 reaction
1, Cellulase	15 mg
2, Mannitol	5 ml
3, KCl 1 M	0.2 ml
4, Pectinase	10 mg
5, MES 100 mM	2 ml
6, dH_2O	2.7 ml
-	-
7, $CaCl_2$ 1 M	0.1 ml
8, BSA	10 mg

Reagents 1 through 6 were added, and the solution was placed on a stirrer until everything was dissolved. Reagents 7 and 8 were then added to the dissolved enzyme solution.
Leaves were harvested from Arabidopsis thaliana wild type, 10-15 leaves depending on the size. The leaves were then cut into thin strips using a sterile scalpel and submerged into the enzyme solution (10 ml). The leaves were incubated in darkness at room temperature for 24 h.
Peg solution was prepared following the below table (Table 30)

Table 30. Peg solution for Protoplast production

Reagent	Amount
PEG 400	2 g
dH_2O	1.5 ml
Mannitol 0.8 M	1.25 ml
$CaCl_2$ 1 M	0.5 ml

The reagents were added to a Falcon tube in the order shown above, then shaken vigorously to make sure the reagents were mixed properly.
Protoplasts were released from the leaves by gentle stirring, using a sterile pipette tip in a sterile environment. The protoplast solution was then filtered using a nylon mesh and cells were checked using a microscope after filtration (10 µl). The protoplast solution was then centrifuged at 100xg for 1 minute and the supernatant was discarded. Ice cold W5 solution (2 ml) was added, and the protoplasts were carefully resuspended into solution. The solution was incubated for 20 minutes on ice. Some cells (10 µl) were again checked using a microscope during this incubation. The solution was centrifuged at 100xg for 1 minute and the supernatant was discarded. The protoplast pellets were then resuspended in MMg (2 ml). The protoplast solution (250 µg) was added to the plasmid to be studied (10 µg) in a small petri

dish and the two solutions were mixed gently by careful pipetting. PEG solution (220 µl) was added drop by drop, and the mixture was incubated for 20 min. at room temperature. Ice cold W5 solution was added (880µl), and the resulting solution was mixed carefully by pipetting. The protoplast solution was then transferred to a falcon tube and centrifuged at 100x g for 1 minute at 4˚C. The supernatant was discarded, and the cells were resuspended in ice cold W5 solution (1 ml) before the solution was transferred to a small petri dish. The protoplasts were then incubated for 25 h. in darkness at room temperature followed by microscopy to study the localization of the fusion-proteins.

2.2.8 Microscopy

The microscope used for this study was Nikon A1R confocal microscope. The localization of the fusion-proteins; PP4-2-EYFP, EYFP-PP4-2, PP4R2L-EYFP and EYFP-PP4R2L were checked using microscopy after one day of incubation and after two days of incubation.

2.2.9 Surface sterilization of *A. thaliana* seeds

Calcium hypochlorite pellets (0.25 g) was added to dH_2O (25 ml) to make a saturated solution. One droplet of tween was added, and the solution mixed rigorously before placed aside to settle. The calcium hypochlorite solution (2.5 ml) was added to 95% ethanol (22.5 ml). One ml. of the solution was then added to dry seeds and incubated for 5 min. The seeds were then washed twice using 95% ethanol, and left in a sterile environment to dry.

2.2.10 Screening of seeds

2.2.10.1 Screening of seeds from T-DNA plants

Seeds were harvested from each plant (already available in the lab). Seeds obtained from homozygous plants were sown directly on soil, to be genotyped when mature enough (full overview of the number of homozygous plants found is given in table 6). Seeds obtained from two of the heterozygous plants (PP4R2L Salk 093041, plant 3 and plant 7) were sown on half strength MS medium containing kanamycin for further screening. The seeds were first surface sterilized, and then sown on kanamycin plates in the sterile hood. Seeds from each plant were sown on four plates, giving a total of eight plates. Three plates containing kanamycin, and one positive control with no antibiotic in the medium. The plates were then placed in darkness at 4˚C for 72 h., before they were moved to a plant growth room with 16 h. light cycle, where they remained until they were ready to be transferred to pots containing soil. Seeds from two different plants were screened twice. The first time four plates were sown for each plant whereas six plates were sown for each plant the second time. Since the second experiment did not reflect the results from the first experiment (Tables 5 and 6), it was decided to transfer 20 plants from each type to soil, and then proceed with genotyping of each individual plant to find homozygous individuals, if present.

2.2.10.2 Screening of seeds from MicroRNA plants

Seeds were harvested from each of the mutant plants. The seeds were then surface sterilized, and sown on half strength MS plates containing the corresponding screening chemical; for pER10 Kanamycin was used and for pBA002 BASTA was used. The plates were placed in the dark at 4˚C for three days, then moved to a plant growth room with a 16 h. light/8 h.

darkness cycle. Any surviving plants were transferred to soil to be observed for any difference in phenotype w.r.t. the wild type.

2.2.11 Protocol for production of growth medium

When cultivating plants sown on soil, Hoagland solution was used as nutrient solution for the growing plants. Seeds sown on plates were sown on half strength MS medium, screening with the corresponding screening agent (Kanamycin or BASTA). Bacterial colonies were cultivated using LB broth for overnight cultures and LB agar for plated colonies. The LB broth and LB agar was prepared following the labels on the containers. Ampicillin was added to the medium as screening agent for bacterial colonies.

2.2.11.1 MS plant medium

Minor I and Minor II were prepared by adding the reagents from table 32 and 33, respectively to 1 l. water.
Normal strength MS solution was prepared by adding the chemicals listed below in order; A, B, C, D, E, Minor I, Minor II, Fe/EDTA to 200 ml water (table 31). The pH was adjusted to reach pH5.8, then more water was added until the solution reached 2 l. in volume to achieve half strength MS medium. For full strength MS, a total volume of 1 l. would be necessary. The liquid medium was divided amongst four bottles, and sucrose was added to three of them. One bottle remained without sucrose for possible future uses. Agar-agar was added to all bottles achieve solidification of the medium to be used in screening of *Arabidopsis thaliana* seeds.

Table 31. Overview of chemicals used for MS nutrient solution

Order	Chemical	Amount
A	KNO_3	20 ml
B	HN_4NO_3	13 ml
C	$MgSO_4 \cdot 7H_2O$	10 ml
D	KH_2PO_4	20 ml
E	$CaCl_2 \cdot 2\ H_2O$	10 ml
Minor I		10 ml
Minor II		10 ml
Fe/EDTA	Fe/EDTA	50 ml

Table 32. Overview of chemicals used for Minor I solution, 1 l.

Chemical	Amount
$ZnSO_4 \cdot 7H_2O$	0.920 g
H_3BO_3	0.620 g
$MnSO_4 \cdot 4H_2O$	2.230 g

Table 33. Overview of chemicals used for Minor II solution, 1 l.

Chemical	Amount
$NaMoO_4 \cdot 2H_2O$	0.025 g
$CuSO_4 \cdot 5\ H_2O$	0.003 g
$CoCl_2 \cdot 6\ H_2O$	0.003 g
KI	0.0083 g

2.2.11.2 Hoagland plant nutrient solution

The chemicals of the micronutrient solution (Table 35) were dissolved in 1 l. water. Water (2 l.) was added to an empty bottle (5 l.). The chemicals listed in table nr 34 were added in the following order; A, B, C, D, E, micronutrient solution (Table 35). Water was added to reach a total volume of 5 l., giving 10x Hoagland solution. 100 ml. 10x Hoagland was added to 900 ml. water for 1x Hoagland solution.

Table 34. Overview of chemicals used for Hoagland solution

Order	Chemical	Amount	Nutrient concentration in 1x Hoagland
A	KH_2PO_4	50 ml	1 m MPO_4^-
B	KNO_3	250 ml	5 mM NO_3^-
C	$Ca(NO_3)_2 \cdot 4H_2O$	250 ml	10 mM NO_3^- 5 mM Ca^{++}
D	$MgSO_4 \cdot 7H_2O$	100 ml	2 mM Mg^{++} 2 mM SO_4^-
E	Fe/EDTA, 1%	50 ml	-
Micronutrients		50 ml	-

Table 35. Overview of chemicals used for 1 l. micronutrient solution

Chemical	Amount in one liter
H_3BO_3	2.86 g.
$MnCl_2 \cdot 4\ H_2O$	1.81 g.
$CuSO_4 \cdot 5\ H_2O$	0.089 g.
$ZnSO_4 \cdot 7H_2O$	0.22 g.
$H_2MoO_4 \cdot 1H_2O$	0.029 g.

3. RESULTS

3.1 Observed phenotype for mutant T-DNA plants, *Arabidopsis thaliana*

The mutant plants (all three lines) displayed shorter stems and fewer stems than the wild type (for mutant PSY2L Salk 125872, see figure 5). The mutants also displayed foliage growth along the length of the stems. Leaves growing like this was not seen with any of the wild type plants. The mutants displayed impaired growth, taking about one month longer to mature and produce seeds when compared to the wild type (impaired growth can be seen in figure 6, 7, 8 and 9). The three mutant types did not display any significant difference when compared to one another.

Figure 5. PSY2L Salk 125872 mutants. The mutants displayed impaired growth when compared to the wild type. The two top rows of pots carry mutant plants whereas the two bottom rows carry wild type plants.

Figure 6. PSY2L Salk 048064 mutants. At the early stage the mutants display a higher degree of impaired growth than that of the PSY2L Salk 125872 mutants when compared to the wild type. The top-left mutant, circled in red, was found to be homozygous after genotyping. Its impaired growth was extreme, and it did not survive into maturity (figure 6).

Figure 7. The PSY2L Salk 048064 from figure 2 at a later stage. The difference in size between the mutants and wild type becomes more evident. The homozygous plant is circled in red.

Figure 8. PSY2L Salk 125872 homozygous and heterozygous plants. The mutant plants are present at the two leftmost rows. The two rows to the right are inhabited by wild type. Shorter stems are clearly visible for the mutants when compared to the wild type.

One homozygous plant for PSY2L Salk 049064 was of particular interest due to its small size when compared to its siblings and the wild type (Figures 6, 7 and 9)

Figure 9. PSY2L Salk 048064 homozygous plant next to heterozygous individuals for the same Salk line. The believed homozygous plant is circled in red. The small size of the homozygous plant is evident. The homozygous plant did not survive to produce seeds.

This small homozygous plant from Salk 048064 did not survive to produce seeds. It died one week after the above photo was taken.

3.2 Observations made for *Arabidopsis thaliana* amiRNA plants

Mutant plants appeared shorter than that of the wild type, the number of leaves and number of stems being fewer than what was observed with the wild type. The length of the stems were also slightly shorter than what is common to see for the wild type. The two mutant types did not differ much in regards to each other. The expectation that plants containing the constitutive vector would display impaired growth was not observed. Mutant plants for both vectors had leaves growing from the stem along the entire length of the plant. The wild type did not exhibit such foliage. The time needed for the mutant plants to produce seeds was also observed to be about one month longer than that of the wild type. One particular individual for pER10 RNA1-2 displayed an abnormally thick and flat stem (Figures 10 and 11).

Figure 10. Abnormal individual for pER10 RNA 1-2. Leaves are growing along the length of the stem. The stem in itself is the interesting part, being much thicker than what was commonly observed. It also displayed a flatness giving it a resemblance to a ribbon.

Figure 11. Close up of the abnormal plant shown in figure 6. The ribbon-like qualities of the stem is evident at the top right of the picture (circled in red), where it is shown to twist displaying the flatness of the stem.

The observations made for these mutants proved to be very similar to any observations made for the mutant plants in the gene expression study for PSY2L Salk 048064, Salk 125872 and PP4R2L Salk 093041 (Table 36). In regards to the stem of the abnormal individual, this was not thought to be caused by the plasmid and/or amiRNA. It is more likely that it was caused by a naturally occurring mutation giving rise to that specific phenotype. Offspring of that particular plant could be used for further studies.

Table 36. Overview of phenotypes of mutant plants

Plant type	**Mutant**	**Phenotype, Homozygous plants**	**Phenotype, Heterozygous plants**
T-DNA plant	PSY2L Salk 125872	Fewer and shorter stems. Impaired growth.	Fewer and shorter stems. Impaired growth.
T-DNA plant	PSY2L Salk 048064	Did not reach maturity. Extremely small size. No seed production due to death of the individual.	Fewer and shorter stems. Impaired growth, more severe than for the other two Salk lines.
T-DNA plant	PP4R2L Salk 093041	Fewer and shorter stems. Impaired growth.	Fewer and shorter stems. Impaired growth.
amiRNA plant	pER10	Fewer and shorter stems. Leaf growth along stems. Impaired growth.*	
amiRNA plant	pBA002	Fewer and shorter stems. Leaf growth along stems. Impaired growth.*	

* No genotyping was performed on amiRNA plants, so discerning homozygous plants from heterozygous ones was not possible**.**

3.3 Genotyping of hetero- and homozygous T-DNA plants*, Arabidopsis thaliana*

Of the plants already provided, none of the PP4R2L T-DNA plants were found to be homozygous. Seeds from both homozygous and heterozygous plants were then surface sterilized, screened on medium containing kanamycin and sown on soil to be used for a second genotyping. The same procedure was performed on plants from the second generation. Homozygous plants were found for the three different Salk lines for the second generation (F1), however time did not allow for expression studies to be done on F_1 plants. Table 37 gives an overview of the total amount of homozygous, heterozygous and wild type plants found from genotyping.

Table 37. Overview of total number of homozygous, heterozygous and wild type plants found when genotyping

Gene	Salk line	Generation	Number of heterozygous plants	Number of WT plants	Number of homozygous plants
PSY2L	Salk 048064	F_0	14	3	0
PSY2L	Salk 048064	F_1	16	2	1*
PSY2L	Salk 125872	F_0	4	4	3
PSY2L	Salk 125872	F_1	5	0	1
PP4R2L	Salk 093041	F_0	5	9	2
PP4R2L	Salk 093041	F_1	5	0	4

Figure 1 shows the result for the first genotyping of plants from seeds harvested from homozygous plants. The plan marked with * died before it could produce any seeds (Figure 6, 7 and 9).

Below follows the results of genotyping of PSY2L Salk 048064 and PSY2L Salk 125872 (Figure 12). Given that there are 15 wells at the top lane and 20 wells at the bottom lane, the result can be tricky to read. Two of the plants that believed to be homozygotes (shown by arrows in figure 12, orange arrow connecting A and B from Salk 048046, and blue arrow connecting A and B from Salk 125872,) were used for expression studies.

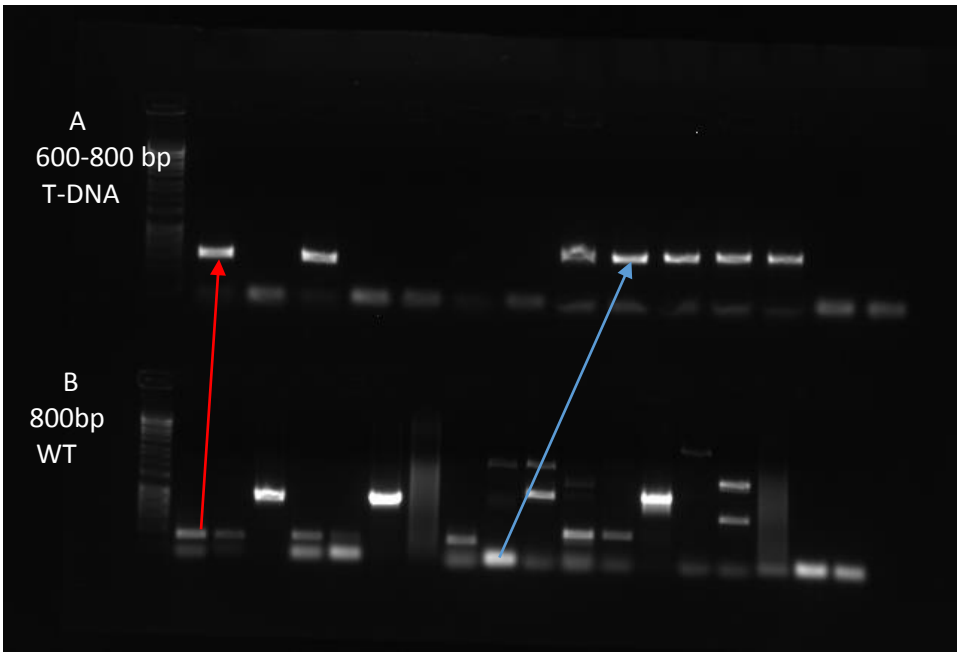

Figure 12. Agarose gel electrophoresis of amplified T-DNA (PSY2L Salk 048064 and PSY2L Salk 125872) and wild type DNA. Top lane (Gel A) shows amplified T-DNA while the bottom lane (Gel B) shows amplified wild type DNA. The wells with plant material used for cDNA production are indicated with a red arrow for Salk 048064 and a blue arrow for Salk 125872. The arrows are connecting wells with plant material from the same plant. Well content is given in table 38.

Table 38. Overview of content of each well from figure 12

Hyperladder is denoted HL, the Salk lines are given by the last two numbers, 64-1 meaning plant 1 for Salk line 048064 and 72-1 meaning plant 1 for Salk line 125872, etc. Negative control is denoted N, and empty wells are denoted E.

Top	HL	64-1	64-2	64-3	64-4	64-5	WT	N	72-1	72-2	72-3	72-4	72-5	WT	N	E	E	E	E
Bottom	HL	64-1	64-2	64-3	64-4	64-5	WT	N	72-1	72-2	72-3	72-4	72-5	WT	N	N	N	N	N

A follow up of the genotyping showed that the believed homozygous plant for Salk line 048064 (Figure 12, red arrow) was heterozygous for the gene (Figure 13, red arrow) as well as confirmed that the homozygous plant for Salk line 72 (Figure 12, blue arrow) was homozygous (Figure 14, blue arrow).

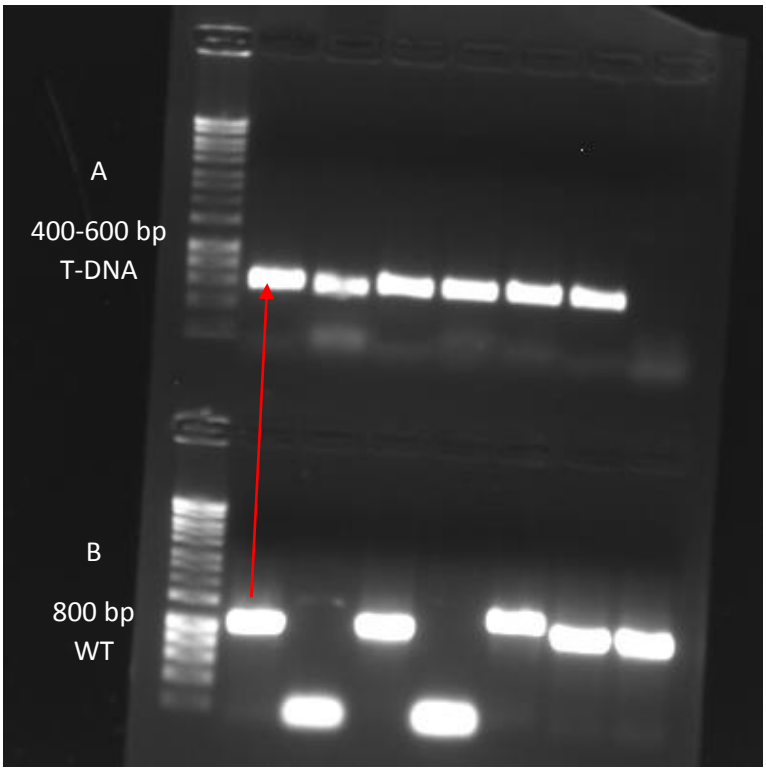

Figure 13. Gel electrophoresis from genotyping of PSY2L Salk 048064. Plant material from the believed homozygous individual indicated by the orange arrow in figure 12 was loaded in well 2, and displays clear bands for both the T-DNA and the wild type DNA (indicated with a red arrow). Plant material from well 3 and 5 could be from homozygous plants. Top lane shows PCR product where primers designed to amplify T-DNA was used and bottom lane contains PCR product where primers designed to amplify wild type DNA was used. Wild type material was used for well 8, and hyperladder was used in well1.

