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Investigation of the Physiological Function of Protein Phosphatase 4 (PP4) in *Arabidopsis thaliana*

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ABSTRACT

Protein Phosphatase 4 (PP4), a member of serine/threonine-specific phospho-protein phosphatases (PPP) family, is remarkably well conserved across eukaryotes. PP4 has been studied mainly in yeast and mammalian cells, and virtually nothing is known about PP4 in plants. In mammalian cells, PP4 plays a role in several processes not relevant to plants. The major objective of this thesis was to investigate the physiological function of PP4 in *Arabidopsis thaliana* as a model plant. *A. thaliana* has two PP4 catalytic subunits, namely PP4-1 (At4G26720) and PP4-2 (At5G55260). In addition, putative regulatory subunits for PP4 were bioinformatically detected in *A. thaliana*: PP4R2L (At5G17070) and PSY2L (At3G06670). Using reverse genetics approach, this thesis focuses on expression studies (semiquantitative RT-PCR) and observation of phenotype of the *A. thaliana* gene encoding PP4 catalytic and putative regulatory subunits in loss-of-function mutants (T-DNA insertional mutagenesis lines), artificial microRNA (amiRNA) stable lines, and gene overexpression lines. Using intensive expression analysis by the in-gel RT-PCR, we succeeded to detect knock down and/or knock out for amiRNA plants and T-DNA plants for *PSY2L* and *PP4R2L*. Moreover, we detected and isolated stable overexpression plants for *PP4-1*, *PP4-2*, and *PP4R2L*. Observation of phenotype showed that knock out of *PSY2L* gene in a T-DNA line, SALK_048064 (insertion in exon 3 of 25), show some interesting phenotypes. The homozygous mutants of this line showed dwarfism, delayed growth, and extended life span. The knock down of *PSY2L* through amiRNA mechanism also showed a phenotype, such as reduced size and twisted leaves. No significant phenotype was found in overexpressor plants. We also investigated the subcellular localization of these subunits in two different plant expression systems: *A. thaliana* mesophyll protoplasts and particle bombardment into onion epidermis cells. Main location of the catalytic subunits, PP4-1 and PP4-2, are in cytoplasm, with few in nucleus. PSY2L is strongly found in nucleus, whereas the other regulatory subunit, PP4R2L is not only found in nucleus but also tend to locate in cytoplasm. *In vivo* investigations of subcellular localization of PP4 subunits show resemblance to *in silico* analysis.

Key words: *Arabidopsis thaliana*, PP4, PSY2L, T-DNA, amiRNA, RT-PCR, Subcellular Localization

CONTENTS

ABBREVIATIONS

ABRC	Arabidopsis Biological Resource Center
AGO	Argonaute
amiRNA	Artificial microRNA
BP	T-DNA border primer
cDNA	Complementary DNA
DNA	Deoxyribonucleic Acid
ER	Endoplasmic Reticulum
EYFP	Enhanced Yellow Fluorescent Protein
LB	Luria-Bertani
LP	Left genomic primer
LS	Linsmaier and Skoog
miRNA	microRNA
mRNA	Messenger Ribonucleic Acid
NASC	Nottingham Arabidopsis Stock Center
PCR	Polymerase Chain Reaction
PP2A	Protein phosphatase 2A
PP4	Protein phosphatase 4
PP6	Protein Phosphatase 6
PPP	Phospho-protein phosphatases (serine/threonine-specific)
RNA	Ribonucleic Acid
RNAi	RNA-mediated Interference
RP	Right genomic primer
RT	Room Temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SNPs	Single Nucleotide Polymorphisms
T-DNA	Transferred DNA
TILLING	Targeting Induced Local Lesions in Genomes
WT	Wild Type

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1. INTRODUCTION

1.1 Protein Phosphatases

1.1.1 Protein Phosphorylation

Protein phosphorylation is recognized as one of the most prevalent and versatile means to regulate protein function. The phosphate group has special properties that can be expected to regulate critical biological function when attached to protein (Hunter, 2012). According to Alberts et al. (2015), a phosphorylation event can affect the protein that is modified in three important ways: first, because addition of two negative charges of a phosphate group to protein can cause a major conformational change in the protein, for instance attracting a cluster of positively changed amino acid chains; second, an attached phosphate group can form part of a structure that the binding site of other proteins recognized; third, masking of a binding site that hold two protein together by addition of a phosphate group can disrupt protein-protein interaction.

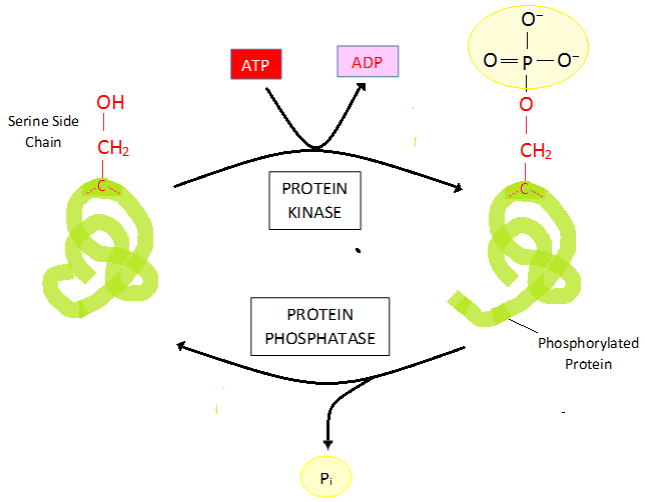


Figure 1-1. Protein phosphorylation. Transfer of a phosphate group from ATP to an amino acid side chain of the target protein is catalyzed by a protein kinase. Removal of the phosphate group is catalyzed by a protein phosphatase. In this case, the phosphate is added to a serine side chain. The figure was redrawn from Alberts et al. (2015).

Protein phosphorylation or the transfer of the terminal phosphate group of an ATP molecule to the hydroxyl group on amino acid side chain of the protein is catalyzed by a protein kinase (Figure 1-1). On the other hand, protein dephosphorylation or removal of the phosphate group is catalyzed by a second enzyme, a protein phosphatase. *Arabidopsis thaliana* has about 1125 protein kinases and 150 protein phosphatases (Tran et al., 2012). For various reasons, the protein phosphatases have been much less studied than protein kinases, mainly because they are difficult

to study since many of them are only functional in the cell as part of complexes with one or more regulatory subunits (Lillo et al., 2014).

1.1.2 Serine/Threonine-Specific Phospho-Protein Phosphatases (PPP) Family

Eukaryotic protein phosphatases can be divided into four distinct gene families, each with different active site signatures: (1) PPP (serine/threonine-specific phospho-protein phosphatases) GDxHG(x)23GDxVDRG(x)25GNHE-; (2) PPM/PP2C (Mg2+-dependent protein phosphatases) -(E/Q)D(x)nDGH(A/G)(x)nD(N/D)-; (3) Asp-based protein phosphatases -DxDx(T/V/I)L-; and (4) phospho-tyrosine phosphatases (PTP) -CX5R- (Kerk et al., 2008, Lillo et al., 2014)

PPP family members catalyze over 90% of all eukaryotic protein dephopshorylation reactions (Heroes et al., 2013). The PPP family can be divided into subgroups: PP1, PP2/PP2A, PP3/PP2B (only in animals), PP4, PP5, PP6, PP7, PPKL/Kelch (only in plants and alveolates), and bacterial-like protein phosphatases (SLP, RLPH, ALPH) (Lillo et al., 2014). Compared with other members of the PPP family, these three proteins, namely PP2A, PP4, and PP6 are phylogenetically distinct by cluster suggestive of a common ancestor (Uhrig et al., 2013). *In vivo*, catalytic subunits of PP2A, PP4, and PP6 in plant are present in trimeric and dimeric complexes with scaffolding and regulatory subunits that control activity and confer substrate specificity to the protein phosphatases (Lillo et al., 2014). List of those catalytic and regulatory subunits in *A. thaliana* is provided in Figure 1-2. Phylogenetic and architectural depiction of the plant (*A. thaliana*) PP2A, PP4, and PP6, including core catalytic domain, is shown in Figure 1-3.

1.1.3 Protein Phosphatase 2A (PP2A) and Protein Phosphatase 6 (PP6)

Trimeric Protein phosphatase 2A (PP2A) in *A. thaliana* (see Figure 1-2) is composed of catalytic subunit C, scaffolding subunit A, and regulatory subunit B (Lillo et al., 2014). The *A. thaliana* genome contains five different gene encoding C subunits (C1 – C5), three genes for A subunits (A1 – A3), and 17 genes encoding the regulatory B subunits, that can be divided into B55 (α,β), B′ (α – κ), and B″ (α – ε) families, giving up to 255 possible combinations of trimers (Lillo et al., 2014).

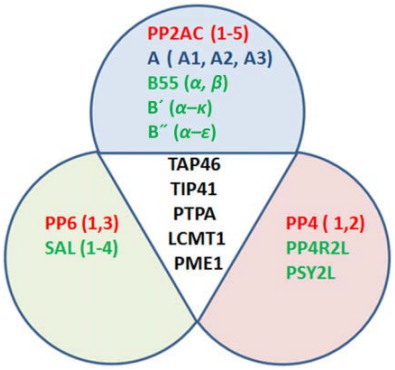


Figure 1-2. List of the catalytic and regulatory subunits of PP2A, PP4, and PP6 in *Arabidopsis thaliana*. Catalytic subunits are shown in red, canonical scaffolding subunits in blue and regulatory subunits are in green. Putative interactors common for PP2A, PP4 and PP6 are shown in black in the central triangle. The figure and description are taken from Lillo et al. (2014)

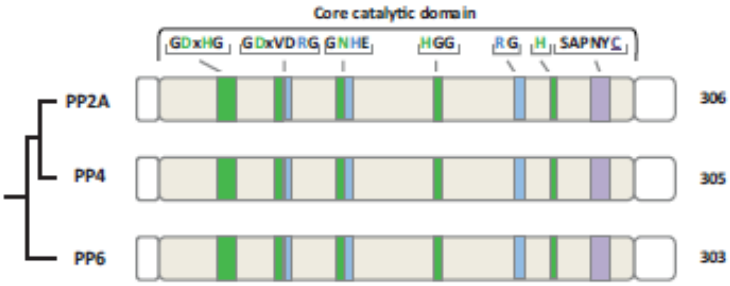


Figure 1-3. Phylogenetic and architectural depiction of the plant (*Arabidopsis thaliana*) phosphoprotein phosphatase (PPP) family, subgroup PP2A, PP4, and PP6. The highly conserved core catalytic domain of subgroup protein is depicted in gray with signature aspects of each motif highlighted. Green and blue represent amino acid involved in metal ion coordination and phosphate binding, respectively. Also describe is the microcystin inhibition docking motif SAPNYC (purple), highlighted by a reactive cysteine (C) to which microcystin covalently attaches. The figure and description are taken from Uhrig et al. (2013).

Lillo et al. (2014) compiled the already known roles of catalytic, regulatory, and scaffolding subunits of PP2A in physiological process in *A. thaliana*, because it is reported that many cellular events are regulated by individual PP2A subunits in plants. For instance, PP2A-B' involves in regulating intracellular response to brassinosteroids (BRs) by dephosphorylation transcription factor BZR1 and BZR2 and influence their movement to the nucleus (Tang et al., 2011). PP2A-A1 has been implicated in the control of microtubule function and regulation of binding and trafficking kinesins (Farkas et al., 2007, Tran et al., 2012). Subunit PP2A-C2 plays role as a negative regulator of ABA (abscisic acid) signaling (Pernas et al., 2007). Regulatory subunit B'γ of PP2A has functional role in light-dependent stress response (Trotta et al., 2011).

Two catalytic Protein Phosphatase 6 (*PP6*) genes in *A. thaliana* are co-expressed throughout the plant with somewhat higher expression levels in shoot meristem, sperm cells, seeds, flowers, and senescent leaves, whereas one or more of the four regulatory subunit (SAL, seen Figure 1-2) genes always show high expression levels in the same tissue as *PP6* (Lillo et al., 2014). Compared to wild type, a pp6-1/pp6-3 Arabidopsis double mutant showed hypersensitivity to abscisic acid (ABA) and lower germination percentage, while the over-expression line showed reduced sensitivity to ABA (Dai et al., 2013). Knockdown of both PP6 catalytic genes resulted in phenotype with shorter main roots, fewer lateral roots, root meristem collapse, abnormal cotyledons, and alter leaf

venation; traits known to be related with auxin (Dai et al., 2012). PP6 was also found to be important for the dephosphorylation of phtyochrome (Kim et al., 2002).

1.1.4 Protein Phosphatase 4 (PP4)

Protein phosphatase 4 (PP4) is remarkably well conserved across eukaryotes. Table 1-1 provides the nomenclature of *A. thaliana* PP4 subunits with functional homologues in *Homo sapiens* and *Saccharomyces cerevisiae*. Table 1-2 shows the detail of each subunits, including location in chromosome and size. As we can see from Figure 1-2 and Table 1-1, *A. thaliana* has two PP4 catalytic subunits, namely PP4-1 (At4G26720) and PP4-2 (At5G55260). In addition to these two catalytic subunits, the active PP4 complex contains also two subunits with regulatory functions, PP4R2L (At5G17070) and PSY2L (At3G06670). Figure 1-4 depicts the genomic organization of them.

Virtually, no clear physiological function for PP4 or Protein Phosphatase 4 has been defined (Lillo et al., 2014, Uhrig et al., 2013). In mammals, PP4 (encoded by the *PPP4C* gene) is implicated in many biological process such as apoptosis regulation (Mourtada-Maarabouni and Williams, 2008), microtubule organization (Han et al., 2009), and recovery from DNA damage checkpoint (Nakada et al., 2008). PP4 also plays a non-redundant role for the differentiation, suppressor activity and gut homeostatis of Treg cells (Liao et al., 2014). Semi-lethal phenotype was produced by disrupting *PP4* gene in *Drosophila melanogaster* (Helps et al., 1998). Depletion of *PP4* gene in *Caenorhabditis elegans* by RNA-mediated interference (RNAi) showed aberration in formation of spindle in both mitosis and sperm meiosis. (Sumiyoshi et al., 2002). Conditional knock out PP4 in mice T-cell inhibited the development of T-cells (Shui et al., 2007). However, recent study by Huang et al. (2016) shows that both up-regulation and inhibition of PP4 inhibited cell proliferation in HepG2 cells, indicated that PP4 plays dual roles during cell proliferation. As it can be seen in mammalian cells, PP4 play a role in several processes not relevant to plants. In yeast, PPH3 (encoded by the *PPH3* gene) together with two members of the protein phosphatase 2C (PP2C) family, Ptc2 and Ptc3, play roles in DNA double-strand break repair (Kim et al., 2011).

Table 1-1. Nomenclature of *Arabidopsis thaliana* PP4 subunits with functional homologues in *Homo sapiens* and *Saccharomyces cerevisiae*. The complete nomenclature of PPP family is provided by Lillo et al. (2014)

Arabidopsis thaliana			***Homo sapiens***		***Saccharomyces cerevisiae***	
AGI Number	**Gene**	**Protein**	**Gene**	**Protein**	**Gene**	**Protein**
Catalytic Subunits						
At4g26720	*PP4-1*	PP4-1	*PPP4C*	Ppp4c/PP4/PPX	*PPH3*	Pph3/PPH3
At5g55260	*PP4-2*	PP4-2				
Regulatory Subunits, PP4R2 domain						
At5g17070	*PP4R2like/ PP4R2L*	PP4R2 domain	*PPP4R2*	PP4R2	*YBL1046W*	Ybl1046w/ YBL1046W
Regulatory Subunits, SMK1 domain						
At3g06670	*PSY2like/PSY2L/ R3like*	SMK1 domain	*PPP4R3α*	Ppp4R3α	*PSY2*	Psy2/PSY2
			PPP4R3β	PPP4R3β	*PSY1*	Psy1/PSY1
					PSY3	Psy3/PSY3

Table 1-2. Detail of *Arabidopsis thaliana* PP4 subunits. The source is NCBI (National Center for Biotechnology) website with address http://www.ncbi.nlm.nih.gov/gene/ (last retrieved: April 2016).

AGI Number	**Name of Gene (Alias)***	**Description of Gene**	**Location of Gene**	**Sequence length of full genomic (bp)**	**Sequence length of full cDNA (bp)**	**Exon Count**
At4g26720	*PP4-1*	Serine/threonine-protein phosphatase PP-X isozyme 1	Chromosome 4	2311	1471	8
At5g55260	*PP4-2*	Serine/threonine-protein phosphatase PP-X isozyme 2	Chromosome 5	2266	1370	8
At5g17070	*PP4R2L*	Hypothetical protein	Chromosome 5	2771	1030	8
At3g06670	*PSY2L*	Component of IIS longevity pathway SMK-1 domain-containing protein	Chromosome 3	7944	3376	25

*: In this thesis, the alias terminology of these genes will be mostly used in order to make it more simple and readable

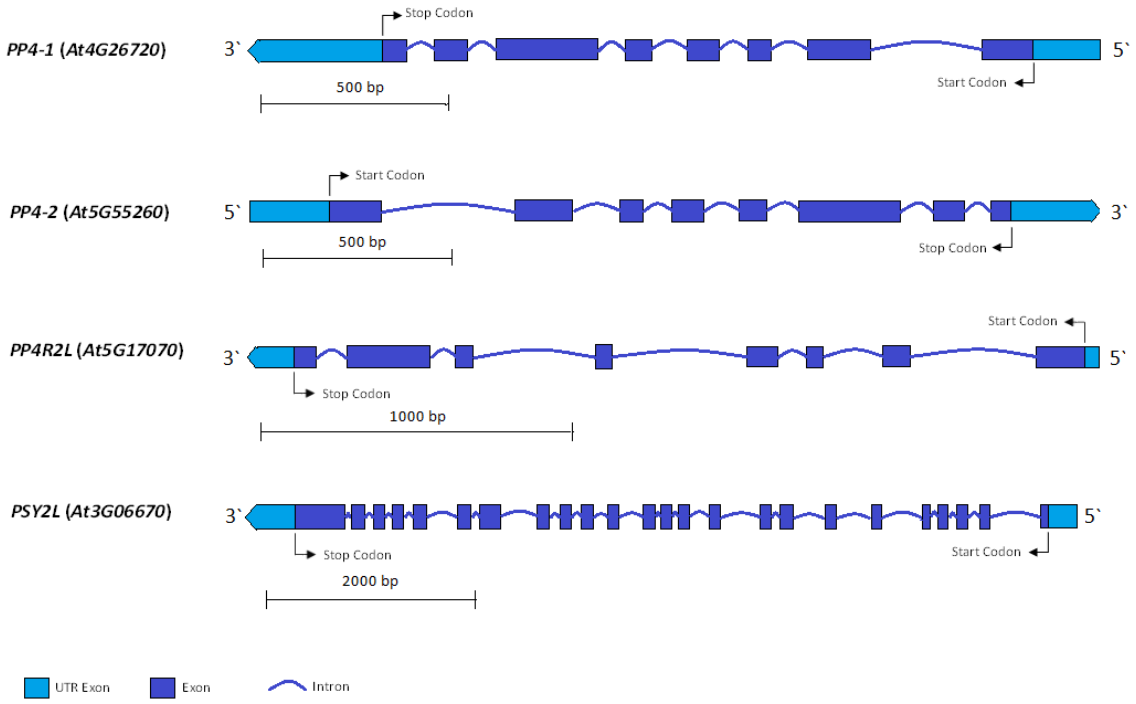


Figure 1-4. Genomic organization of *PP4-1* (*At4G26720*), *PP4-2* (*At5G55260*), *PP4R2L* (*At5G17070*), and *PSY2L* (*At3G06670*). The figures were redrawn based on data in TAIR (Arabidopsis Information Resource) website with address https://www.arabidopsis.org (last retrieved: April 2016).

Putative regulatory proteins for PP4 in *A. thaliana* were bioinformatically detected, namely PP4R2L (At5G17070) and PSY2L (At3G06670). SMK-1, putative homolog of PSY2L in *C. elegans*, is reported to promote longevity by modulating DAF-16 (FOXO transcriptional factor) without affecting other processes regulated by IIS or Insulin/IGF-1 signaling (Wolff et al., 2006). The functional homolog of PSY2L in *Saccharomyces cerevisiae*, PSY2, in coordination with other protein, plays role in the DNA damage response (O'Neill et al., 2004). Moreover, in *Homo sapiens*, Ppp4R3 in complex with Ppp4c and PP4R2 involved in anticancer cisplatin sensitivity, linked to the DNA damage response (Gingras et al., 2005). The functional homolog of PPR2L in *Saccharomyces cerevisiae*, YBL1046W, binds the catalytic subunits PPH3 and also shows resistance to cisplatin (Hastie et al., 2006).

In this thesis, the possibility to understand the physiological function of PP4 in plants will be tested by using reverse genetics approach and investigation of subcellular localization of catalytic and regulatory subunits of PP4. We investigated each catalytic and regulatory subunit individually. The genes to be examined in this study were the *A. thaliana* gene encoding PP4 catalytic subunits, which are *PP4-1*, and *PP4-2* as well as the putative regulatory subunits, namely *PP4R2L* and *PSY2L*.

1.2 Studying Gene Function: Reverse Genetics

Arabidopsis thaliana was the first plant that had its genome completely sequence, more than 25,000 genes have been identified (Bevan and Walsh, 2005), but many of its genes' functions are not yet well understood. Computational approach offers viable solution to this problem; numerous bioinformatics methods continue to be develop to infer protein function, most commonly based on evolutionary history, sequence similarity, genomic location, and the presence of certain small sequence motifs (Petrey et al., 2015). Even though this *in silico* analysis offers valuable information in investigating the function of these genes, the elucidation of gene function must always be verified *in vivo* using genetic analysis (Bolle et al., 2011). In order to observe the function of genes, two main strategy have been introduced, classical or forward genetics (from-phenotype-to-gene) and reverse genetics (from-gene-to-phenotype). Figure 1-5 for overview of the both approaches.

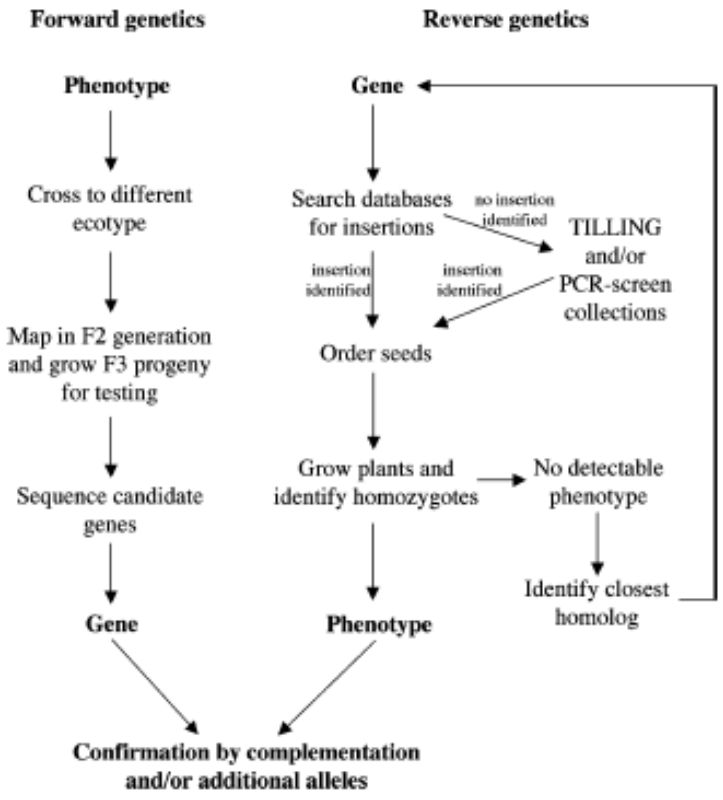


Figure 1-5. Overview of difference approach to establish gene function: forward genetics and reverse genetics. Taken from Ostergaard and Yanofsky (2004).

Reverse genetics is a powerful tool that establishes a direct link between the biochemical function of a gene product and its role *in vivo* (Gilchrist and Haughn, 2010). Several methods have been developed that enable to change the nature of gene products for reverse genetics: loss-of-function approach, approach with reduced gene expression, and gain-of-function approach (Bolle et al., 2011)

Using reverse genetics approach, this thesis focuses on expression studies of the *A. thaliana* gene encoding PP4 catalytic and putative regulatory subunits in several transgenic lines: T-DNA insertional mutagenesis lines, artificial microRNA (amiRNA) stable lines, and gene overexpression lines by using semiquantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Finally, we observed the phenotype of the selected mutants from these transgenic lines.

1.2.1 Loss-of-Function Approach: T-DNA Insertional Mutagenesis

Loss of gene function can be achieved by several techniques, such as chemical mutagenesis/TILLING and Insertional Mutagenesis. The total inactivation of a certain gene that cause alteration of phenotype is the most straightforward approach to investigating the function of gene (Bouche and Bouchez, 2001).

Chemical mutagenesis using ethyl methanesulfonate (EMS) induces point mutations of DNA in *A. thaliana* target genes (Greene et al., 2003). For reverse genetics, the difficulty with using point mutations is that there have not been many direct, cost effective ways of screening for individuals from the mutagenized population that carry mutations in specific genes of interest (Gilchrist and Haughn, 2010). Therefore, TILLING (Targeting Induced Local Lesions in Genomes) has been introduced for screening. This techniques, based on Colbert et al. (2001), “employs a mismatch-specific endonuclease for identifying single nucleotide polymorphisms (SNPs) in a gene of interest”. In this methods, DNA from several different plants is pooled and then used as a template for PCR amplification with fluorescently tagged, gene-specific primers.

Insertional mutagenesis is an alternative means of disrupting gene function and is based on the insertion of foreign DNA into a gene of interest. In *Arabidopsis*, this involves the use of either transposable elements or T-DNA/Transferred DNA (Krysan et al., 1999). Instead of transposons, the advantage of using T-DNAs as the insertional mutagen is that T-DNA insertions will not transpose subsequent to integration within the genome and therefore chemically and physically stable through multiple generations (Krysan et al., 1999). The foreign DNA has function as disruptor for the expression of gene interest. Besides that, it also plays role as a marker for identification of mutation. Insertional mutagenesis has two major advantages: the mutations are labelled by the inserted fragments of known sequence (“tags”) and insertions within the coding region have a high probability of eliminating the gene function (Bolle et al., 2011). Some of the disadvantages include the facts that phenotypes may not be obvious if the gene function is

redundant and insertions in essential genes will typically result in lethality making these types of genes difficult to examine using this technique (Gilchrist and Haughn, 2010).

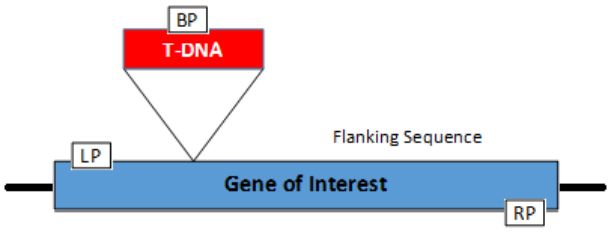


Figure 1-6. Simplified depiction of T-DNA insertion in gene of interest and the primers for genotyping. A specific primer pair are used for genotyping (explain in Figure 1-7) in order to check for the presence of a wild type and homozygous individual. LP: Left genomic primer of gene of interest; RP: Right genomic primer of gene of interest; BP: T-DNA border primer. The figure was redrawn based on http://signal.salk.edu/tdnaprimers.2.html (last retrieved: May 2016)

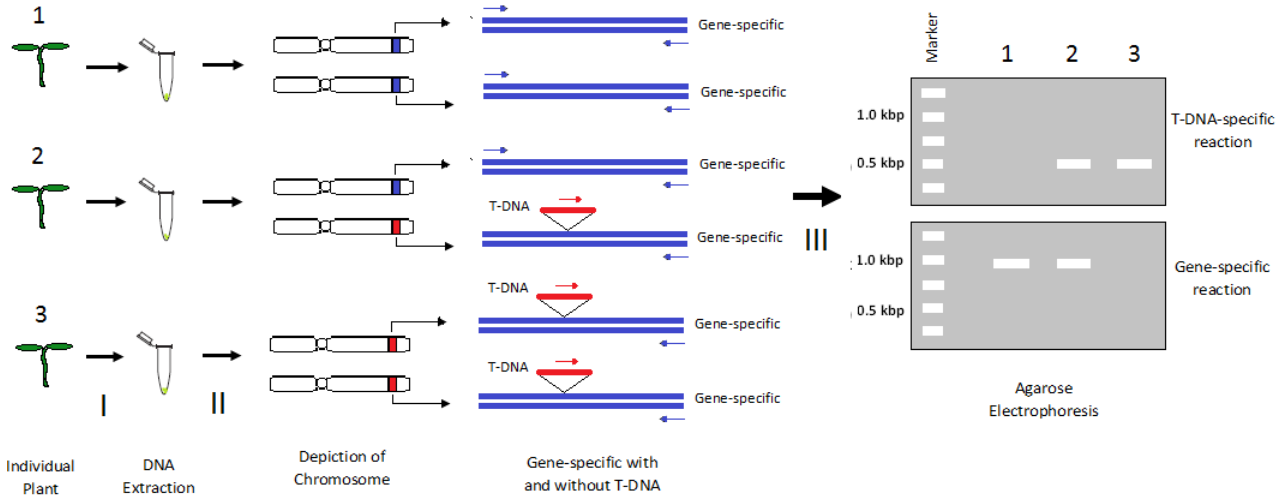


Figure 1-7. Genotyping of segregating T-DNA insertion lines to identify homozygous individual. Here, example of three individual plants were examined and labelled (1, 2, and 3). A single leaf of each plants was transferred to separate tubes and DNA is extracted from these leaf (**I**). PCR (**II**) and gel (agarose) electrophoresis (**III**) are used to genotype the individual DNA samples. A primer pair specific for regions flanking the insertion site are used to check for the presence of a wild type, undisrupted allele of the gene (Figure 1-6). A separate PCR reaction using a T-DNA-specific primer (red arrow) and gene-specific primer (blue arrow) are used to test for the presence of a T-DNA insertion in gene of interest. A wild type (depicted in chromosome as two blue locus) will produce a gene-specific product only (size 1 kbp), as can be seen for individual 1 in the electrophoresis image. A homozygous plant (depicted in chromosome as two red locus) will produce a T-DNA insertion product (size 0.5 kbp), but no wild type product as can be seen for individual 3 in the electrophoresis image. A heterozygous plant (depicted in chromosome as red and blue locus) will produce both T-DNA insertion product and wild type product as can be seen for individual 2 in the electrophoresis image. The figure was remodeling based on O'Malley and Ecker (2010)

T-DNA, any DNA segment flanked by specific 25 bp direct repeats which can be transferred from *Agrobacterium* into plants by the help of Ti and Ri plasmid-encoded virulence (vir) gene functions, have become a universal tool for plant molecular biology (Koncz et al., 1992, Gelvin, 2009). Because *Arabidopsis* introns are small and because there is very little intergenic material, insertion of a piece of T-DNA (Figure 1-6) produces a dramatic disruption of gene function

(O'Malley and Ecker, 2010, Krysan et al., 1999). Even though T-DNA insertional mutagenesis is a knockout approach (Bolle et al., 2011), the insertion of T-DNA element into an *Arabidopsis* chromosome can lead to many different outcomes (Krysan et al., 1999).

In order to identify a homozygous plant for the analysis of phenotypes, a genotyping steps is necessary. Figure 1-7 shows overview the genotyping of segregating T-DNA insertion lines to identify homozygous individual. Polymerase chain reaction (PCR) methods have been developed that allow one to easily isolate individual plants that carry T-DNA mutation of gene interest (May et al., 2002). A specific primer pair are used to genotyping in order to check for the presence of a wild type and homozygous individual (see Figure 1-6).

In this thesis, the lines of the T-DNA plants that were examined are *psy2l* SALK_125872, *psy2l* SALK_048064, *pp4r2l* SALK_093041, *pp4-1* GK_651B07, *pp4-1* SALK_070977, *pp4-2* SAIL_569_H09, and *pp4-2* SALK_049725.

1.2.2 Approach with Reduced Gene Expression: Artificial MicroRNA (amiRNA)

One of the drawbacks of loss-of-function technique is the mutation result in lethality. To overcome this, transgene-gene mediated gene silencing can be used to decrease but not completely abolish (knocked-down approach) the expression of gene of interest (Bolle et al., 2011). Silencing is normally achieved by post-transcriptional down-regulation of transcript accumulation via small RNAs that act in a sequence-specific manner by base pairing to complementary mRNA molecules. Based on gene silencing by small RNA, many strategies have been developed (Ossowski et al., 2008). A widely used approach involves the activation of RNA interference (RNAi) pathway, particularly by using microRNA (miRNA).

Endogenous microRNA or miRNA is a small noncoding RNA that, in complex with Argonaute (AGO) protein, uses seed sequences near its 5’ end to base pair with target mRNA to induce deadenylation and decay or translational regulation (Cech and Steitz, 2014). As a key regulator of gene expression, miRNAs contribute to regulate a broad range processes, including development, differentiation, and disease processes (Gurtan and Sharp, 2013). However, certain viruses have acquired and manipulate host miRNA genes to augment their replication potential in order to enhance their infection (Skalsky and Cullen, 2010).

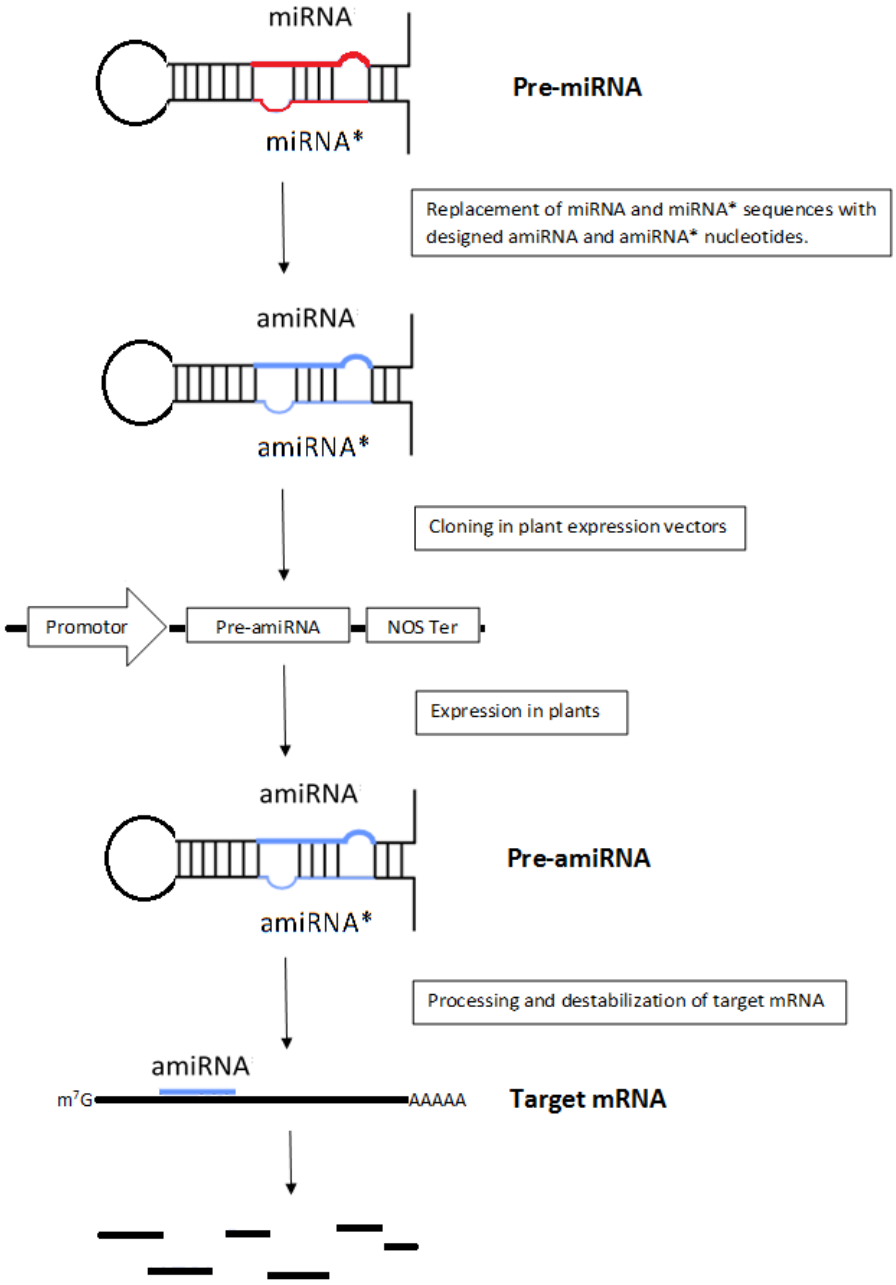


Figure 1-8. Simplified depiction of systematic representation of amiRNA cloning and action. Designed amiRNA is inserted into endogenous miRNA precursor by replacing miRNA/miRNA* sequence with respective amiRNA-amiRNA* nucleotides. In plants, amiRNA inhibits target mRNA expression either by mRNA translation inhibition or degradation of mRNA. The figure was remodeled from (Tiwari et al. (2014), Schwab et al., 2006)

Similar to miRNA, artificial microRNA (amiRNA) are single stranded, approximately 21 nt long, and designed by replacing the mature miRNA sequence with duplex within pre-miRNAs and these amiRNAs are processed via small RNA biogenesis and silencing machinery and deregulate target expression (Tiwari et al., 2014). The systematic representation of amiRNA cloning and action of amiRNA is depicted in Figure 1-8. Expression of miRNAs can be efficiently silenced in *Arabidopsis thaliana* using amiRNA technology (Eamens et al., 2011). In this thesis, the studies were performed to amiRNA against *PP4-1* (the same target as *PP4-2*), *PP4R2L*, and *PSY2L* mRNA.

1.2.3 Gain-of-Function Approach: Overexpression Line

An observation from knock-out approach and knock-down approach are insufficient to deduce the function of gene. Additional genetic approach are needed, which is called gain-of-function (over-expression) approach. Increasing expression of a wild type gene can also be disruptive to a cell or organism, thereby alteration of the phenotype (Prelich, 2012). Gain-of-function is achieved by increasing gene expression levels through the random activation of endogenous genes by transcriptional enhancers or the expression of individual transgenes by transformation (Kondou et al., 2010)

Often the mutant phenotypes induced by loss-of-function and gain-of-function approaches are complementary to each other (Bolle et al., 2011). The mechanism that result in alteration of phenotype due to the over-expression of certain gene are inhibition of another protein to simply reduce the amount that protein or activation of a step in a pathway (Prelich, 2012). In this thesis, the studies were performed to *pp4-1* overexpression, *pp4-2* overexpression, and *pp4r2l* overexpression in *Arabidopsis*.

1.3 Expression Analysis: Semiquantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Several technologies have been developed to measure the level of transcription. They can be divided according to their underlying principle: PCR-based, sequencing-based, and hybridization-based technology (Busch and Lohmann, 2007). Reverse transcription polymerase chain reaction (RT-PCR) is a suitable method for studying transcription of gene with relatively low expression levels; however, this semiquantitative method is labor-intensive and gives data for only one or a very few genes each assay (Etienne et al., 2004). To determine the gene encoding catalytic and regulatory subunits of PP4 (*PP4-1, PP4-2, PP4R2L,* and *PSY2L*) transcript levels in their homozygous T-DNA mutant lines, amiRNA lines, and over-expression lines, total RNAs were isolated from young leaves of wild type and those aforementioned lines, and the gene transcript levels were analyzed by semiquantitative RT-PCR (in short, RT-PCR). Onate-Sanchez and Vicente-Carbajosa (2008) provided DNA-free RNA isolation protocols for *A. thaliana* leaf, seeds, and also siliques. For RT-PCR, mRNA was reverse transcribed, amplified, and electrophoresed (See Figure 1-9 for simplified protocol of semiquantitative RT-PCR). For more advanced method, such as

quantitative RT-PCR (qRT-PCR), other next steps, such as blotted and probed with ^{32}P-labelled internal oligonucleotides, are needed (Caldana et al., 2007).

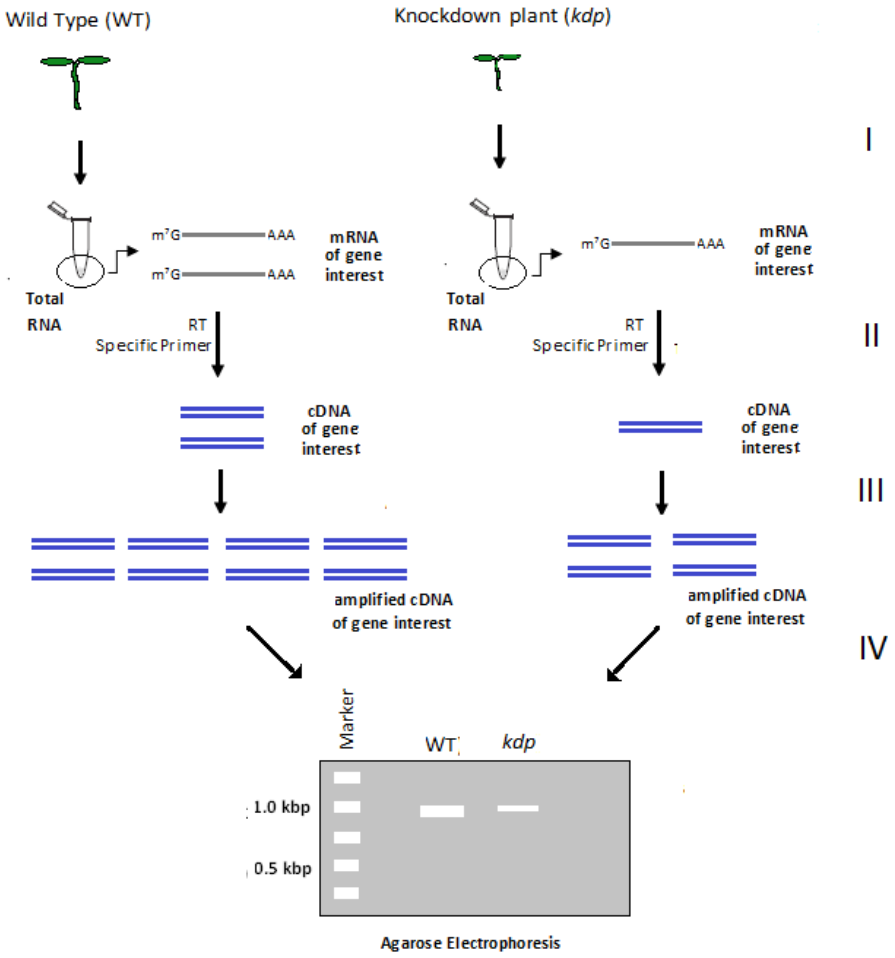


Figure 1-9. Simplified depiction of RT-PCR method to study expression of a gene in plants. Here, we use an example of "knockdown plant" along with control (wild type). **I**: Isolation of total RNA are followed by removing genomic DNA using DNAase. **II**: Synthesis of cDNA from mRNA (depicted the mRNA from gene interest only) by Reverse Transcriptase (RT) with help of primer (i.e. specific primer). **III**: Next step is amplification of cDNA by PCR. **IV**: Finally, electrophoresed of PCR product in agarose electrophoresis, showing the expression of gene of interest in wild type is higher than knockdown plant.

1.4 Subcellular Localization of Protein

Subcellular localizations determine the environments in which proteins operate. As such, subcellular localization influences protein function by controlling access to and availability of all types of molecular interaction partners (Scott et al., 2005). In eukaryotes, protein function may also depend on the protein final destination within the cell complex compartmentalization (Casadio et al., 2008). Localization data are also useful as a means of evaluating protein information inferred from genetic data and reveal protein mechanism of action (Kumar et al., 2002).

Because the protein's function is usually related to its subcellular localization, the ability to predict subcellular localization will be useful on the characterization of the expressed sequences of unknown functions of protein (Yu et al., 2006). *In silico*, many bioinformatics tools have been implemented for predicting subcellular localization, based either on similarity search, motif detection, and sequence analysis with machine learning (ML) methods (Casadio et al., 2008).

In vivo, many methods have been developed to characterize subcellular localization of protein. Traditionally, immunolocalization has been the classical choice for determination of subcellular localization of protein. Although this approach has high specificity and sensitivity, its expensive cost is not suitable for a genome-wide scale (Koroleva et al., 2005). Immunolocalization study of *Arabidopsis* PP4 shows that the protein is a luminal component of root plastids (Pujol et al., 2000).

Systematic localization of proteins can be achieved on a global scale, via expression of proteins tagged with fluorescent markers used to monitor location and movement in living cells (Koroleva et al., 2005). Because proteins are usually not visible in a microscope, the protein to be studied can be visualized by tagging it with a fluorescent marker. Typically the corresponding gene is cloned into recombinant plasmids that encode a fluorescent protein. For instance, Enhanced Yellow Fluorescent Protein (EYFP), one of the fluorescent protein, has proven invaluable as *in vivo* marker for subcellular localization of Arabidopsis Rab GTPase RabA4b to the tips of growing root hair cells (Preuss et al., 2004). The fluorescent protein is fused routinely to either the N or C termini of target genes (Kumar et al., 2002). Many targeting signals may be disrupted by using this approach. Specifically, with N-terminal fusions, endoplasmic reticulum (ER) signal peptides may be masked and mitochondrial or chloroplast transit peptides can be obscured (Tian et al., 2004). With C-terminal fusions, many proteins may also mislocalize, such as the CesA1 family protein that encodes the catalytic subunits of cellulose in Arabidopsis (Tian et al., 2004).

In order to study the protein localization on a large scale, it is very essential to express the tagged proteins in cells in an efficient, rapid, versatile, and non-destructive way (Marion et al., 2008). Several methods of transient transformation have been described in plants, such as mesophyll protoplasts and particle bombardment/biolistic. To complement, the localization of the fusion-proteins were visualized using confocal microscopy after one or two days of incubation. In this thesis, those two methods are used, and the subcellular localization studies were performed toward fusion protein of PP4-1, PP4-2, PP4R2L, and PSY2L into fluorescent protein EYFP on the N–Terminus and C-terminus.

1.4.1 Transient Transformation via *Arabidopsis* Mesophyll Protoplasts

Plant protoplast without cell walls offer a versatile cell-based experimental system (Yoo et al., 2007). Although plant protoplasts show physiological perceptions and responses similar to cell-autonomous in intact tissues and plants (Sheen, 2001), they are considered not sufficiently able to reproduce the in planta situation (Faraco et al., 2011). As describes by Sheen (2001), mesophyll protoplast isolated from fresh leaves have many practical advantages, for example no sterile procedure is needed for storage and it also has high transformation efficiency reaching 90% using Arabidopsis mesophyll protoplasts. Beside advantages, the method also has limitations, for instance the inadequacy to isolate active protoplasts from each plant cell type or from all growth conditions and the fact that cell walls, plasmodesmata, and cell-cell interactions are lost and interrupted (Sheen, 2001). Yoo et al. (2007) described an efficient and versatile transient assay involving transformation of mesophyll protoplast, where the DNA is delivered into protoplasts using PEG-calcium fusion. One example of the application of this method is the study of PP2A holoenzyme, that targeted peroxisomes by piggybacking (Kataya et al., 2015).

1.4.2 Transient Transformation via Particle Bombardment

Particle bombardment or “gene gun” employs high-velocity microprojectiles to deliver substances into cells and tissues in which DNA is coated onto the surface of micro-sized tungsten or gold particles by precipitation with calcium chloride and spermidine (Kikkert et al., 2005). Particle bombardment allows direct transformation of plant cells, usually to *Allium cepa* (onion) epidermal cells, but shows relatively poor efficiency and indulges significant mechanical stress (Marion et al., 2008). *Agrobacterium*-mediated transformation of onion epidermal cells may provide higher efficiency and more simplified subcellular localization (Sun et al., 2007).

1.5 Objectives of the Present Study

Protein phosphatase 4 (PP4) is remarkably well conserved across eukaryotes. PP4 has been studied mainly in yeast and mammalian cells, and virtually nothing is known about PP4 in plants. In mammalian cells PP4 play a role in several processes not relevant to plants. As a continuous work from previous master student, Sætre (2015), the overall aim of this thesis is to understand the physiological function of PP4 in plants. *Arabidopsis thaliana* as a model plant has two PP4 catalytic subunits, namely PP4-1 (At4G26720) and PP4-2 (At5G55260). In addition, putative regulatory proteins for PP4 were bioinformatically detected: PP4R2L (At5G17070) and PSY2L (At3G06670) (Lillo et al., 2014). Therefore, in order to achieve this aim, the main objectives of the present study were:

- Using reverse genetics approach, this thesis focuses on expression studies by using semiquantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) of the *A. thaliana* gene encoding PP4 catalytic and putative regulatory subunits in several transgenic lines: T-DNA insertional mutagenesis lines, artificial microRNA (amiRNA) stable lines, and gene overexpression lines. We also observed the phenotype the selected plants of these transgenic mutants.
- Investigation of the subcellular localization of PP4 catalytic and putative regulatory subunits in two different plant expression systems: *Arabidopsis* mesophyll protoplasts and particle bombardment into onion epidermis cells.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Materials

All work was done on plants of the species *Arabidopsis thaliana*. For this study, one type of wild type plant and three types of plant mutants were used; T-DNA plants, amiRNA plants, and overexpression gene plants.

2.1.1.1 Wild Type Plant

Arabidopsis thaliana ecotype Columbia was used as the wild type.

2.1.1.2 T-DNA Plants

The T-DNA plant materials (*Arabidopsis thaliana*) used for this study were received from previous employee (Zek Ginbot) and master student (Christine Sætre). The genes to be examined in this study were the *PSY2L*, *PP4R2L*, *PP4-1*, and *PP4-2* gene. Two lines were used for the *PSY2L* gene, one for the *PP4R2L* gene, two for the *PP4-1* gene, and two for *PP4-2* gene (Table 2-1).
The seed of the plants that already available in the lab were to be tested to find homozygous individual by genotyping.

Table 2-1. Overview of T-DNA insert in the plant materials. The source is TAIR (Arabidopsis Information Resource) website with address https://www.arabidopsis.org (Last Retrieved: March 2016).

Name of gene	AGI number	Line	T-DNA Location
PSY2L	At3g06670	SALK_125872	Flank-tagged downstream of translation, segregating (see Figure 3-5)
		SALK_048064	Insert in exon 3 of 25, segregating (see Figure 3-1)
PP4R2L	At5g17070	SALK_093041	Insert in exon 7 of 8, segregating (see Figure 3-7)
PP4-1	At4g26720	GK_651B07	Flank-tagged upstream of translation, segregating (see Figure 3-11)
		SALK_070977	Flank-tagged downstream of translation, segregating (see Figure 3-11)
PP4-2	At5g55260	SAIL_569_H09	Insert in intron 1 of 7, segregating (see Figure 3-9)
		SALK_049725C	Insert in exon 8 of 8, segregating (see Figure 3-9)

2.1.1.3 Artificial MicroRNA Plants (amiRNA Plants)

2.1.1.3.1 amiRNA Plants against *PP4-1*/*PP4-2*

Two different amiRNAs that designed to target *PP4-1* as well as *PP4-2* were introduced to the plants with the following sequence:

amiRNA1: 5′ TAATGAGAGTTATACGGTCTA 3′

amiRNA2: 5′ TTAAAAGACGTAACAACGCTG 3′

The amiRNAs were transformed using the constitutive pBA002 vector (Appendix 2-1). The plants that carrying these amiRNA were already available in the lab as well**.** Two plants were used for each amiRNA, giving rise to the following classification: 1-1 being amiRNA1-plant 1; 1-2 being amiRNA1-plant 2; 2-1 being amiRNA2-plant 1; and 2-2 being amiRNA2-plant 2. Plants transformed with only the vector with no amiRNA present were also available for this study (vector only).

2.1.1.3.2 amiRNA Plants against *PSY2L*

The amiRNAs for targeting *PSY2L* were transformed using pBA002 vector. The plants that carrying these amiRNA were already available in the lab as well**.** Two different amiRNAs were introduced to the plants 5A and 7A. Plants transformed with only the vector with no amiRNA present were also available for this study (vector only).

2.1.1.3.3 amiRNA Plants against *PP4R2L*

The amiRNAs for targeting *PP4R2L* were transformed using pBA002 vector. The plants that carrying these amiRNA were already available in the lab as well (plants 9A). Plants transformed with only the vector with no amiRNA present were also available for this study (vector only).

2.1.1.4 Overexpression Plants

2.1.1.4.1 *pp4-1* Overexpression Plants

Mutant plants transformed using the constitutive pBA002 vector carrying the cDNA of *PP4-1*. The plants that carrying these were already available in the lab as well (plants A1/1A).

2.1.1.4.2 *pp4-2* Overexpression Plants

Mutant plants transformed using the constitutive pBA002 vector carrying the cDNA of *PP4-2*. The plants that carrying these were already available in the lab as well (plants A2/2A).

2.1.1.4.3 *pp4r2l* Overexpression Plants

Mutant plants transformed using the constitutive pBA002 vector carrying the cDNA of *PP4R2L*. The plants that carrying these were already available in the lab as well (plants A3/3A).

2.1.2 Vectors for Molecular Cloning

PSY2L and *PP4-1* gene were examined to clone into two different vectors; pCAT-EYFP-1 and pCAT-EYFP-2. Both vectors share the same nucleotide sequence. The difference is pCAT-EYFP-1 will ensure that the resulting fusion-protein will carry the EYFP tag on the N–Terminus, whereas the pCAT-EYFP-2 will give the resulting fusion-protein an EYFP tag at the C–terminus. See Appendix 2-2 for pCAT-EYFP-1 map (and also for pCAT-EYFP-2).

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 (Promega).

2.1.4 Kits

Table 2-2 gives an overview of the kits used during this theses.

Table 2-2. Overview of all kits used

Kit	Function	Supplier	City, Country
Phire® Plant Direct PCR Kit	Genotyping of plants	New England Biolabs	Ipswich, MA, USA
Plasmid miniprep kit	Plasmid isolation	Sigma Aldrich	St. Louis, MO, USA
High Fidelity PCR kit	Amplification of gene	Roche	Mannheim, Germany
DreamTaq™ DNA Polymerase	PCR for targeted cDNA	Thermo Scientific	Carlsbad, CA, USA
SuperScript® III Reverse Transcriptase	Reverse Transcriptase for synthesizing cDNA	Invitrogen	Carlsbad, CA, USA
Nucleospin® Gel and PCR clean up	Isolation of DNA after PCR and digestion	Machery Nagel	Düren, Germany

2.1.5 Chemical Lists

Table 2-3 gives an overview of the reagents used during this theses.

Table 2-3. Overview of the reagents used during this thesis.

Reagan	Classification	Supplier	City, Country
BSA, Purified BSA 100x (10 mg/ml)	Protein	New England Biolabs	Ipswich, MA, USA
NcoI	Restriction Enzyme	New England Biolabs	Ipswich, MA, USA
NotI	Restriction Enzyme	New England Biolabs	Ipswich, MA, USA
SacI	Restriction Enzyme	New England Biolabs	Ipswich, MA, USA
SacII	Restriction Enzyme	New England Biolabs	Ipswich, MA, USA
Taq Polymerase	DNA Polymerase Enzyme	Invitrogen	Carlsbad, CA, USA
High Fidelity Polymerase	DNA Polymerase Enzyme	Roche	Mannheim, Germany
T4 DNA Polymerase	DNA Polymerase Enzyme	Promega	Madison, MI, USA
rSap	Enzyme	New England Biolabs	Ipswich, MA, USA
NEBuffer 1	Buffer	New England Biolabs	Ipswich, MA, USA
NEBuffer 3	Buffer	New England Biolabs	Ipswich, MA, USA
PCR buffer, 10X PCR rxn buffer -$MgCl_2$	Buffer	Invitrogen	Carlsbad, CA, USA
High fidelity PCR buffer, Expand high fidelity PlusPCR system Reaction Buffer (5x) with $MgCl_2$	Buffer	Roche	Mannheim, Germany
T4 DNA Ligase 10X buffer	Buffer	Promega	Madison, MI, USA
rSap buffer	Buffer	New England Biolabs	Ipswich, MA, USA
1x TAE Buffer	Buffer	In-house	-
6x Loading buffer	Buffer	In-house	-
Molecular Biology Grade Water	Chemical	Thomas Scientific	Swedesboro, NJ, USA
dNTP's, 2.5 mM of each NTP, total 10 mM	Chemical	Bioline	Luckenwalde, Germany
Gel red 10,000x in water	Chemical	Biotium	Hayward, CA, USA
Linsmaier and Skoog (LS)	Chemical	Caisson Laboratories	Smithfield, UT, USA

Agarose	Chemical	Sigma Aldrich	St. Louis, MO, USA
Agar-agar	Chemical	Merck	Darmstadt Germany
LB agar	Chemical	Merck	Darmstadt Germany
LB Broth	Chemical	Merck	Darmstadt Germany
Triton X-100	Chemical	Sigma Aldrich	St. Louis, MO, USA
HyperLadder™ 1kb	Marker gel electrophoresis	Bioline	Luckenwalde, Germany
Quick-Load® 100 bp	Marker gel electrophoresis	New England Biolabs	Ipswich, MA, USA
P-jord	Planting Soil	Tjerbo	Rakkestad, Norway
Agra-Vermiculite	Phyllosilicate group of minerals	RHP	Rhenen, The Netherland

2.2 Methods

2.2.1 Polymerase Chain Reaction (PCR)

The PCR is performed to amplify DNA based on a template strand. To make sure the DNA sample will be processed in correct amplification step at the correct time, a thermocycler is utilized. For this study, PCR was used for genotyping T-DNA plants to find homozygous individuals, amplifying genes used for molecular cloning (high fidelity PCR), checking if transformation of bacterial colonies from the molecular cloning contained the insert (colony PCR), and amplification of targeted cDNA (RT-PCR).