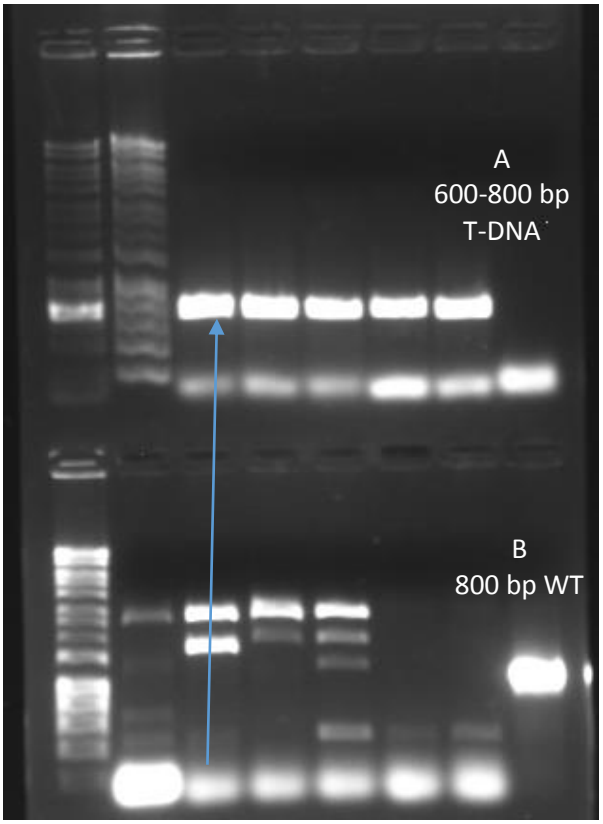

Figure 14. Gel electrophoresis from second genotyping for PSY2L Salk 125872. Top lane shows PCR product where primers designed to amplify T-DNA was used and bottom lane contains PCR product where primers designed to amplify wild type DNA was used. Wild type material was used for well 8, and hyperladder was used in well 1.The top lane has hyperladder in both wells 1 and 2 as well as PCR product from plant 1 due to a loading error. The homozygous plants from figure 12 is retested in well 2, shown by a blue arrow.

There are clear bands with the correct size for the T-DNA for all plant material tested, wild type excluded. The bands shown in the bottom lane of figure 14 are not of the correct wild type size, so the plants are believed to be homozygotes despite the presence of these bands. The specificity of the primers used to amplify T-DNA for Salk line 125872 could be questioned. Due to the presence of these ghost bands in some wells but not all, contamination of any chemicals used is not in question. A negative control could be preferable to add. Figure 15 shows one result from the genotyping of PP4R2L Salk 093041 mutant plants

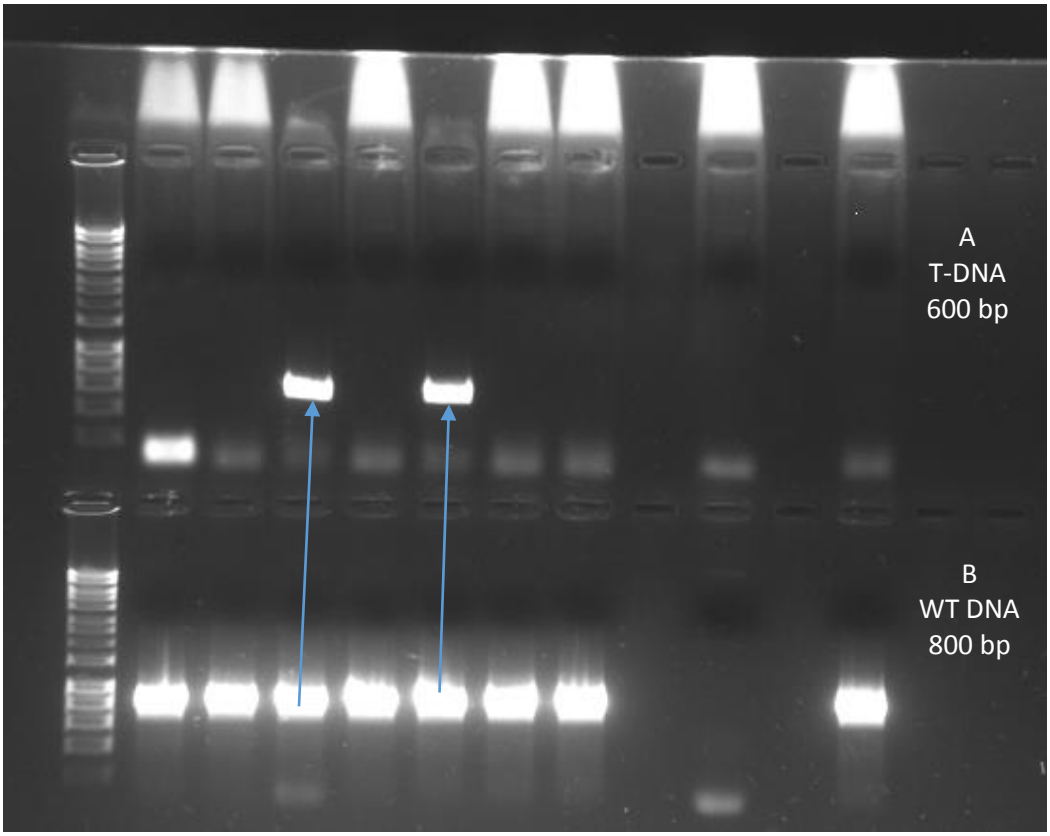

Figure 15. Gel electrophoresis of genotyping of PP4R2L Salk 093041. Samples to detect T-DNA were loaded in the top lane and samples to detect WT DNA was loaded in the bottom lane. Two believed homozygous individuals were found shown by the blue arrows.

The genotyping had to be performed multiple times on the same plants due to some contradictory results. A negative control was added for many of the attempts, but these proved to be positive some times and negative other times. Any genotyping results obtained with bands showing up for the negative control were retested at a later time to ensure its validity.

3.4 Molecular cloning, and localization of fusion-proteins

The molecular cloning attempts resulted in the production of fusion-proteins PP4-2-EYFP, PP4R2L-EYFP and EYFP-PP4R2L. The EYFP-PP4-2 fusion-protein was already available in the lab, produced by Dr. Amr Kataya.

3.4.1 Colony PCR and plasmid check for PP4-2, PP4R2L and PSY2L

Agarose gel electrophoresis results of one colony PCR check for PSY2L with vector pCAT-EYFP is shown in figure 16, PSY2L with vector pCAT-DECR-EYFP is shown in figure 17, PP4R2L with vector pCAT-DECR-EYFP is shown in figure 19, PP4R2L with vector pCAT-EYFP is shown in figure 20, and PP4-2 with vector pCAT-DECR-EYFP is shown in figure 22. Figure 16 shows no positive colonies.

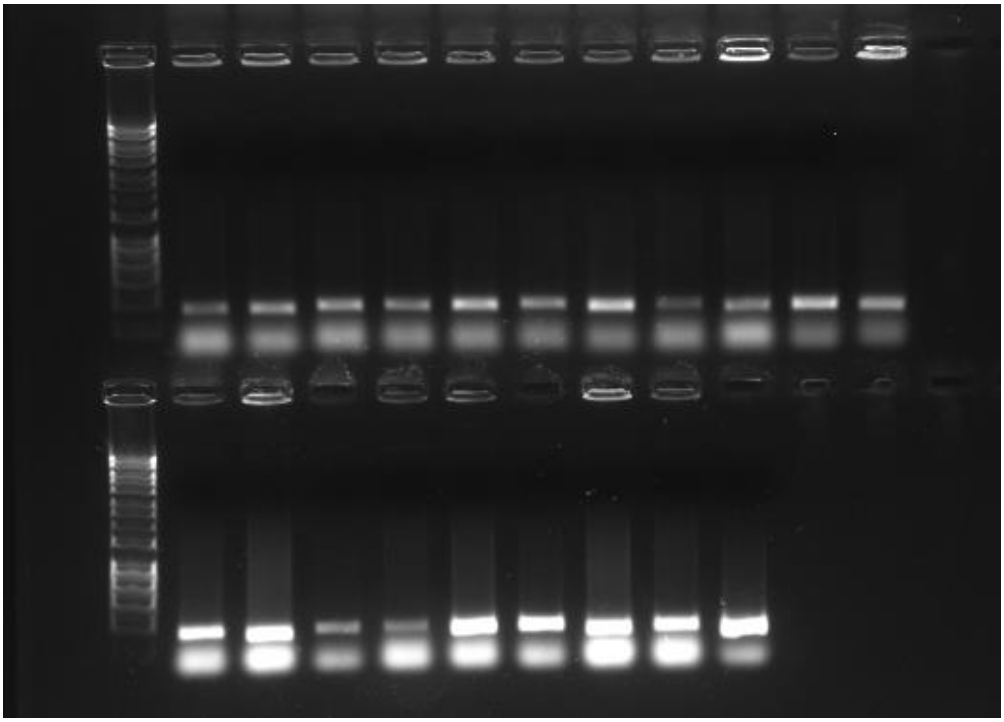

Figure 16. Agarose gel electrophoresis results of colony PCR of PSY2L with vector pCAT-EYFP, no positive colonies found. Vector specific primers were used. The higher of the two bands is believed to be remains of the vector. The lower of the two bands is though to be primer-dimers.

Colony PCR of PSY2Lwith vector pCAT-DECR-EYFP showed one positive colony (Figure 17).

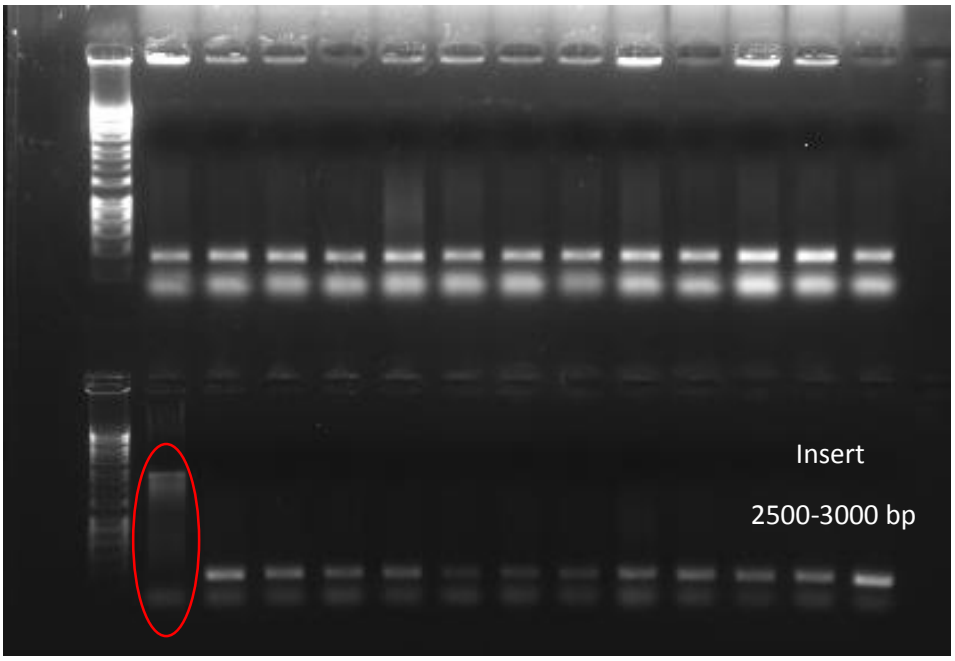

Figure 17. Agarose gel electrophoresis results of colony PCR of PSY2L with vector pCAT-DECR-EYFP, one believed positive colony found, colony 38 circled in red.

After positive colonies were found, plasmid isolation was performed, and a small amount of the plasmid was digested using the corresponding restriction enzymes. The digestion was then run on gel to make sure the plasmid contained the insert. Digestion of pCAT-DECR-EYFP

with insert PSY2L is shown in figure 18, two clear bands are visible where the plasmid has been cut correctly.

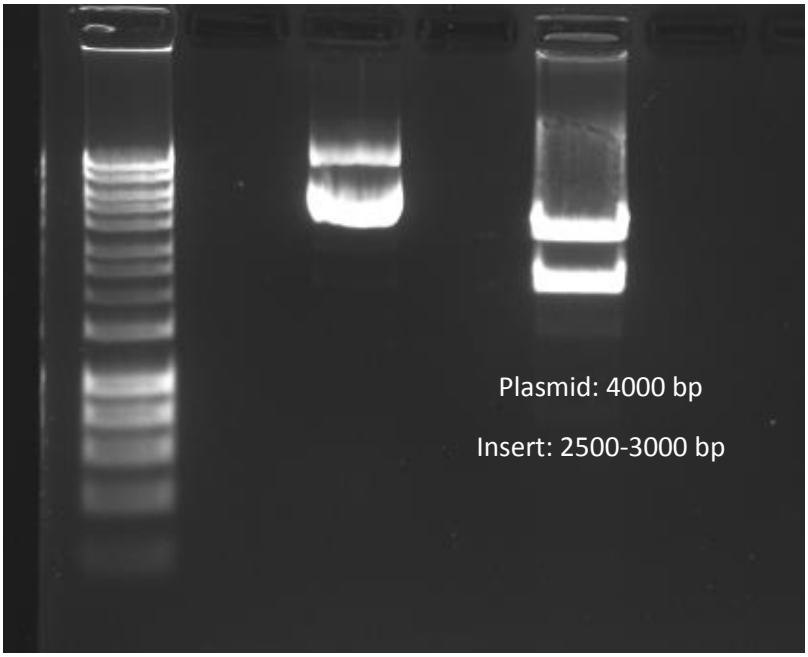

Figure 18. Agarose gel electrophoresis of plasmid digestion, PSY2L with vector pCAT-DECR-EYFP (figure 13). Well 3 contains uncut plasmid and well 5 contains digested plasmid and insert.

Colony PCR check of PP4R2L with vector pCAT-DECR-EYFP is shown in figure 19, where two colonies were thought to be positive. Plasmid check of these two, plus an additional plasmid from a believed positive colony found at a later time, is shown in figure 23.

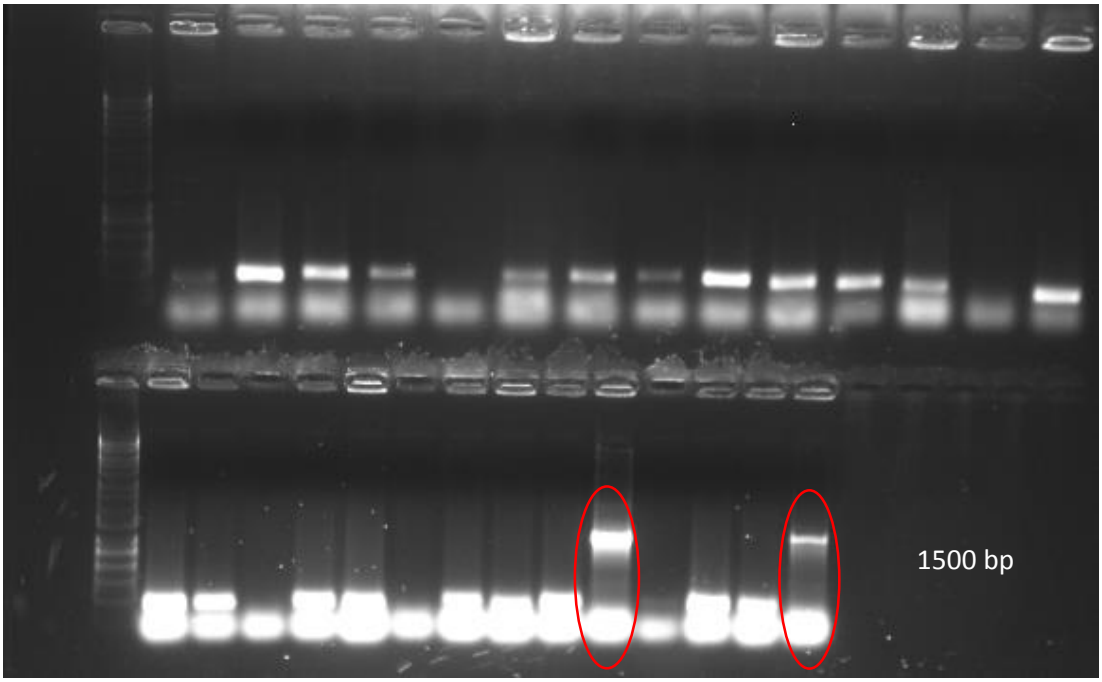

Figure 19. Agarose gel electrophoresis of colony PCR for PP4R2L with vector pCAT-DECR-EYFP. Two believed positive results found, colonies 24 and 28, both circled in red.

Figure 20 shows results from colony PCR of PP4R2L with vector pCAT-EYFP. Positive colonies were found as described in the figure text.

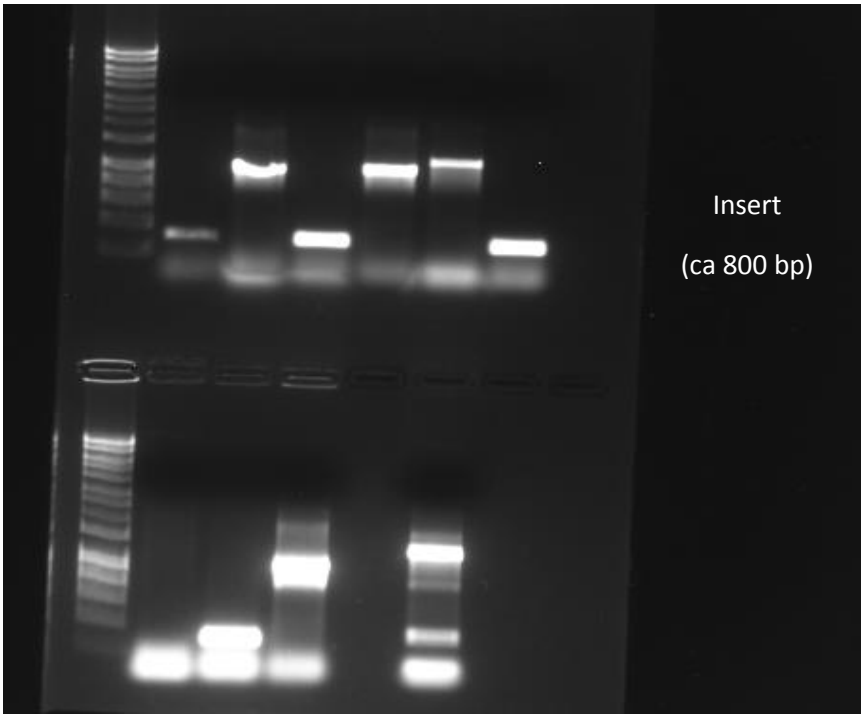

Figure 20. Agarose gel electrophoresis of Colony PCR for PP4R2L with vector pCAT-EYFP. Insert specific primers used. Positive results for colonies 2, 4, 5 and 9. Negative control yielded positive results. The plasmid was isolated from the colonies, cut with restriction enzymes and run again on another gel.

Plasmid check of the believed positive colonies shown in figure 20 was performed and results are displayed in figure 21. Three of the four plasmids from believed positive colonies were cut correctly.

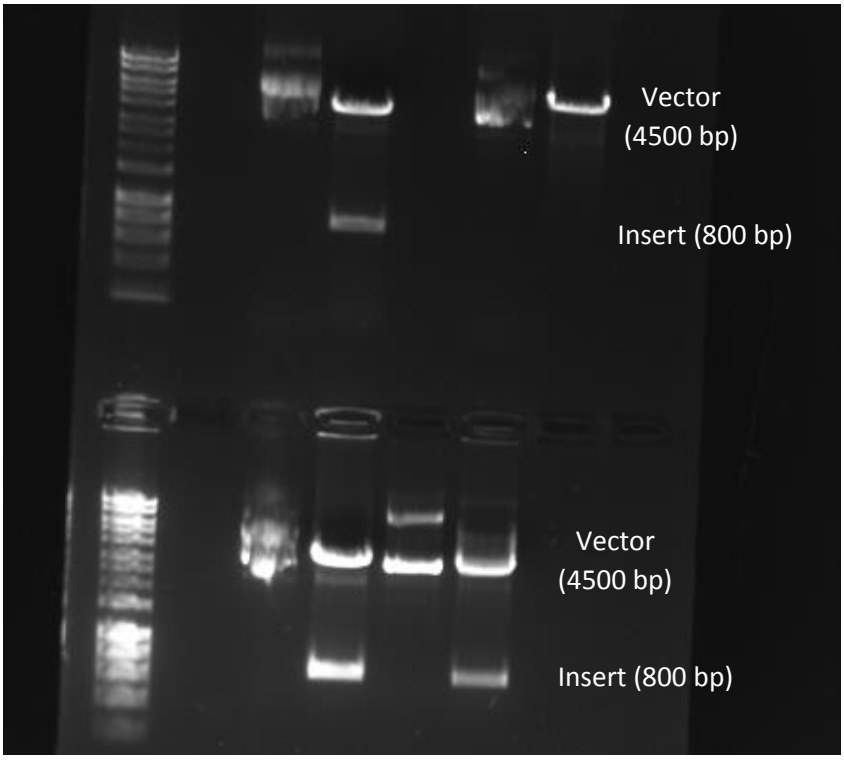

Figure 21. Agarose gel electrophoresis of plasmid digestion from the positive colonies from figure 20, PP4R2L with vector pCAT-EYFP. Colonies 2, 6 and 10 gave rise to one band at the insert size and one band at the vector size. The smears visible for top wells 2, 5 and bottom wells 2 are uncut plasmids with insert.

Results from colony PCR of PP4-2 with vector pCAT-DECR-EYFP is shown in figure 22. One colony was believed to be positive for the plasmid with insert, however on closer inspection of the plasmid (digestion with restriction enzymes) that assumption did not appear to hold true (figure 23).

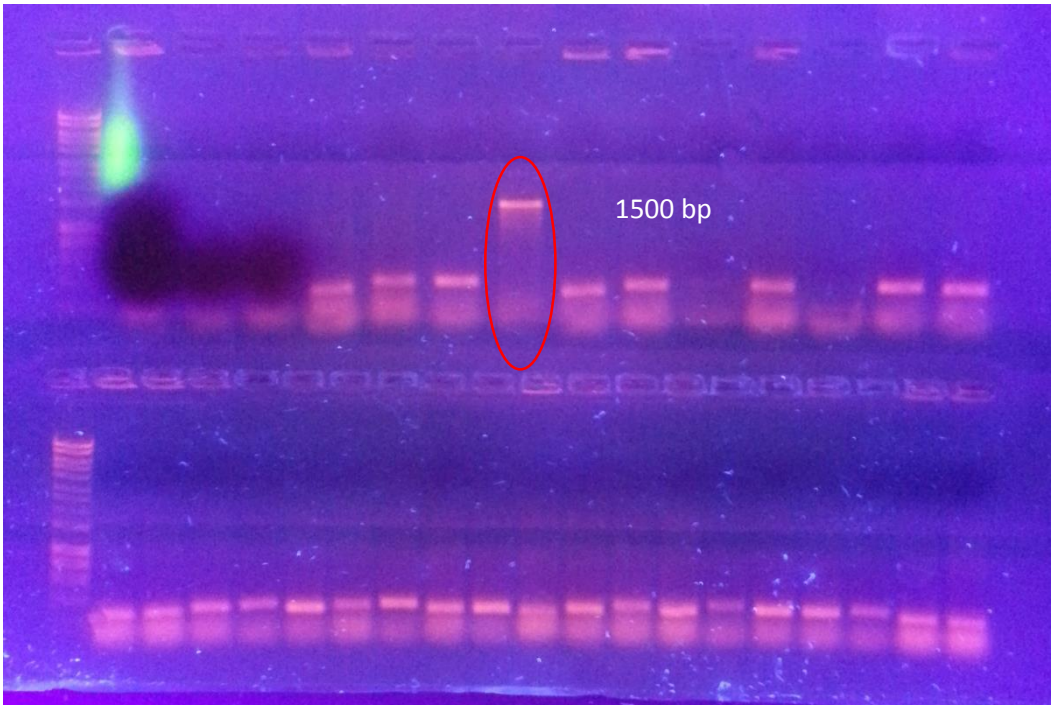

Figure 22. Agarose gel electrophoresis for colony PCR of PP4-2 with vector pCAT-DECR-EYFP. One positive, colony 8, circled in red. Imaging from the correct UV device was impossible due to the mechanical errors.

Digestion of plasmids from believed positive colonies for PP4-2 with vector pCAT-DECR-EYFP (Figure 22), PSY2L with vector pCAT-DECR-EYFP (Figure 17) and PP4R2L with vector pCAT-EYFP (Figure 19) is shown in figure 23.

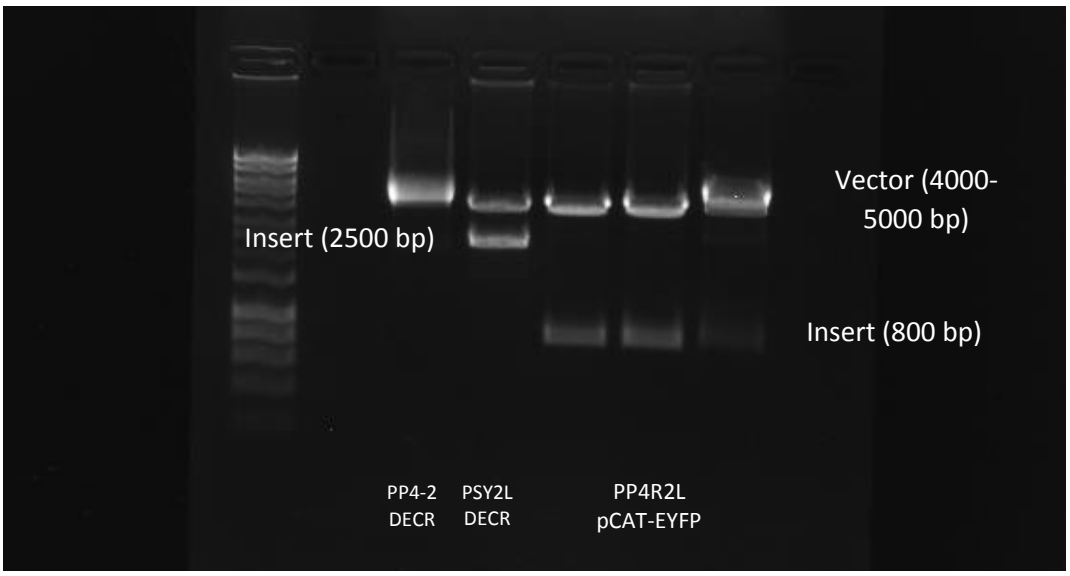

Figure 23. Gel electrophoresis of plasmid digestion for PP4-2, PSY2L and PP4R2L. Correct digestion can be observed for PSY2L with vector pCAT-DECR-EYFP as well as for the tree samples containing PP4R2L with the pCAT-EYFP vector. The sample containing PP4-2 was not digested, indicating that the plasmid did not contain the insert.

A new colony PCR check of PP4-2 was performed, and one positive colony was found. Digestion of the isolated plasmid from this colony is shown in figure 24.

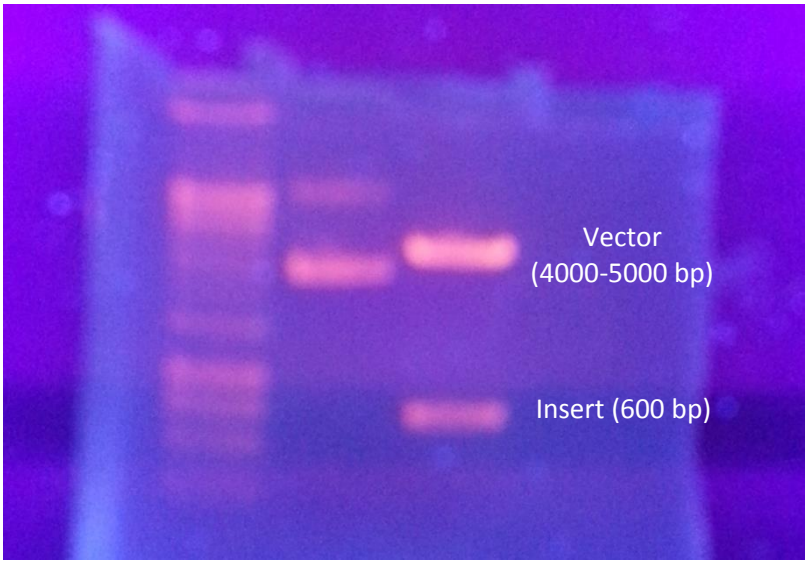

Figure 24. Gel electrophoresis of plasmid digestion for PP4-2 with vector pCAT-DECR-EYFP. Uncut plasmid was loaded in well 2, and digested plasmid was loaded in well 3. Well 3 shows two clear bands, indicative of digestion having occurred. Imaging from the correct UV device was impossible due to the mechanical errors.

Cloning of PP4R2L into the vector pCAT-EYFP yielded three positive results after being sent to sequencing. PP4-2 displayed one mutation after sequencing, due to time restrictions this sample was still used for localization studies even though a mutation was present.
No positive colonies were found for the PSY2L with pCAT-EYFP or the PSY2L with pWEN25 plasmids. One positive colony was found for the PSY2L pCAT-DECR-EYFP plasmid. This plasmid proved to contain a stop codon at the wrong location due to an error occurring when ordering the primer being used (Table 39, the plasmid with the wrongfully placed stop codon is marked in blue).

Table 39. Overview of cloning results for genes; PP4-2, PP4R2L and PSY2L

Gene	Vector	Nr of confirmed positive colonies (after digestion of plasmid)	Nr of samples sent to sequencing	Nr of positive samples confirmed by sequencing
PP4-2	pCAT-DECR-EYFP	1	1	0
PSY2L	pCAT-EYFP	0	0	0
PSY2L	pWEN25	0	0	0
PSY2L	pCAT-DECR-EYFP	1	1	1
PP4R2L	pCAT-EYFP	3	3	3
PP4R2L	pCAT-DECR-EYFP	2	2	2

Due to the low number of positive colonies found for the PSY2L gene (Table 37), multiple ligation attempts were made for ligation of this gene into the vectors where the Vector:insert ratio was altered. This was done in an attempt to find the optimal ratio for this insert to vector combination. Below follows an overview of the number of colonies tested and the number of positive colonies found for each gene. The table also gives an overview of the ratios used for each cloning attempt (Table 40).

Table 40. Overview of number of colonies tested by colony PCR to find positive colonies (colonies from successful cloning)

Gene	Vector	Vector: insert ratio	Nr of colonies tested	Nr of positive colonies before sequencing
PP4-2	pCAT-DECR-EYFP	1:6	112	1
PSY2L	pCAT-EYFP	1:8	107	0
PSY2L	pWEN25	1:8	184	0
PSY2L	pCAT-DECR-EYFP	1:8	60	1*
PSY2L	pCAT-DECR-EYFP	1:6	33	0
PSY2L	pCAT-DECR-EYFP	1:3	66	0
PP4R2L	pCAT-EYFP	1:6	9	3
PP4R2L	pCAT-DECR-EYFP	1:6	67	2

Some problems were encountered when cloning the PSY2L gene. The high fidelity PCR performed on this large gene yielded a smear in all occasions (Figure 25). The resulting concentration was also somewhat lower than that of the smaller genes; PSY2L concentration being in the 27-44 range, whereas PP4R2L was in the 67-206 and PP4-2 had a range between 34-65. This low yield is thought to be due to the large size of PSY2L, being larger than 2600 bp. This size can also pose some problems i.r.t. the actual sequencing as the lab performing the sequencing has a limit of 1000 bp. A primer that binds in the middle of the large PSY2L gene was prepared for this particular instance.

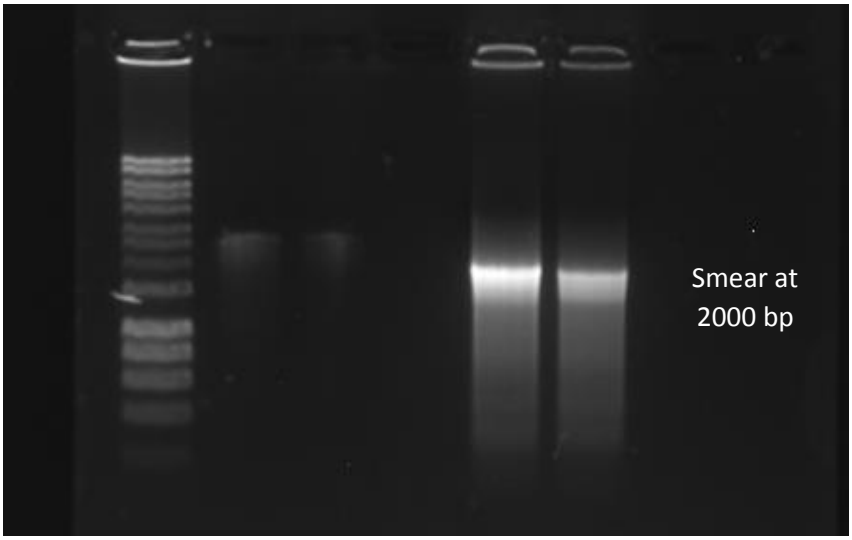

Figure 25. Agarose gel electrophoresis of PSY2L gene to be used for cloning. A smear is evident for all four wells, well 1 containing hyperladder with gel-red. Wells 2 and 3 containing 3 µl sample, well 4 is empty and wells 5 and 6 contains 15 µl sample. The lighting is reduced significantly to improve the quality of the image.

3.4.2 Cellular localization predictions using software

Today many software programs exists that can predict the subcellular localization of proteins. This prediction can give an indication as to where the protein might be located, but empirical evidence is needed to be able to conclude with more certainty where the actual localization the protein is. Subcellular localization predicitons for PP4-2, PP4R2L and PSY2L from the following software has been added to the table below; SubLoc v1.0, CELLO, BaCeLo , Euk-mPLoc 2.0 and SUBA (Table 41).

Table 41. Overview of localization predictions performed using software for PP4-2, PP4R2L and PSY2L

Gene	Locus	SubLoc v1.0	CELLO	BaCeLo	Euk-mPLoc 2.0	SUBA
PP4-2	AT5G55260	Cytoplasmic	Cytoplasmic	Nuclear	Cytoplasmic nuclear mitochondrial	Cytoplasmic ER Golgi Nuclear mitochondrial extracellular
PP4R2L	AT5G17070	Nuclear	Cytoplasmic	Nuclear	Nuclear	Cytoplasmic mitochondrial nuclear
PSY2L	AT3G06670	Nuclear	Cytoplasmic	Nuclear	Nuclear	Cytoplasmic mitochondrial nuclear

3.4.3 Microscopy, protein localization results

Microscopy was only performed once for this study. The PP4-2 fusion-proteins both displayed aggregation in the cells. The aggregation was prominent enough to lead to pixel saturation for samples analysed after one day of incubation as well as after two days. The fusion-proteins seem to be widespread throughout the cells, giving the impression of PP4-2 being a cytosolic protein (Figures 26, 27, 28 and 29). The PP4R2L fusion-proteins do not display any aggregation in the cell. They do, in similarity to PP4-2, appear cytosolic due to the proteins being distributed evenly along the inside of the cell (Figures 30, 31, 32 and 33).

3.4.3.1 PP4-2-EYFP fusion-protein localization

Figures 26 and 27 show microscopy results for the PP4-2-EYFP fusion-protein. Figure 26 was taken after one day of incubation and figure 27 was taken after two days of incubation. Both samples were taken from the same plant material. Aggregation of the protein can be seen in both samples, but the signal is stronger after a two day incubation period.

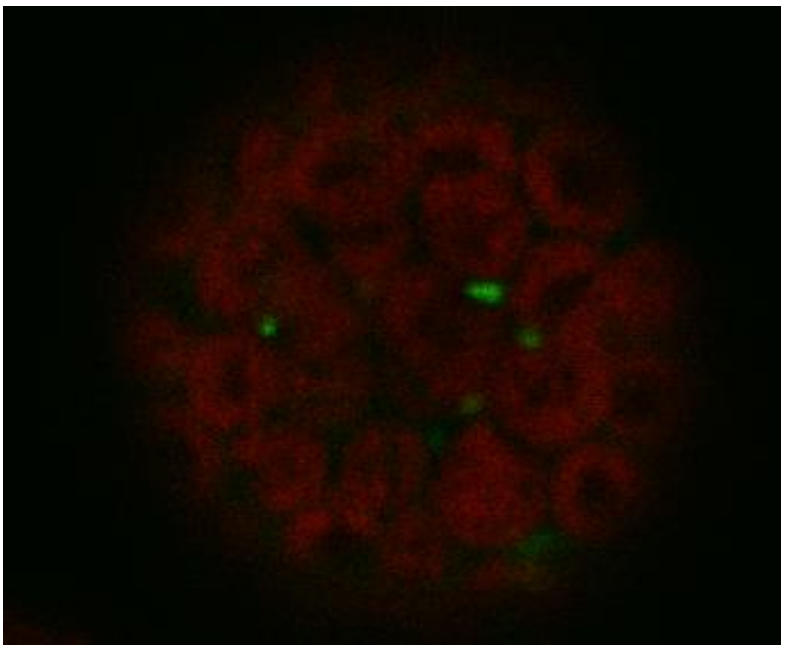

Figure 26. Microscopy of the fusion-protein PP4-2-EYFP in protoplasts taken after one day of incubation. Aggregation of the protein is evident as saturated areas of green. The plastids are visible in red.