2.2.1.1 PCR for Genotyping T-DNA Plants to Find Homozygous Individuals

Genotyping was performed on individual plants of *Arabidopsis thaliana* T-DNA plants for the following Salk lines: *psy2l* SALK_125872, *psy2l* SALK_048064, *pp4r2l* SALK_093041, *pp4-1* GK_651B07, *pp4-1* SALK_070977, *pp4-2* SAIL_569_H09, and *pp4-2* SALK_049725C.

Two types of master mix was prepared, one designed using a specific primer pair that would amplify T-DNA inserted with gene of interest by using BP (T-DNA border primer) and RP (Right genomic primer of gene of interest). The other using primers that would amplify only the wild type DNA by using LP (Left genomic primer of gene of interest) and RP (Right genomic primer of gene of interest). The general location of these primers toward gene of interest and T-DNA is shown in Figure 1-6.

A ten-fold dilution of the primer stock solution (concentration 10 µM) was used as the primer working solution for all primers. They were made by added 10 µl primer stock solution (concentration 100 µM) to 90 µl dH_2O.

2.2.1.1.1 Primers Used for Genotyping

List of primers used when genotyping the T-DNA plants can be seen in Table 2-4

Table 2-4. List of primers used when genotyping the T-DNA plants

Line	Name of Primer	Nucleotide Sequence (5' → 3')
BP for all SALK lines	LBb1.3 New Salk	ATTTTGCCGATTTCGGAAC
SALK_125872	PSY2L Salk RP 125872	AAARGAATATGGCTTTTGGGG
	PSY2L Salk LP 125872	AAGCCTCTGAGGATGAGGAAG
SALK_048064	PSY2L Salk RP 048064	TGTTGAATTGAGATGGAAGGG
	PSY2L Salk LP 048064	ATGTTTCGCCTGTTCAATCAC
SALK_093041	PP4R2 Salk RP 093041	TGTTCAACAGATCCTTTTGGC
	PP4R2 Salk LP 093041	CAACATATTTGGCATTTTGGC
SAIL_569_H09	PP4-1 Sail RP 569H09	ATAGAGCAGCTTAAACGCTGC
	PP4-1 Sail LP 569H09	TGGAGAGAGACCTCCATGAAC
GK_651B07	PP4-1 GK RP 651B07	TAAAGACACAAAATCTCCGCG
	PP4-1 GK LP 651B07	ACAAAATCAAAGACACGGTCG
SALK_070977	PP4-1 Salk RP 07977	AGAATTCCGTGTGTTTGATGC
	PP4-1 Salk LP 07977	TTGTATCCAGCCCGTTATTTG
SALK_049725C	PP4-2 Salk RP 049725C	AACGATGACCTTGCAATTTTG
	PP4-2 Salk LP 049725C	TTCATGGAGGTCTCTCTCCAG

2.2.1.1.2 PCR Mix and PCR Program Used when Genotyping

For genotyping, Thermo Scientific Phire Plant Direct PCR kit was used.

PCR mix used for genotyping of T-DNA plants per tube sample can be seen in Table 2-5 and Table 2-6 shows PCR program used for genotyping of T-DNA plants

Table 2-5. PCR mix used for genotyping of T-DNA plants per tube sample

Reagent	Amount (µL)	Final Concentration
2x Buffer	10	1x
Primer 1 (10 µM)	1*	0.5 µM
Primer 2 (10 µM)	1*	0.5 µM
DNA Polymerase	0.4	-
Plant material (Template DNA)**	0.5	-
Water	7.1	-
Total	***20***	

*: Amount of primer was determined by using this calculation = total volume x final concentration of primer)/concentration of diluted primer. In this case total volume is 20 µL, final concentration of primer is 0.5 µM, and concentration of diluted primer is 10 µM.

**: Template DNA was obtained by crushing approximately 1x2 mm young leaf of plant in 15 µL Dilution Buffer.

Table 2-6. PCR program used for genotyping of T-DNA plants

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

Note: denaturation, annealing, and extension were repeated 40 times.

2.2.1.2 PCR for Amplifying Genes Used for Molecular Cloning (High Fidelity PCR)

High fidelity PCR was performed to ensure that the amplified constructs were correct in regards to the original sequence**.** The *PSY2L* and *PP4-1* gene were amplified using high fidelity PCR. Both genes were examined to clone into two different vectors; pCAT-EYFP-1 and pCAT-EYFP-2.

2.2.1.2.1 Primers Used for High Fidelity PCR

List of primers used when amplifying *PSY2L* and *PP4-1* gene can be seen at Table 2-7 and 2-8, respectively:

Table 2-7. List of primers used when amplifying *PSY2L* gene

Destination Vector	Primer	Nucleotide Sequence (5’ → 3’)
pCAT-EYFP-1	Forward Primer : EYFPPSY2Lf	AAAGCGGCCGCTTATGGGCGCTCCGGAAAAGTCT
	Reversed Primer : EYFPPSY2Lr	ATTGCGGCCGCTCAGGATCCATTTACAGCCAT
pCAT-EYFP-2	Forward Primer : PSY2LEYFPf	ATCCATGGGTATGGGCGCTCCGGAAAAGTCT
	Reversed Primer : PSY2LEYFPr	ATTGCGGCCGCGGATCCATTTACAGCCAT

Table 2-8. List of primers used when amplifying *PP4-1* gene

Destination Vector	Primer	Nucleotide Sequence (5’ → 3’)
pCAT-EYFP-1	Forward Primer : AK72f	ATGCGGCCGCTATGTCAGACCTAGATCGGCAAA
	Reversed Primer : AK73r	ATCCGCGGTTATAGGAAGTAATCAGGGG
pCAT-EYFP-2	Forward Primer : AK74f	ATGAGCTCTCATGTCAGACCTAGATCGGCA
	Reversed Primer : AK75r	ATGCGGCCGCTATGTCAGACCTAGACAAGCAA

2.2.1.2.2 PCR Mix and PCR Program Used High Fidelity PCR

The amount of reagents used when performing high fidelity PRC to amplify constructs used for molecular cloning is shown in Table 2-9 and Table 2-10 shows The PCR program used follows.

Table 2-9. The amount of reagents used when performing high fidelity PRC to amplify constructs used for molecular cloning

Reagent	Amount (µL) per tube sample	Final Concentration
Expand Hifi^{PLUS} reaction buffer 5x	10	1x
Upstream primer (10 µM)	2*	0.4 µM
Downstream primer (10 µM)	2*	0.4 µM
Expand HifiPLUS Enzyme Blend	0.5	2.5 U
Template DNA	0.5	5-500 ng (genomic DNA) 100 pg-10 ng (plasmid DNA)
Water	34	-
Total	***50***	

*: Amount of primer was determined by using this calculation = (total volume x final concentration of primer)/concentration of diluted primer. In this case total volume is 50 µL, final concentration of primer is 0.4 µM, and concentration of diluted primer is 10 µM.

Table 2-10. The PCR program high fidelity PRC to amplify constructs used for molecular cloning

Step	Temperature	Time
Initial denaturation	94˚C	2 min
Denaturation	94˚C	15 s
Annealing	60˚C	30 s
Extension	70˚C	2.5 min
Final extension	72˚C	7 min
Hold	4˚C	∞

Note: denaturation, annealing, and extension were repeated 35 times

2.2.1.3 PCR for Checking Transformation of Bacterial Colonies from the Molecular Cloning if Contained the Insert (Colony PCR)

Colony PCR was performed on transformed bacterial colonies using both vector-specific primers and gene-specific primers.

2.2.1.3.1 Primers Used for Colony PCR

List of primers used for colony PCR of transformed bacteria can be seen at Table 2-11 and 2-12:

Table 2-11. List of primers used for colony of transformed bacteria with *PSY2L* gene

Destination Vector	Primer	Nucleotide Sequence (5′ → 3′)
pCAT-EYFP-1	Forward Primer : PSY2L Middle	ATACACCAGACGTACAGAATTGG
	Reversed Primer : EYFPPSY2Lr	ATTGCGGCCGCTCAGGATCCATTTACAGCCAT
pCAT-EYFP-2	Forward Primer : PSY2L Middle	ATACACCAGACGTACAGAATTGG
	Reversed Primer : EYFPPSY2Lr	ATTGCGGCCGCTCAGGATCCATTTACAGCCAT

Table 2-12. List of primers used for colony of transformed bacteria with *PP4-1* gene

Destination Vector	Primer	Nucleotide Sequence (5′ → 3′)
pCAT-EYFP-1	Forward Primer : EYFP-C-Terf	ACTACCTGAGCTACCAGTCC
	Reversed Primer : AK73r	ATCCGCGGTTATAGGAAGTAATCAGGGG
pCAT-EYFP-2	Forward Primer : AK74f	ATGAGCTCTCATGTCAGACCTAGATCGGCA
	Reversed Primer : AK94r	AACTTCAGGGTCAGCTTGCCGT

2.2.1.3.2 PCR Mix and PCR Program Used in Colony PCR

The amount of reagents used when performing colony PCR is shown in Table 2-13 also the program that is used in Table 2-14.

Table 2-13. The amount of reagents used when performing colony PCR

Reagent	Amount (µL) per tube sample	Final Concentration
10x Buffer –$MgCl_2$	2.5	1x
50 mM $MgCl_2$	0.75	1.5
10 mM dNTP mixture	0.5	0.2
10 µM Primer forward primer	1.25*	0.5
10 µM Primer reversed primer	1.25*	0.5
Template DNA	From colony	-
Taq DNA polymerase	0.1	-
Water	18.65	-
Total	***25***	

*: Amount of primer was determined by using this calculation = total volume x final concentration of primer)/concentration of diluted primer. In this case total volume is 25 µL, final concentration of primer is 0.5 µM, and concentration of diluted primer is 10 µM.

Table 2-14. The PCR program used for colony PCR

Step	Temperature	Time
Initial denaturation	94˚C	3 min
Denaturation	94˚C	45 s
Annealing	55˚C	30 s
Extension	72˚C	3 min
Final extension	72˚C	10 min
Hold	4˚C	∞

Note: denaturation, annealing, and extension were repeated 35 cycles

2.2.1.4 Semiquantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

2.2.1.4.1 Primers Used for RT-PCR

List of primers used for RT-PCR of transformed bacteria can be seen at Table 2-15:

Table 2-15. List of primers used for RT-PCR

cDNA	Primer	Nucleotide Sequence (5' → 3')
PP4-1	Forward Primer : AK74f	ATGAGCTCTCATGTCAGACCTAGATCGGCA
	Reversed Primer : AK75r	ATGCGGCCGCTATGTCAGACCTAGACAAGCAA
PP4-2	Forward Primer : AK77f	ATCCATGGGTATGTCAGACCTAGACAAGCA
	Reversed Primer : AK75r	ATGCGGCCGCTATGTCAGACCTAGACAAGCAA
PP4R2L	Forward Primer : PP4R2LEYFPf	ATCCATGGGTATGGAGAATCCGTCATCATCG
	Reversed Primer : PP4R2LEYFPr	AAGCGGCCGCGGCACACGTTGTAGGCAACCG
PSY2L	Forward Primer : RT PSY2L-LP	CTCATGGAGAGACCCAGAGC
	Reversed Primer : RT PSY2L-RP	CCATCAGATTCCGGAAGAAA

2.2.1.4.2 PCR Mix and PCR Program Used in RT-PCR

The amount of reagents used when performing RT-PCR is shown in Table 2-16 also the program that is used in Table 2-17.

Table 2-16. The amount of reagents used when performing RT-PCR

Reagent	Amount (µL) per tube sample	Final Concentration
10x Dream taq Buffer	2.0	-
10 mM dNTP mixture	2.0	-
10 µM Primer forward primer	2.0*	1.0 µM
10 µM Primer reversed primer	2.0*	1.0 µM
Template DNA	2.0	-
Dream Taq DNA polymerase	0.1	0.5 U
Water	9.9	-
Total	***20***	

*: Amount of primer was determined by using this calculation = total volume x final concentration of primer)/concentration of diluted primer. In this case total volume is 20 µL, final concentration of primer is 1.0 µM, and concentration of diluted primer is 10 µM.

Table 2-17. The PCR program used for RT-PCR

Step	Temperature	Time
Initial denaturation	95˚C	3 min
Denaturation	95˚C	30 s
Annealing	60˚C	30 s
Extension	72˚C	2 min 45 s
Final extension	72˚C	10 min
Hold	4˚C	∞

Note: denaturation, annealing, and extension were repeated 31 times

2.2.2 Agarose Gel Electrophoresis

Concentration of Agarose gel that was used for genotyping PCR, high fidelity PCR, and colony PCR is 1 %. To make the gel, 1 g agarose was used in 100 ml 1x TAE buffer. The agarose-buffer mixture was heated until all agarose was solved, then poured into the cast with proper combs to solidify.

Concentration of Agarose gel that was used for digestion and RT-PCR is 1.5 %. To make the gel, 1.5 g agarose was used in 100 ml 1x TAE buffer. The agarose-buffer mixture was heated until all agarose was solved, then poured into the cast with proper combs to solidify.

For visualizing the DNA bands, gel-red 1:50 (in water) was used. Loading buffer 6x was added to ensure that the sample maintained its position in the correct well. 2 µl of gel red 1:50 and 2 µl of loading buffer 6x were added to each 10 µl of PCR sample before running it on agarose gel.

Marker was used for determination of size. In this thesis, HyperLadder™ 1kb and Quick-Load® 100 bp were used. Figure 2-1 provides the overview of these markers. For every electrophoresis running, 5 µl of marker was mixed with 2 µl gel-red 1:50. The gel was run for 40 min at 90 V and analyzed using UV light to visualize the DNA bands.

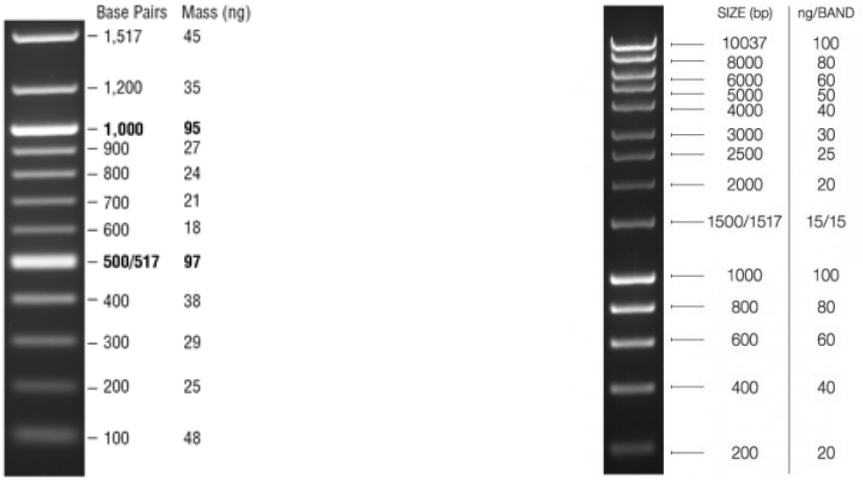


Figure 2-1. Overview of the markers, Quick-Load® 100 bp (**A**) and HyperLadder™ 1kb (**B**), with size and also concentration (per 5 µl). This figure will be shown in gel electrophoresis of sample, depends on kind of marker that was used.

2.2.3 Molecular Cloning

Overview of the entire molecular cloning procedure:

1. Amplification of the construct gene using high fidelity PCR. Further, the sample was analyzed using gel electrophoresis. The DNA band in gel was then extracted using gel filtration kit, and the concentration of construct was measured using nanodrop.
2. Ligation of gene with vector plasmid
3. Transformation to competent cells and incubation overnight into selected medium
4. Checking the survival colonies using colony PCR and gel electrophoresis
5. Isolation of the plasmid from survival colonies
6. Single or and double digestion of the plasmid using selected restriction endonuclease and checking the digested product using gel electrophoresis.
7. The positive sample was sent to sequencing.

2.2.3.1 Amplification of Gene Construct

For amplifying gene construct, high fidelity PCR was used. Further, the sample was analyzed using gel electrophoresis. The target band in gel was cut, and extracted using Nucleo spin® Gel & PCR Clean-up (Machoy Nagel) following this procedure: 200 µL NTI solution was added to each 100 mg gel, incubated in 50^{O}C for 10 min. The liquid was transferred to silica column in collection tube, centrifuged at 11,000 g for 30 sec, and the flowthrough was discarded. 700 µL NT3 solution was added into column, centrifuged at 11,000 g for 30 s, and and the flowthrough was discarded. This step was done twice. Dried the column by centrifuging at 11,000 g for 60 s. Collection tube was replaced. 200 µL water was added into column, kept in column for 60 s, and centrifuged at 11,000 g for 60 s. The liquid was collected, labelled, and stored at -20^{o}C. By using nanodrop, the concentration of gene was measured.

2.2.3.2 Ligation of Gene with Vector Plasmid

Ligation of insert was performed into 2 different vectors, pCAT-EYFP-1 and pCAT-EYFP-2.

For ligating the insert into the vector, the amount of insert and vector was calculated following this equation:

$$amount\ of\ insert\ (ng) = \frac{amount\ of\ vector\ (ng) x\ size\ of\ insert\ (kb)}{size\ of\ vector\ (kb)}\ x\ molar\ ratio\ of\ \frac{insert}{vector}$$

The recommended molar ratio of insert/vector is 3/1. This value means that the insert amount was multiplied with 3. Increasing of ratio can be done based on ligation result.

The amount of reagents used for ligation follows in Table 2-18.

Table 2-18. The amount of reagents used for ligation

Reagent	Amount (µL) reagent	
	Sample	**Control**
10x ligation buffer*	1	1
pGEMT Vector	1	1
T4 DNA Ligase	1	1
Control insert DNA	-	2
PCR product (sample)	7**	-
Water		5
Total	***15***	***15***

*: 10x ligation buffer must be vortex vigorously before each use.

**: Volume of PCR product (µL) was determined by using this calculation = expected amount of DNA for reaction (ng) / concentration of PCR product (ng/µL). Water was added until reach 15 µL volume reaction.

Following the above list, reagents for each ligation mix was prepared. The ligation mixture was left at 4^{o}C overnight before it was used in transformation of bacterial cells.

2.2.3.3 Transformation to Competent Cells

The heat-shock method was used for transformation of competent bacterial cells, *Escherichia coli* JM109. The following procedure was followed to transform the plasmid to competent cells: The competent cells were first thawed on ice for 10 min. DNA material from ligation mixture 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 using water bath for 50 sec. The bacterial mixture then was incubated on ice for 5 mi and was added with 0.5 mL LB medium. The bacterial suspension was shake and incubated at 37˚C for 2 h. 600 µL bacterial suspension were then transferred to LB agar plates containing Ampicillin that was added 40 µL X-gal and 40 µL IPTG solution before the plates were incubated at 37˚C overnight.

2.2.3.4 Confirmation and Colony PCR

Figure 2-2 describe the following procedures for sampling selected colonies (white colonies) and checking them using colony PCR. Here, we used blue-white screening with X-gal.

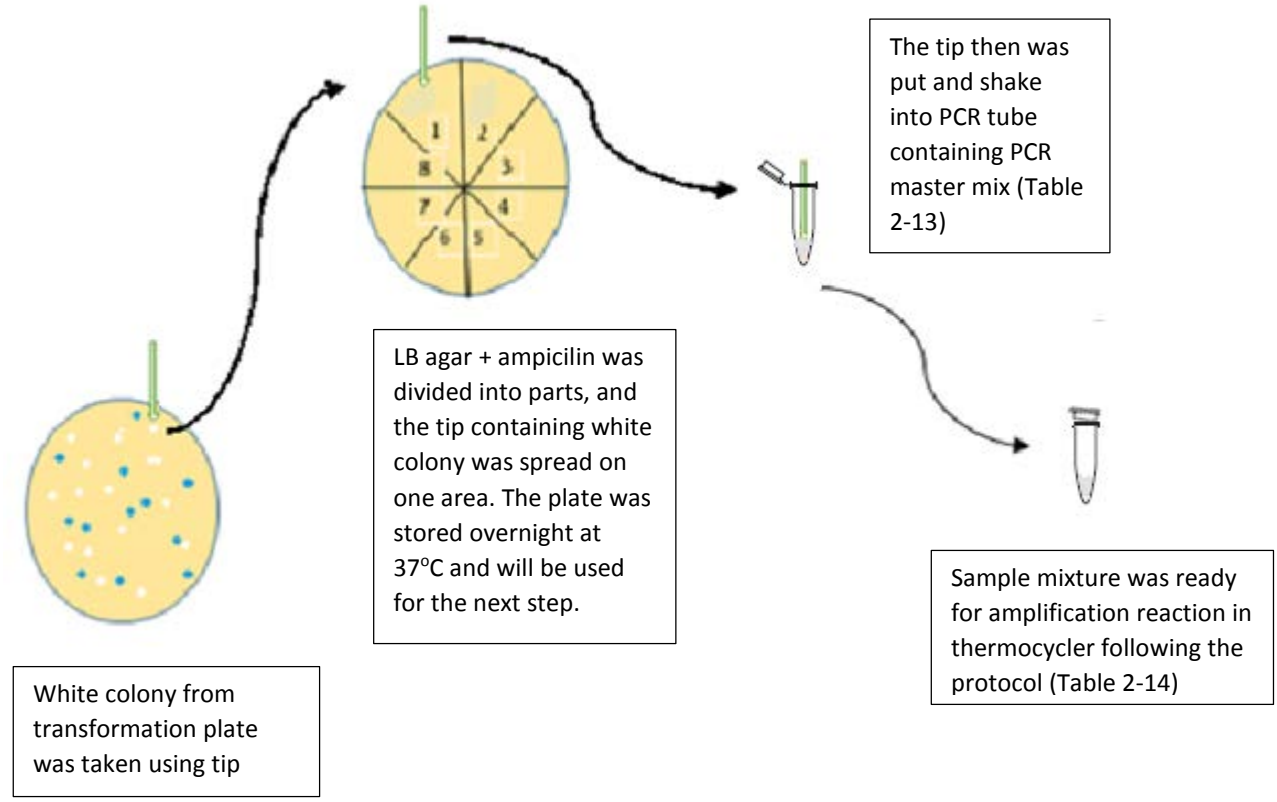


Figure 2-2. The procedures for sampling selected colonies (white colonies) and checking them using colony PCR. Here, we used blue-white screening with X-gal.

2.2.3.5 Isolation of the Plasmid from Survival Colonies

Figure 2-3 shows the procedure for cultivating colony into LB Broth.

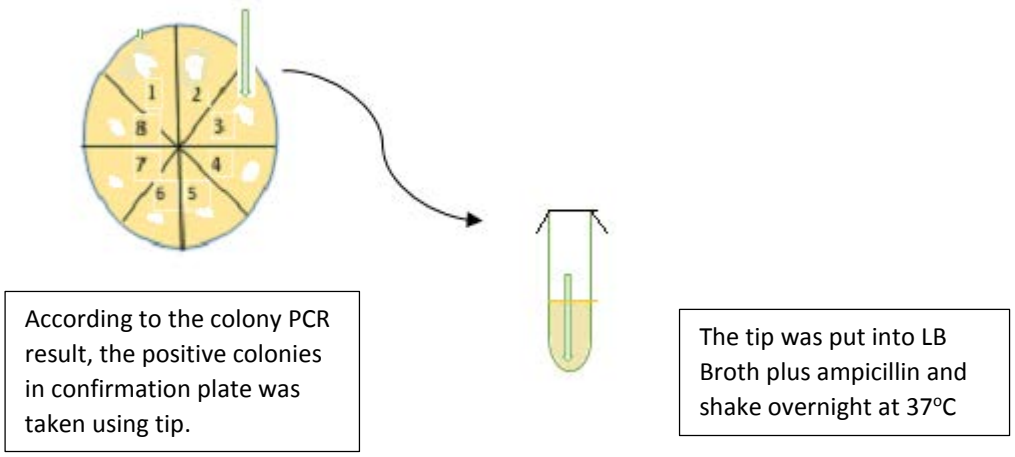


Figure 2-3. The procedure for cultivating colony from confirmation plate into LB Broth

The selected colonies were transferred to a tube containing 7 mL LB Broth plus 7 µL ampicillin 100 mg/mL and shake overnight at 37°C (picture).

Plasmid isolation was performed using the plasmid miniprep kit from Sigma-Aldrich/Promega. The protocol given by the supplier was followed. After gel electrophoresis, by using nanodrop, the concentration of samples were measured.

2.2.3.6 Digestion with One Type of Restriction Enzyme

The following table gives an overview of the reagents and restriction enzymes used for digestion with one type of restriction enzyme. All mixture were incubated at 37°C for 2 h. It was followed by running sample to agarose gel electrophoresis for single digestion.

Table 2-19. The reagents for digestion with one type of restriction enzyme

Reagent	Amount (µL) per tube sample
Purified BSA 100x	1.5
NE buffer*	1.5
Restriction Enzyme (10.000 U/µL)	1
DNA	11**
Water	
Total	***15***

*: The type of NE buffer was determined based on suitability with used restriction enzymes.

**: Volume of DNA sample (µL) was determined by using this calculation = expected amount of DNA for reaction (ng) / concentration of DNA sample (ng/µL). Water was added until reach 15 µL volume reaction.

2.2.3.7 Digestion with Two Types of Restriction Enzyme

The following table gives an overview of the reagents and restriction enzymes used for digestion with two types of restriction enzyme. All mixture were incubated at 37°C for 2 h. Following by running sample to agarose gel electrophoresis for single digestion.

Table 2-20. The reagents for digestion with two types of restriction enzyme.

Reagent	Amount (µL) per tube sample
Purified BSA 100x	1.5
NE buffer*	1.5
Restriction Enzyme I (10.000 U/µL)	1
Restriction Enzyme II (10.000 U/µL)	1
DNA	10**
Water	
Total	***15***

*: The type of NE buffer was determined based on suitability with used restriction enzymes.

**: Volume of DNA sample (µL) was determined by using this calculation = expected amount of DNA for reaction (ng) / concentration of DNA sample (ng/µL). Water was added until reach 15 µL volume reaction.

For further investigation, such as sequencing, the targeted band in gel was extracted using Nucleo spin® Gel & PCR Clean-up (Machoy Nagel) following this procedure: 200 µL NTI solution was added to each 100 mg gel, incubated in 50^{O}C for 10 min. The liquid was transferred to silica column in collection tube, centrifuge in 11,000 g for 30 s, and discard through flow. 700 µL NT3 solution was added into column, centrifuge in 11,000 g for 30 s, and discard throughflow. This step was done twice. Dried the column by centrifuging in 11,000 g for 60 s. Collection tube was replaced. 200 µL water was added into column, kept in column for 60 s, then centrifuged in 11,000 g for 60 s. The liquid was collected, labelled, and stored at -20^{o}C. By using nanodrop, the concentration of gene was measured.

2.2.3.8 Sequencing of the Positive Sample

The plasmids that was intended for sequencing were sent to Seqlab-Sequence Laboratories Göttingen GmBh, address; Hannah-Vogt-Str.1 37085 Göttingen Postfach 3343 37023 Göttingen Germany.

Premixed DNA sequencing was applied for all samples by mixing the plasmid (containing DNA template) and primer in a tube before sending them. Table 2-21 shows the list of primer that is used for sequencing.