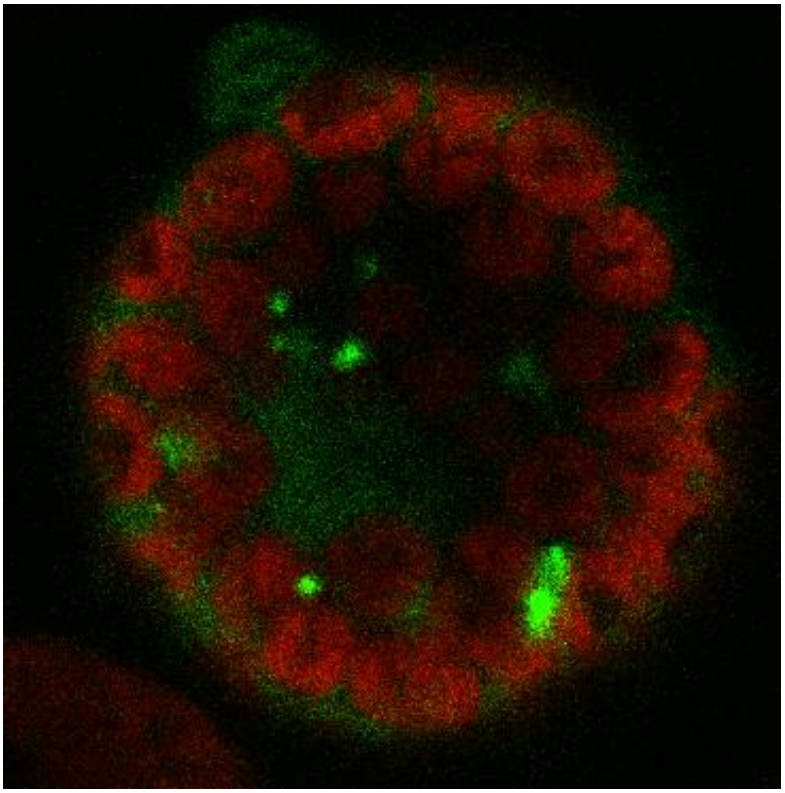

Figure 27. Microscopy of the PP4-2EYFP fusion-protein in protoplasts taken after two days of incubation.

3.4.3.2 EYFP-PP4-2 fusion-protein localization

Results from localization study of fusion-protein EYFP-PP4-2 follows in figure 28 and 29. Figure 28 shows the results from one day incubation, giving the impression of some cytosolic activity and some aggregation, and figure 29 shows results after incubating the same sample for two days. Figure 29 displays strong aggregation of the protein.

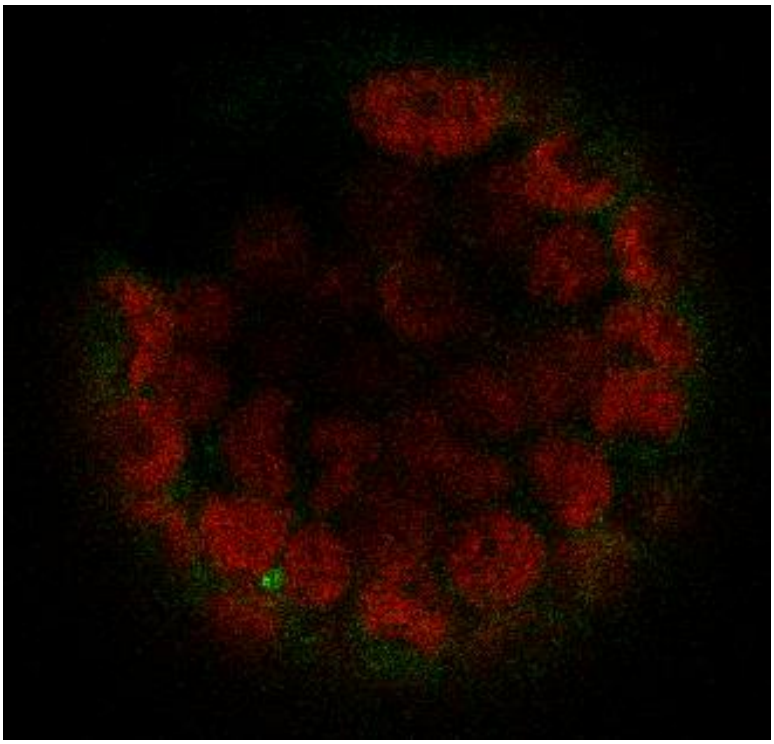

Figure 28. Microscopy of the fusion-protein EYFP-PP4-2 in protoplasts taken after one day of incubation. The fusion-protein displays aggregation shown by the bright green spot.

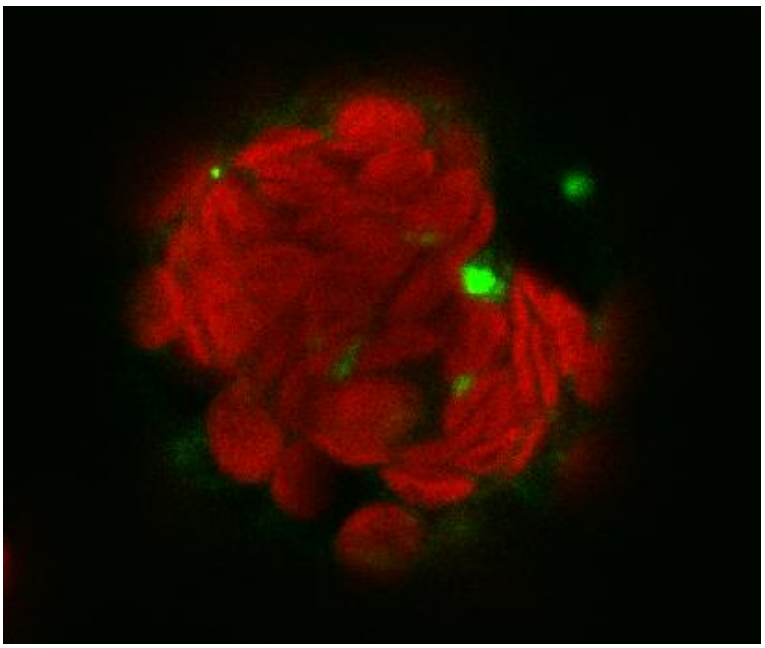

Figure 29. Microscopy of EYFP-PP4-2 fusion-protein in protoplasts taken after two days of incubation. Aggregation of the protein is more evident for the second day than the first in this instance.

3.4.3.3 PP4R2L-EYFP fusion-protein localization

Localization of fusion-protein PP4R2L-EYFP. Figure 30 shows believed cytosolic distribution of the protein after one day of incubation. Figure 31 displays a stronger signal after two days of incubation, also cytosolic in nature. No aggregation is seen for this fusion protein.

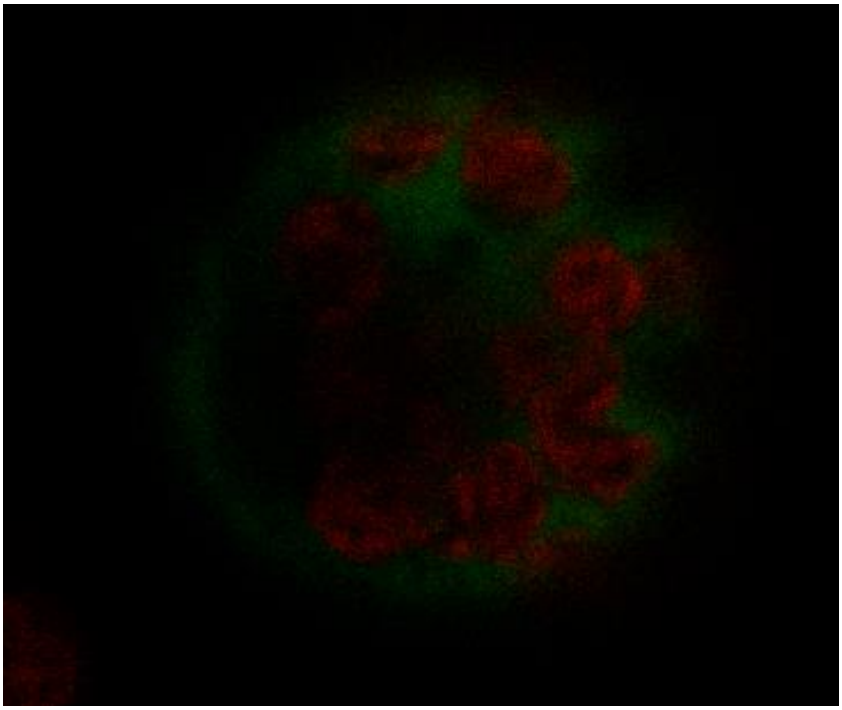

Figure 30. Microscopy of the fusion-protein, PP4R2L-EYFP, in protoplasts taken after one day of incubation. No protein-aggregation is seen for this fusion-protein. It appears widespread throughout the cell.

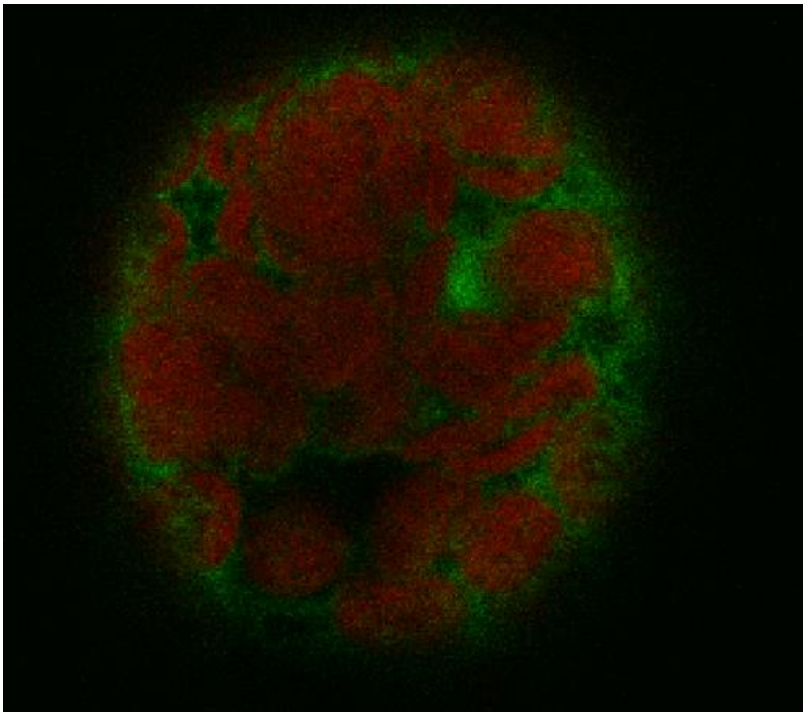

Figure 31. Microscopy of PP4R2L-EYFP fusion-protein after two days of incubation. The protein is more abundant after incubating for two days, as was expected. It still displays a cytosolic localization.

3.4.3.4 EYFP-PP4R2L fusion-protein localization

Believed cytosolic localization of fusion-protein EYFP-PP4R2L can be seen in figure 32 and 33. The signal from figure 33 is stronger, which is to be expected after a two-day incubation period. Similar to the PP4R2L-EYFP fusion-protein, the EYFP-PP4R2L fusion-protein does not display any aggregation in the cell.

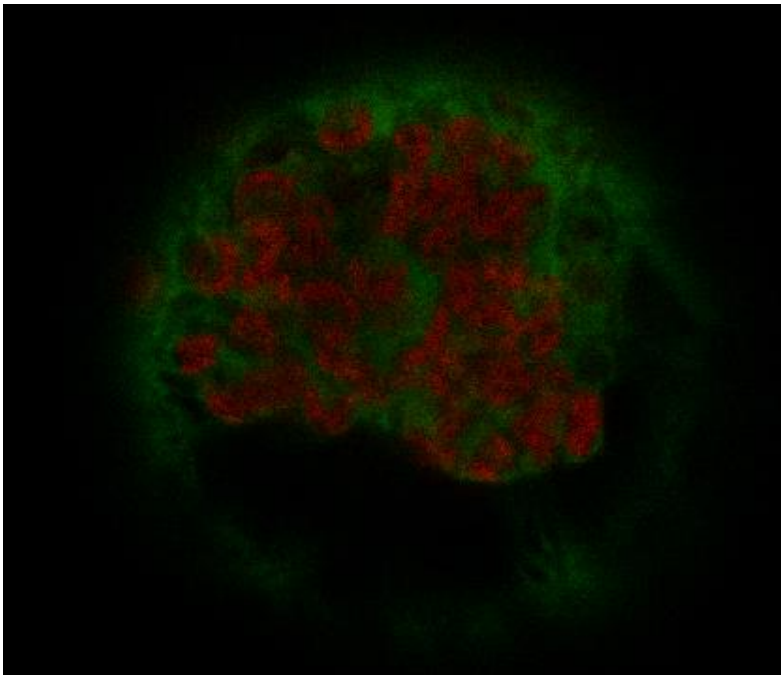

Figure 32. Microscopy of the EYFP-PP4R2L fusion-protein in protoplasts. Taken after incubation for one day. The regulatory protein displays cytosolic localization, the dark area at the bottom is believed to be a vacuole.

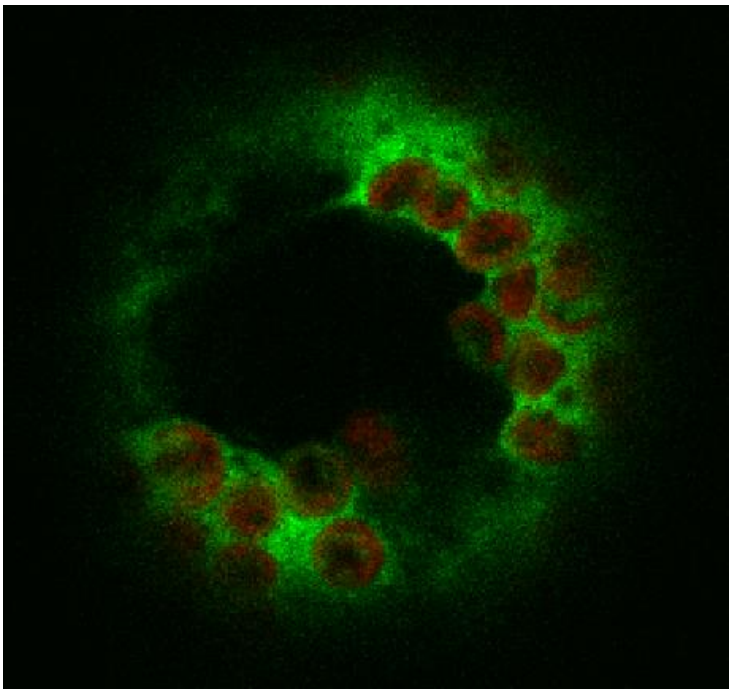

Figure 33. Microscopy of the EYFP-PP4R2L fusion-protein in protoplasts taken after a two-day incubation period. The regulatory protein does not display any aggregation, even after two days of incubation.

3.5 PSY2L and PP4R2L, a study of protein expression in T-DNA plants

The heterozygous PSY2L Salk 048064 mutant plant appear to have about the same amount of RNA as the wild type, and the homozygous PSY2L Salk 125872 mutant sample appears to have less RNA (Figures 34 and 35).

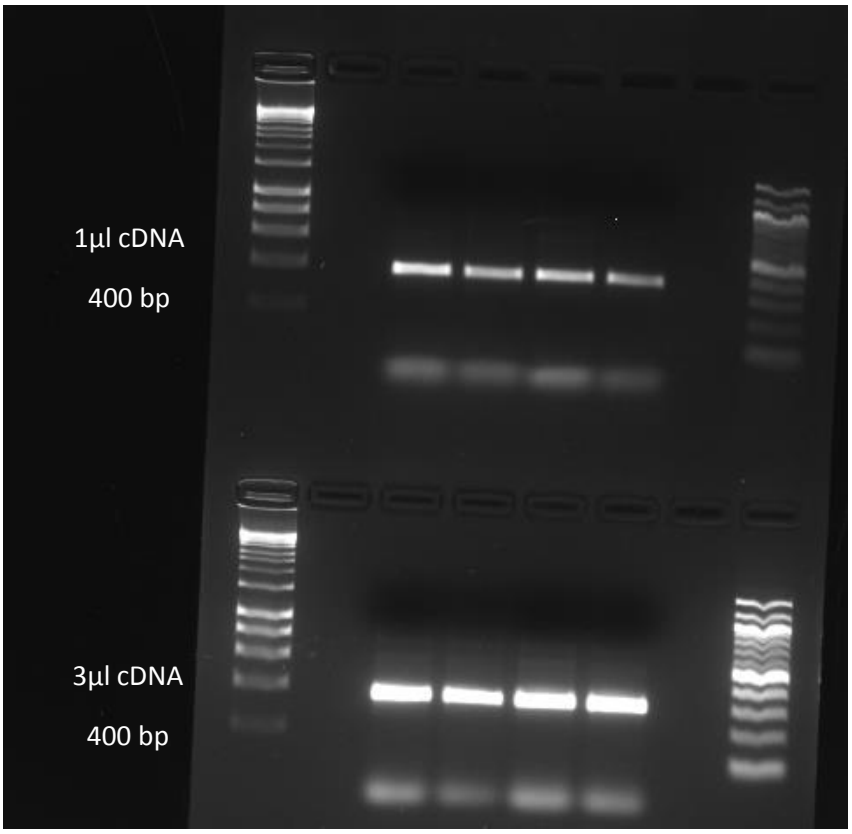

Figure 34. Agarose gel electrophoresis of product of PCR performed after production of cDNA. The gel contains samples run at 30 cycles. Top lane: Well 1-hyperladder, well 2-empty, well 3-Wild type 1 1 µl, well 4-Wild type 2 1 µl, well 5-Salk 048064 1 µl, well 6-Salk 125872 1 µ, well 7-empty, well 8-100 bp ladder. Bottom lane: Well 1-hyperladder, well 2-empty, well 3-Wild type1 3 µl, well 4-Wild type 2 3 µl, well 5-Salk 048064 3 µl, well 6-salk 125872 3 µl, well 7-empty, well 8-100 bp ladder.

The bottom wells of figure 34 displays saturation for the bands. This indicates that 3µl at 30 cycles can not be used to correctly determine the amount of cDNA present. Below follows the results obtained when using plant material from the same plants with only 25 cycles for the PCR (Figure 35).

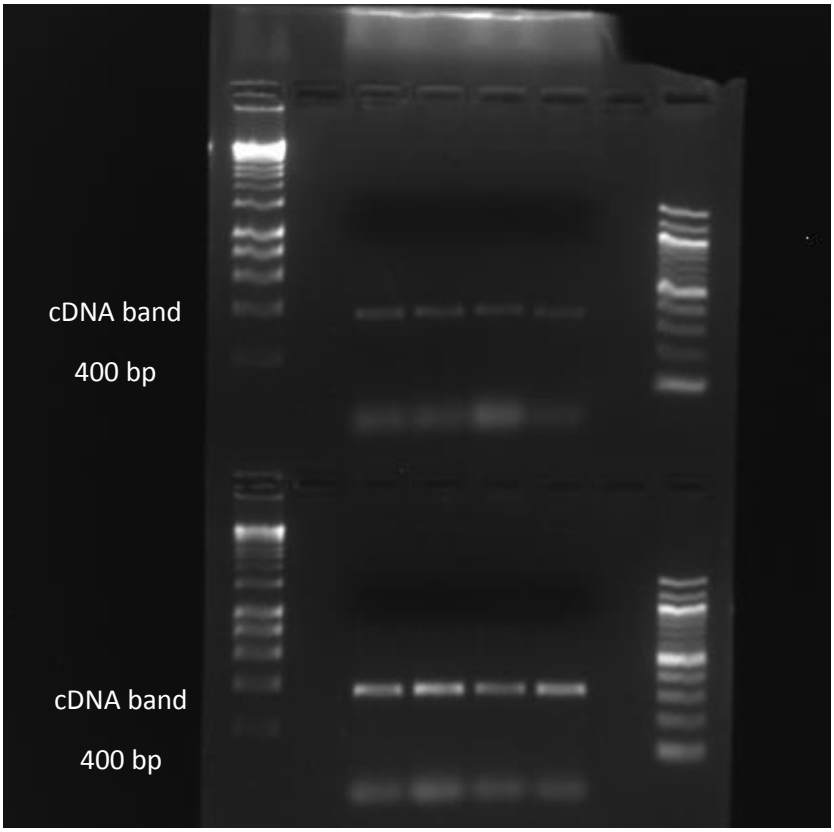

Figure 35. Agarose gel electrophoresis of product of PCR performed after production of cDNA. The gel contains samples run at 25 cycles. Top lane: Well 1-hyperladder, well 2-empty, well 3-Wild type 1 1 µl, well 4-Wild type 2 1 µl, well 5-Salk 048064 1 µl, well 6-Salk 125872 1 µ, well 7-empty, well 8-100 bp ladder. Bottom lane: Well 1-hyperladder, well 2-empty, well 3-Wild type1 3 µl, well 4-Wild type 2 3 µl, well 5-Salk 048064 3 µl, well 6-salk 125872 3 µl, well 7-empty, well 8-100 bp ladder.