Table 2-21. List of primers used for sequencing

Name of primer	Nucleotide Sequence (5′ → 3′)	cDNA Template
T7	TAATACGACTCACTATAGGG	*PSY2L*
SP6	ATTTAGGTGACACTATAG	*PSY2L*
PSY2L Middle	ATACACCAGACGTACAGAATTGG	*PSY2L*
EYFPPSY2Lf	ATTGCGGCCGCTCAGGATCCATTTACAGCCAT	*PSY2L*
PSY2LEYFPf	ATCCATGGGTATGGGCGCTCCGGAAAAGTCT	*PSY2L*
AK92r	CCTTATCTGGGAACTACTCAC	*PSY2L, PP4-1*
Ak93f	GCATTCTACTTCTATTGCAGC	*PSY2L, PP4-1*
Ak94r	AACTTCAGGGTCAGCTTGCCGT	*PSY2L, PP4-1*
EYFP-C-Terf	ACTACCTGAGCTACCAGTCC	*PSY2L, PP4-1*

2.2.4 Transient Transformation

Transient transformations for subcellular localization studies have been done by using two methods: *Arabidopsis* mesophyll protoplasts and particle bombardment/biolistic. The subcellular localization studies were performed for PP4-1, PP4-2, PSY2L, and PP4R2L. To complement, the localization of the fusion-proteins were visualized using confocal microscopy after one or two days of incubation.

2.2.4.1 Methods of Transient Transformation via *Arabidopsis* Mesophyll Protoplasts

Arabidopsis protoplasts were transfected for transient gene expression using a method described in 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 et al., 2007). Table 2-22 provides the solutions and their contents for this method.

2.2.4.1.1 Protoplast Isolation

Arabidopsis thaliana ecotype Colombia plants were grown under short photoperiod condition for 3-4 weeks. Fifteen to twenty well expanded leaves, were cut into narrow stripe from the middle part of the leaf with a sharp-razor blade, were transferred into a fresh Petri dish. The leaves then were submerged immediately and incubated with 7 mL Enzyme Solution at room temperature (RT) overnight in the dark room. After incubation, the plate was shake briefly. In the sterile bench, the protoplast suspension was filtered with a 10-ml pipette (wide opening) through a steel-mesh

combination with 125 µm on 63 µm mesh width into a conical flask. The Petri dish and filter then were rinsed with total 8 ml 0.2 M $CaCl_2$. The protoplasts suspension was transferred with a fresh pipette into a screw-cap tube and centrifuged (in a pre-cooled centrifuge, 20^oC) for 5 min at 60 x g. The protoplast pellets then were resuspended in W5 medium (an artificial seawater) and counted by haemocytometer (15 µl to be added, so each 4 squares will be equal to 0.1 µl). The protoplasts in W5-medium were incubated for 1 – 2 h on ice. Then, the protoplast were centrifuged (in a pre-cooled centrifuge, 20oC) for 5 min at 60 x g. The supernatant was discarded and the cell number was adjusted to 1.67 Mio/ml with ice cold MaMg solution (500.000/300 µl).

Table 2-22. List of solutions used for Transient Gene Expression Analysis

Solution	Content and Concentration
Enzyme Solution	0.45 M Mannitol
	10 mM $CaCl_2$ x $2H_2O$
	1 % Cellulase
	0.25 % Macerozyme
0.2 M $CaCl_2$	0.2 M $CaCl_2$ x $2H_2O$
W5 Medium	145 mM NaCl
	125 mM $CaCl_2$ x $2H_2O$
	5 mM KCl
	5 mM Glucose
	(pH 5 – 6)
MaMg Solution	0.5 M Mannitol
	15 mM $MgCl_2$ x $6H_2O$
	0.1 % MES
	(pH 5.7, adjust with 0.1 N KOH)
PEG Solution	0.4 M Mannitol
	0.1 M $Ca(NO_3)_2$ x $4H_2O$
	0.1 % MES
	(pH 8, adjust with NaOH)
	PEG 6000
Gamborg B5 Medium	3.17 g/L Gamborg
	0.45 M Glucose
	(pH 5.7, adjust with 0.1 N KOH)

2.2.4.1.2 PEG Transfection

PEG transfection was used to deliver DNA into protoplasts. For each transformation, around 5 µg plasmid DNA was placed centrally in a Greiner Petri dish. The plasmid solution was mixed with 300 µl protoplast suspension by pipetting gently up and down with 1-ml blue tip whose opening has been enlarged by cutting the tip end. To each transformation, 500 µl PEG solution was added drop-wise from a distance of about 5 cm height slowly. The plate then was covered and incubated for 30 min at RT under the sterile bench. After that, the suspension was diluted stepwise each 5 min with in total circa 7 ml W5 medium (i.e. + 0.5 ml, + 1 ml, + 2 ml, + 3 ml = 6.5 ml) and transferred to a screw-cap tube. The petri dish was also rinsed with W5 and collect it to the tube. Then, the suspension was centrifuged for 5 min at 60 g. The pellets were resuspend in 3 ml Gamborg B5 medium, transferred to small Petri dishes, and incubated overnight in dark room at RT. Finally, the expression of gene was checked by using fluorescence microscope.

2.2.4.2 Methods of Transient Transformation via Particle Bombardment

2.2.4.2.1 DNA Precipitation onto Gold Particles

The DNA precipitation onto gold particles for cell bombardment is following these steps. Fifty mg gold particles were resuspended in 1 ml ethanol and vortexed for 3-5 min. The gold particles were then sedimented by centrifugation at 10,000 ×g for 3 s and the supernatant was removed. This step was repeated two times. The gold particles were resuspended in 1 ml water and vortexed for 3-5 min and then centrifuged at 10,000 ×g for 3 s. The supernatant was removed and the gold particles resuspended in 1 ml water. The suspension was aliquoted in 50 µl aliquots. The next steps were performed on ice. These following components were added one after the other in orderly and vortexed thoroughly for 1 min after each addition: 5 µg plasmid DNA (calculated based on the the concentration of plasmid DNA), 50 µl 2.5 M $CaCl_2$ (final concentration 1 M) and 20 µl 0.1 M Spermidine (final concentration 10 mM). The DNA was precipitated onto the gold particles by centrifugation at 10,000 × g for 3 s, and the supernatant was removed. After that, the particles were resuspended in 250 µl ethanol, vortexed, and sedimented by centrifugation at 10,000 × g for 3 s. The supernatant was removed. This step was repeated thrice. The particles were finally resuspended in 50 µl ethanol and ready to use for the transformation of onion epidermal cell by bombardment (gene shooting).

2.2.4.2.2 Transformation of Onion (*Allium cepa* L.) Epidermal Cells by Particle Bombardment

A biolistic system (PDS 1000/He Particle Delievery system, Biorad) was used in the transformation of onion epidermal cells (*Allium cepa* L.). The biolistic system uses highly pressurized helium, which builds up above a rupture disk that bursts at a defined pressure. A helium shock wave is generated into the cell bombardment chamber when the rupture disk bursts. The helium shock wave will force the macrocarrier loaded with DNA coated gold particles toward the target cells at high velocity. The macrocarrier is stopped by a stopping screen while the DNA coated particles continue to the target and transform the onion epidermal cells.

Onion has to be made ready for bombardment before putting it in the biolistic system. A healthy onion was peeled and cut into well-sized slices. A quarter of a slice with the epidermal cell layer still attached was placed in a Petri dish on a wet piece of paper. The gun chamber was sterilized with 70% ethanol. The helium bottle was opened and the pressure adjusted to 1400 PSI. The pressure should be adjusted a little higher than where the disc ruptures. Before using, the suspension of gold particles coated with the desired plasmids was vortexed thoroughly and 7 µl were loaded onto the macrocarrier holder in the shooting device. The gold particles were spread with the side of a pipette tip over an area of about 1 cm^2. A rupture disk was sterilized in ethanol and loaded into the retaining cap. The retaining cap was secured to the end of the gas acceleration tube and tightened with a torque wrench. The macrocarrier containing the DNA and the stopping screen were loaded into the micro carrier launch assembly. Inside the bombardment chamber, the micro carrier launch assembly was placed into the top shelf and the targeted onion cells placed into the third shelf. The chamber room was closed tightly, the vacuum pump turned, and the power switch on the device turned on. The vacuum was lowered to about 270 inches Hg and then held. The fire button was pushed until rupture of the disc. This can be marked by cracking sound and decreasing the pressure rapidly. After that, the pump was turned off and the vacuum slowly released. The vacuum pump was turned off, the chamber door opened, and the dish with the onion removed. The macro carrier and stopping screen from the microcarrier launch assembly were unloaded and discarded as well as the spent rupture disk. When all the experiments were completed the helium bottle was closed. The helium pressure was released from the tubing by applying vacuum and shooting a couple of times. The onion was left in the Petri dish and incubated in the dark for about 18 - 24 h. After incubation, the epidermal cell layer was peeled off and analyzed by fluorescence microscopy.

2.2.4.3 Fluorescence Microscopy

The microscope used for this study was Nikon A1R confocal microscope. The localization of the fusion-proteins PP4-1-EYFP, EYFP-PP4-1, PSY2L-EYFP, and EYFP-PSY2L, were checked using microscopy after one day of incubation for both method Biolistic Transfection of Onion Cells and Transient Gene Expression Analysis. For PP4-2-EYFP, EYFP-PP4-2, PP4R2L-EYFP, and EYFP-PP4R2L, the experiment was done by previous Master student, Christine Sæetre. List of fusion protein for subcellular localization is shown in Table 3-25.

2.2.5 Expression Studies for T-DNA plants, amiRNA Plants, and Overexpression Plants

The plant materials were used for expression studies are *psy2l* SALK_125872*, psy2l* SALK_048064*, pp4r2l* SALK_093041*, pp4-1* GK_651B07*, pp4-1* SALK_070977*, pp4-2* SAIL_569_H09*,* and *pp4-2* SALK_049725C. These plants were first genotyped to find homozygous individuals. Expression study of T-DNA plants was done by following three steps: isolation of total RNA, synthesis of cDNA, and RT PCR.

2.2.5.1 Isolation of Total RNA

The isolation of total RNA sample was done by using method DNA-free RNA isolation protocols for *Arabidopsis thaliana* by (Onate-Sanchez and Vicente-Carbajosa, 2008). Table 2-23 provides the solutions and their contents for this step.

Table 2-23. List of solutions used for Isolation of total RNA step in Expression Studies

Solution	Content and Concentration
Cell Lysis Solution	2 % Sodium Dodecyl Sulfate (SDS)
	68 mM Sodium Citrate
	132 mM Citric Acid
	1 mM EDTA
Protein-DNA precipitation solution	4 M NaCl
	16 mM Sodium Citrate
	32 mM Citric Acid
7.5 M Ammonium Acetate	7.5 M Ammonium Acetate

The preparation of sample is following these steps: Approximately 80 mg of leaf samples were freeze and grind in liquid nitrogen with the help of a mortar and pestle or other appropriate tool. For dwarf plants, as much as 20 mg of leaves were used. 300 µL of cell lysis solution was added to

ground tissue and homogenized quickly using vortex for 60 s and inverting and flicking the tube gently. The tubes were then left at room temperature for 5 min. 100 µL of protein-DNA precipitation solution was added to the cell lysate. The tubes were mixed inverting gently and incubated at 4^{o}C for 10 min. The tubes were then spin at 4^{o}C using maximum speed for 10 min. The supernatant was transferred to new tube and added 300 µL isopropanol. The sample was mixed by inverting gently the tube, then spin 4^{o}C for 10 min. The supernatant was carefully pour off remaining pellet in the bottom and around inner wall of tube. The pellet was washed with 70% ethanol, air-dried, and resuspend in 25 µL water. The concentration of total RNA was measured. If needed, the samples were diluted to give a final concentration about 200 ng/µL for 25 µL.

After that, 3 µL 10X DNase buffer and 5 µL of DNase I (RQ1 RNase-free DNase) were added to RNA samples and incubated 30 min at 37^{o}C. 70 µL water, 50 µL of 7.5 M Ammonium Acetate and 400 µL 99.5% ethanol were added to the RNA samples, mixed well, and spin 20 min at 4^{o}C. The pellet was washed with 70% ethanol, air-dried, and resuspend in 20 µL water. The concentration of total RNA was measured.

2.2.5.2 Synthesis of cDNA and RT-PCR

The following 10 µL volume reaction was used for 500 ng total RNA sample from isolation step. cDNA was synthesized by using Reverse Transcriptase following these procedure: Amount of total RNA (calculated to achieve 500 ng total RNA in 10 µL volume reaction), 2 pmol specific primer (Table 2-24), 0.5 µL dNTP mix (each concentration 0.2 mM) were mixed by pipetting up abd down and then added water until 6.5 µL, mixed again. The mixture was heated at 60^{o}C for 5 min, incubated on ice for 1 min, and centrifuged briefly. For each tube, 2 µL 5x first strand buffer, 0.5 µL DTT 0.1 M, 0.5 µL water, and 0.5 µL Superscript^{TM} III Reverse Transcriptase 200 unit/µL was added and mixed well. The mixtures was incubated at 55^{o}C for 60 min, following by inactivation enzyme at 70^{o}C for 15 min. The cDNA was ready to polymerase through RT PCR step.

Table 2-24. List of primers used for synthesising cDNA

cDNA	Specific Primer	Nucleotide Sequence (5' → 3')
PP4-1	AK75r	ATGCGGCCGCTATGTCAGACCTAGACAAGCAA
PP4-2	AK75r	ATGCGGCCGCTATGTCAGACCTAGACAAGCAA
PP4R2L	RT PSY2L-RP	CCATCAGATTCCGGAAGAAA
PSY2L	PP4R2LEYFPr	AAGCGGCCGCGGCACACGTTGTAGGCAACCG

The RT-PCR procedure is explain in section 2.2.1.4 (RT-PCR) and followed by running the PCR result on agarose gel electrophoresis (section 2.2.2).

2.2.6 Protocol for Production of Growth Medium

Seeds of plants sown on plates containing half strength Linsmaier and Skoog (LS) medium, screening with the corresponding screening agent (Phosphinothricin/BASTA 50 mg/mL). Bacterial colonies were cultivated using LB broth for overnight cultures and LB agar for plated colonies.

2.2.6.1 Half strength Linsmaier and Skoog (LS) medium

Half strength Linsmaier and Skoog (LS) medium was prepared by adding 5 gram agar-agar and 1.2 gram Linsmaier and Skoog (from Caisson) to 500 mL water. For screening amiRNA plants in pBA002, 75 µL of BASTA 50 mg/mL was added each 250 mL half LS medium.

2.2.6.2 Luria-Bertani (LB) broth and Luria-Bertani (LB) agar

The LB broth and LB agar was prepared following the labels on the containers. 1 µL Ampicillin 100 mg/mL was added each 1 mL LB Broth and LB agar for screening agent for bacterial colonies.

2.2.7 Surface Sterilization of *Arabidopsis thaliana* Seeds and Screening of the Seeds

2.2.7.1 Surface Sterilization of *Arabidopsis thaliana* Seeds

Triton X-100 (10 µl) was added to 10 ml Ethanol 70% to make a 0.1 % Triton X-100 solution in Ethanol 70%. One ml of the solution was then added to dry seeds and incubated for 15 min. The seeds were then washed using 1 ml ethanol 95 % and incubated for 10 min. The washing process was done twice. The seeds then were left in a sterile environment to dry.

2.2.7.2 Screening of the Seeds for T-DNA Plants and Overexpression Plants

Seeds were harvested from each of the mutant plants. The seeds were then surface sterilized, and sown on half strength LS plates. 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 compare to the wild type.

2.2.7.3 Screening of the Seeds for amiRNA Plants

Seeds were harvested from each of the mutant plants. The seeds were then surface sterilized, and sown on half strength LS plates containing the corresponding screening chemical, for pBA002, BASTA 15 mg/ml 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 compare to the wild type.

2.2.8 Growing Plants Condition and Hoagland Plant Nutrient Solution

2.2.8.1 Condition for Growing Plants

The plants were grown in mixture of planting soil and agra-vermiculite in the ratio 3:1. Usually, after sowing the seeds or small plants, they were covered with plastic dome for 5 – 7 days to maintain humidity. The plants were growing mainly in the growth chamber, 16 h light / 8 h dark cycle at 22^{o}C. However, some plants were growing in the growth chamber, 12 h light / 12 h dark cycle at 22^{o}C. If not mentioned, the condition of growing plants is 16 h light / 8 h dark cycle at 22^{o}C.

2.2.8.2 Hoagland Plant Nutrient Solution

Hoagland plant nutrient solution was used as nutrient solution for plants during growth time in soil. The chemicals that used for making Hoagland plant nutrient solution are listed in Table 2-25. The working solution to be applied to plants is 1x concentration solution. Usually, the stock solution in lab is 10x concentration solution. In order to make 1x concentration solution, 100 ml of 10x concentration solution was added to 900 ml of water. The plants were watered with this solution two times a week.

Table 2-25. The chemicals that used for making Hoagland plant nutrient solution

Chemical	Nutrient concentration in 1x concentration solution
KH_2PO_4	1 mM
KNO_3	5 mM
$Ca(NO_3)_2{\cdot}4H_2O$	5 mM
$MgSO_4 \cdot 7H_2O$	2 mM
Fe-EDTA	1 µM
H_3BO_3	46.23 µM
$MnCl_2.4H_2O$	9.2 µM
$CuSO_4.5H_2O$	0.36 µM
$ZnSO_4.7H_2O$	0.77 µM
$Na_2MoO_4.H_2O$	0.12 µM

3. RESULTS

3.1 Genotyping, Expression Studies, and Observation of Phenotype for T-DNA plants

To investigate the function of *PP4-1, PP4-2, PP4R2L,* and *PSY2L* gene in Arabidopsis, we searched for loss-of-function mutants for each aforementioned genes. The mutants were found in Salk Institute T-DNA insertion collection plants and delivered from NASC (Nottingham Arabidopsis Stock Centre). The lines of the T-DNA plants are *psy2l* SALK_125872, *psy2l* SALK_048064, *pp4r2l* SALK_093041, *pp4-1* GK_651B07, *pp4-1* SALK_070977, *pp4-2* SAIL_569_H09, and *pp4-2* SALK_049725. Genotyping to identify the homozygous plants and then continue to expression studies of the targeted gene were performed to the T-DNA plants. Finally, the obtained results were complemented and compared by examining the visible character of the T-DNA plants (phenotyping).

3.1.1 Genotyping of T-DNA Plants to Identify Homozygous Plant

Genotyping was performed on individual plant of *Arabidopsis thaliana* of abovementioned lines. In order to identify the homozygous plants, analysis of two independent alleles is required. Therefore, the DNA sample of plants was amplified through PCR as two separate reactions using two set of primers, (Left Primer/LP – Right Primer/RP) pair and (Right Primer/RP – T-DNA primer/BP) pair (see Figure 1-6 and Figure 1-7). The position of each T-DNA insertion at gene of interest will be shown in the following figure of genomic organization of each lines.

3.1.1.1 Genotyping *psy2l* SALK_048064

Figure 3-1 provides the detailed characterization of the T-DNA insertion at the *PSY2L* gene for *psy2l* SALK_048064 mutant plant.

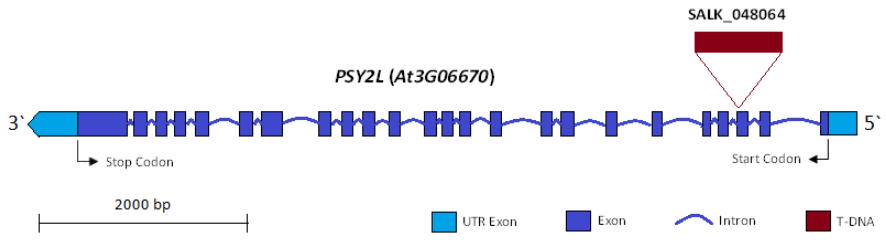


Figure 3-1. Characterization of the T-DNA insertion at the *PSY2L* gene for *psy2l* SALK_048064 mutant plant (Insert in exon 3 of 25).

Three pots of mutant *psy2l* SALK_048064, namely "Original", "Chris-3", "Chris-16", were analysed. "Original" means they are the segregating seeds delivered directly from NASC. "Chris " ("Chris-3" and "Chris-16") means these seeds are from a previous master student, Christine Sætre (2015), who tried to select the homozygous plants, but only found heterozygous plants, except one homozygous plant that did not survive. For each pot, 3 plants were labelled and tissue samples (young leaves) were used in order to perform genotyping. Total 9 plant samples plus 1 wild type as control were analysed. Those samples were divided into 2 parts based on primers that were added. Table 3-1 shows the detail of samples.

Figure 3-2 shows the gel electrophoresis of genotyping plant *psy2l* SALK_048064 and Table 3-1 shows the interpretation of bands in gel each sample.

Table 3-1. Samples and their result for first trial genotyping mutant *psy2l* Salk_048064. Primers for lane A are LBb1.3 New Salk (T-DNA primer) and PSY2L Salk RP 048064 (right primer). Primers for lane B are PSY2L Salk LP 048064 (left primer) and PSY2L Salk RP 048064 (right primer).

Mutant *psy2l*	**Sample**		**Result**	
SALK_048064	**Lane A**	**Lane B**	**Band(s)**	**Interpretation**
Pot: Original				
1	1 A	1 B	B	Wild Type
2	2 A	2 B	A and B	Heterozygous
3	3 A	3 B	B	Wild Type
Pot: Chris-3				
4	4 A	4 B	A and B	Heterozygous
5	5 A	5 B	Non-specific bands	-
6	6 A	6 B	A and B	Heterozygous
Pot : Chris-16				
7	7 A	7 B	A and B	Heterozygous
8	8 A	8 B	A and B	Heterozygous
9	9 A	9 B	No band	-
Wild Type (Control)				
10	10 A	10 B	B	Wild Type (Control)

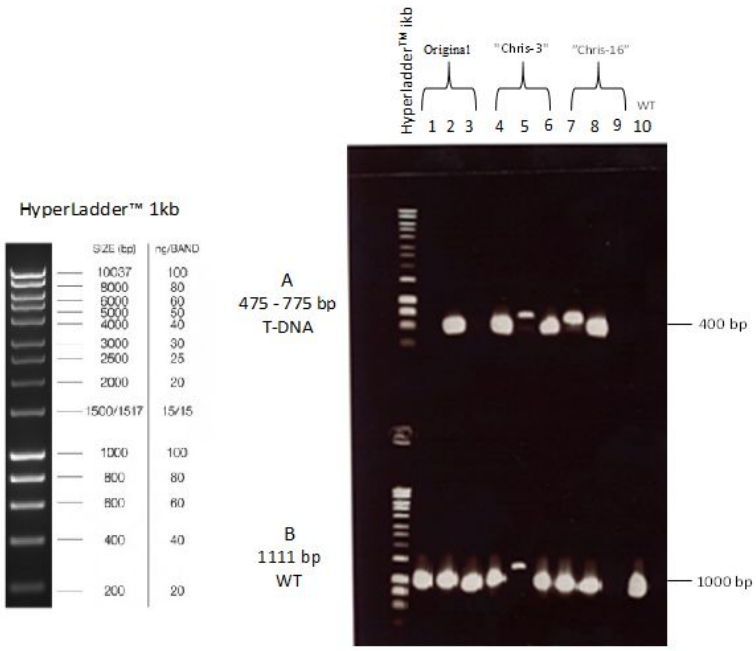


Figure 3-2. The gel electrophoresis of first trial genotyping plant *psy2l* SALK_048064. Lane A (T-DNA primer: LBb1.3 New Salk and Right Primer: PSY2L Salk RP 048064) has expected band at 475 – 775 bp T-DNA and Lane B (Left Primer: PSY2L Salk LP 048064 and Right Primer: PSY2L Salk RP 048064) has expected band at 1111 bp wild type. The size of expected bands at A and B are based on SALK website tool that can be got from http://signal.salk.edu/tdnaprimers.2.html (last retrieved: May 2016).

It can be seen from Table 3-1 that no homozygous plant can be identified from selected samples. Therefore, other *psy2l* SALK_048064 sample plants were analyzed. Three pots of *psy2l* SALK_048064 plants, namely “Original”, “Chris-3”, and “Chris-16” were examined for this second trial of genotyping. Four sample plants from “Original“ and “Chris-3“, respectively, and 5 sample plants from “Chris-16“ were labelled and tissue samples (young leaves) were used in order to perform genotyping. Total 13 *psy2l* SALK_048064 plants plus 1 wild type plant as control were analysed. Those samples were divided into 2 parts based on primers that were added. Table 3-2 shows the detail of analysed samples.

Table 3-2. Samples and their results for second trial genotyping mutant *psy2l* SALK_048064. Primers for lane A are LBb1.3 New Salk (T-DNA primer) and PSY2L Salk RP 048064 (right primer). Primers for lane B are PSY2L Salk LP 048064 (left primer) and PSY2L Salk RP 048064 (right primer).

Mutant *psy2l* SALK_048064	**Sample**		**Result**	
	Lane A	**Lane B**	**Band(s)**	**Interpretation**
Pot: Original				
33	33 A	33 B	A and B	Heterozygous
34	34 A	34 B	A and B	Heterozygous
35	35 A	35 B	A and B	Heterozygous
36	36 A	36 B	A and B	Heterozygous
Pot: Chris-3				
37	37 A	37 B	No Band	-
38	38 A	38 B	A and B	Heterozygous
39	39 A	39 B	A and B	Heterozygous
40	40 A	40 B	No Band	-
Pot : Chris-16				
41	41 A	41 B	A and B	Heterozygous
42	42 A	42 B	A and B	Heterozygous
43	43 A	43 B	A and B	Heterozygous
44	44 A	44 B	A and B	Heterozygous
45	45 A	45 B	A and B	Heterozygous
Wild Type (Control)				
46	46 A	46 B	No Band	-

Figure 3-3 shows the gel electrophoresis of second trial genotyping mutant *psy2l* SALK_048064 and Table 3-2 shows the interpretation of bands in gel each sample.

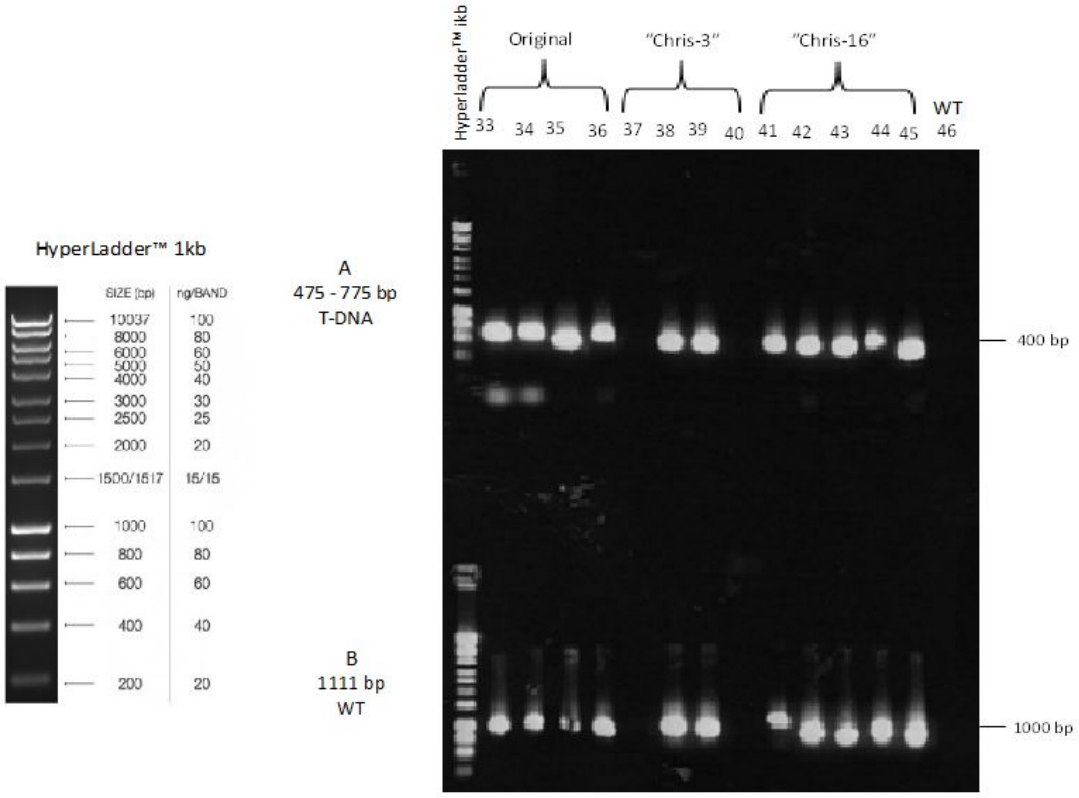


Figure 3-3. The gel electrophoresis of second trial genotyping mutant *psy2l* SALK_048064. Lane A (T-DNA primer: LBb1.3 New Salk and Right Primer: PSY2L Salk RP 048064) has expected band at 475 – 775 bp T-DNA and Lane B (Left Primer: PSY2L Salk LP 048064 and Right Primer: PSY2L Salk RP 048064) has expected band at 1111 bp wild type. The size of expected bands at A and B are based on SALK website tool that can be got from http://signal.salk.edu/tdnaprimers.2.html (last retrieved: May 2016).