The bands shown in figure 35 are very weak, however the trend seen in figure 34 remains true also here. The fourth band on the top lane, belonging to the homozygous PSY2L Salk 125872 plant, seems weaker than the rest. For the bottom lane, all four bands seem to be of somewhat equal intensity.

3.6 Screening results

3.6.1 T-DNA plants

Screening results of PP4R2L Salk 093041 follows below in table 42. The seeds were sown on half strength MS medium with the corresponding screening agent, Kanamycin for pER10 and BASTA for pBA002.

Table 42. Overview of screening results for seeds from heterozygous plants of PP4R2L Salk 093041 for a total of eight plates

Plant nr and plate nr.	Total number of green plants	Total number of yellow plants	Number of seeds sown	Number of surviving plants (fraction)	Number of surviving plants (%)
Plant 3, dish 1	29	20	49	29/49	59%
Plant3, dish 2	34	14	49	34/49	69%
Plant 3, dish 3	37	15	52	37/52	71%
Total for plant 3	100	49	148	100/148	67%
Plant 7, dish 1	38	9	47	38/47	80%
Plant 7, dish 2	32	15	48	32/48	66%
Plant 7, dish 3	40	10	50	40/50	80%
Total for plant 7	110	34	145	110/145	75%
Plant 3, positive control-no kanamycin	40	0	40	40/40	100%
Plant 7, positive control- no kakamycin	40	0	40	40/40	100%

Results obtained form the above experiment indicates the presence of homozygous individuals from the offspring of plant 7, whereas the percentage obtained from plant 3 indicates the presence of heterozygotes (3/4, 75% survival rate being indicative of homozygous and heterozygous plants being present in the offspring, whereas 2/3, 66% survival rate is theorized to indicate only heterozygous plants present in the offspring). The positive control displayed normal growth for all seeds sown on medium containing no antibiotic. A second experiment was conducted to check if the above results were reproducible (results in table 43). The second time six plates were sown for each plant, one positive control with no antibiotic present and five screening plates with antibiotic in the medium, giving a total of 12 plates. The results obtained from the second screening did not reflect the results obtained from the first screening.

Table 43. Overview of screening results for seeds from heterozygous plants of PP4R2L Salk 093041 for a total of 12 plates

Plant nr and plate nr.	Total number of green plants	Total number of yellow plants	Number of seeds sown	Number of surviving plants (fraction)	Number of surviving plants (%)
Plant 3, dish 1	43	6	49	43/49	87%
Plant3, dish 2	43	6	49	43/49	87%
Plant 3, dish 3	36	13	49	36/49	73%
Plant 3, dish 4	37	11	48	37/48	77%
Plant 3, dish 5	43	6	49	43/49	87%
Total for plant 3	202	42	244	202/244	82%
Plant 7, dish 1	37	6	43	37/43	86%
Plant 7, dish 2	43	4	47	43/47	91%
Plant 7, dish 3	37	11	48	37/48	77%
Plant 7, dish 4	41	7	48	41/48	88%
Plant 7, dish 5	38	8	46	38/46	84%
Total for plant 7	196	36	232	196/232	84%
Plant 3, positive control-no kanamycin	40	0	40	40/40	100%
Plant 7, positive control-no kanamycin	40	0	40	40/40	100%

The screening worked efficiently as shown in figure 36, 37 and 38. All wild type plants showed impaired growth and whitening of leaves, whereas only a fraction of the mutant plants displayed the same.

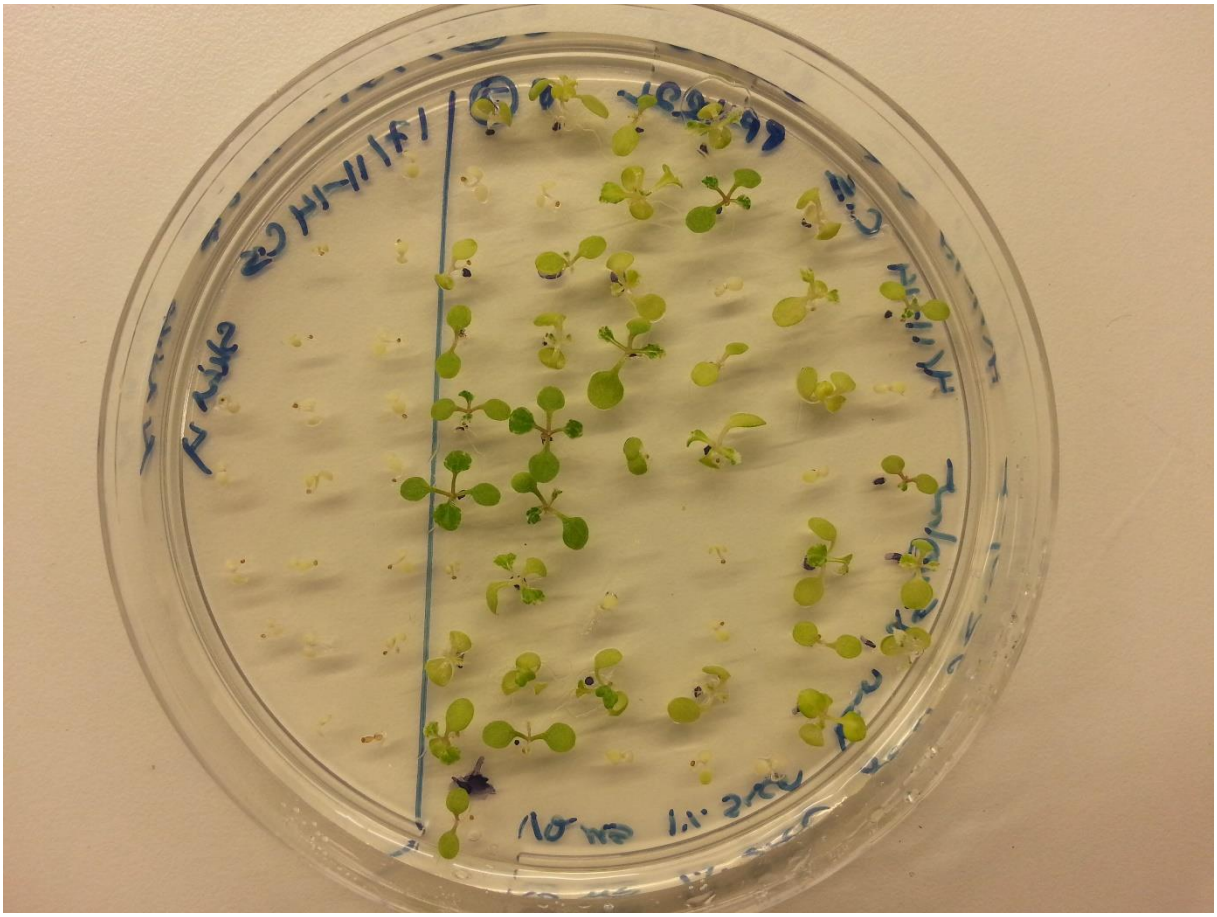

Figure 36. Screening of heterozygous PP4R2L seeds from plant 7. Seeds from the wild type are visible on the left, evidently not surviving on medium containing kanamycin. Green plants are marked with a black marker.

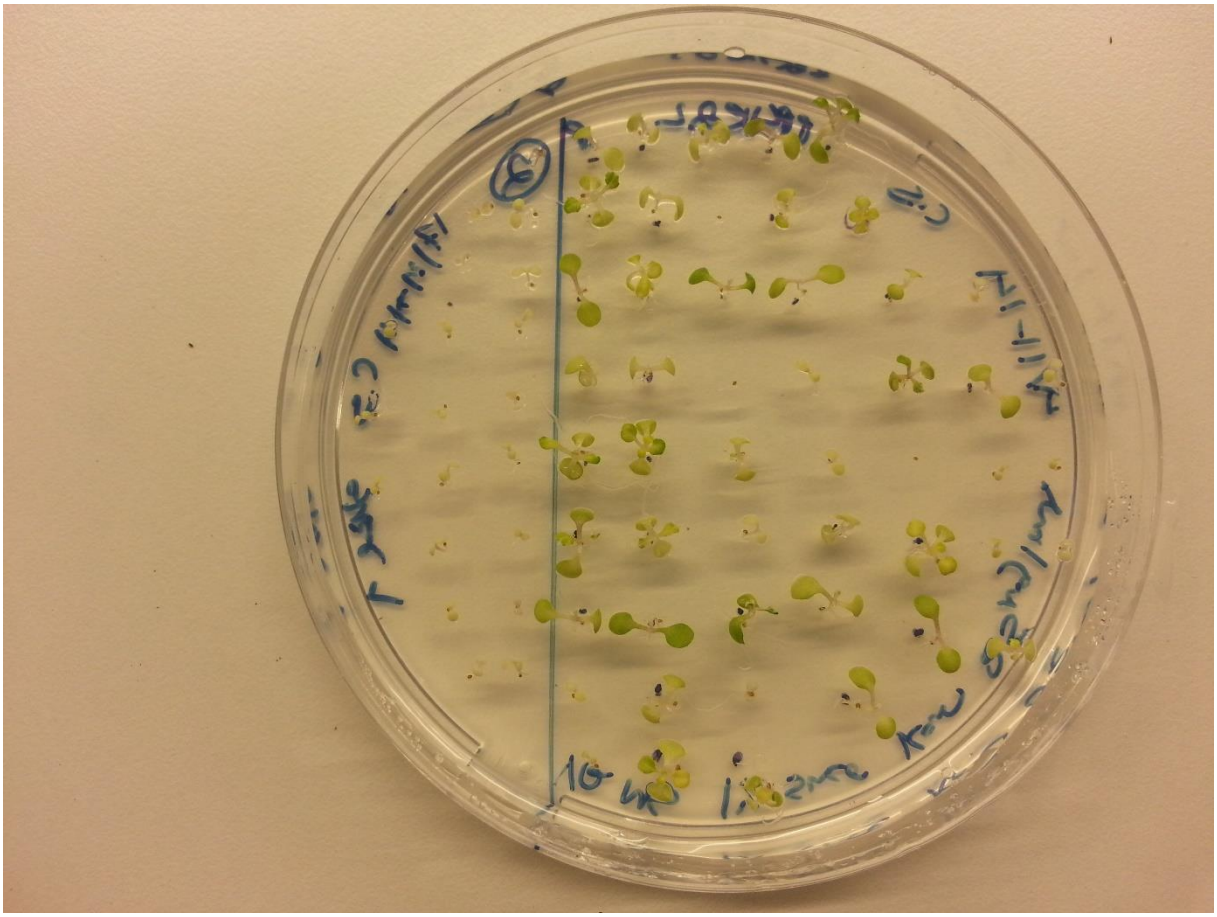

Figure 37. Screening of heterozygous PP4R2L seeds from plant 3. Seeds from the wild type are visible on the left. Green plants are also here marked with a black marker on the backside of the plate.

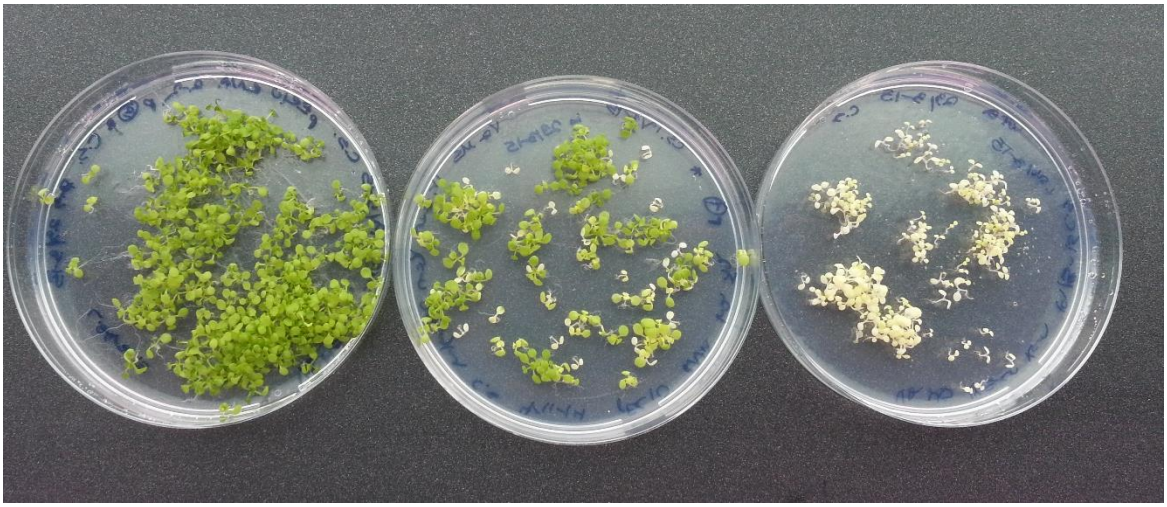

Figure 38. This figure shows an example of one seed batch with all surviving seeds, one seed batch with a fraction of surviving seeds and one negatice control (wild type seeds on screening medium).

3.6.2 MicroRNA plants

Screening of seeds obtained from the first generation (f_1, the original plants being regarded as f_0) yielded no seed batches where every seed survived, one plant giving rise to one batch of seeds. Screening of seed batches from the second generation (f_2) gave the results shown in

table 2 and 3. Expression studies for any plants from a seed batch with only surviving plants (f_2) was not possible to do in the time frame of this thesis, due to the plants still being too young at the time the thesis was being finished. The mutant plants needed more time to produce seeds than their wild type counterparts, as mentioned earlier. Below follows table 44, giving an overview of the second-generation seed screening.

Table 44. Overview of the amount of seed batches (one batch harvested from one plant) used when screening for surviving plants (f_2)

Micro RNA	Vector	Nr. of seed batches with some surviving plants.	Nr. of seed batches with all surviving plants.
1-2	pER10	14	1
2-1	pER10	5	6
2-2	pER10	9	9
NA (vector only)	pER10	7	0
1-1	pBA002	3	2
1-2	pBA002	2	3
2-1	pBA002	3	4
2-2	pBA002	11	6
NA (Vector only)	pBA002	0	3

Phenotype and expression studies for plants from seed batches with all surviving plants could not be done within the time frame of this thesis.

4. DISCUSSION AND OUTLOOK

4.1 T-DNA plants

Some larger differences were expected to become evident for both phenotype and expression studies in regards to the mutants versus the wild type. More so for the PSY2L Salk 048064 and the PP4R2L Salk 093041 mutant due to the inserts being located within the expressed regions of the gene. The survival of the homozygous mutant plants for two of the Salk lines can indicate that T-DNA insertion at those two locations for this study, PSY2L Salk 125872 and PP4R2L Salk 093041 does not prove fatal to the plant in question. It is instead thought to merely impair the growth of the plants. The mechanisms behind this impaired growth remains unclear. Since the only confirmed homozygous plant for PSY2L Salk 048064 died without producing any seeds, it is possible that the T-DNA insert at this location could be fatal. For this Salk line, the insert was in exon 3 of a total of 25 exons. More work needs to be done to try to breed forth a surviving individual confirmed to be a homozygous individual for this particular line. If it is indeed impossible to procure a homozygous individual for this line, the logical conclusion remains that it is important for exon 3 to be intact for the regulator to work properly. With only one individual to refer to, no real conclusion can be drawn at this time. Repetition of this study where no homozygous plants for this line were to be found can confirm the lethality of an insertion at exon 3 for PSY2L. Seeing a similar phenotype for the mutants of all Salk lines, shows that incorrect regulation of PP4-2 impairs the plant's growth, and is less than ideal for the plant. The expression of PP4-2 for PSY2L Salk line 125872 was slightly lower than that of the wild type, meaning that the insert at that particular location (downstream of translation) impairs mRNA production to some degree. Due to this being a homozygous plant, no wild type allele was present to make up for the decrease in protein production. The decrease of mRNA production introduced by the T-DNA insert does not appear to be very substantial when looking at the results of the expression study. A repetition of the experiment is needed to be able to further strengthen this theory and to be able to say anything about the effect on expression for the other two Salk lines. The difficulty in finding a homozygous plant for PSY2L Salk 048064 could indicate that the T-DNA insert is more damaging to the plant for that location (exon 3) than for the PSY2L Salk 125872 location (downstream of translation). However, it could also be a random effect due to not all seeds from a mother plant being sown on soil. The possibility that there are homozygous plants within the remaining seeds, but none of them were sown is present. Or, as aforementioned; the insert at exon 3 could prove to be fatal to a homozygous plant. More research is needed to be able to say something about this theoretical fatality, whether it can occur early or later in the plants lifecycle. Time did not allow for expression studies to be done upon the PP4R2L Salk 093041 T-DNA mutants. Checking the expression of PP4-2, and not the expression of the regulators as was done in this thesis, in the same T-DNA plants can also be done at a later time. Seeing if the disruption of the regulators cause a higher expression for PP4-2 than what is normally seen for the wild type could give some useful information, and is a possible way forward in regards to this project.

When screening plants obtained from a homozygous plant (confirmed by genotyping), it was commonly found that not all plants survived on the screening medium. This low number of surviving individuals after screening these plants obtained from a homozygous individual can indicate that there could have been some cross contamination occurring during the harvesting

procedure. After being harvested from the mother plant, the seeds were left in open containers to dry for 24 h. This period is thought to be a window for possible cross contamination. Cross contamination could also have occurred during the actual harvesting process, however, this is not believed to happen at such a high rate as the one observed here.

4.2 AmiRNA plants

No significant differences were observed when watching the mutant plants growth and phenotype in regards to each other. The mutants for both vectors displayed impaired growth when compared to the wild type. The inducible pER10 vector mutants were expected to appear identical to the wild type plants until the amiRNA production was induced by the plant being exposed to estradiol. The fact that these plants displayed impaired growth without being exposed to the chemical gave rise to new questions. Is the vector in itself somehow responsible for the impaired growth of the plants? Given that the mutant plants were kept in the same room and given the same treatment as the wild type plants, when the wild type plants displayed normal growth, the idea that the environment was responsible for the impaired growth was discarded. The mutant plants were also sown in a similar fashion as the wild type; an equal amount of seeds in each pot. Overpopulation and competing for nutrients would not be an issue, and was not thought to have an impact on the growth of the mutant plants. No significant differences were observed for the mutants bearing only the vectors when compared to the mutants with both vector and amiRNA. These results were not as expected, as the mutants bearing only vectors should display a phenotype similar to the wild type. Observations like the ones stated above gave rise to the idea that the vector in itself might have some influence on the plant growth, as mentioned earlier. The two vectors do not in theory contain any elements that could cause this effect, however, theory is often tested by practice. Further repetitions of the study are needed to decide whether or not the vector might impair the growth of the mutant plants, as this study only brought forth two generations of mutant plants. Not all seeds were screened and sown on soil, so the road ahead could be to check if these results are reproducible before drawing any conclusion. During the development of this thesis, time did not allow for any expression studied to be performed on these plants, and no attempt was done to induce the inducible plants with estradiol. The decreased growth rate of the mutant plants made it impossible to grow forth more than two generations of the mutants. Seeds are available for all plants of both generations to continue this study. Producing more plant material for expression studies, and study the effects of inducing the mutant plants with estradiol could be a step forwards in regards to this project.

4.3 Molecular cloning and localization study

The localization results for PP4-2 and PP4R2L showed that both fusion-proteins for both genes was located in the cytosol with PP4-2 aggregating in the cell.
The aggregation of PP4-2 displayed in this study can be thought to be caused by the protein being toxic in large amounts. If the protein did appear to have a detrimental effect in large amounts, the cell would benefit from collecting the abundant proteins in vacuoles, to keep them away from any cytosolic elements, or in an attempt to have the excess proteins destroyed. Such an action would give rise to areas of high protein content as seen in figures 26, 27, 28 and 29. Due to the regulatory protein, PP4R2L, not being seen in higher amounts at certain places in the cell, one could surmise that PP4-2 does not act upon said areas. Due to the nature of the localization study, these areas were not identified in the cell. Tagging other localizations in the cell along with the proteins to be studied could prove useful for future studies. Any conclusion could not be drawn due to the localization study only being

performed once. Further studies are needed to be able to say anything with any certainty regarding the localization of the fusion-proteins in question. The time allotted for this thesis did not allow for further studies on localization for PP4-2 or PP4R2L. These results only give a small indication to the localization of the protein. The fact that the results are quite uniform gives rise to the thought that they might give an indication comparable to the actual event. The results obtained from this localization study can indicate that both PP4-2 and the one regulator studied here; PP4R2L, are present in the cytosol at a specific time. Whether they are active there throughout the cell cycle, or are transported to different locations at different times remains to be examined. Doing localization studies at different times for many more protoplasts is ideal to see if the protein is localized at different places at different times. After localization studies have been done, studying if the protein does indeed interact with the believed regulators would be the next logical step. This can be achieved by protein-protein interaction analysis. Protein-protein interaction analysis procedures available today are, among others; techniques such as bimolecular fluorescence complementation (proteins believed to interact are tagged with one fluorescent tag and one activator tag that will give off light if they come in close proximity to each other), protein complex immunoprecipitation (protein complexes or interacting proteins are precipitated out of solution using an antibody targeting one of the proteins in the complex), label transfer (a label is transferred from one protein to the interacting protein) or tandem affinity purification (beads are used to pull the protein to be examined out of solution, any interacting protein will be pulled out with it). The discrepancy found between the results obtained using subcellular localization prediction software and the results from the microscopy, emphasizes the fact that actual biological events are difficult to predict given the tools available today. Continuing with the molecular cloning for the PSY2L gene can give useful information about the localization of this putative regulator for PP4-2, and should be done for future studies.

As it stands now, no definite conclusion can be drawn for any of the three experiments performed throughout this thesis. More work is needed, and many more repetitions must be performed to be able to confidently say anything with some degree of certainty, in regards to the workings of PP4-2 and its regulators in *Arabidopsis thaliana*.

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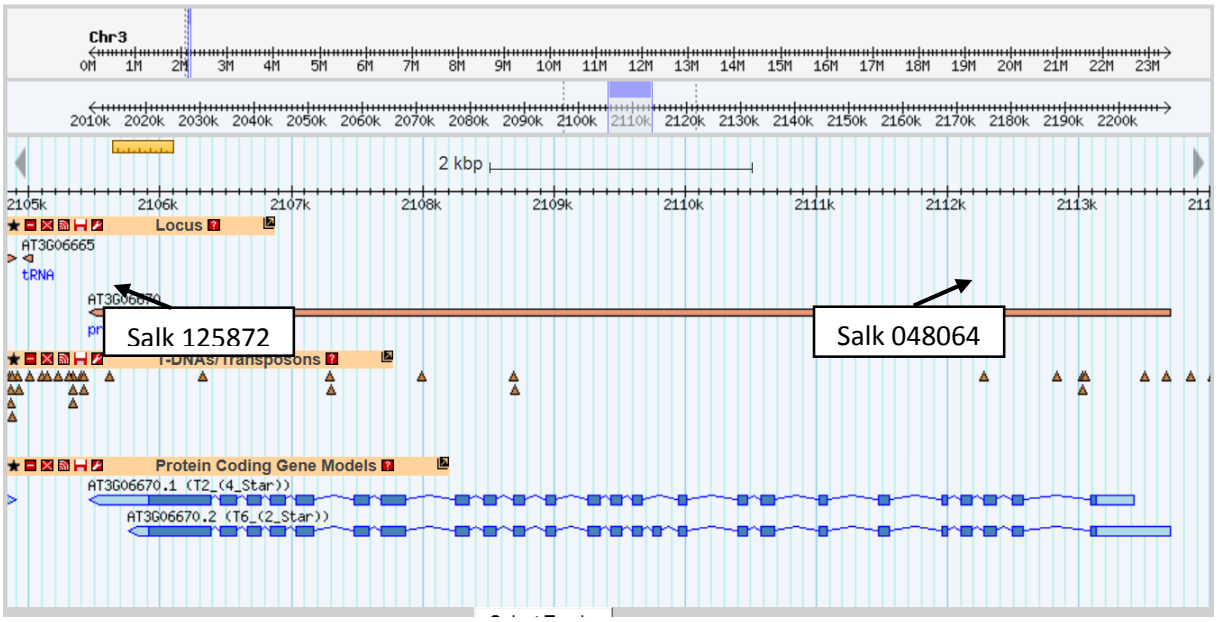
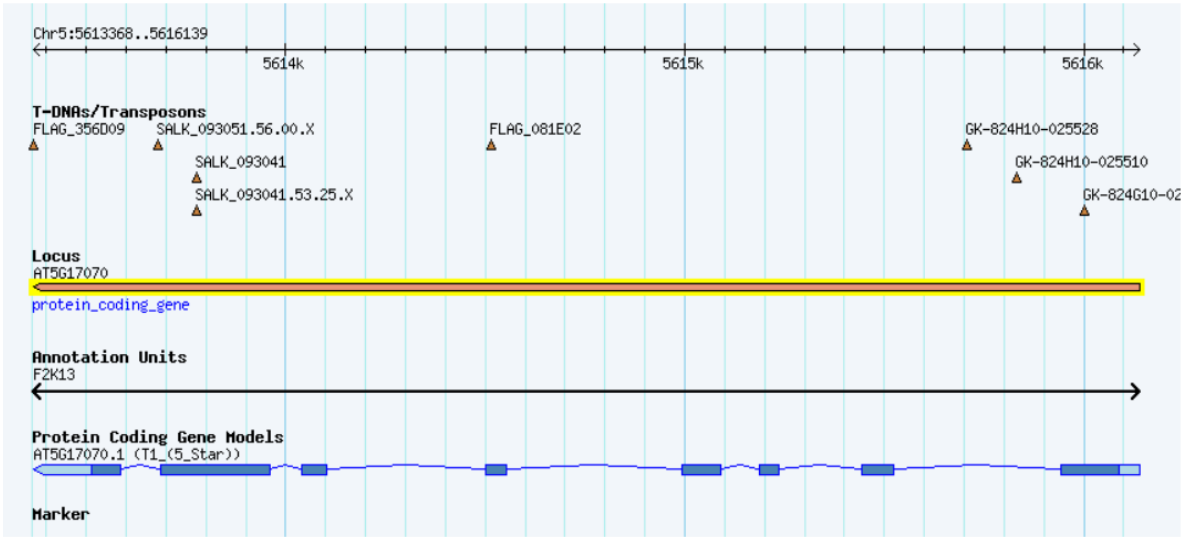
APPENDIX

A1- Overview of *Arabidopsis thaliana* At3g06670 PSY2L T-DNA insertion lines: Salk 048064 and Salk 125872



A-2 Overview of *Arabidopsis thaliana* At5g17070 PP4R2L T-DNA insertion line Salk 093041



A-3 Multiple sequence alignment from sequencing, PP4-2-EYFP result; one mutation

```
3_PP4_2_VF_1_AK93f  ACCATTTACGAACGATAGCCATGGGTATGTCAGACCTAGACAAGCAAATA
PP4_2               --------------------------ATGTCAGACCTAGACAAGCAAATA
                                               ************************

3_PP4_2_VF_1_AK93f  GAGCAGCTTAAACGCTGCGAGGCTTTGAAGGAATCAGAAGTGAAGGCTCT
PP4_2               GAGCAGCTTAAACGCTGCGAGGCTTTGAAGGAATCAGAAGTGAAGGCTCT
                    **************************************************

3_PP4_2_VF_1_AK93f  TTGTCTTAAAGCTATGGAGATTCTAGTTGAAGAGAGCAATGTTCAAAGAG
PP4_2               TTGTCTTAAAGCTATGGAGATTCTAGTTGAAGAGAGCAATGTTCAAAGAG
                    **************************************************

3_PP4_2_VF_1_AK93f  TCGATGCTCCTGCCACTATATGTGGCGACATTCATGGACAGTTCTATGAC
PP4_2               TCGATGCTCCTGTCACTATATGTGGCGACATTCATGGACAGTTCTATGAC
                    ************ ***********************************

3_PP4_2_VF_1_AK93f  ATGAAAGAGCTTTTCAAAGTTGGGGGTGATTGCCCTAAGACCAATTATTT
PP4_2               ATGAAAGAGCTTTTCAAAGTTGGGGGTGATTGCCCTAAGACCAATTATTT
                    **************************************************

3_PP4_2_VF_1_AK93f  GTTTCTTGGAGATTTTGTTGACCGAGGTTTTTATTCGGTTGAGACATTTC
PP4_2               GTTTCTTGGAGATTTTGTTGACCGAGGTTTTTATTCGGTTGAGACATTTC
                    **************************************************

3_PP4_2_VF_1_AK93f  TACTTCTTCTAGCTCTCAAGGTTAGATATCCAGACCGTATAACTCTCATT
PP4_2               TACTTCTTCTAGCTCTCAAGGTTAGATATCCAGACCGTATAACTCTCATT
                    **************************************************

3_PP4_2_VF_1_AK93f  AGAGGGAACCACGAGAGCCGGCAGATTACGCAGGTATATGGATTTTATGA
PP4_2               AGAGGGAACCACGAGAGCCGGCAGATTACGCAGGTATATGGATTTTATGA
                    **************************************************

3_PP4_2_VF_1_AK93f  TGAGTGTCTGCGTAAATATGGCTCTGTAAATGTTTGGAGATACTGCACAG
PP4_2               TGAGTGTCTGCGTAAATATGGCTCTGTAAATGTTTGGAGATACTGCACAG
                    **************************************************

3_PP4_2_VF_1_AK93f  ATATCTTTGACTACTTGAGTCTTTCAGCTCTTGTCGAGAACAAGATATTT
PP4_2               ATATCTTTGACTACTTGAGTCTTTCAGCTCTTGTCGAGAACAAGATATTT
                    **************************************************

3_PP4_2_VF_1_AK93f  TGTGTTCATGGAGGTCTCTCTCCAGCTATTATGACTCTAGACCAGATCAG
PP4_2               TGTGTTCATGGAGGTCTCTCTCCAGCTATTATGACTCTAGACCAGATCAG
                    **************************************************

3_PP4_2_VF_1_AK93f  GGCTATTGATCGAAAGCAAGAAGTACCACATGATGGTGCTATGTGTGATC
PP4_2               GGCTATTGATCGAAAGCAAGAAGTACCACATGATGGTGCTATGTGTGATC
                    **************************************************

3_PP4_2_VF_1_AK93f  TTTTATGGTCTGATCCAGAAGATATTGTCGATGGTTGGGGATTGAGTCCC
PP4_2               TTTTATGGTCTGATCCAGAAGATATTGTCGATGGTTGGGGATTGAGTCCC
                    **************************************************

3_PP4_2_VF_1_AK93f  CGTGGTGCCGGATTCCTTTTCGGCGGCAGTGTTGTTACGTCTTTTAACCA
PP4_2               CGTGGTGCCGGATTCCTTTTCGGCGGCAGTGTTGTTACGTCTTTTAACCA
                    **************************************************

3_PP4_2_VF_1_AK93f  CTCAAACAACATTGATTACATATGTCGAGCTCATCAGCTAGTGATGGAAG
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PP4_2               CTCAAACAACATTGATTACATATGTCGAGCTCATCAGCTAGTGATGGAAG
                    **************************************************

3_PP4_2_VF_1_AK93f  GTTACAAATGGATGTTCAATAGCCAGATAGTCACTGTTTGGTCTGCCCCA
PP4_2               GTTACAAATGGATGTTCAATAGCCAGATAGTCACTGTTTGGTCTGCCCCA
                    **************************************************

3_PP4_2_VF_1_AK93f  AATTACTGTTATAGATGCGGTAATGTAGCTGCAATTCTAGAGCTCGATGA
PP4_2               AATTACTGTTATAGATGCGGTAATGTAGCTGCAATTCTAGAGCTCGATGA
                    **************************************************

3_PP4_2_VF_1_AK93f  GAATCTAAACAAAGAGTTTCGTGTCTTCGATGCAGCCCCACAAGAATCGA
PP4_2               GAATCTAAACAAAGAGTTTCGTGTCTTCGATGCAGCCCCACAAGAATCGA
                    **************************************************

3_PP4_2_VF_1_AK93f  GAGGAGCTCTAGCCAAGAAACCTGCACCTGATTATTTCCTGGCGGCCGCT
PP4_2               GAGGAGCTCTAGCCAAGAAACCTGCACCTGATTATTTCCTG---------
                    ****************************************

3_PP4_2_VF_1_AK93f  GCCGCGGCAATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCC
PP4_2               -------------------------------------------------


3_PP4_2_VF_1_AK93f  CATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCMCAAGTTCAGCGTGT
PP4_2               ------------------------------------------------T--
                                                                    *

3_PP4_2_VF_1_AK93f  CCGGCRAGGGCAAGGGCGATGCCMCCTACGGCAAGYTGACCCTGAAATTC
PP4_2               -------------------------------------------------


3_PP4_2_VF_1_AK93f  AT
PP4_2               GA
```

A-4 Multiple sequence alignment from sequencing, PP4R2L2-EYFP; positive result

```
7_PP4R2L_DECR_57_AK93  AATTTTCACCATTTACGAACGATAGCCATGGGTATGGAGAATCCGTCATC
PP4R2L                 ---------------------------------ATGGAGAATCCGTCATC
                                                         *****************

7_PP4R2L_DECR_57_AK93  ATCGGAAACTTCCGAGATTTCCTCCGTCGTTCATCCCAATGACGGCGTTC
PP4R2L                 ATCGGAAACTTCCGAGATTTCCTCCGTCGTTCATCCCAATGACGGCGTTC
                       **************************************************

7_PP4R2L_DECR_57_AK93  ATCCCAATGACGGCGTTCATCCCAATGACGGCGTTCAACGCCAGGATCAC
PP4R2L                 ATCCCAATGACGGCGTTCATCCCAATGACGGCGTTCAACGCCAGGATCAC
                       **************************************************

7_PP4R2L_DECR_57_AK93  GCCGTCCTTCCCGAAGTTCTTGAGCATCCTGGAGCTGAGCAGATAGCAGA
PP4R2L                 GCCGTCCTTCCCGAAGTTCTTGAGCATCCTGGAGCTGAGCAGATAGCAGA
                       **************************************************

7_PP4R2L_DECR_57_AK93  TATGTCTGAGGAAGAAGTAAAGCGCACATTAGAAGCTGTAGCATCTACTG
PP4R2L                 TATGTCTGAGGAAGAAGTAAAGCGCACATTAGAAGCTGTAGCATCTACTG
                       **************************************************

7_PP4R2L_DECR_57_AK93  GGAAGTTCTGGCAGGACTGGGAGATACTAAAGGGAACGCTATCGTACTGG
PP4R2L                 GGAAGTTCTGGCAGGACTGGGAGATACTAAAGGGAACGCTATCGTACTGG
                       **************************************************

7_PP4R2L_DECR_57_AK93  TTGAAGAAGGTTCTATCGGAATATTCTGAGGCAAAAATGACGGATGAGCA
PP4R2L                 TTGAAGAAGGTTCTATCGGAATATTCTGAGGCAAAAATGACGGATGAGCA
                       **************************************************

7_PP4R2L_DECR_57_AK93  ACAAAAGGAAGCTCTTGGAGAACCATATTCAGAGCTGGTTAGTCGATTGG
PP4R2L                 ACAAAAGGAAGCTCTTGGAGAACCATATTCAGAGCTGGTTAGTCGATTGG
                       **************************************************
```

```
7_PP4R2L_DECR_57_AK93  ATGAAGCCCTTCTTAGATTCGATGATGGACCTCCATTTACATTGCAGAGA
PP4R2L                 ATGAAGCCCTTCTTAGATTCGATGATGGACCTCCATTTACATTGCAGAGA
                       **************************************************

7_PP4R2L_DECR_57_AK93  CTCTGTGAGATCCTTTTGGCTGCAAGGAGCATCTACCCAAAGCTCTCAAA
PP4R2L                 CTCTGTGAGATCCTTTTGGCTGCAAGGAGCATCTACCCAAAGCTCTCAAA
                       **************************************************

7_PP4R2L_DECR_57_AK93  ACTCGCTCTTGCATTAGAAAAGAATCTGTTGGTTACTTCTATGTTAGCCA
PP4R2L                 ACTCGCTCTTGCATTAGAAAAGAATCTGTTGGTTACTTCTATGTTAGCCA
                       **************************************************

7_PP4R2L_DECR_57_AK93  TCAGTACAGAGCCACAATCACAAACCACTGAGGATCCAAACACAGCAACC
PP4R2L                 TCAGTACAGAGCCACAATCACAAACCACTGAGGATCCAAACACAGCAACC
                       **************************************************

7_PP4R2L_DECR_57_AK93  TCAGAGACAATAACATCTGCTGCAAGTTGCGATCCAAATGTAATTGAGTC
PP4R2L                 TCAGAGACAATAACATCTGCTGCAAGTTGCGATCCAAATGTAATTGAGTC
                       **************************************************

7_PP4R2L_DECR_57_AK93  AATGGGAGGCGATAAGGATGAGATAATGACAGAGGTAGAAGAAGCAGATG
PP4R2L                 AATGGGAGGCGATAAGGATGAGATAATGACAGAGGTAGAAGAAGCAGATG
                       **************************************************

7_PP4R2L_DECR_57_AK93  TTGATGACGCAATGACTGTTGACATGGAAACAATCGATGAACCATCAGAG
PP4R2L                 TTGATGACGCAATGACTGTTGACATGGAAACAATCGATGAACCATCAGAG
                       **************************************************

7_PP4R2L_DECR_57_AK93  ACAATGACGACCACGAGTGAGAGTGAGACTCTAAGCGAAAACACTGCTGC
PP4R2L                 ACAATGACGACCACGAGTGAGAGTGAGACTCTAAGCGAAAACACTGCTGC
                       **************************************************

7_PP4R2L_DECR_57_AK93  ACAACCATTATCGGATTCAATGGTGGCAGAGGAAGGAGATTCACGGTTGC
PP4R2L                 ACAACCATTATCGGATTCAATGGTGGCAGAGGAAGGAGATTCACGGTTGC
                       **************************************************

7_PP4R2L_DECR_57_AK93  CTACAACGTGTGCCGCGGCCGCTGCCGCGGCAATGGTGAGCAAGGGCGAG
PP4R2L                 CTACAACGTGTGCC--------------------------------------
                       **************

7_PP4R2L_DECR_57_AK93  GAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGT
PP4R2L                 --------------------------------------------------


7_PP4R2L_DECR_57_AK93  AAACGGCCACAAGTTCAGCGTGTCCGGCAAGGGCCAAGGCGATGCCACCT
PP4R2L                 -------------------------------------------------T
                                                                       *

7_PP4R2L_DECR_57_AK93  ACGGCAAGCTGACCCTGAAGTTCATCTGCMCCM
PP4R2L                 AG-------------------------------
                       *
```