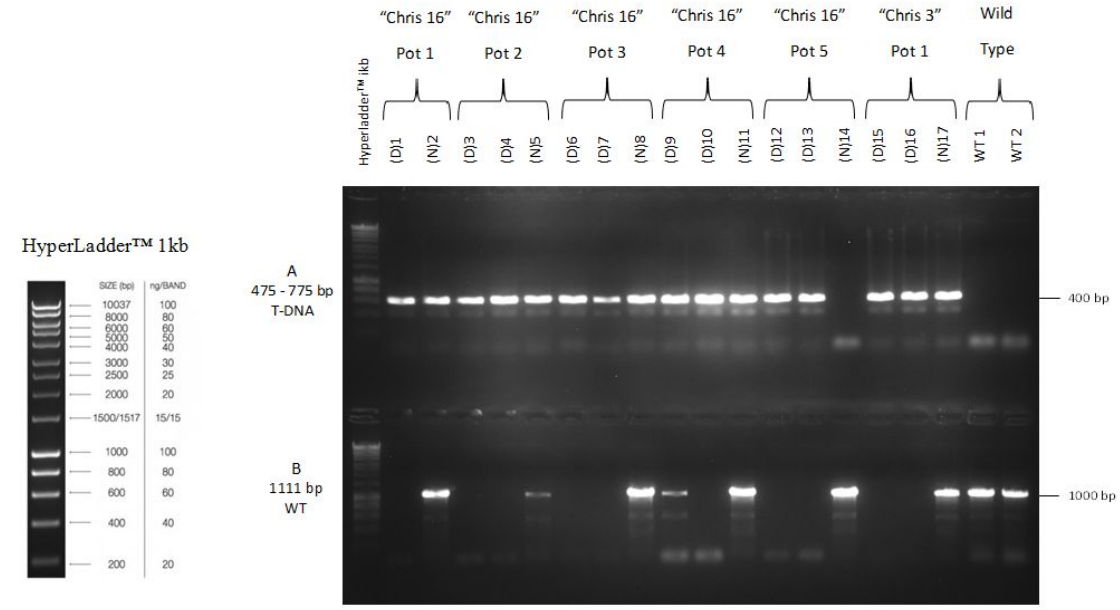
According to the result in Table 3-1 and Table 3-2, twenty two *psy2l* SALK_048064 plants had been tested, but still no homozygous plant was detected. Plant number 41 (see Figure 3-3 and Table 3-2) was dwarf and died when was 4 – 6 weeks old (in 12 h light / 12 h dark room). It had a T-DNA specific band, but because of a probable non-specific band (see Figure 3-3, lane B), we thought that this plant was heterozygous. Therefore, we collected seeds from "Chris-3" and "Chris-16" again, and sow them in several pots. Then, genotyping was performed to this next generation plants. One pot of mutant *psy2l* SALK_048064 "Chris-3" and five pots of mutant *psy2l* SALK_048064 "Chris-16", were analysed. Interestingly, we found two different phenotypes for each pot in this next generation mutant plants, one is smaller size which we called "dwarf" and the other which size is resemble to wild type, so we called them "Normal Size" (refer also Figure 3-4 left side to see the difference appearance of "dwarf" and "normal size" of the mutants in one of the pots). For each pot, the "dwarf" (D) and "normal size" (N) representative plants were labelled and tissue samples (young leaves) were used in order to perform genotyping. Those

samples were divided into 2 parts based on primers that were added. Table 3-3 shows the detail of samples.

Table 3-3. Samples and their results for genotyping further generation of mutant *psy2l* SALK_048064 "Chris-3" and "Chris-16". D and N stand for "Dwarf" and "Normal size", respectively. Primers for lane A are LBb1.3 New Salk (T-DNA primer) and PSY2L Salk RP 048064 (right primer). Primers for lane B are PSY2L Salk LP 048064 (left primer) and PSY2L Salk RP 048064 (right primer).

Mutant *psy2l*	**Sample**		**Result**	
SALK_048064	**Lane A**	**Lane B**	**Band(s)**	**Interpretation**
Pot: Chris-16 (1)				
(D)1	(D)1 A	(D)1 B	A	Homozygous
(N)2	(N)2 A	(N)2 B	A and B	Heterozygous
Pot: Chris-16 (2)				
(D)3	(D)3 A	(D)3 B	A	Homozygous
(D)4	(D)4 A	(D)4 B	A	Homozygous
(N)5	(N)5 A	(N)5 B	A and B	Heterozygous
Pot: Chris-16 (3)				
(D)6	(D)6 A	(D)6 B	A	Homozygous
(D)7	(D)7 A	(D)7 B	A	Homozygous
(N)8	(N)8 A	(N)8 B	A and B	Heterozygous
Pot: Chris-16 (4)				
(D)9	(D)9 A	(D)9 B	A and B	Heterozygous
(D)10	(D)10 A	(D)10 B	A	Homozygous
(N)11	(N)11 A	(N)11 B	A and B	Heterozygous
Pot: Chris-16 (5)				
(D)12	(D)12 A	(D)12 B	A	Homozygous
(D)13	(D)13 A	(D)13 B	A	Homozygous
(N)14	(N)14 A	(N)14 B	B	Wild Type
Pot: Chris-3 (1)				
(D)15	(D)15 A	(D)15 B	A	Homozygous
(D)16	(D)16 A	(D)16 B	A	Homozygous
(N)17	(N)17 A	(N)17 B	A and B	Heterozygous
Pot: Wild Type				
WT1	WT1 A	WT1 B	B	Wild Type (Control)
WT2	WT2 A	WT2 B	B	Wild Type (Control)

Figure 3-4 above side shows the gel electrophoresis of genotyping of this further generation mutants of *psy2l* SALK_048064 "Chris-3" and "Chris-16". Table 3-3 shows the interpretation of bands in gel each sample.



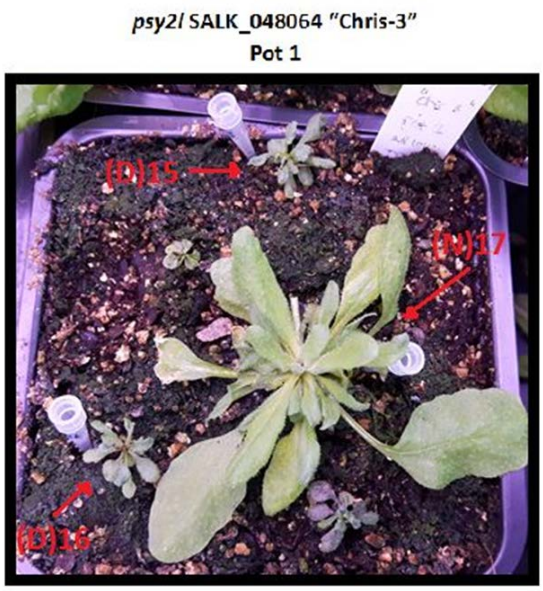


Figure 3-4. The genotyping result and phenotype of further generation of mutant *psy2l* SALK_048064 "Chris-3" and "Chris-16".
Above: The gel electrophoresis of genotyping further generation of mutant *psy2l* SALK_048064 "Chris-3" and "Chris-16". Lane A (T-DNA primer: LBb1.3 New Salk and Right Primer: PSY2L Salk RP 048064) has expected band at 475 – 775 bp T-DNA and Lane B (Left Primer: PSY2L Salk LP 048064 and Right Primer: PSY2L Salk RP 048064) has expected band at 1111 bp wild type. The size of expected bands at A and B are based on SALK website tool that can be got from http://signal.salk.edu/tdnaprimers.2.html (last retrieved: May 2016).
Left: The phenotype of so called "Dwarf" (D) and "Normal Size" (N) of further generation of mutant *psy2l* SALK_048064. Here, we provide example from "Chris-3" pot 1, containing two samples of "dwarf" plants, (D)15 and (D)16, and one "normal size" (N)17. According to genotyping result above, (D)15 and (D)16 are homozygous mutants and (N)17 is a heterozygous mutant.

According to the result of genotyping mutant *psy2l* SALK_048064 "Chris-3" and "Chris-16", mostly so-called "dwarf" plants were proved to be homozygous and "normal size" plants either were heterozygous or not. The "dwarf" plants survived up to 6 months, but remain dwarf in compare to wild type. The heterozygous plants showed different phenotypes in different plants, for instance darker-coloured leaves, curly leaves, and later showing delayed flowering (see Figure 3-4 left side).

3.1.1.2 Genotyping *psy2l* SALK_125872

Figure 3-5 provides the detailed characterization of the T-DNA insertion at the *PSY2L* gene for *psy2l* SALK_125872 mutant plant.

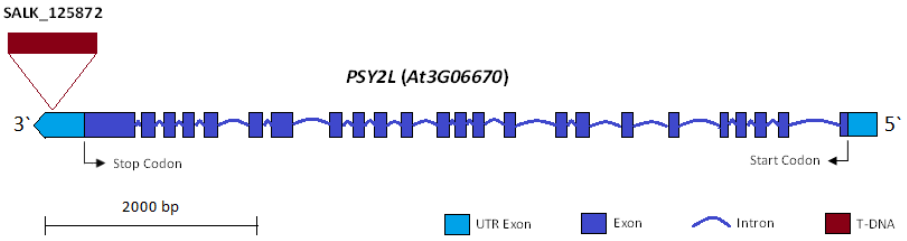


Figure 3-5. Characterization of the T-DNA insertion at the *PSY2L* gene for *psy2l* SALK_125872 mutant plant (flank-tagged downstream of translation).

Two pots of mutant *psy2l* SALK_125872 (Pot 3_1 and Pot 3_2) were analyzed. Each pot, 2 samples were labelled and tissue samples (young leaves) were used in order to perform genotyping. Total 4 mutants of *psy2l* SALK_125872 plus 1 wild type as control were analyzed. Those samples were divided into 2 parts based on primers that were added. Table 3-4 shows the detail of samples.

Table 3-4. Samples and their results for genotyping mutant *psy2l* Salk_125872. Primers for lane A are LBb1.3 New Salk (T-DNA primer) and PSY2L Salk RP 125872 (right primer). Primers for lane B are PSY2L Salk LP 125872 (left primer) and PSY2L Salk RP 125872 (right primer).

Mutant *psy2l* SALK_125872	**Sample**		**Result**	
	Lane A	**Lane B**	**Band(s)**	**Interpretation**
Pot: 3_1				
16	16A	16B	A	Homozygous
17	17A	17B	A and B	Heterozygous
Pot: 3_2				
18	18A	18B	A	Homozygous
19	19A	19B	A	Homozygous
Wild Type (Control)				
20	20A	20B	Non-specific bands	-

Figure 3-6 shows the gel electrophoresis of genotyping mutant *psy2l* SALK_125872 and Table 3-4 shows the interpretation of bands in gel each sample.

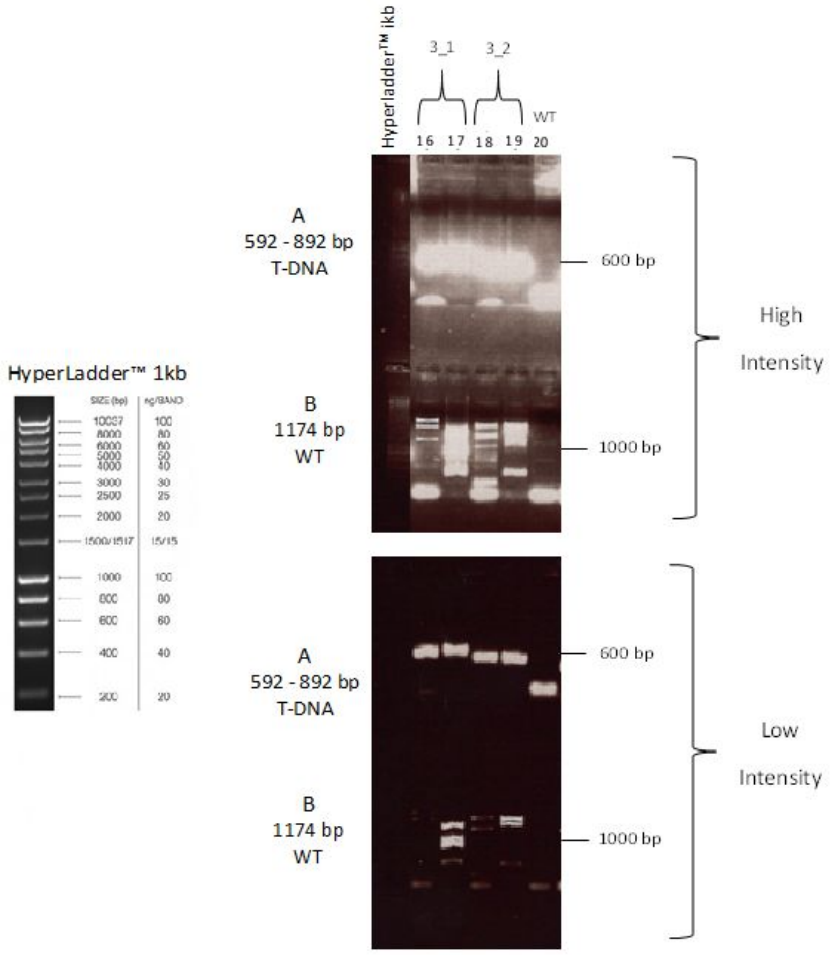


Figure 3-6. The gel electrophoresis of genotyping mutant *psy2l* SALK_125872. The result is provided in high intensity and low intensity. Lane A (T-DNA primer: LBb1.3 New Salk and Right Primer: PSY2L Salk RP 125872) has expected band at 592 – 892 bp T-DNA and Lane B (Left Primer: PSY2L Salk LP 125872 and Right Primer: PSY2L Salk RP 125872) has expected band at 1174 bp wild type. The size of expected bands at A and B are based on SALK website tool that can be seen from http://signal.salk.edu/tdnaprimers.2.html (last retrieved: May 2016).

From Table 3-4, for this Salk line, homozygous plants were identified. According to this result, *psy2l* SALK_125872 plant pot 3_1 #18 and #19 were selected for expression analysis.

3.1.1.3 Genotyping *pp4r2l* SALK_093041

Figure 3-7 provides the detailed characterization of the T-DNA insertion at the *PP4R2L* gene for *pp4r2l* SALK_093041 mutant plant.

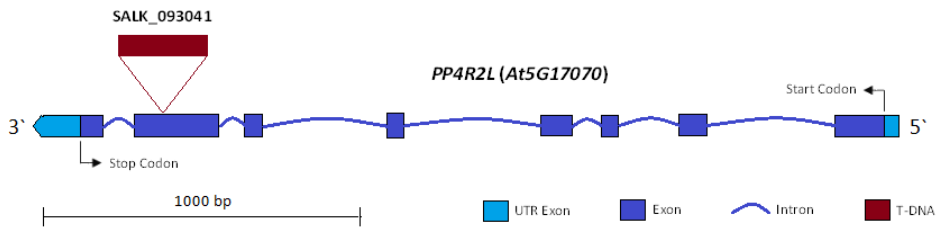


Figure 3-7. Characterization of the T-DNA insertion at the *PP4R2L* gene for *pp4r2l* SALK_093041 mutant plant (insert in exon 7 of 8).

Two pots of mutant *pp4r2l* SALK_093041 (Pot 1 and Pot 2) were analyzed. Each pot, 2 samples were labelled and tissue samples (young leaves) were used in order to perform genotyping. Total 4 mutants of *pp4r2l* SALK_093041 plus 1 wild type as control were analyzed. Those samples were divided into 2 parts based on primers that were added. Table 3-5 shows the detail of samples.

Table 3-5. Samples and their results for genotyping mutant *pp4r2l* SALK_093041. Primers for lane A are LBb1.3 New Salk (T-DNA primer) and PP4R2L Salk RP 093041 (right primer). Primers for lane B are PP4R2L Salk LP 093041 (left primer) and PP4R2L Salk RP 093041 (right primer).

Mutant *pp4r2l* SALK_093041	**Sample**		**Result**	
	Lane A	**Lane B**	**Band(s)**	**Interpretation**
Pot: 1				
11	11A	11B	A	Homozygous
12	12A	12B	No band	-
Pot: 2				
13	13A	13B	A and B	Heterozygous
14	14A	14B	A and B	Heterozygous
Wild Type (Control)				
15	15A	15B	B	Wild Type (Control)

Figure 3-8 shows the gel electrophoresis of genotyping mutant *pp4r2l* SALK_093041 and Table 3-5 shows the interpretation of bands in gel each sample.

From Table 3-5, for all this Salk line, homozygous plant were identified. According to this result, *pp4r2l* SALK_093041 plant pot 1 #1 was selected for expression analysis.

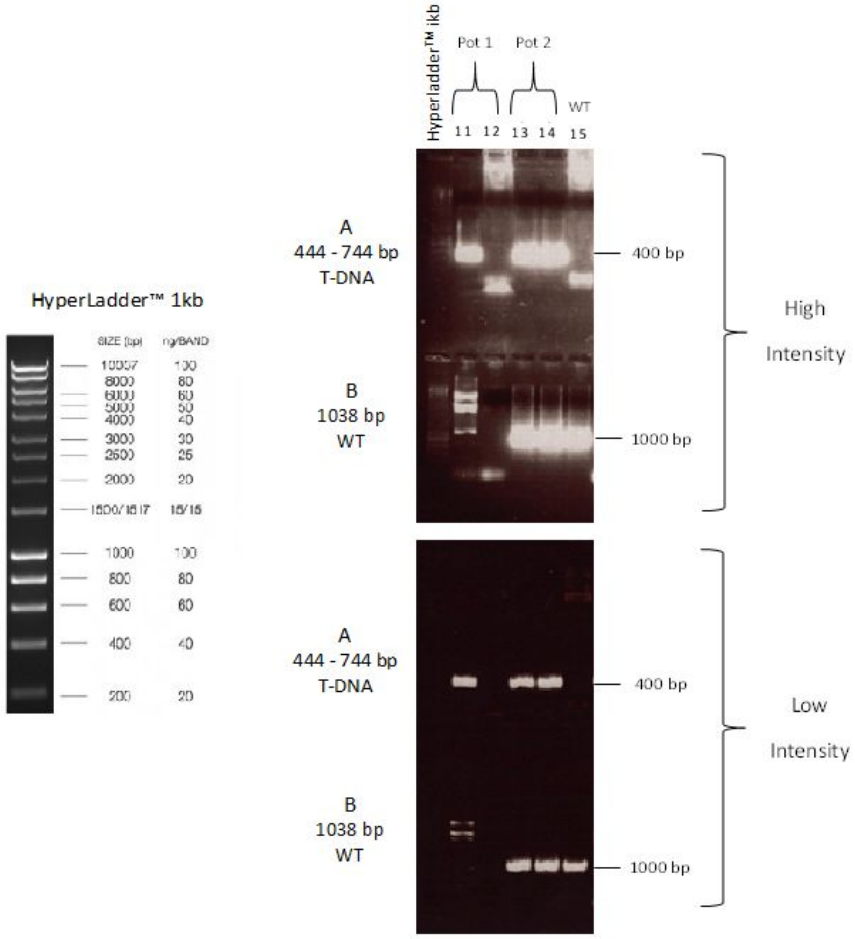


Figure 3-8. The gel electrophoresis of genotyping mutant *pp4r2l* SALK_093041. The result is provided in high intensity and low intensity. Lane A (T-DNA primer: LBb1.3 New Salk and Right Primer: PP4R2L Salk RP 093041) has expected band at 444 – 744 bp T-DNA and Lane B (Left Primer: PP4R2L Salk LP 093041 and Right Primer: PP4R2L Salk RP 093041) has expected band at 1038 bp wild type. The size of expected bands at A and B are based on SALK website tool that can be seen from http://signal.salk.edu/tdnaprimers.2.html (last retrieved: May 2016).

3.1.1.4 Genotyping *pp4-2* SAIL_569_H09 and *pp4-2* SALK_049725C

Figure 3-9 provides the detailed characterization of the T-DNA insertion at the *PP4-2* gene for *pp4-2* SAIL_569_H09 *and pp4-2* SALK_049725 mutant plant.

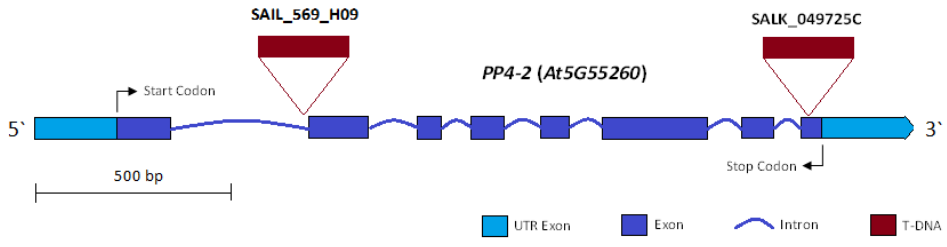


Figure 3-9. Characterization of the T-DNA insertion at the *PP4-2* gene for *pp4-2* SAIL_569_H09 mutant plant (insert in intron 1 of 7) and *pp4-2* SALK_049725C mutant plant (insert in exon 8 of 8).

Two pots of mutant *pp4-2* Sail_569_H09 (Pot 3 and Pot 5) were analyzed. One sample from pot 3 and two samples from pot 5 were labelled and tissue samples (young leaves) were used in order to perform genotyping. Total 3 mutants of *pp4-2* Sail_569H09 plus 1 wild type as a control were

analyzed. Those samples were divided into 2 parts based on primers that were added. Table 3-6 shows the detail of samples.

A pot of *pp4-2* Salk_049725C plant was examined. Two plants of that pot were labelled and tissue samples (young leaves) were used in order to perform genotyping. One wild type sample as control for each plant was analyzed. Those samples were divided into 2 parts based on primers that were added. Table 3-7 shows the detail of samples.

Table 3-6. Samples for genotyping mutant *pp4-2* SAIL_569_H09. Primers for lane A are LBP3 New Sail (T-DNA primer) and PP4-2 Sail RP 569_H09 (right primer). Primers for lane B are PP4-2 Sail LP 569_H09 (left primer) and PP4-2 Sail RP 569_H09 (right primer).

Mutant *pp4-2*	**Sample**		**Result**	
SAIL_569_H09	**Lane A**	**Lane B**	**Band(s)**	**Interpretation**
Pot: 3				
21	21A	21B	A	Homozygous
Pot: 5				
22	22A	22B	A	Homozygous
23	23A	23B	A	Homozygous
Wild Type (Control)				
24	24A	24B	B	Wild Type (Control)

Table 3-7. Samples for genotyping mutant *pp4-2* SALK_049725C. Primers for lane A are LBb1.3 New Salk (T-DNA primer) and PP4-2 Salk RP 049725 (right primer). Primers for lane B are PP4-2 Salk LP 049725 (left primer) and PP4-2 Salk RP 049725 (right primer).

Mutant *pp4-2*	**Sample**		**Result**	
SALK_049725C	**Lane A**	**Lane B**	**Band(s)**	**Interpretation**
Pot 1				
31	31A	31B	A	Homozygous
32	32A	32B	No band	-
Wild Type (Control)				
33	33A	34B	No band	-

Figure 3-10 shows the gel electrophoresis of genotyping mutant *pp4-2* SAIL_569_H09 and *pp4-2* SALK_049725C and Table 3-6 and Table 3-7 shows the interpretation of bands in gel each sample of *pp4-2* SAIL_569_H09 and *pp4-2* SALK_049725C lines, respectively.

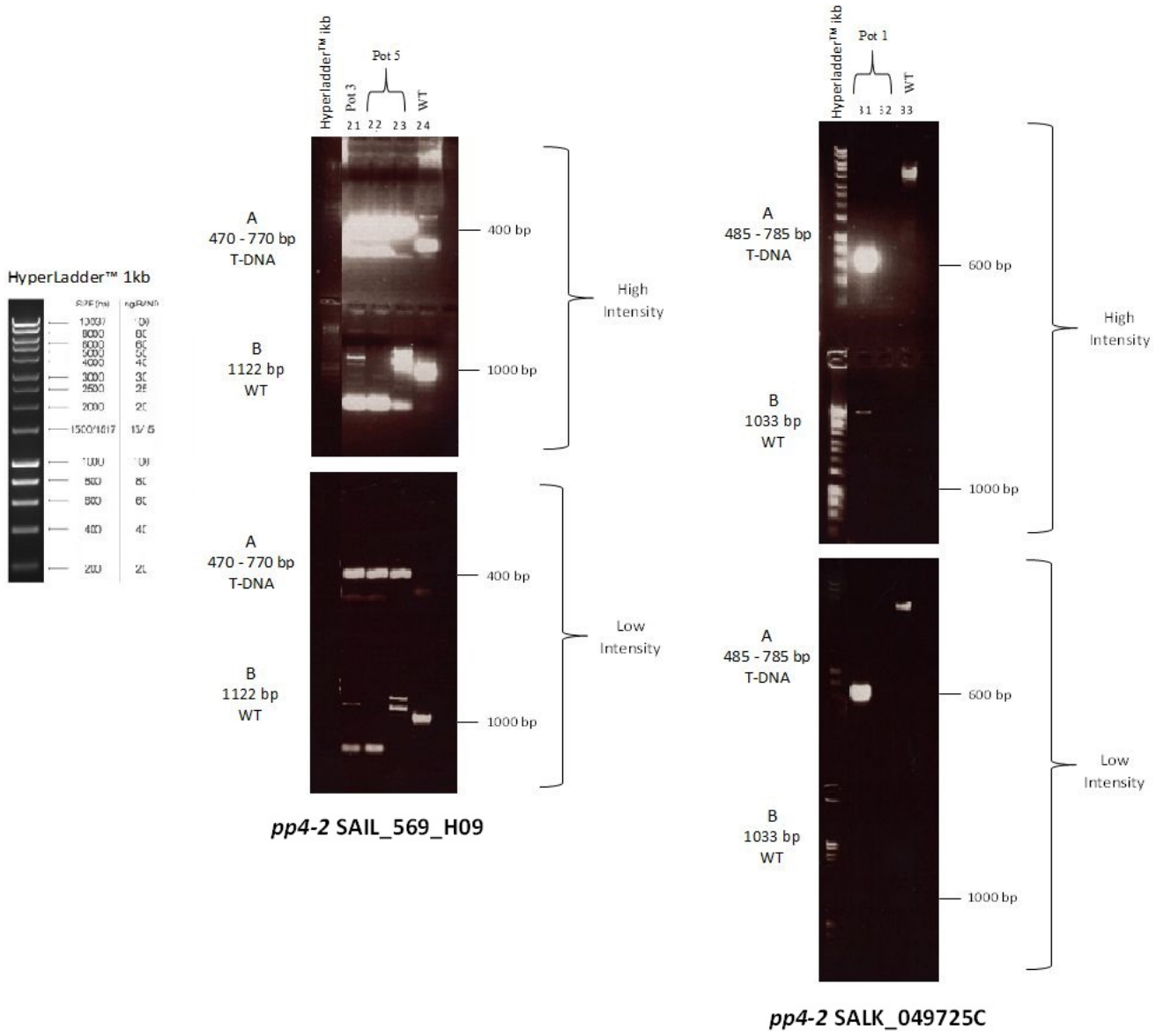


Figure 3-10. The gel electrophoresis of genotyping mutant *pp4-2* SAIL_569_H09 and *pp4-2* SALK_049725C. The result is provided in high intensity and low intensity. For *pp4-2* SAIL_569_H09, Lane A (T-DNA primer: LBP3 New Sail and Right Primer: PP4-2 Sail RP 569_H09) has expected band at 470 – 770 bp T-DNA and Lane B (Left Primer: PP4-2 Sail LP 569_H09 and Right Primer: PP4-2 Sail RP 569_H09) has expected band at 1122 bp wild type. For *pp4-2* SALK_049725C, Lane A (T-DNA primer: LBb1.3 New Salk and Right Primer: PP4-2 Salk RP 049725) has expected band at 485 – 785 bp T-DNA and Lane B (Left Primer: PP4-2 Salk LP 049725 and Right Primer: PP4-2 Salk RP 049725) has expected band at 1033 bp wild type. The size of A and B for both lines are based on SALK website tool that can be seen from http://signal.salk.edu/tdnaprimers.2.html (last retrieved: May 2016).

From Table 3-6 and Table 3-7, for all these lines, homozygous plants were identified. According to this result, all *pp4-2* SAIL_569_H09 plants and *pp4-2* SALK_049725C plant pot 1 #31 were selected for expression analysis.

3.1.1.5 Genotyping *pp4-1* GK_651B07 and *pp4-1* SALK_070977

Figure 3-11 provides the detailed characterization of the T-DNA insertion at the *PP4-1* gene for *pp4-1* GK_651B07 and *pp4-1* SALK_070977 mutant plant.