A-5 Multiple sequence alignment from sequencing, EYFP-PP4R2L; positive result

```
2_Ey_PP4R2_6_EYFP_F  TCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCA
PP4R2L_CDS           ------------------------------------------------


2_Ey_PP4R2_6_EYFP_F  TGGACGAGCTGTACAAGGCGGCCGCTATGGAGAATCCGTCATCATCGGAA
PP4R2L_CDS           ---------------------------ATGGAGAATCCGTCATCATCGGAA
                                                 ************************

2_Ey_PP4R2_6_EYFP_F  ACTTCCGAGATTTCCTCCGTCGTTCATCCCAATGACGGCGTTCATCCCAA
PP4R2L_CDS           ACTTCCGAGATTTCCTCCGTCGTTCATCCCAATGACGGCGTTCATCCCAA
                     **************************************************
```

```
2_Ey_PP4R2_6_EYFP_F  TGACGGCGTTCATCCCAATGACGGCGTTCAACGCCAGGATCACGCCGTCC
PP4R2L_CDS           TGACGGCGTTCATCCCAATGACGGCGTTCAACGCCAGGATCACGCCGTCC
                     **************************************************

2_Ey_PP4R2_6_EYFP_F  TTCCCGAAGTTCTTGAGCATCCTGGAGCTGAGCAGATAGCAGATATGTCT
PP4R2L_CDS           TTCCCGAAGTTCTTGAGCATCCTGGAGCTGAGCAGATAGCAGATATGTCT
                     **************************************************

2_Ey_PP4R2_6_EYFP_F  GAGGAAGAAGTAAAGCGCACATTAGAAGCTGTAGCATCTACTGGGAAGTT
PP4R2L_CDS           GAGGAAGAAGTAAAGCGCACATTAGAAGCTGTAGCATCTACTGGGAAGTT
                     **************************************************

2_Ey_PP4R2_6_EYFP_F  CTGGCAGGACTGGGAGATACTAAAGGGAACGCTATCGTACTGGTTGAAGA
PP4R2L_CDS           CTGGCAGGACTGGGAGATACTAAAGGGAACGCTATCGTACTGGTTGAAGA
                     **************************************************

2_Ey_PP4R2_6_EYFP_F  AGGTTCTATCGGAATATTCTGAGGCAAAAATGACGGATGAGCAACAAAAG
PP4R2L_CDS           AGGTTCTATCGGAATATTCTGAGGCAAAAATGACGGATGAGCAACAAAAG
                     **************************************************

2_Ey_PP4R2_6_EYFP_F  GAAGCTCTTGGAGAACCATATTCAGAGCTGGTTAGTCGATTGGATGAAGC
PP4R2L_CDS           GAAGCTCTTGGAGAACCATATTCAGAGCTGGTTAGTCGATTGGATGAAGC
                     **************************************************

2_Ey_PP4R2_6_EYFP_F  CCTTCTTAGATTCGATGATGGACCTCCATTTACATTGCAGAGACTCTGTG
PP4R2L_CDS           CCTTCTTAGATTCGATGATGGACCTCCATTTACATTGCAGAGACTCTGTG
                     **************************************************

2_Ey_PP4R2_6_EYFP_F  AGATCCTTTTGGCTGCAAGGAGCATCTACCCAAAGCTCTCAAAACTCGCT
PP4R2L_CDS           AGATCCTTTTGGCTGCAAGGAGCATCTACCCAAAGCTCTCAAAACTCGCT
                     **************************************************

2_Ey_PP4R2_6_EYFP_F  CTTGCATTAGAAAAGAATCTGTTGGTTACTTCTATGTTAGCCATCAGTAC
PP4R2L_CDS           CTTGCATTAGAAAAGAATCTGTTGGTTACTTCTATGTTAGCCATCAGTAC
                     **************************************************

2_Ey_PP4R2_6_EYFP_F  AGAGCCACAATCACAAACCACTGAGGATCCAAACACAGCAACCTCAGAGA
PP4R2L_CDS           AGAGCCACAATCACAAACCACTGAGGATCCAAACACAGCAACCTCAGAGA
                     **************************************************

2_Ey_PP4R2_6_EYFP_F  CAATAACATCTGCTGCAAGTTGCGATCCAAATGTAATTGAGTCAATGGGA
PP4R2L_CDS           CAATAACATCTGCTGCAAGTTGCGATCCAAATGTAATTGAGTCAATGGGA
                     **************************************************

2_Ey_PP4R2_6_EYFP_F  GGCGATAAGGATGAGATAATGACAGAGGTAGAAGAAGCAGATGTTGATGA
PP4R2L_CDS           GGCGATAAGGATGAGATAATGACAGAGGTAGAAGAAGCAGATGTTGATGA
                     **************************************************

2_Ey_PP4R2_6_EYFP_F  CGCAATGACTGTTGACATGGAAACAATCGATGAACCATCAGAGACAATGA
PP4R2L_CDS           CGCAATGACTGTTGACATGGAAACAATCGATGAACCATCAGAGACAATGA
                     **************************************************

2_Ey_PP4R2_6_EYFP_F  CGACCACGAGTGAGAGTGAGACTCTAAGCGAAAACACTGCTGCACAACCA
PP4R2L_CDS           CGACCACGAGTGAGAGTGAGACTCTAAGCGAAAACACTGCTGCACAACCA
                     **************************************************

2_Ey_PP4R2_6_EYFP_F  TTATCGGATTCAATGGTGGCAGAGGAAGGAGATTCACGGTTGCCTACAAC
PP4R2L_CDS           TTATCGGATTCAATGGTGGCAGAGGAAGGAGATTCACGGTTGCCTACAAC
                     **************************************************

2_Ey_PP4R2_6_EYFP_F  GTGTGCCTAGCCGCGGTCTAGAGTCCGCAAAAATCACCAGTCTCTCTCTA
PP4R2L_CDS           GTGTGCCT----------------------------------------
                     ********

2_Ey_PP4R2_6_EYFP_F  CAAATCTATCTCTCTCTATTTTCTCCAGAATAATGTTGTGAGTAGTTCCA
PP4R2L_CDS           ----------------------------------------------------
```

```
2_Ey_PP4R2_6_EYFP_F  GATAAGGGAATTAGGGTYCTTATAGGGTTTCGCTCATGTGTTGAGCATAT
PP4R2L_CDS           --------------------------------------------------

2_Ey_PP4R2_6_EYFP_F  AAGAACCCTTARGAAGGATTTGGATTTGGAAAATACTTCTATCAATAAAA
PP4R2L_CDS           --------------------------------------------------

2_Ey_PP4R2_6_EYFP_F  TTT
PP4R2L_CDS           -AG
```

A-6 Multiple sequence alignment from sequencing, PSY2L-EYFP; positive result

Forward primer

```
1_PSY2L_DECR_35F_AK93  AATTTTCACCATTTACGAACGATAGCCATGGGTATGGGCGCTCCGGAAAA
PSY2L                  ---------------------------------ATGGGCGCTCCGGAAAA
                                                         *****************

1_PSY2L_DECR_35F_AK93  GTCTCAATCTAATACCAATTCGATGCAGAGAGTGAAAGTCTATCATTTGA
PSY2L                  GTCTCAATCTAATACCAATTCGATGCAGAGAGTGAAAGTCTATCATTTGA
                       **************************************************

1_PSY2L_DECR_35F_AK93  ATGAAGATGGTAAATGGGATGATCGAGGAACTGGGCACGTAAGCATCGAC
PSY2L                  ATGAAGATGGTAAATGGGATGATCGAGGAACTGGGCACGTAAGCATCGAC
                       **************************************************

1_PSY2L_DECR_35F_AK93  TTTGTGGAGCGATCTGAAGAACTCAGTCTATGTGTAATTGATGAAGAAGA
PSY2L                  TTTGTGGAGCGATCTGAAGAACTCAGTCTATGTGTAATTGATGAAGAAGA
                       **************************************************

1_PSY2L_DECR_35F_AK93  TAACGAGACGTTACTTGTTCATCCCATCAACCCTGAGGATATTTACAGGA
PSY2L                  TAACGAGACGTTACTTGTTCATCCCATCAACCCTGAGGATATTTACAGGA
                       **************************************************

1_PSY2L_DECR_35F_AK93  AACAAGAAGACACAATAATCTCATGGAGAGACCCAGAGCGCTCAACAGAA
PSY2L                  AACAAGAAGACACAATAATCTCATGGAGAGACCCAGAGCGCTCAACAGAA
                       **************************************************

1_PSY2L_DECR_35F_AK93  TTGGCTTTAAGCTTTCAAGAGACTGCAGGGTGCTCTTATGTATGGGATCA
PSY2L                  TTGGCTTTAAGCTTTCAAGAGACTGCAGGGTGCTCTTATGTATGGGATCA
                       **************************************************

1_PSY2L_DECR_35F_AK93  AATCTGCACTATGCAACGAAATTTGCATTTCAGCTCTCTAAACAGCGAAA
PSY2L                  AATCTGCACTATGCAACGAAATTTGCATTTCAGCTCTCTAAACAGCGAAA
                       **************************************************

1_PSY2L_DECR_35F_AK93  CATTTCACAGCTTGAACAGTGAGTTGAGGGAGCTTCCTGCTGTAGAGCTT
PSY2L                  CATTTCACAGCTTGAACAGTGAGTTGAGGGAGCTTCCTGCTGTAGAGCTT
                       **************************************************

1_PSY2L_DECR_35F_AK93  ACTACTCTTCCCCTAATACTGAAGATTGTTACAGAGAGTGGCATTACAGA
PSY2L                  ACTACTCTTCCCCTAATACTGAAGATTGTTACAGAGAGTGGCATTACAGA
                       **************************************************

1_PSY2L_DECR_35F_AK93  TCAGATGCGCCTAACTGAACTTATTTTGAAGGATCATGATTTCTTCCGGA
PSY2L                  TCAGATGCGCCTAACTGAACTTATTTTGAAGGATCATGATTTCTTCCGGA
                       **************************************************

1_PSY2L_DECR_35F_AK93  ATCTGATGGGTGTTTTTAAAATATGCGAGGACTTGGAAAATGTTGATGGC
PSY2L                  ATCTGATGGGTGTTTTTAAAATATGCGAGGACTTGGAAAATGTTGATGGC
                       **************************************************

1_PSY2L_DECR_35F_AK93  CTTCACATGATATTCAACATTGTCAAGGGAATCATTTTGCTTAACAGTTC
PSY2L                  CTTCACATGATATTCAACATTGTCAAGGGAATCATTTTGCTTAACAGTTC
                       **************************************************
```

```
1_PSY2L_DECR_35F_AK93  TCAGATCTTGGAGAAAATATTTGGAGATGAATTGATTATGGAGATTATCG
PSY2L                  TCAGATCTTGGAGAAAATATTTGGAGATGAATTGATTATGGAGATTATCG
                       *************************************************

1_PSY2L_DECR_35F_AK93  GATGCCTTGAATATGATCCTGGTGTTCCTCACTCTCAGCATCACCGGAAT
PSY2L                  GATGCCTTGAATATGATCCTGGTGTTCCTCACTCTCAGCATCACCGGAAT
                       *************************************************

1_PSY2L_DECR_35F_AK93  TTTCTGAAGGAGCATGTTGTTTTTAAGGAGGCTATACCAATCAAAGATCC
PSY2L                  TTTCTGAAGGAGCATGTTGTTTTTAAGGAGGCTATACCAATCAAAGATCC
                       *************************************************

1_PSY2L_DECR_35F_AK93  CTTAGTCC----------------------------------------
PSY2L                  CTTAGTCCTGTCAAAGATACACCAGACGTACAGAATTGGTTACTTGAAGG
                       ********

1_PSY2L_DECR_35F_AK93  -------------------------------------------------
PSY2L                  ATGTTGTTTTGGCTAGAGTACTAGATGATGCTATTGTTGCAAACTTGAAT

1_PSY2L_DECR_35F_AK93  -------------------------------------------------
PSY2L                  TCTGTAATCCATGCGAACAATGCCATAGTAGTTTCATTGCTGAAGGACGA

1_PSY2L_DECR_35F_AK93  -------------------------------------------------
PSY2L                  TAGCACTTTTATTCAAGAGTTATTTGCAAGGTTGAGGTCGCCTTCTACTT

1_PSY2L_DECR_35F_AK93  -------------------------------------------------
PSY2L                  CTATGGAATCCAAGAAAAATTTGGTATATTTCTTGCACGAATTTTGTAGT

1_PSY2L_DECR_35F_AK93  -------------------------------------------------
PSY2L                  TTAAGCAAGAGCCTCCAGGTGGTGCAGCAGCTGCGACTTTTTAGGGACCT

1_PSY2L_DECR_35F_AK93  -------------------------------------------------
PSY2L                  TATTAATGAAGGCATTTTTCATGTCATAGAAGAAGTCTTGCAGATTCCAG

1_PSY2L_DECR_35F_AK93  -------------------------------------------------
PSY2L                  ACAAAAAACTCGTATTGACTGGGACAGATATCCTGATTCTTTTCTTGACT

1_PSY2L_DECR_35F_AK93  -------------------------------------------------
PSY2L                  CAAGACCCCAACCTTTTACGTTCTTATGTTGTTCGGACAGAAGGAAACCC

1_PSY2L_DECR_35F_AK93  -------------------------------------------------
PSY2L                  CCTCCTCGGTCTCCTGGTCAAGGGAATGATGGAAGACTTTGGTGATAAGA

1_PSY2L_DECR_35F_AK93  -------------------------------------------------
PSY2L                  TGCACTGCCAATTTCTAGAAATTATCCGTACCTTACTAGATGCAAATGCA

1_PSY2L_DECR_35F_AK93  -------------------------------------------------
PSY2L                  TTGTCTGGTGGAGCTCAGAGAGCAAATATCATGGATATTTTCTACGAGAA

1_PSY2L_DECR_35F_AK93  -------------------------------------------------
PSY2L                  GCATCTACCTGAGTTAGTGGATGTTATTACTGCCTCATGTCCTGAGAAGT

1_PSY2L_DECR_35F_AK93  -------------------------------------------------
PSY2L                  CGAGCAACGCATCTGAAGGTGCTGCCAGAAGGATTTTCACAAAGCCTGAA
```

```
1_PSY2L_DECR_35F_AK93  ----TGTCAAAGATACACCAGACGTACAGAATTGGTTACTTGAAG-----
PSY2L                  GTCCTGTTGAACATATGTGAATTGTTGTGCTTTTGCATTATGCAAGATGC
                           ***  ** ***    *   **   *  ** *     ** *

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  ATCCAGGACAAAATGCAGTTTTCTCCAAAACAATGTGACTGAAAAGGTTT

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  TGCATCTCACACGGAGAAAGGAAAAATACCTAGTGGTCGCTGCTATACGA

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  TTTGTCCGTACTCTCCTCTCTGTCCATGATGATTATGTCCAGAATTACGT

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  GGTTAAAAACAACTTGTTGAAACCGATCATAGATGTCTTCATTGCCAATG

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  GAACCCGGTACAATCTGCTGAACTCTGCAGTCTTGGATCTGCTTGAGCAC

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  ATACGCAAGGGAAATGCAACTCTGTTGCTCAAATACATAGTTGATACGTT

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  CTGGGACCAGTTGGCCCCATTTCAGTGCTTGACCTCCATCCAGGCTTTCA

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  AGGTTAAGTATGAACAGTGTTTAGAAAGTGCCGGACCAAAAAGCACTTCT

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  GATGCGGTTGATCCAAGAAGAAGAGTTGACGAGCGGGCATTGGAGAAAGA

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  GGAAGAAGATTATTTCAATGAAGACAGCGATGAAGAAGATTCAGCCTCTG

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  CTTCTAATACACAAAAGGAAAAACCTGCTTCTAATATACAGAAAGAACAA

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  CCTAAGCCTCATCTCTCCAATGGAGTGGCTGCAAGCCCTACTTCTTCAAG

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  TCCGAGGTCTGGAGGCTTGGTTGATTATGAGGACGATGAAGATGATGAAG

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  ACTATAAACCTCCTCCGCGGAAACAGCCAGAAGCCTCTGAGGATGAGGAA

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  GGCGAGCTCCTGAGGCTGAAACGAAAATCCGCTCTTGTAGAAAGAGAACA

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  AGAGCCGTCCAAGAAACCACGGCTGGGGAAAAGTTCGAAAAGGGAAAATG
```

```
1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  TATTTGCTGTGCTATGTTCGACACTGAGCCATGCAGTGCTTACGGGTAAG

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  AAAAGTCCAGGCCCCGCTGGATCAGCAGCCCGGTCAATAGTAGCGAAAGG

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  AGCTGAGGATTCAAAAAGTAGTGAAGAGAATAATAGCAGCAGTTCAGATG

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  ATGAGAATCATAAGGATGATGGAGTATCGAGTTCTGAACATGAAACATCA

1_PSY2L_DECR_35F_AK93  --------------------------------------------------
PSY2L                  GACAATGGAAAGCTAAATGGGGAAGAATCTCTGGTAGTAGCTCCAAAATC

1_PSY2L_DECR_35F_AK93  -------------------------------
PSY2L                  ATCACCTGAAATGGCTGTAAATGGATCCTGA
```