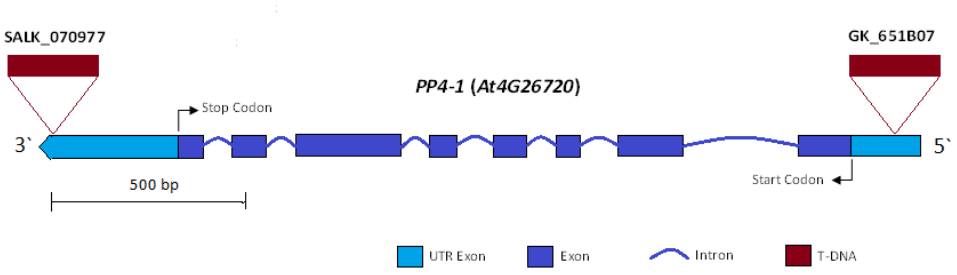


Figure 3-11. Characterization of the T-DNA insertion at the *PP4-1* gene for *pp4-1* GK_651B07 mutant plant (Flank-tagged upstream of translation) and *pp4-1* SALK_070977 (Flank-tagged downstream of translation) mutant plant

A pot of *pp4-1* GK 651B07 plant and *pp4-1* SALK_070977 plant, respectively, were examined. Two plants of each pot were labelled and tissue samples (young leaves) were used in order to perform genotyping. One wild type sample as control for each plant were analyzed. Those samples were divided into 2 parts based on primers that were added. Table 3-8 and Table 3-9 shows the detail of sample of pp4-1 GK 651B07 line and pp4-1 SALK_070977 line, respectively.

Table 3-8. Samples for genotyping mutant *pp4-1* GK_651B07. Primers for lane A are GABI kat (T-DNA primer) and PP4-1 GK RP 651B07 (right primer). Primers for lane B are PP4-1 GK LP 651B07 (left primer) and PP4-1 GK RP 651B07 (right primer).

Mutant *pp4-1* GK_651B07	**Sample**		**Result**	
	Lane A	**Lane B**	**Band(s)**	**Interpretation**
Pot 1				
25	25A	25B	A	Homozygous
26	26A	26B	A	Homozygous
Wild Type (Control)				
27	27A	27B	B	Wild Type (Control)

Figure 3-12 shows the gel electrophoresis of genotyping mutant *pp4-1* GK_651B07 and *pp4-1* SALK_070977, and Table 3-8 and Table 3-9 shows the interpretation of bands in gel each sample of *pp4-1* GK 651B07 line and *pp4-1* SALK_070977 line, respectively.

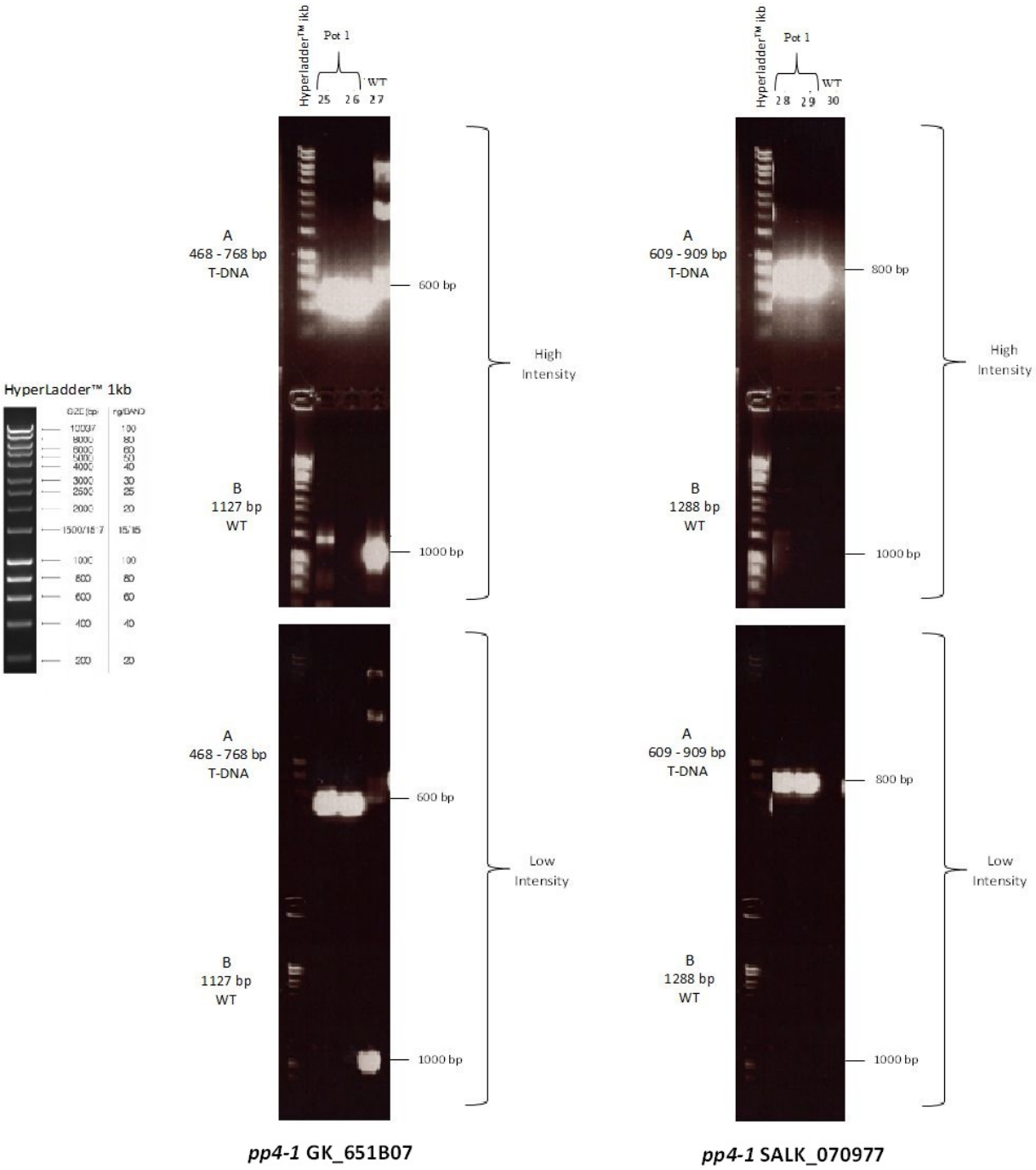


Figure 3-12. The gel electrophoresis of genotyping mutant *pp4-1* GK_651B07 and *pp4-1* SALK_070977. The result is provided in high intensity and low intensity. For *pp4-1* GK_651B07, Lane A (T-DNA primer: GABI kat and Right Primer: PP4-1 GK RP 651B07) has expected band at 468 – 768 bp T-DNA and Lane B (Left Primer: PP4-1 GK LP 651B07 and Right Primer: PP4-1 GK RP 651B07) has expected band at 1127 bp wild type. For *pp4-1* SALK_070977, Lane A (T-DNA primer: LBb1.3 New Salk and Right Primer: PP4-1 Salk RP 07977) has expected band at 609 – 909 bp T-DNA and Lane B (Left Primer: PP4-1 Salk LP 07977 and Right Primer: PP4-1 Salk RP 07977) has expected band at 1288 bp wild type.. The size of A and B for both lines are based on SALK website tool that can be got from http://signal.salk.edu/tdnaprimers.2.html (last retrieved: May 2016).

Table 3-9. Samples for genotyping mutant *pp4-1* SALK_070977. Primers for lane A are LBb1.3 New Salk (T-DNA primer) and PP4-1 Salk RP 07977 (right primer). Primers for lane B are PP4-1 Salk LP 07977 (left primer) and PP4-1 Salk RP 07977 (right primer).

Mutant *pp4-1* SALK_070977	Sample		Result	
	Lane A	Lane B	Band(s)	Interpretation
Pot 1				
28	28A	28B	A	Homozygous
29	29A	29B	A	Homozygous
Wild Type (Control)				
30	30A	30B	No band	-

From Table 3-8 and Table 3-9, for all these lines, GK and SALK line, homozygous plants were identified. According to this result, all *pp4-1* GK_651B07 plants and *pp4-1* SALK_070977 plants were selected for expression analysis.

3.1.1.6 Summary of Genotyping T-DNA Plant Samples

Table 3-10 provides the summary of genotyping examination to identify homozygous plant for various plant lines that has been done.

Table 3-10. Summary of genotyping of individual plants to identify homozygous plant.

Line	Number of plants examined	Heterozygous	Homozygous	No bands and or Wild Type
psy2l SALK_125872	4	1	3	0
psy2l SALK_048064	39	24	10	5
pp4r2l SALK_093041	4	2	1	1
pp4-1 GK_651B07	2	0	2	0
pp4-1 SALK_07977	2	0	2	0
pp4-2 SAIL_569_H09	3	0	3	0
pp4-2 SALK_049725C	2	0	2	0

3.1.2 Expression Studies and Observation of Phenotype for T-DNA Plants

Expression studies of T-DNA plants were performed on individual plants of *Arabidopsis thaliana* T-DNA plants that had been genotyped. The following lines are *psy2l* SALK_125872, *psy2l* SALK_048064, *pp4r2l* SALK_093041, *pp4-1* GK_651B07, *pp4-1* SALK_070977, *pp4-2* SAIL_569_H09, and *pp4-2* SALK_049725C. For each line, representative plants were selected to perform the expression studies, with considering the phenotype of sample plants. Table 3-11 shows the list of plants that were selected.

Table 3-11. The list of T-DNA plants that were selected for initial expression study

Name of Gene	Line	Pot	Plant Number	Genotyping Result	Phenotype
psy2l	SALK_048064	"Chris-3"	38	Heterozygous	Green, Dwarf
			39	Heterozygous	Green, Normal Size
		"Chris-16"	41	Probably Homozygous (?)	Brown (dying), Dwarf
			44	Heterozygous	Green, Normal Size
			45	Heterozygous	Green, Normal Size
	SALK_125872	3_2	18	Homozygous	Green, Normal Size
			19	Homozygous	Green, Normal Size
pp4r2l	SALK_093041	1	11	Homozygous	Green, Normal Size
			12	-	Green, Normal Size
pp4-1	GK_651B07	1	25	Homozygous	Green, Normal Size
			26	Homozygous	Green, Normal Size
	SALK_070977	1	28	Homozygous	Green, Normal Size
			29	Homozygous	Green, Normal Size
pp4-2	SAIL_569_H09	3	21	Homozygous	Green, Normal Size
			22	Homozygous	Green, Normal Size
	SALK_049725C	5	31	Homozygous	Green, Normal Size
			32	-	Green, Dwarf

3.1.2.1 Expression Studies and Observation of Phenotype for T-DNA plants *psy2l* SALK_125872 and *psy2l* SALK_048064

cDNA was created from isolated RNA. Agarose gel electrophoresis for PCR product of the cDNA for *psy2l* SALK_125872 and *psy2l* SALK_048064 is shown in Figure 3-13 I. Further generations were also checked and the results are provided in Figure 3-14. All the mutant plants were compared to wild type.

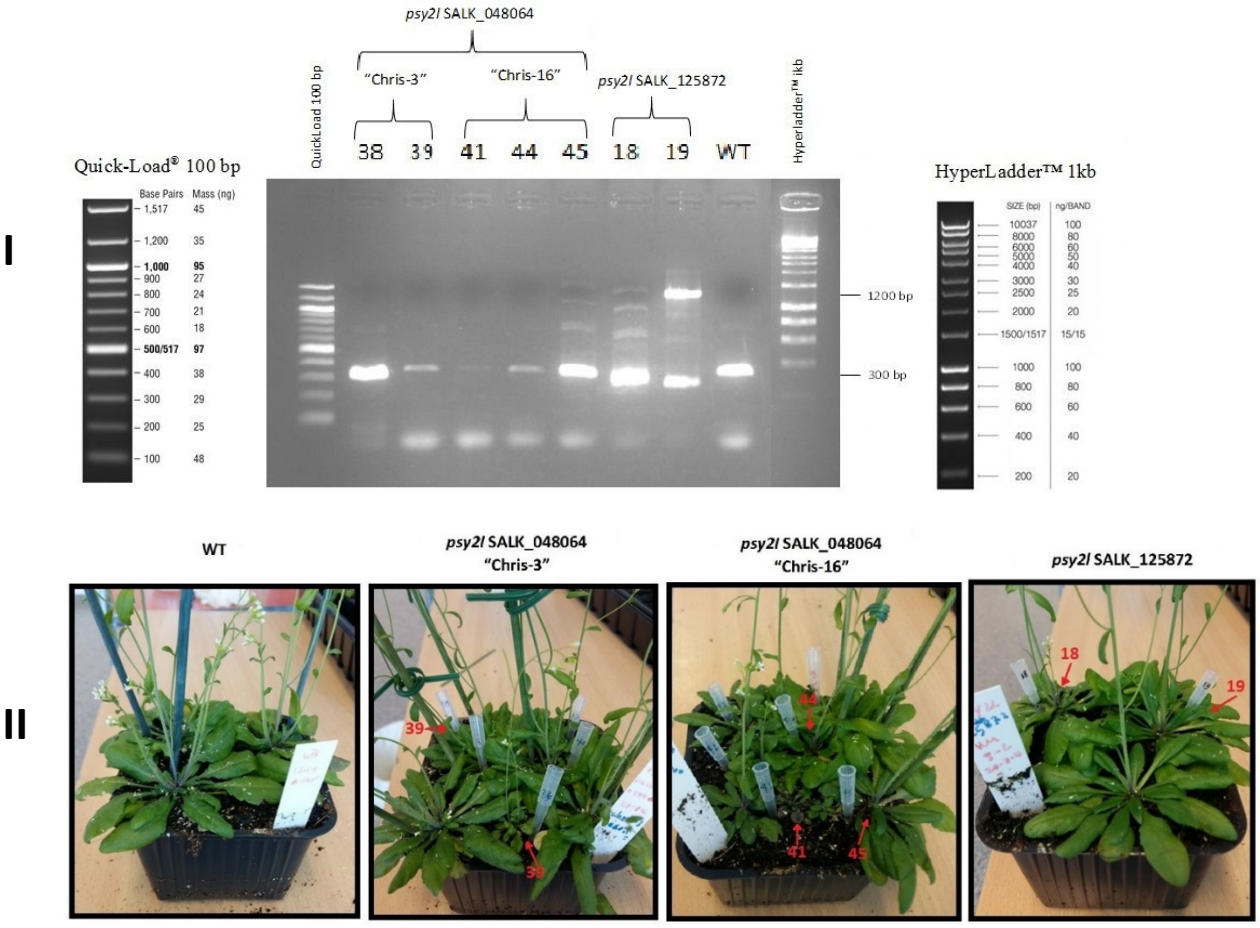


Figure 3-13. The expression analysis and phenotype of *psy2l* SALK_125872 and psy2l SALK_048064 plants. **I:** The agarose gel electrophoresis of product of RT PCR performed after production of cDNA for *psy2l* SALK_125872 and psy2l SALK_048064 plants. Expected PCR products is about 300 – 400 bp. The Primers were RT PSY2L LP as forward Primer and RT PSY2L RP as reversed Primer. These primers were designed by previous master thesis. **II:** The phenotype of the plant sample of *psy2l* SALK_125872 and psy2l SALK_048064. Notice that the lower expression level of the plant number 41, the more dwarf the plant phenotype is, for instance plant number 39 and 41.

As provided in Figure 3-13 I, the heterozygous *psy2l* SALK_048064 mutant plants appear to have various amount of RNA when compare with the wild type. The phenotype of the plant sample is shown in Figure 3-13 II. The brownish dwarf plant (number 41) shows the least amount of RNA, while the dark green dwarf plant (number 38) has most abundant amount of product

amplification. Heterozygous green normal-sized plants (number 39 and 44) have little *PSY2L* expression compare to wild type. These variations in *PSY2L* expressions are urge of interest to further investigation in this study. The homozygous *psy2l* SALK_125872 mutant plants (number 18 and 19) appear to have more *PSY2L* expression compared to wild type.

As we can see in Table 3-3, the genotyping was also performed to the next generation of *psy2l* SALK_048064 mutant plants "Chris-3" and "Chris-16". Four "dwarf" and "Normal Size" of this next generation of *psy2l* SALK_048064 mutant plants "Chris-16" were selected. Moreover, further generation of heterozygous *psy2l* SALK_048064 mutant pot "Chris-3" (plant number 39 and 44 from Table 3-2) were also checked for expression analysis without performing genotyping. As comparison, two further generation of homozygous *psy2l* SALK_125872 mutant plants 3_2 (number 19) from Table 3-4 were also analysed for expression studies without performing genotyping as well. Table 3-12 shows the list of plant that was selected for repetition of expression study of *psy2l* SALK_048064 and *psy2l* SALK_125872 mutant plants.

Table 3-12. The list of T-DNA plants that were selected for repetition of expression study of *psy2l* SALK_048064 and *psy2l* SALK_125872 mutant plants

Name of Gene	Line	Pot	Plant Number	Genotyping Result	Phenotype
PSY2L	SALK_048064	Next generation of "Chris-16" seeds from Table 3-3	4 "Dwarf" plants	Homozygous (see Table 3-3 and Figure 3-4)	Green, Dwarf
			4 "Normal size" plants	Heterozygous (see Table 3-3 and Figure 3-4)	Green, Normal Size
		Next generation of "Chris-3" seeds from Table 3-2	39	No genotyping	Green, Normal Size
			44	No genotyping	Green, Normal Size
	SALK_125872	Next generation of 3_2 seeds from Table 3-4	19 (1)	No genotyping	Green, Normal Size
			19 (2)	No genotyping	Green, Normal Size

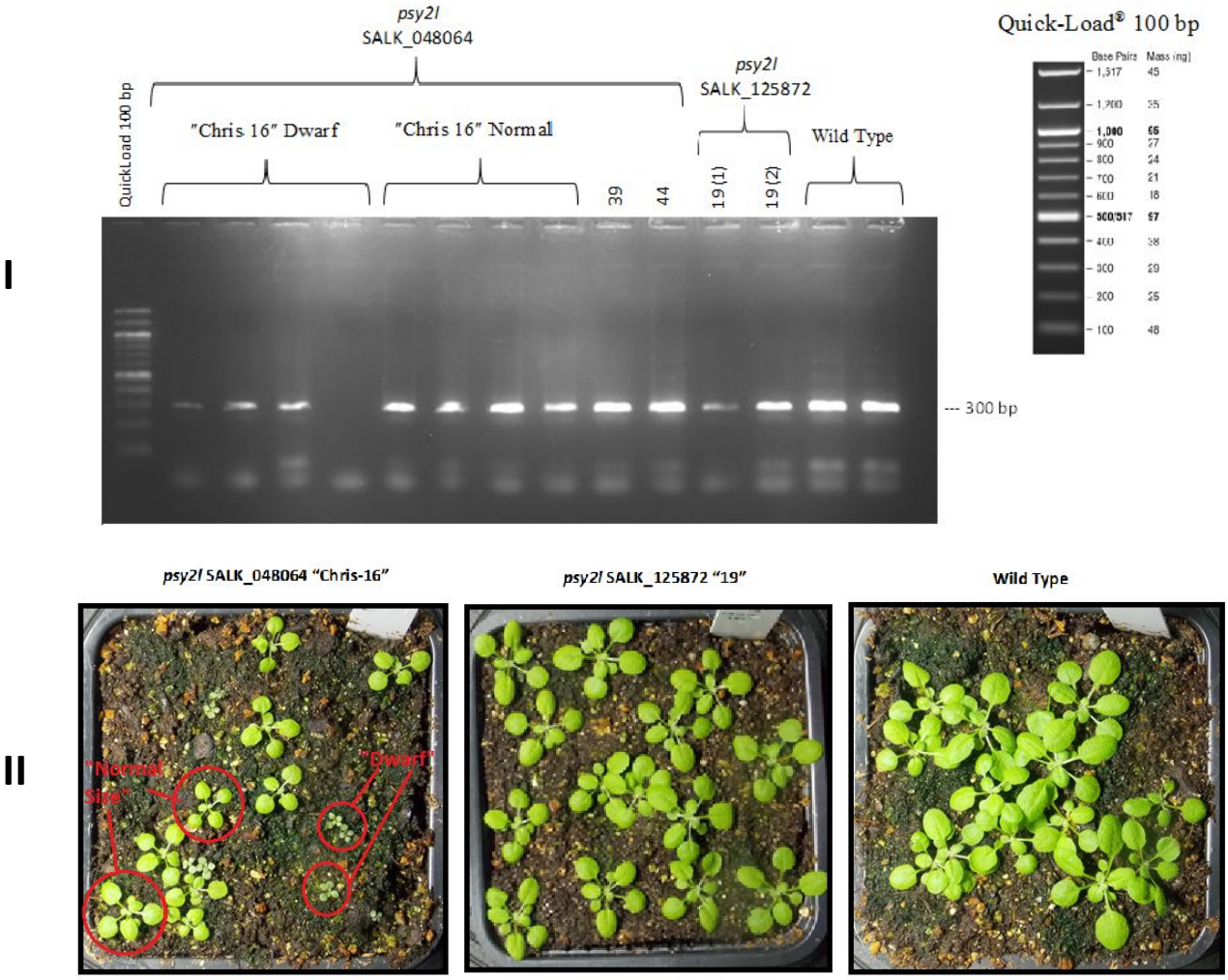


Figure 3-14. The repetition expression analysis and phenotype of *psy2l* SALK_125872 and psy2l SALK_048064 plants. **I:** The agarose gel electrophoresis of product of repetition RT PCR performed after production of cDNA for *psy2l* SALK_125872 and psy2l SALK_048064 plants. Expected PCR products is about 300 – 400 bp. The Primers were RT PSY2L LP as forward Primer and RT PSY2L RP as reversed Primer. These primers were designed by previous master thesis, Christine Sætre. **II:** The phenotype of *psy2l* SALK_048064 mutant plants "Chris 16" shows the "Dwarf" and "Normal Size" plants. The phenotype of *psy2l* SALK_125872 mutant plants shows slightly bigger than "Normal size" of *psy2l* SALK_048064, but smaller than wild type.

From Figure 3-14 I, some of the *psy2l* SALK_048064 mutant plants ("Chris-16" "Normal size") and the homozygous *psy2l* SALK_048064 mutant plants ("Chris-16" "Dwarf") appear to have weaker band than wild type and most probably represent the knockout or knockdown gene (the successful mutant). However, the "Chris-16" "Normal size" showed approximately similar *PSY2L* expression as the wild type. Also, notice the phenotype of *psy2l* SALK_048064 mutant plants "Chris 16" in Figure 3-14 II shows two different size for the homozygous "Dwarf" and heterozygous "Normal Size" plants. Other *psy2l* SALK_048064 mutant plants, the next generation of "Chris-3" (number 39 and 44) appear to have about the same *PSY2L* expression as the wild type as well, but not low as their mother plants (see Figure 3-13 I). The *psy2l* SALK_125872, the next generation of 3_2, shows various result. Number 19(1) has less *PSY2L* expression than wild type,

probably because of the amplification problem. On the other hand, number 19(2) is appeared to have same *PSY2L* expression as the wild type. The phenotype of *psy2l* SALK_125872 mutant plants shows slightly bigger than the heterozygous "Normal size" of *psy2l* SALK_048064, but smaller than wild type (Figure 3-14 II).

According to the result in Figure 3-14, we decided to more focus on SALK_048064 mutant plants "Chris 16", and repeated the examination. We then sow "Chris 16" seeds in the soil and put them in two different place, one in climatic chamber and the other in plant room 12 h light / 12 h dark cycle at 22^{o}C. We also selected and distinguished the plants based on the size, namely "dwarf" (homozygous) and "semi dwarf" (heterozygous), and "normal size" (heterozygous and wild type). After approximately one month, we investigated the expression study of *PSY2L* for selected "dwarf" and "semi dwarf" plants in two different treatment. The procedure was same as we did before. Figure 3-15 provides the result of this expression study.

According to Figure 3-15, the *PSY2L* expression of "dwarf" plants is lower than wild type as well as "semi dwarf" in two different conditions. For "semi dwarf" plants, the *PSY2L* expression apparently shows slightly less to similar than wild type. Overall, the *PSY2L* expression of wild type and "semi dwarf" (allegedly heterozygous) shows no significant difference. The phenotype of this mutant line in plant room 12 h light / 12 h dark has been monitored intensively, and it can be seen in Figure 3-16.

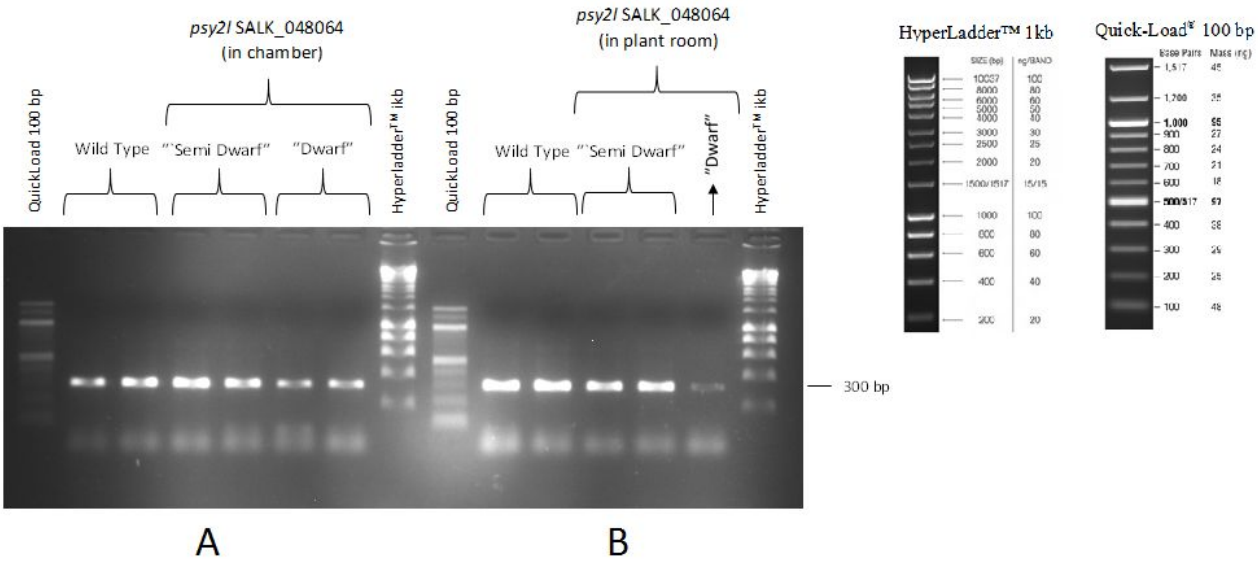


Figure 3-15. The agarose gel electrophoresis of product of repetition RT-PCR performed after production of cDNA for *psy2l* SALK_048064 plants in two different conditions, chamber (**A**) and plant room 12 h light / 12 h dark (**B**). The "dwarf" and "semi dwarf" plants for each condition were selected and compared it with wild type. Expected PCR products is about 300 – 400 bp. The Primers were RT PSY2L LP as forward Primer and RT PSY2L RP as reversed Primer. These primers were designed by previous master thesis, Christine Sætre.

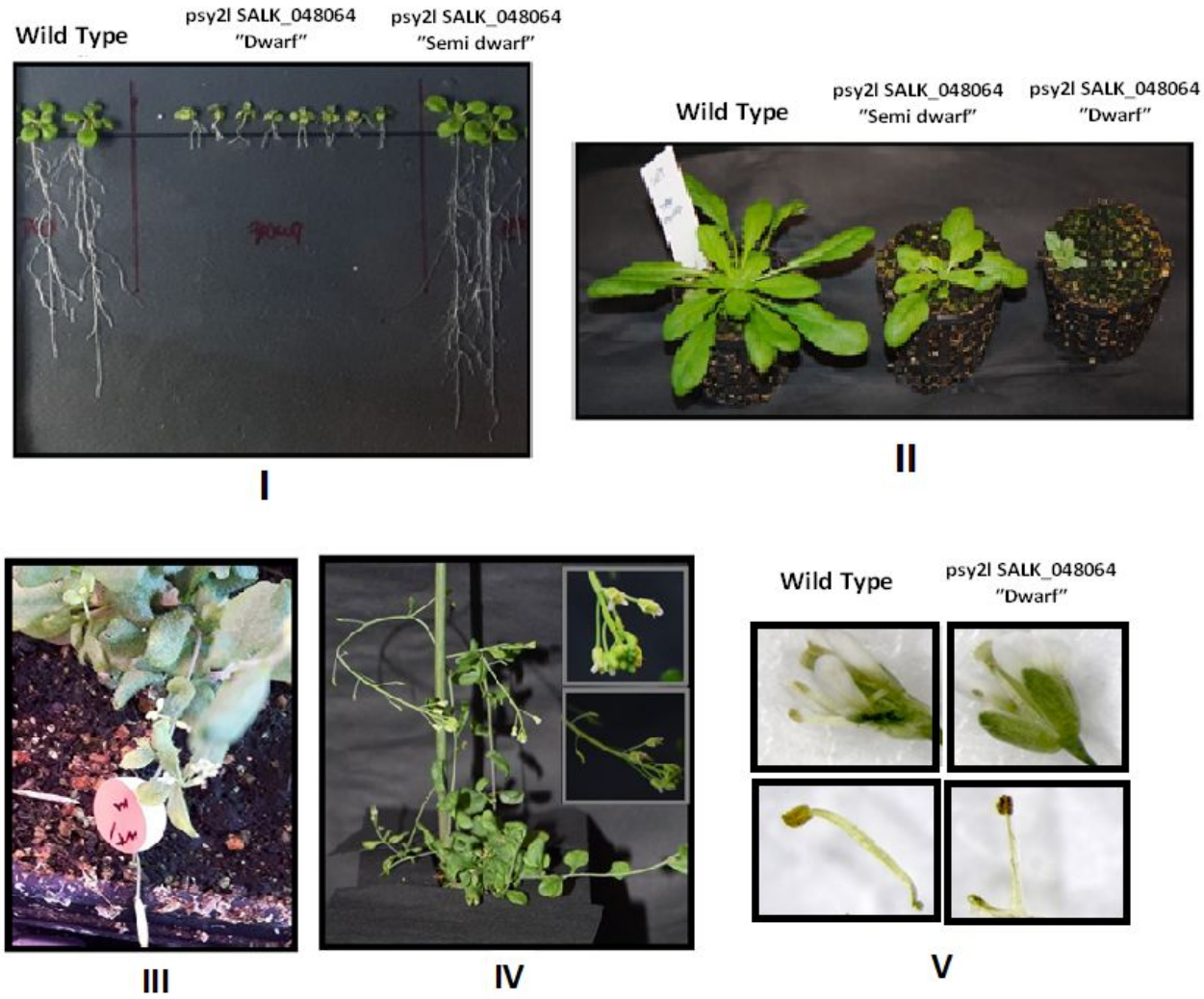


Figure 3-16. The monitoring phenotype of *psy2l* SALK_048064 mutants in plant room 12 h light / 12 h dark. **I:** Comparison of phenotype of dwarf (homozygous) and semi dwarf (heterozygous) *psy2l* SALK_048064 mutant plants with wild type. These plants was sown in half LS agar, and photographed at about 8 days after germination. The wild type and semi dwarf plants show resemble size, while the dwarf plants were clearly had smaller size. **II:** Three week plants in soil show more obvious phenotype. The semi dwarf has smaller size than wild type, but bigger than the dwarf. **III**: The image of 3 month dwarf (homozygous) *psy2l* SALK_048064 mutant plant is captured two weeks after cross-polination, where the wild type and semi dwarf mutants were already died. Wild type pollens successfully cross-polinate this mutant plant, which is not able to develop any seeds by its own. **IV**: The image of 4 month dwarf (homozygous) *psy2l* SALK_048064 mutant plant is captured five weeks after cross-polination. **V**: Overlook of different phenotype between wild type and homozygous *psy2l* SALK_048064 mutants. All pictures were photographed by Dr. Amr Kataya.

3.1.2.2 Expression Studies and Observation of Phenotype for T-DNA plants *pp4r2l* SALK_093041, *pp4-1* GK_651B07, *pp4-1* SALK_070977, *pp4-2* SAIL_569_H09, and *pp4-2* SALK_049725

Agarose gel electrophoresis of product of PCR performed after production of cDNA for *pp4r2l* SALK_093041*, pp4-1* GK_651B07*, pp4-1* SALK_070977*, pp4-2* SAIL_569_H09*,* and *pp4-2* SALK_049725 is shown in Figure 3-17 and for the repetition is provided in Figure 3-18. All the mutant plants are compared to wild type. Figure 3-19 shows the phenotype of all of these mutant lines.

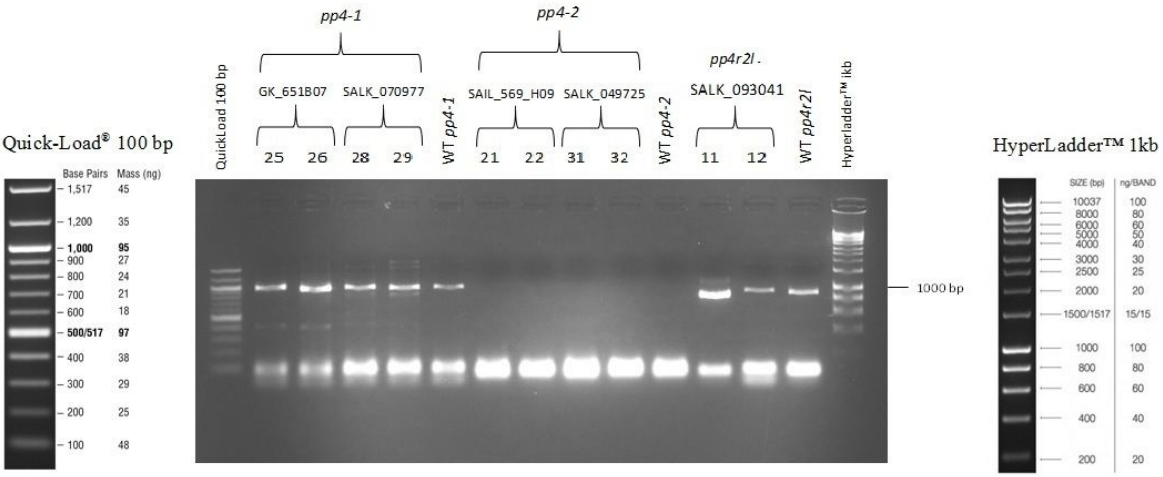


Figure 3-17. The agarose gel electrophoresis of product of RT-PCR performed after production of cDNA for *pp4r2l* SALK_093041, *pp4-1* GK_651B07, *pp4-1* SALK_070977, *pp4-2* SAIL_569_H09, and *pp4-2* SALK_049725 plants. Expected PCR products is about 1000 bp. For *pp4r2l* mutant, the primers were PP4R2LEYPFf as forward primer and PP4R2LEYPFr as reversed primer. For *pp4-1* mutants, the primers were AK74Bf as forward Primer and AK75Br as reversed primer. For *pp4-2* mutants, the primers were AK77Bf as forward Primer and AK75Br as reversed primer. Primers for *pp4r2l* mutant were designed by previous master thesis (Christine Sætre), while primers for *pp4-1* and *pp4-2* mutant were designed by Dr. Amr Kataya.

From Figure 3-17, the homozygous *pp4-1* GK_651B07 and SALK_070977 mutant plants (number 25, 26, 28, and 29) appear to show similar *PP4-1* expression as the wild type. On the other hand, no band appeared for all the *pp4-2* mutant plants, including wild type. This probably needed RT-PCR optimization. The homozygous *pp4r2l* SALK_093041 (number 11) shows more *PP4R2L* expression than wild type. The unidentified genotyping *pp4r2l* SALK_093041 (number 12) has slightly *PP4R2L* expression than wild type, and it needs further investigation.

After analyzing result from Figure 3-17, it was decided to repeat the RT-PCR for sample of *PP4-2* and *PP4R2L*. While using the same samples, we tried to use different primers (List in Table 3-13). We examined samples of *pp4-2* plants (number 21, 22, 31, and 32) by using AK 110f as forward primer and AK111r as reverse primer. We also examined the samples of *pp4r2l* plants (number

11 and 12) by using “Front1” primers and “Back2” primers. “Front1” indicates the position of primers near 5` orientation of gene sequence given, while “Back2” means the primers are placed in the middle of gene sequence more near 3` orientation. Therefore, “Front1” primers are expected to give bigger size of product than “Back2”.

Table 3-13. The list of primers for repeating RT-PCR of *pp4-2* and *pp4r2l* T-DNA plants.

Name of Gene	Position of Primer	Name of Primer	Sequence (5’ → 3’)
pp4-2	-	Forward : AK 110f	TTCTAGCTCTCAAGGTTAGATATCCAGAC
		Reverse : AK111r	TAGCTGATGAGCTCGACATATGTAATCA
pp4r2l	“Front1”	Forward : RT_PP4R2L_Front1_LP	GAGATTTCCTCCGTCGTTCA
		Reverse : RT_PP4R2L_Front1_RP	ATCCGTCATTTTTGCCTCAG
	“Back2”	Forward : RT_PP4R2L_Back2_LP	GCAATGACTGTTGACATGGA
		Reverse : RT_PP4R2L_Back2_RP	TAGGCAACCGTGAATCTCCT

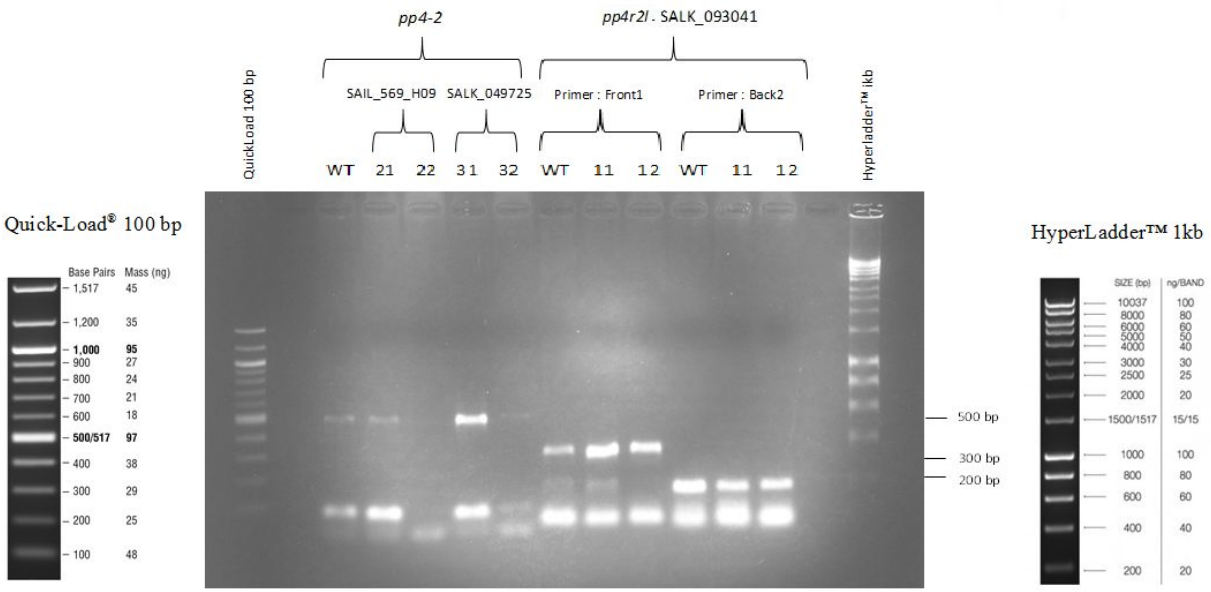


Figure 3-18. The agarose gel electrophoresis of product of RT-PCR performed after repeating production of cDNA for *pp4r2l* SALK_093041, *pp4-2* SAIL_569_H09, and *pp4-2* SALK_049725C plants. Expected PCR products is about 500 bp, 350 bp, and 200 bp for *pp4-2* mutants, *pp4r2l* SALK_093041 using “Front1” primers, and *pp4r2l* Salk_093041 using “Back2” primers, respectively. The list of primers for repeating RT PCR of *pp4-2* and *pp4r2l* mutant are provided in Table 40.

Figure 3-18 shows the agarose gel electrophoresis of product of RT PCR performed after repeating production of cDNA for *pp4-2* and *pp4r2l* plants. From Figure 3-18, the homozygous *pp4-2* SAIL_569_H09 and SALK_049725 mutant plants appear to have variable result. When all sample plants show similar or even weaker band than wild type, one *pp4-2* SALK_049725 mutant plant (number 31) show stronger band than control (wild type). After performing RT PCR for homozygous *PP4R2L* SALK_093041 by using different primers, “Front1” and “Back2”, we could

explain that the expression upstream of the T-DNA insertion is maximized which was not the case with primers downstream of insertion. By using “Front1” primer, both mutants (number 11 and 12) show stronger band than wild type. However, they appear to have weaker band than wild type when using “Back2” primer.

The phenotypes of all sample plants from *pp4r2l* SALK_093041, *pp4-1* GK_651B07, *pp4-1* SALK_070977, *pp4-2* SAIL_569_H09, and *pp4-2* SALK_049725C line are shown in Figure 3-19.

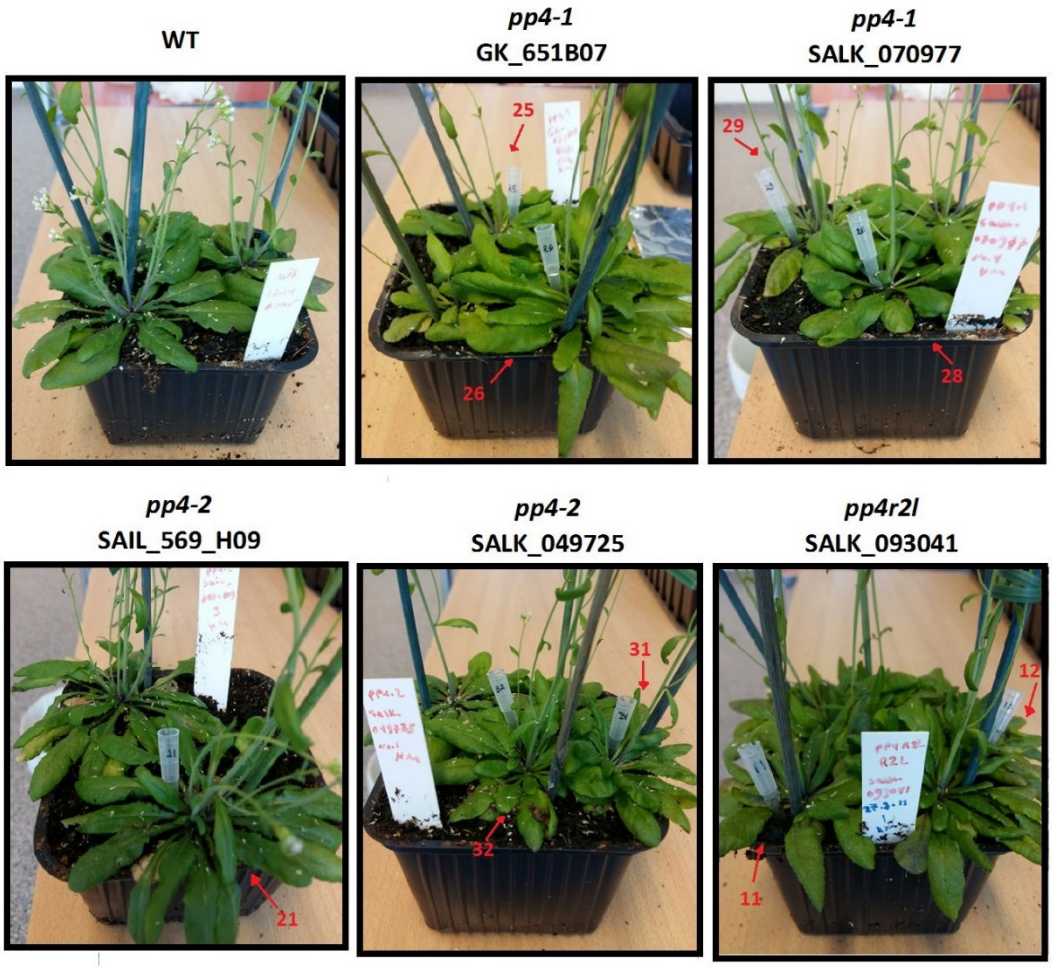


Figure 3-19. The phenotypes of all sample plants from *pp4r2l* SALK_093041, *pp4-1* GK_651B07, *pp4-1* SALK_070977, *pp4-2* SAIL_569_H09, and *pp4-2* SALK_049725C line. No significant difference was observed between these mutant plants and wild type.

3.1.3 Conclusion of Genotyping, Expression Studies, and Phenotyping for T-DNA plants

- We have succeeded to find homozygous "dwarf" plants from *psy2l* SALK_048064 that showed low expression being "knock out" or "knock down" plants. The phenotype of homozygous mutants of this line showed dwarfism, delayed growth, and extended life span.
- The homozygous plants of the other genes (*psy2l* SALK_125872, *pp4r2l* SALK_093041*, pp4-1* GK_651B07*, pp4-1* SALK_070977*, pp4-2* SAIL_569_H09*,* and *pp4-2* SALK_049725) were isolated and attempted to investigate their expression using RT-PCR. The expression of *psy2l* SALK_125872, *pp4-1* GK_651B07*,* and *pp4-1* SALK_070977 is not different from wild type. However, the expression of *pp4-2* SAIL_569_H09*, pp4r2l* SALK_093041, and *pp4-2* SALK_049725 is still not clear and they need more investigation to be understood.

3.2 Expression Studies and Observation of Phenotype for amiRNA plants

Expression studies of artificial microRNA (amiRNA) plants were performed on individual mutant plants of *Arabidopsis thaliana* initial (T0) seeds from previous employee (Zek Ginbot) and third generation seeds (F3) from previous master student (Christine Sætre) for *pp4-1/pp4-2* amiRNA. The study also included *psy2l* amiRNA Plants and *pp4r2l* amiRNA Plants, which the seeds were established and provided by Dr. Amr Kataya.

3.2.1 Expression Studies and Observation of Phenotype for *pp4-1/pp4-2* amiRNA Plants

The expression studies is more focused on *PP4-1* gene. Mutant plants transformed the constitutive pBA002 vector (contains 35s promotor) carrying artificial micro RNA (amiRNA). Two different amiRNAs were introduced to the plants with the following sequence:

amiRNA1: 5' TAATGAGAGTTATACGGTCTA 3'

amiRNA2: 5' TTAAAAGACGTAACAACGCTG 3'

Both microRNAs are designed to target *PP4-1* as well as *PP4-2*. For each generation, representative plants were selected to perform the expression study. *Arabidopsis thaliana* that contains vector without inserted amiRNA (NA vector) is used as control, similar role to wild type.

The amiRNAs were designed to target both *PP4-1* and *PP4-2* mRNA. The cDNA was created from isolated RNA. Agarose gel electrophoresis showing PCR product of F1 generation for *PP4-1* cDNA is shown in Figure 3-20. Further generation (F3) was also checked and the result is described in the next section. All the mutant plants were compared to NA vector. Table 3-14 shows the list of plant that were selected.

3.2.1.1 Expression Studies for First Generation (F1) of *pp4-1/pp4-2* amiRNA Plants

Table 3-14 lists the first generation (F1) of amiRNA plants transformed with vector pBA002 that were selected for expression study, and Figure 3-15 shows the agarose gel electrophoresis of RT-PCR product (*PP4-1*) for first generation (F1) of amiRNA with vector pBA002.

Table 3-14. The list of first generation (F1) of *pp4-1/pp4-2* amiRNA plants with vector pBA002 that were selected for expression study of *PP4-1*. The amiRNA 1 has two plant number 1-1 and 1-2, while the amiRNA 2 has only one plant number 2-2. Every plant number was only kept in one tube. Then, we sowed the seed from each plant number into plate containing half LS medium with herbicide BASTA 15 mg/mL. According to each plant number, we sowed the seeds into one plate. After 2 weeks, we selected all the survivor plants that we can found and sowed them into pot containing soil and labelled them with numbers in sequence that preceded by "P", one plant in one pot.

Vector	Generation	Type of amiRNA	Plant Number	Pot Number
pBA002	F1	amiRNA 1	1-1	P1
			1-2	P1
				P2
				P3
				P4
				P5
		amiRNA 2	2-2	P1
		NA Vector (Control)	NA Vector	P1
				P2

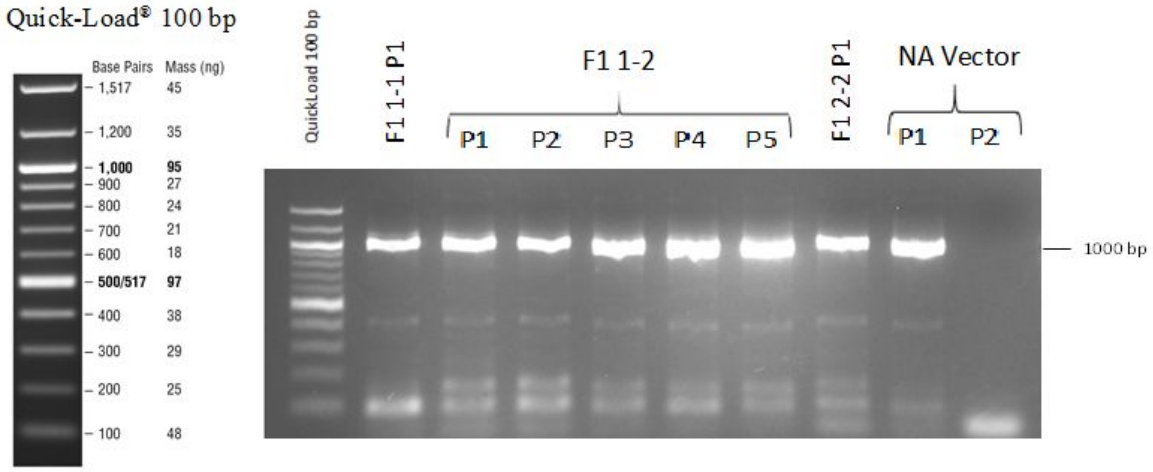


Figure 3-20. The agarose gel electrophoresis of RT-PCR product (*PP4-1*) for first generation (F1) of amiRNA with vector pBA002. The gel was loaded in 5 µL amount of PCR product. Expected PCR products is about 1000 bp. The Primers were AK74bf as forward Primer and AK75br as reversed Primer.

Figure 3-20 shows that the first generation (F1) of amiRNA plants with vector pBA002 that appear to have various *PP4-1* expression in comparison to NA vector plants (control). amiRNA 1 plant F1 1-1 (1) had less *PP4-1* expression than control. Some of amiRNA 1 plants F1 1-2, number (1) and (2), show less *PP4-1* expression as well, while the others (number (3), (4), and (5)) show the same expression with the control. amiRNA 2 plant, F1 2-2 (1), appear to have weaker band than control. With these variations, still it is not clear that we have a real knockdown/knockout of *PP4-1* and *PP4-2*.

3.2.1.2 Expression Studies and Observation of Phenotype for Third Generation (F3) of *pp4-1/pp4-2* amiRNA Plants

Beside first generation of amiRNA plants with vector pBA002, we also examined the expression study of *PP4-1* for the third generation plants (F3). Table 3-15 provide the list of the initial samples that were tested, which also divided into two different kind of amiRNAs, 1 and 2. The result can be seen in Figure 3-21. It can be seen that three plants have weaker band than NA Vector plants (control), while the others show similar or even stronger band than control. Those plants are amiRNA 1 plant F3 1-1 (1) P1, amiRNA 1 plant F3 1-2 (20) P1, and amiRNA 2 plant 2-1 (3) P1. These representative plants show less *PP4-1* expression than NA Vector plants (control). Therefore we decided to perform expression analysis to the other plants from the same pot number. Table 3-16 list the selected tube number and also pot number for this second examination of expression analysis. The result can be seen in Figure 3-22.

Table 3-15. The list of third generation (F3) of *pp4-1/pp4-2* amiRNA plants with vector pBA002 that were initially selected for expression study of *PP4-1.* Both the type of amiRNA 1 and 2 were divided into two plant numbers. Notice that in every plant number, we divided the seeds into some tubes, from 6 to 21 tubes, and also labelled them in parenthesis. Then, we only selected some tubes and sowed the seed into plate containing half LS medium with herbicide BASTA 15 mg/mL. For every one tube, we sowed the seeds into one plate. After 2 weeks, in every plate, we selected 3 to 5 survivor plants and transferred them into pot containing soil, one plant in one pot and also labelled them with numbers in sequence that preceded by "P". Finally, we only selected one pot/plant as a representative for every tube to perform PP4-1 expression analysis.

Vector	Generation	Type of amiRNA	Plant Number	Tube Number	Pot Number
pBA002	F3	amiRNA 1	1-1	(1)	P1 (out of P2 and P3)
				(2)	P1 (out of P2, P3, P4 and P5)
				(3)	P1 (out of P2, P3, P4 and P5)
			1-2	(14)	P1 (out of P2, P3, P4 and P5)
				(20)	P1 (out of P2, P3, P4 and P5)
		amiRNA 2	2-1	(1)	P1 (out of P2, P3, P4 and P5)
				(2)	P1 (out of P2 and P3)
				(3)	P1 (out of P2, P3, P4 and P5)
				(11)	P1 (out of P2 and P3)
			2-2	(1)	P1 (out of P2, P3, P4 and P5)
				(3)	P1 (out of P2, P3, P4 and P5)
		NA Vector (control)	NA Vector	(1)	P1 (out of P2, P3, P4 and P5)
				(2)	P1 (out of P2, P3, P4 and P5)

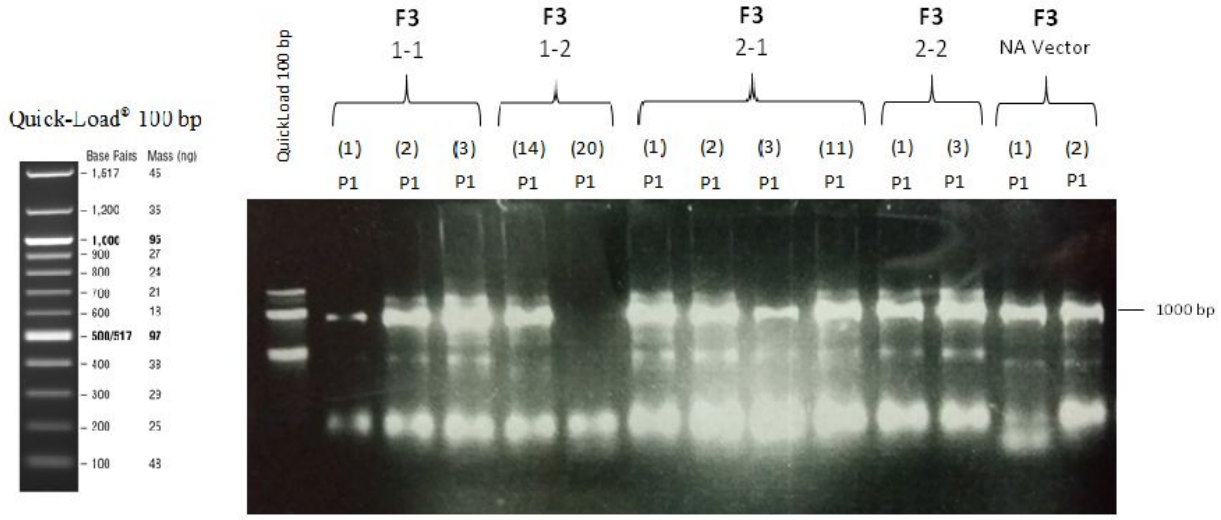


Figure 3-21. The agarose gel electrophoresis of RT-PCR product (*PP4-1*) of initial examination of expression analysis for third generation (F3) of amiRNA with vector pBA002. Expected PCR product is about 1000 bp. The Primers were AK74bf as forward Primer and AK75br as reversed Primer.

Table 3-16. The list of third generation (F3) *pp4-1/pp4-2* amiRNA plants that were selected for second examination of expression study based on the result of the initial examination (Figure 3-21).

Vector	Generation	Type of amiRNA	Plant Number	Tube Number	Pot Number
pBA002	F3	amiRNA 1	1-1	(1)	P1
					P2
					P3
			1-2	(20)	P1
					P2
					P3
					P4
					P5
		amiRNA 2	2-1	(3)	P1
					P2
					P3
					P4
					P5
		NA Vector (control)	NA Vector	(1)	P1
					P2
					P3
					P4
					P5

As expected, it can be seen from Figure 3-22 that all the selected third generation (F3) of amiRNA plants with vector pBA002 for the second examination of *PP4-1* expression analysis appear to give

weaker bands in comparison to most of NA vector plants (control), although the NA vector plants show non uniformity band. Re-investigation has similar intensity band in compare to previous analysis (Figure 3-21).