Reverse primer

```
2_PSY2L_reverse  --------------------------------------------------
PSY2L            ATGGGCGCTCCGGAAAAGTCTCAATCTAATACCAATTCGATGCAGAGAGT

2_PSY2L_reverse  --------------------------------------------------
PSY2L            GAAAGTCTATCATTTGAATGAAGATGGTAAATGGGATGATCGAGGAACTG

2_PSY2L_reverse  --------------------------------------------------
PSY2L            GGCACGTAAGCATCGACTTTGTGGAGCGATCTGAAGAACTCAGTCTATGT

2_PSY2L_reverse  --------------------------------------------------
PSY2L            GTAATTGATGAAGAAGATAACGAGACGTTACTTGTTCATCCCATCAACCC

2_PSY2L_reverse  --------------------------------------------------
PSY2L            TGAGGATATTTACAGGAAACAAGAAGACACAATAATCTCATGGAGAGACC

2_PSY2L_reverse  --------------------------------------------------
PSY2L            CAGAGCGCTCAACAGAATTGGCTTTAAGCTTTCAAGAGACTGCAGGGTGC

2_PSY2L_reverse  --------------------------------------------------
PSY2L            TCTTATGTATGGGATCAAATCTGCACTATGCAACGAAATTTGCATTTCAG

2_PSY2L_reverse  --------------------------------------------------
PSY2L            CTCTCTAAACAGCGAAACATTTCACAGCTTGAACAGTGAGTTGAGGGAGC

2_PSY2L_reverse  --------------------------------------------------
PSY2L            TTCCTGCTGTAGAGCTTACTACTCTTCCCCTAATACTGAAGATTGTTACA

2_PSY2L_reverse  --------------------------------------------------
PSY2L            GAGAGTGGCATTACAGATCAGATGCGCCTAACTGAACTTATTTTGAAGGA

2_PSY2L_reverse  --------------------------------------------------
```

```
PSY2L            TCATGATTTCTTCCGGAATCTGATGGGTGTTTTTAAAATATGCGAGGACT

2_PSY2L_reverse  --------------------------------------------------
PSY2L            TGGAAAATGTTGATGGCCTTCACATGATATTCAACATTGTCAAGGGAATC

2_PSY2L_reverse  --------------------------------------------------
PSY2L            ATTTTGCTTAACAGTTCTCAGATCTTGGAGAAAATATTTGGAGATGAATT

2_PSY2L_reverse  --------------------------------------------------
PSY2L            GATTATGGAGATTATCGGATGCCTTGAATATGATCCTGGTGTTCCTCACT

2_PSY2L_reverse  --------------------------------------------------
PSY2L            CTCAGCATCACCGGAATTTTCTGAAGGAGCATGTTGTTTTTAAGGAGGCT

2_PSY2L_reverse  --------------------------------------------------
PSY2L            ATACCAATCAAAGATCCCTTAGTCCTGTCAAAGATACACCAGACGTACAG

2_PSY2L_reverse  --------------------------------------------------
PSY2L            AATTGGTTACTTGAAGGATGTTGTTTTGGCTAGAGTACTAGATGATGCTA

2_PSY2L_reverse  --------------------------------------------------
PSY2L            TTGTTGCAAACTTGAATTCTGTAATCCATGCGAACAATGCCATAGTAGTT

2_PSY2L_reverse  --------------------------------------------------
PSY2L            TCATTGCTGAAGGACGATAGCACTTTTATTCAAGAGTTATTTGCAAGGTT

2_PSY2L_reverse  --------------------------------------------------
PSY2L            GAGGTCGCCTTCTACTTCTATGGAATCCAAGAAAAATTTGGTATATTTCT

2_PSY2L_reverse  --------------------------------------------------
PSY2L            TGCACGAATTTTGTAGTTTAAGCAAGAGCCTCCAGGTGGTGCAGCAGCTG

2_PSY2L_reverse  --------------------------------------------------
PSY2L            CGACTTTTTAGGGACCTTATTAATGAAGGCATTTTTCATGTCATAGAAGA

2_PSY2L_reverse  --------------------------------------------------
PSY2L            AGTCTTGCAGATTCCAGACAAAAAACTCGTATTGACTGGGACAGATATCC

2_PSY2L_reverse  --------------------------------------------------
PSY2L            TGATTCTTTTCTTGACTCAAGACCCCAACCTTTTACGTTCTTATGTTGTT

2_PSY2L_reverse  --------------------------------------------------
PSY2L            CGGACAGAAGGAAACCCCCTCCTCGGTCTCCTGGTCAAGGGAATGATGGA

2_PSY2L_reverse  --------------------------------------------------
PSY2L            AGACTTTGGTGATAAGATGCACTGCCAATTTCTAGAAATTATCCGTACCT

2_PSY2L_reverse  --------------------------------------------------
PSY2L            TACTAGATGCAAATGCATTGTCTGGTGGAGCTCAGAGAGCAAATATCATG

2_PSY2L_reverse  --------------------------------------------------
```

```
PSY2L             GATATTTTCTACGAGAAGCATCTACCTGAGTTAGTGGATGTTATTACTGC

2_PSY2L_reverse   --------------------------------------------------
PSY2L             CTCATGTCCTGAGAAGTCGAGCAACGCATCTGAAGGTGCTGCCAGAAGGA

2_PSY2L_reverse   --------------------------------------------------
PSY2L             TTTTCACAAAGCCTGAAGTCCTGTTGAACATATGTGAATTGTTGTGCTTT

2_PSY2L_reverse   --------------------------------------------------
PSY2L             TGCATTATGCAAGATGCATCCAGGACAAAATGCAGTTTTCTCCAAAACAA

2_PSY2L_reverse   --------------------------------------------------
PSY2L             TGTGACTGAAAAGGTTTTGCATCTCACACGGAGAAAGGAAAAATACCTAG

2_PSY2L_reverse   --------------------------------------------------
PSY2L             TGGTCGCTGCTATACGATTTGTCCGTACTCTCCTCTCTGTCCATGATGAT

2_PSY2L_reverse   --------------------------------------------------
PSY2L             TATGTCCAGAATTACGTGGTTAAAAACAACTTGTTGAAACCGATCATAGA

2_PSY2L_reverse   --ATTTCATTGCCAATGGAACCCGGTACAATTTGCTGAACTCTGCAGTCT
PSY2L             TGTCTTCATTGCCAATGGAACCCGGTACAATCTGCTGAACTCTGCAGTCT
                      *************************** ******************

2_PSY2L_reverse   TGGATCTGCTTGAGCACATACGCAAGGGAAATGCAACTCTGTTGCTCAAA
PSY2L             TGGATCTGCTTGAGCACATACGCAAGGGAAATGCAACTCTGTTGCTCAAA
                  **************************************************

2_PSY2L_reverse   TACATAGTTGATACGTTCTGGGACCAGTTGGCCCCATTTCAGTGCTTGAC
PSY2L             TACATAGTTGATACGTTCTGGGACCAGTTGGCCCCATTTCAGTGCTTGAC
                  **************************************************

2_PSY2L_reverse   CTCCATCCAGGCTTTCAAGGTTAAGTATGAACAGTGTTTAGAAAGTGCCG
PSY2L             CTCCATCCAGGCTTTCAAGGTTAAGTATGAACAGTGTTTAGAAAGTGCCG
                  **************************************************

2_PSY2L_reverse   GACCAAAAAGCACTTCTGATGCGGTTGATCCAAGAAGAAGAGTTGACGAG
PSY2L             GACCAAAAAGCACTTCTGATGCGGTTGATCCAAGAAGAAGAGTTGACGAG
                  **************************************************

2_PSY2L_reverse   CGGGCATTGGAGAAAGAGGAAGAAGATTATTTCAATGAAGACAGCGATGA
PSY2L             CGGGCATTGGAGAAAGAGGAAGAAGATTATTTCAATGAAGACAGCGATGA
                  **************************************************

2_PSY2L_reverse   AGAAGATTCAGCCTCTGCTTCTAATACACAAAAGGAAAAACCTGCTTCTA
PSY2L             AGAAGATTCAGCCTCTGCTTCTAATACACAAAAGGAAAAACCTGCTTCTA
                  **************************************************

2_PSY2L_reverse   ATATACAGAAAGAACAACCTAAGCCTCATCTCTCCAATGGAGTGGCTGCA
PSY2L             ATATACAGAAAGAACAACCTAAGCCTCATCTCTCCAATGGAGTGGCTGCA
                  **************************************************

2_PSY2L_reverse   AGCCCTACTTCTTCAAGTCCGAGGTCTGGAGGCTTGGTTGATTATGAGGA
PSY2L             AGCCCTACTTCTTCAAGTCCGAGGTCTGGAGGCTTGGTTGATTATGAGGA
                  **************************************************

2_PSY2L_reverse   CGATGAAGATGATGAAGACTATAAACCTCCTCCGCGGAAACAGCCAGAAG
PSY2L             CGATGAAGATGATGAAGACTATAAACCTCCTCCGCGGAAACAGCCAGAAG
                  **************************************************

2_PSY2L_reverse   CCTCTGAGGATGAGGAAGGCGAGCTCCTGAGGCTGAAACGAAAATCCGCT
```

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PSY2L            CCTCTGAGGATGAGGAAGGCGAGCTCCTGAGGCTGAAACGAAAATCCGCT
                 *********************************************

2_PSY2L_reverse  CTTGTAGAAAGAGAACAAGAGCCGTCCAAGAAACCACGGCTGGGGAAAAG
PSY2L            CTTGTAGAAAGAGAACAAGAGCCGTCCAAGAAACCACGGCTGGGGAAAAG
                 *********************************************

2_PSY2L_reverse  TTCGAAAAGGGAAAATGTATTTGCTGTGCTATGTTCGACACTGAGCCATG
PSY2L            TTCGAAAAGGGAAAATGTATTTGCTGTGCTATGTTCGACACTGAGCCATG
                 *********************************************

2_PSY2L_reverse  CAGTGCTTACGGGTAAGAAAAGTCCAGGCCCCGCTGGATCAGCAGCCCGG
PSY2L            CAGTGCTTACGGGTAAGAAAAGTCCAGGCCCCGCTGGATCAGCAGCCCGG
                 *********************************************

2_PSY2L_reverse  TCAATAGTAGCGAAAGGAGCTGAGGATTCAAAAAGTAGTGAAGAGAATAA
PSY2L            TCAATAGTAGCGAAAGGAGCTGAGGATTCAAAAAGTAGTGAAGAGAATAA
                 *********************************************

2_PSY2L_reverse  TAGCAGCAGTTCAGATGATGAGAATCATAAGGATGATGGAGTATCGAGTT
PSY2L            TAGCAGCAGTTCAGATGATGAGAATCATAAGGATGATGGAGTATCGAGTT
                 *********************************************

2_PSY2L_reverse  CTGAACATGAAACATCAGACAATGGAAAGCTAAATGGGGAAGAATCTCTG
PSY2L            CTGAACATGAAACATCAGACAATGGAAAGCTAAATGGGGAAGAATCTCTG
                 *********************************************

2_PSY2L_reverse  GTAGTAGCTCCAAAATCATCACCTGAAATGGCTGTAAATGGATCCTGAGC
PSY2L            GTAGTAGCTCCAAAATCATCACCTGAAATGGCTGTAAATGGATCCT----
                 ******************************************

2_PSY2L_reverse  GGCCGCTGCCGCGGCAATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
PSY2L            ---------------------------------------------

2_PSY2L_reverse  TGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTCA
PSY2L            ---------------------------------------------

2_PSY2L_reverse  GCGT
PSY2L            --GA
                   *
```

Middle primer

```
3_PSY2L_DECR_35M_Middle  ------------------------------------------------
PSY2L                    ATGGGCGCTCCGGAAAAGTCTCAATCTAATACCAATTCGATGCAGAGAGT

3_PSY2L_DECR_35M_Middle  ------------------------------------------------
PSY2L                    GAAAGTCTATCATTTGAATGAAGATGGTAAATGGGATGATCGAGGAACTG

3_PSY2L_DECR_35M_Middle  ------------------------------------------------
PSY2L                    GGCACGTAAGCATCGACTTTGTGGAGCGATCTGAAGAACTCAGTCTATGT

3_PSY2L_DECR_35M_Middle  ------------------------------------------------
PSY2L                    GTAATTGATGAAGAAGATAACGAGACGTTACTTGTTCATCCCATCAACCC

3_PSY2L_DECR_35M_Middle  ------------------------------------------------
PSY2L                    TGAGGATATTTACAGGAAACAAGAAGACACAATAATCTCATGGAGAGACC

3_PSY2L_DECR_35M_Middle  ------------------------------------------------
PSY2L                    CAGAGCGCTCAACAGAATTGGCTTTAAGCTTTCAAGAGACTGCAGGGTGC
```

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3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    TCTTATGTATGGGATCAAATCTGCACTATGCAACGAAATTTGCATTTCAG

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    CTCTCTAAACAGCGAAACATTTCACAGCTTGAACAGTGAGTTGAGGGAGC

3_PSY2L_DECR_35M_Middle  ---------------------------------GATGATGCTATTGTT---
PSY2L                    TTCCTGCTGTAGAGCTTACTACTCTTCCCCTAATACTGAAGATTGTTACA
                                                            ******

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    GAGAGTGGCATTACAGATCAGATGCGCCTAACTGAACTTATTTTGAAGGA

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    TCATGATTTCTTCCGGAATCTGATGGGTGTTTTTAAAATATGCGAGGACT

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    TGGAAAATGTTGATGGCCTTCACATGATATTCAACATTGTCAAGGGAATC

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    ATTTTGCTTAACAGTTCTCAGATCTTGGAGAAAATATTTGGAGATGAATT

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    GATTATGGAGATTATCGGATGCCTTGAATATGATCCTGGTGTTCCTCACT

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    CTCAGCATCACCGGAATTTTCTGAAGGAGCATGTTGTTTTTAAGGAGGCT

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    ATACCAATCAAAGATCCCTTAGTCCTGTCAAAGATACACCAGACGTACAG

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    AATTGGTTACTTGAAGGATGTTGTTTTGGCTAGAGTACTAGATGATGCTA

3_PSY2L_DECR_35M_Middle  -----GCAAACTTGAATTCTGTAATCCATGCGAACAATGCCATAGTAGTT
PSY2L                    TTGTTGCAAACTTGAATTCTGTAATCCATGCGAACAATGCCATAGTAGTT
                              ******************************************

3_PSY2L_DECR_35M_Middle  TCATTGCTGAAGGACGATAGCACTTTTATTCAAGAGTTATTTGCAAGGTT
PSY2L                    TCATTGCTGAAGGACGATAGCACTTTTATTCAAGAGTTATTTGCAAGGTT
                         **************************************************

3_PSY2L_DECR_35M_Middle  GAGGTCGCCTTCTACTTCTATGGAATCCAAGAAAAATTTGGTATATTTCT
PSY2L                    GAGGTCGCCTTCTACTTCTATGGAATCCAAGAAAAATTTGGTATATTTCT
                         **************************************************

3_PSY2L_DECR_35M_Middle  TGCACGAATTTTGTAGTTTAAGCAAGAGCCTCCAGGTGGTGCAGCAGCTG
PSY2L                    TGCACGAATTTTGTAGTTTAAGCAAGAGCCTCCAGGTGGTGCAGCAGCTG
                         **************************************************

3_PSY2L_DECR_35M_Middle  CGACTTTTTAGGGACCTTATTAATGAAGGCATTTTTCATGTCATAGAAGA
PSY2L                    CGACTTTTTAGGGACCTTATTAATGAAGGCATTTTTCATGTCATAGAAGA
                         **************************************************

3_PSY2L_DECR_35M_Middle  AGTCTTGCAGATTCCAGACAAAAAACTCGTATTGACTGGGACAGATATCC
PSY2L                    AGTCTTGCAGATTCCAGACAAAAAACTCGTATTGACTGGGACAGATATCC
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M

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                                **************************************************

3_PSY2L_DECR_35M_Middle  TGATTCTTTTCTTGACTCAAGACCCCAACCTTTTACGTTCTTATGTTGTT
PSY2L                    TGATTCTTTTCTTGACTCAAGACCCCAACCTTTTACGTTCTTATGTTGTT
                         **************************************************

3_PSY2L_DECR_35M_Middle  CGGACAGAAGGAAACCCCCTCCTCGGTCTCCTGGTCAAGGGAATGATGGA
PSY2L                    CGGACAGAAGGAAACCCCCTCCTCGGTCTCCTGGTCAAGGGAATGATGGA
                         **************************************************

3_PSY2L_DECR_35M_Middle  AGACTTTGGTGATAAGATGCACTGCCAATTTCTAGAAATTATCCGTACCT
PSY2L                    AGACTTTGGTGATAAGATGCACTGCCAATTTCTAGAAATTATCCGTACCT
                         **************************************************

3_PSY2L_DECR_35M_Middle  TACTAGATGCAAATGCATTGTCTGGTGGAGCTCAGAGAGCAAATATCATG
PSY2L                    TACTAGATGCAAATGCATTGTCTGGTGGAGCTCAGAGAGCAAATATCATG
                         **************************************************

3_PSY2L_DECR_35M_Middle  GATATTTTCTACGAGAAGCATCTACCTGAGTTAGTGGATGTTATTACTGC
PSY2L                    GATATTTTCTACGAGAAGCATCTACCTGAGTTAGTGGATGTTATTACTGC
                         **************************************************

3_PSY2L_DECR_35M_Middle  CTCATGTCCTGAGAAGTCGAGCAACGCATCTGAAGGTGCTGCCAGAAGGA
PSY2L                    CTCATGTCCTGAGAAGTCGAGCAACGCATCTGAAGGTGCTGCCAGAAGGA
                         **************************************************

3_PSY2L_DECR_35M_Middle  TTTTCACAAAGCCTGAAGTCCTGTTGAACATATGTGAATTGTTGTGCTTT
PSY2L                    TTTTCACAAAGCCTGAAGTCCTGTTGAACATATGTGAATTGTTGTGCTTT
                         **************************************************

3_PSY2L_DECR_35M_Middle  TGCATTATGCAAGATGCATCCAGGACAAAATGCAGTTTTCTCCAAAACAA
PSY2L                    TGCATTATGCAAGATGCATCCAGGACAAAATGCAGTTTTCTCCAAAACAA
                         **************************************************

3_PSY2L_DECR_35M_Middle  TGTGACTGAAAAGGTTTTGCATCTCACACGGAGAAAGGAAAAATACCTAG
PSY2L                    TGTGACTGAAAAGGTTTTGCATCTCACACGGAGAAAGGAAAAATACCTAG
                         **************************************************

3_PSY2L_DECR_35M_Middle  TGGTCGCTGCTATACGATTTGTCCGTACTCTCCTCTCTGTCCATGATGAT
PSY2L                    TGGTCGCTGCTATACGATTTGTCCGTACTCTCCTCTCTGTCCATGATGAT
                         **************************************************

3_PSY2L_DECR_35M_Middle  TATGTCCAGAATTACGTGGTTAAAAACAACTTGTTGAAACCGATCATAGA
PSY2L                    TATGTCCAGAATTACGTGGTTAAAAACAACTTGTTGAAACCGATCATAGA
                         **************************************************

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    TGTCTTCATTGCCAATGGAACCCGGTACAATCTGCTGAACTCTGCAGTCT

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    TGGATCTGCTTGAGCACATACGCAAGGGAAATGCAACTCTGTTGCTCAAA

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    TACATAGTTGATACGTTCTGGGACCAGTTGGCCCCATTTCAGTGCTTGAC

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    CTCCATCCAGGCTTTCAAGGTTAAGTATGAACAGTGTTTAGAAAGTGCCG

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    GACCAAAAAGCACTTCTGATGCGGTTGATCCAAGAAGAAGAGTTGACGAG

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    CGGGCATTGGAGAAAGAGGAAGAAGATTATTTCAATGAAGACAGCGATGA
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N

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3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    AGAAGATTCAGCCTCTGCTTCTAATACACAAAAGGAAAAACCTGCTTCTA

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    ATATACAGAAAGAACAACCTAAGCCTCATCTCTCCAATGGAGTGGCTGCA

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    AGCCCTACTTCTTCAAGTCCGAGGTCTGGAGGCTTGGTTGATTATGAGGA

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    CGATGAAGATGATGAAGACTATAAACCTCCTCCGCGGAAACAGCCAGAAG

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    CCTCTGAGGATGAGGAAGGCGAGCTCCTGAGGCTGAAACGAAAATCCGCT

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    CTTGTAGAAAGAGAACAAGAGCCGTCCAAGAAACCACGGCTGGGGAAAAG

3_PSY2L_DECR_35M_Middle  ---------------TGTCTTCATTGCCAA--------------------
PSY2L                    TTCGAAAAGGGAAAATGTATTTGCTGTGCTATGTTCGACACTGAGCCATG
                                        *** **   **

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    CAGTGCTTACGGGTAAGAAAAGTCCAGGCCCCGCTGGATCAGCAGCCCGG

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    TCAATAGTAGCGAAAGGAGCTGAGGATTCAAAAAGTAGTGAAGAGAATAA

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    TAGCAGCAGTTCAGATGATGAGAATCATAAGGATGATGGAGTATCGAGTT

3_PSY2L_DECR_35M_Middle  --------------------------------------------------
PSY2L                    CTGAACATGAAACATCAGACAATGGAAAGCTAAATGGGGAAGAATCTCTG

3_PSY2L_DECR_35M_Middle  ----------------------------------------------
PSY2L                    GTAGTAGCTCCAAAATCATCACCTGAAATGGCTGTAAATGGATCCTGA
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