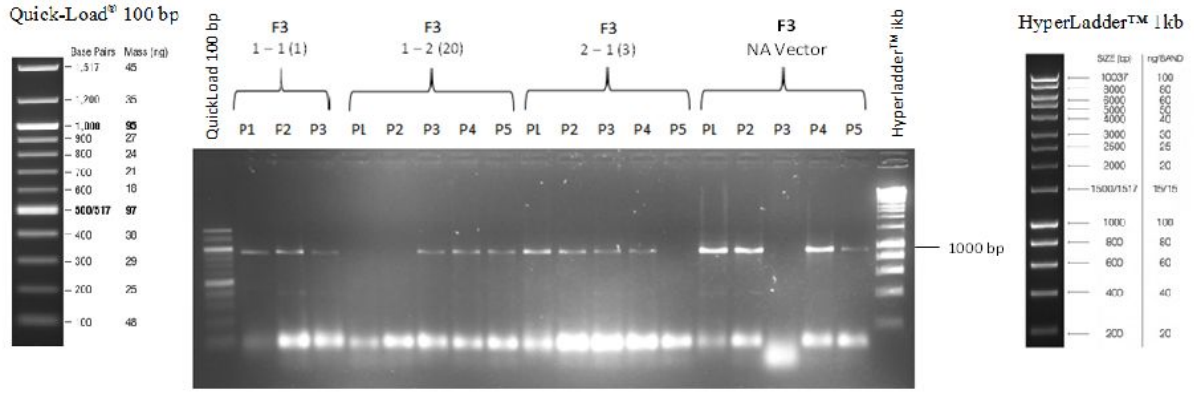


Figure 3-22. The agarose gel electrophoresis of RT-PCR product (*PP4-1*) of second examination of expression analysis for third generation (F3) of amiRNA with vector pBA002.Expected PCR product is about 1000 bp. The Primers were AK74bf as forward Primer and AK75br as reversed Primer.

To confirm and strengthen this result, we tried to do RT-PCR by using different primers for the same cDNA of these third generation amiRNA. We used AK108f and AK109r as forward and reversed primer, respectively. These primers will only amplify the *PP4-1* cDNA partially, resulting the expected PCR product is only about 300 kb instead of full length *PP4-1* cDNA (1000 kb). Figure 3-23 shows the result of the agarose gel electrophoresis of this RT-PCR. Along with that, we also tried to investigate the effect of both amiRNAs, 1 and 2, to the expression of *PP4-2* in this F3 plants. By using the same cDNA, we used different primers, AK111f and AK112r as forward and reversed primer, respectively. The result of this investigation is presented in Figure 3-24.

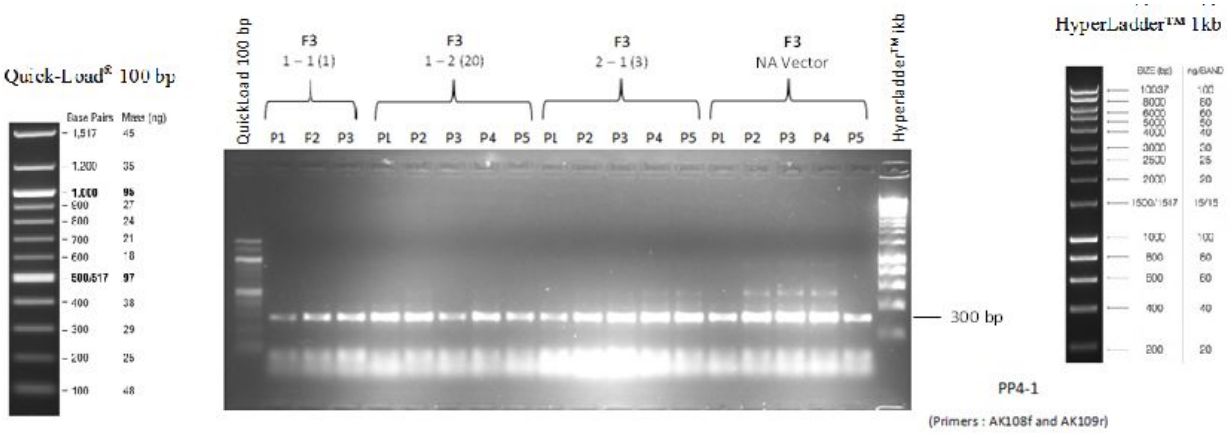


Figure 3-23. The agarose gel electrophoresis of RT-PCR product (*PP4-1*) of second examination of expression analysis for third generation (F3) of amiRNA with vector pBA002 by using AK108f as forward Primer and AK109r as reversed Primer. Expected PCR products is about 300 bp.

From Figure 3-23, the result of gel electrophoresis of RT PCR product (*PP4-1*) of second examination of expression analysis for third generation (F3) of amiRNA with vector pBA002 by using AK108f as forward Primer and AK109r as reversed Primer do not seem correlated to each other. Each group of plants, including F3 NA Vector as control, show non uniformity band compare to other band in the same group. However, all the amiRNA 1 plants, F3 1-1 (1) and F3 1-2 (20), appear to have weaker band than control. This is different from amiRNA 2 plants (F3 2-1 (3)) which seems to indicate similarity with control´s bands.

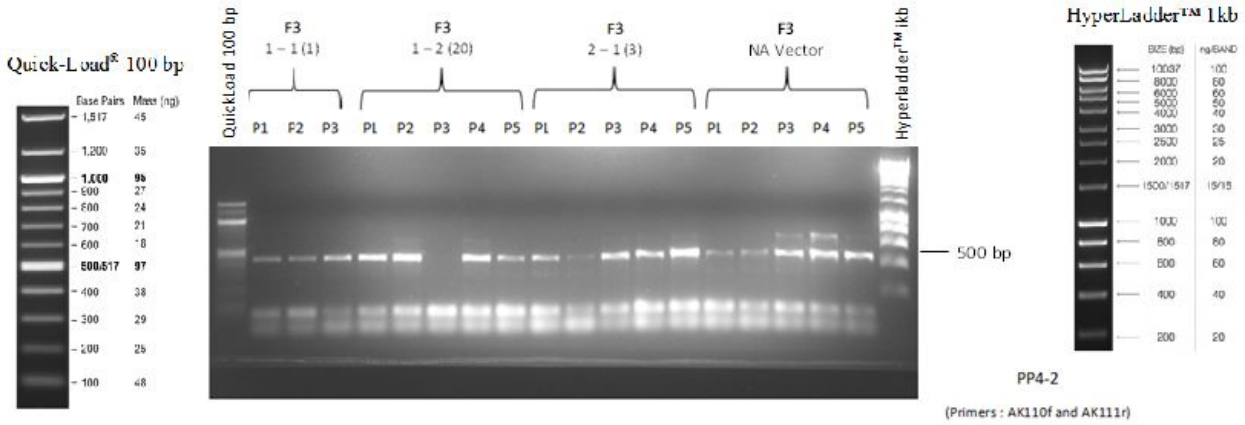


Figure 3-24. The agarose gel electrophoresis of RT-PCR product (*PP4-2*) of second examination of expression analysis for third generation (F3) of amiRNA with vector pBA002 by using AK110f as forward Primer and AK111r as reversed Primer. Expected PCR products is about 300 bp.

Besides examining the effect of amiRNAs to *PP4-1*, we also tried to investigate the influence of them to another target namely *PP4-2*. Figure 3-24 provides the result of agarose gel electrophoresis of RT-PCR product (*PP4-2*) of second examination of expression analysis for third generation (F3) of amiRNA with vector pBA002 by using AK110f as forward Primer and AK111r as reversed Primer. Each group of plants, including F3 NA Vector as control, show non uniformity band compare to other band in the same group.

After analysing the result of second examination of expression study of both *PP4-1* and *PP4-2* in the third generation (F3) amiRNA plants with vector pBA002, we decided to do third examination. According to the result of second examination in Figure 3-22, we selected two pots/plants from each tube number that have shown little expression of *PP4-1*. Table 3-17 lists these chosen plants.

Table 3-17. The list of third generation (F3) *pp4-1/pp4-2* amiRNA plants that were selected for third examination of expression study based on the result of the second examination (in Figure 3-22).

Vector	Generation	Type of amiRNA	Plant Number	Tube Number	Pot Number
pBA002	F3	amiRNA 1	1-1	(1)	P1
					P3
			1-2	(20)	P1
					P2
		amiRNA 2	2-1	(3)	P4
					P5
		NA Vector (control)	NA Vector	(1)	P1

We first sowed the seeds of these selected plants in plate containing half LS media with sucrose. In every plate, NA vector plant as control also sow to make it easy for comparison of phenotype with the amiRNA plant. Figure 3-26 shows the phenotype of the plants and to control (NA Vector) after 10 days. At a glance, it can be seen that either the amiRNA plants or control have no significant difference in appearance. We then collected the seedlings (10 day old) from the plate directly and examined the expression analysis of *PP4-1*. After isolation of RNA, the cDNA was synthesized. The RT-PCR then performed for this cDNA by using the AK74bf as forward primer and AK75br as reversed primer (the same condition as Figure 3-22) and by using the AK108f as forward primer and AK109f as reversed primer (the same condition as Figure 3-23). The result of this investigation is shown in Figure 3-25.

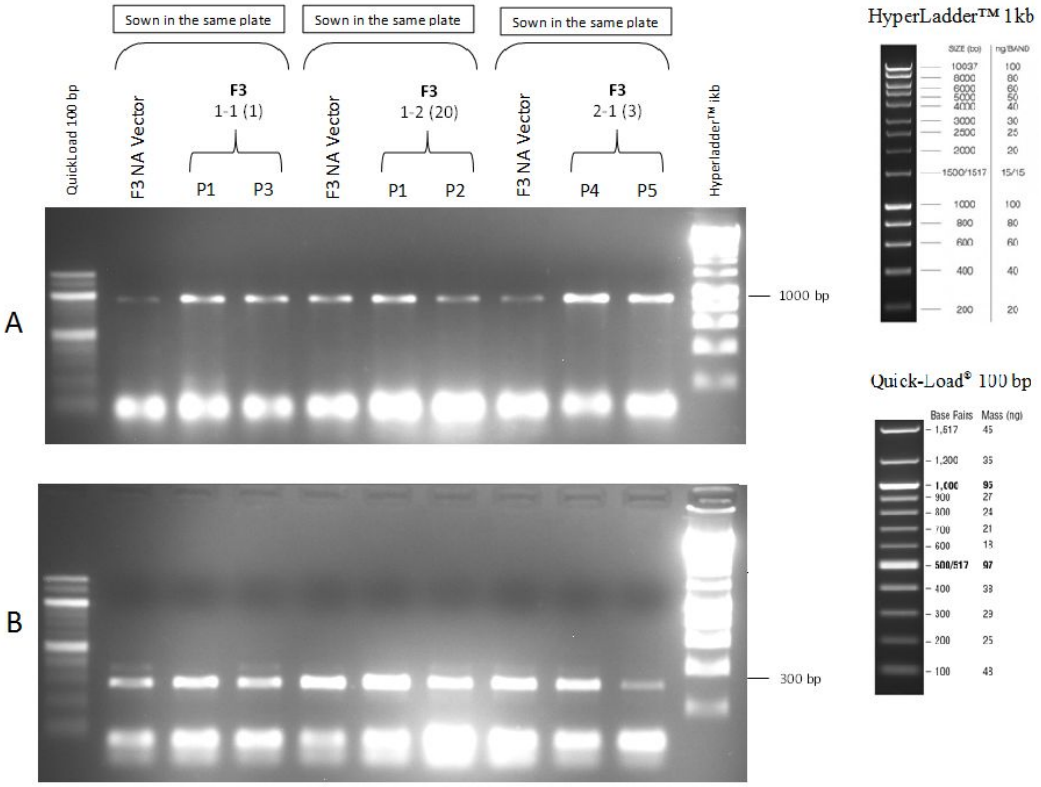
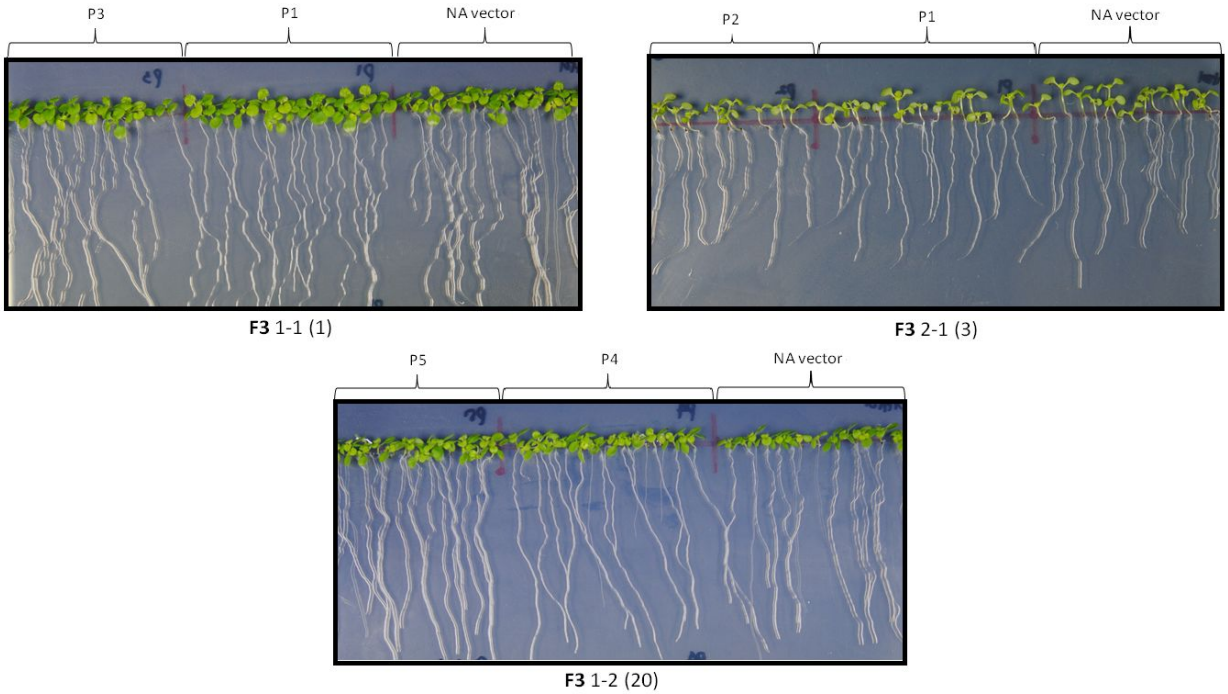


Figure 3-25. The agarose gel electrophoresis of RT-PCR product (*PP4-1*) of third examination of expression analysis for third generation (F3) of amiRNA with vector pBA002. NA vector plant as a control was shown in the same plate with each selected amiRNA plant.
A: The Primers were AK74bf as forward Primer and AK75br as reversed Primer (Expected PCR products is about 1000 bp).
B: The Primers were AK108f as forward Primer and AK109r as reversed Primer (Expected PCR products is about 300 bp). These primers were designed by Dr. Amr Kataya.



F3 1-1 (1)

F3 2-1 (3)

F3 1-2 (20)

Figure 3-26. The phenotype of third generation (F3) amiRNA plants that were selected for third examination of expression study. The expression analysis results are shown in Figure 3-20. The plants were 10 day old, and sown in half LS agar. Plants with pBA002 vector only (NA Vector) were used as control. No significant different phenotype between control and these amiRNA mutant plants was observed.

As can be seen in Figure 3-25A, the comparison of band between NA vector plant (control) against the amiRNA plant seems variable. The amiRNA 1 plant F3 1-2 (20) P1 have quite similar band to control, but the other plant (F3 1-2 (20) P1) shows a weaker band than control. On the other hand, the amiRNA 1 plant F3 1-1 (1) and the amiRNA 2 plant F3 2-1 (3) show stronger band than control. It also can be seen that the band of the NA Vector plants are not uniform. To complement this result, we also attempted to perform RT-PCR by using AK108f and AK109r as forward and reversed primer, respectively. The result is displayed in Figure 3-25B. From Figure 3-25B, the amiRNA 1 plant F3 1-1 (1) P3 have quite similar band to control, but the other plant (F3 1-1 (2) P1) shows a stronger band than control. For the F3 1-2 (20), the P2 also shows similar band to control and the P1 shows a stronger band than control as well. However, both the amiRNA 1 plant F3 2-1 (3) P4 and P5 have weaker bands than control. No significant different phenotype between control and these amiRNA mutant plants was observed (Figure 3-26).

3.2.2 Expression Studies and Observation of Phenotype for *psy2l* amiRNA Plants

Expression study of *psy2l* artificial microRNA (amiRNA) plants were performed on individual mutant plants of *Arabidopsis thaliana* which was more focused on *PSY2L* gene as main target of the amiRNAs. Mutant plants were transformed using the constitutive pBA002 vector carrying amiRNA. Two different amiRNAs had been introduced to the plants 5A and 7A. Representative plants were selected to perform the expression study and *Arabidopsis thaliana* wild type is used as control. Figure 3-27 shows the agarose gel electrophoresis of RT-PCR product (*PSY2L*) of amiRNA with vector pBA002, and Figure 3-28 provides the phenotype of some mutant plants.

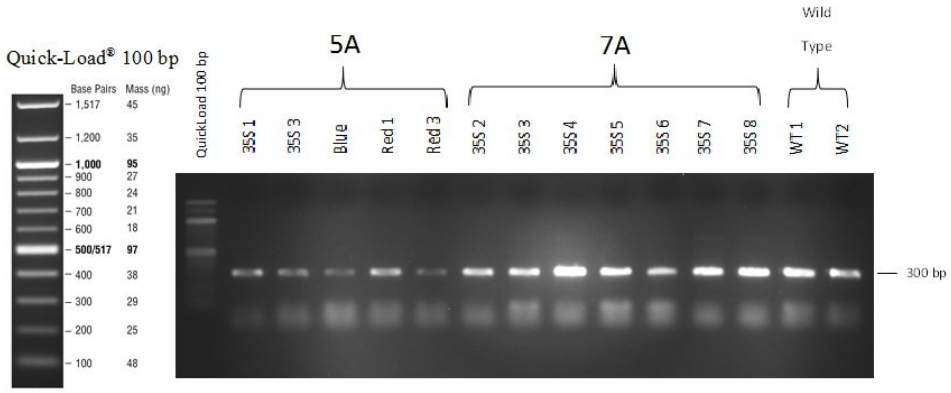


Figure 3-27. The agarose gel electrophoresis of RT-PCR product (*PSY2L*) of amiRNA with vector pBA002. Expected PCR products is about 300 bp. The Primers were RT PSY2L LP as forward Primer and RT PSY2L RP as reversed Primer.

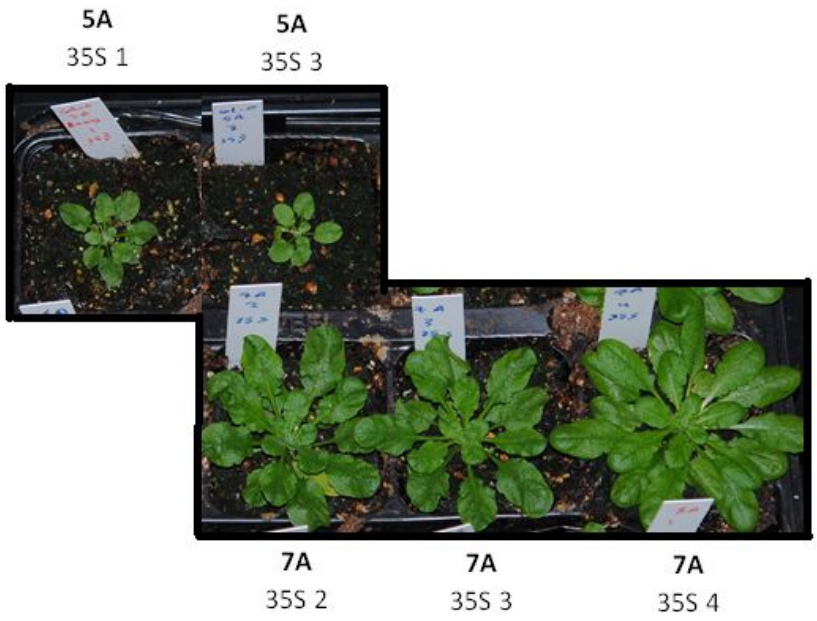


Figure 3-28. The phenotype of some selected *psy2l* amiRNA mutant plants. Showed in this figure, 5A and 7A plants.

Figure 3-27 shows clearly that the 5A amiRNA plants with vector pBA002 appear to have less *PSY2L* expression compared to wild type. However, the other amiRNA plants (7A) apparently show various level of expression with wild type. Some such as 7A 35S 2 and 7A 35S 3 show slightly lower expression than wild type, while 7A 35S 4 is seemingly overexpress. The phenotype of some of the sample plants show some interesting characteristic. The highly knocked down plants (5A 35S 1 and 5A 35S 3) have dwarf appearance and twisted leaf. The slightly knocked down plants, 7A 35S 2 and 7A 35S 3, also show twisted leaf, even though the size is bigger than 5A plants.

3.2.3 Expression Studies and Observation of Phenotype for *pp4r2l* amiRNA Plants

Expression study of *pp4r2l* artificial microRNA (amiRNA) plants were performed on individual mutant plants of *Arabidopsis thaliana* which was more focused on *PP4R2L* gene as main target of the amiRNAs. Mutant plants transformed using the constitutive pBA002 vector carrying amiRNA. Eight representative *pp4r2l* artificial microRNA (amiRNA) plants (9A) were selected to perform the expression study and *Arabidopsis thaliana* with pBA002 vector only as well as the wild type were used as control. Figure 3-29 shows the agarose gel electrophoresis of RT-PCR product (*PP4R2L*) of amiRNA with vector pBA002.

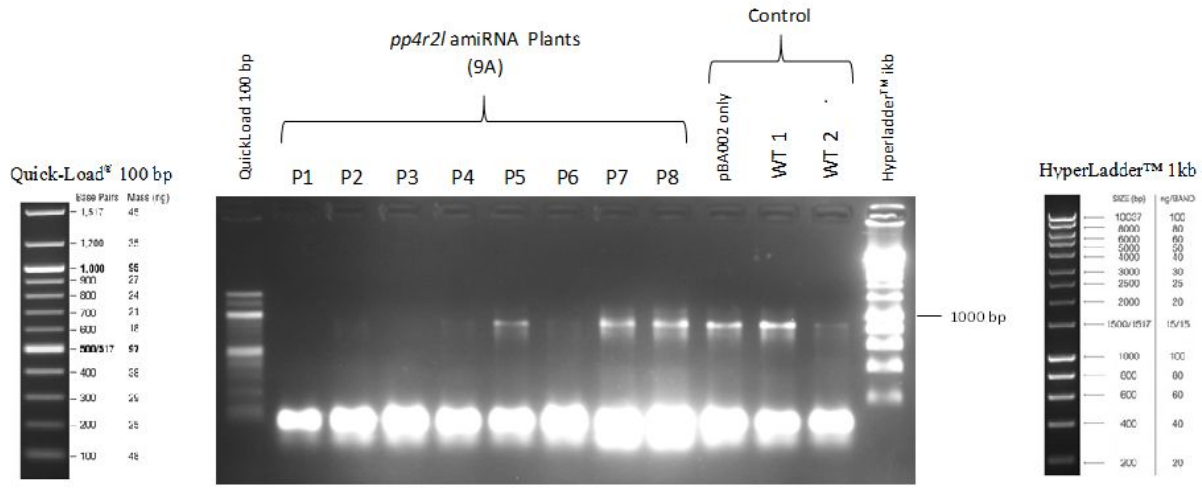


Figure 3-29. The agarose gel electrophoresis of RT-PCR product (*PP4R2L*) of amiRNA with vector pBA002. Expected PCR products is about 1000 bp. The Primers were PP4R2LEYFPf as forward Primer and PP4R2LEYFPr as reversed Primer.

Figure 3-29 shows clearly that all three 9A amiRNA plants, namely P5, P7, and P8 have relatively similar *PP4R2L* expression with control. The other plants (P1, P2, P3, P4, and P6) show lower expression than control. Overall, no significant different phenotype was shown by the knocked down plant compared to wild type.

3.2.4 Conclusion of Expression Studies and Observation of Phenotype for amiRNA plants

- Laborious works have done for searching the knocked down mutant *pp4-1/pp4-2* amiRNA plants. The level of expression is seemingly various and unstable between sample in the same generation and even between different generations. Not only that, their phenotype is also similar to the control or showed relatively weak phenotype.
- The knocked down *psy2l* amiRNA plants show some interesting phenotype, such as reduced size and twisted leaf.
- No significant different phenotype was shown by the knocked down plant of *pp4r2l* amiRNA plants compared to wild type.

3.3 Expression Studies and Observation of Phenotype for Gene Overexpression in Plants

Expression study of gene overexpression in plants were performed on individual mutant plants of *Arabidopsis thaliana* seeds from Dr. Amr Kataya. The study was performed to *pp4-1* overexpression, *pp4-2* overexpression, and *pp4r2l* overexpression in *Arabidopsis thaliana*.

3.3.1 Expression Studies and Observation of Phenotype for *pp4-1* Overexpression Plants

Expression study of *pp4-1* overexpression in plants were performed on individual mutant plants of *Arabidopsis thaliana* which was focused on analysis expression of *PP4-1*. Mutant plants transformed using the constitutive pBA002 vector carrying the cDNA of *PP4-1*. Representative plants were selected to perform the expression study and *Arabidopsis thaliana* with pBA002 vector only as well as the wild type were used as control. Figure 3-30 shows the agarose gel electrophoresis of RT-PCR product of *pp4-1* Overexpression in plants with vector pBA002.

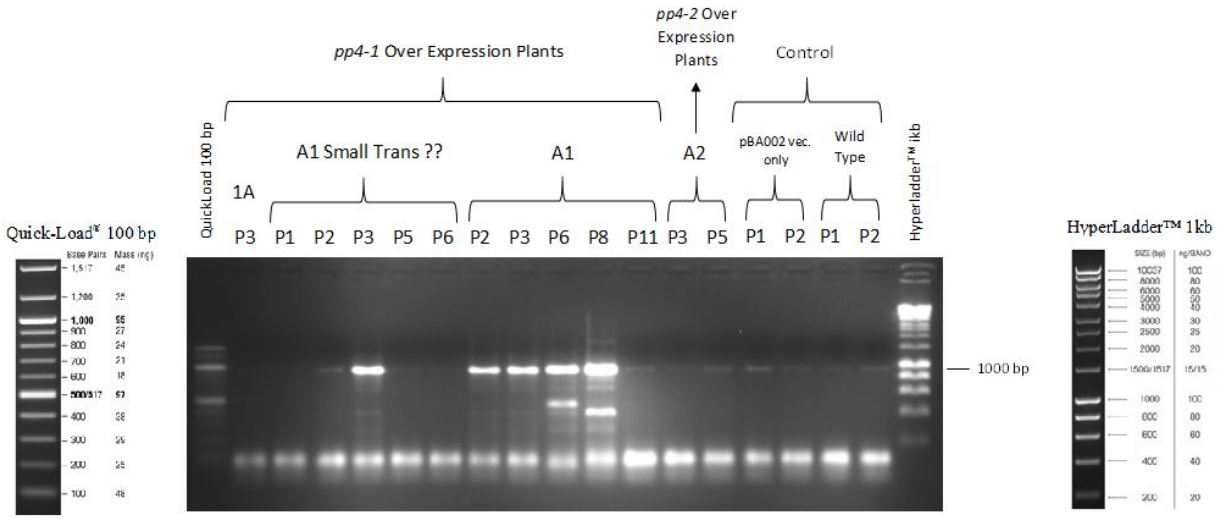


Figure 3-30. The agarose gel electrophoresis of RT-PCR product of *pp4-1* overexpression plants with vector pBA002. 1A, A1, and also A1 small trans ?? are the same *pp4-1* overexpression plants. However, 1A plant was planting earlier than A1 plants. The label "small trans (??)" plant means these plants were very small and the vector as well as the cDNA were doubted transform into the plants. Besides examine *pp4-1* overexpression in plants, we also tried to investigate the expression of *PP4-1* in *PP4-2* overexpression plants (A2). *Arabidopsis thaliana* with pBA002 vector only as well as the wild type were used as control. Expected PCR products for pp4-1 is about 1000 bp. For *pp4-1*, the Primers were AK74bf as forward Primer and AK75br as reversed Primer.

Figure 3-30 shows that expression of *PP4-1* in *PP4-1* overexpression plants is variable among the selected plants. All plants that we selected were relatively small. As expected, some of the A2

plants (P2, P3, P6, and P8) and one A1 small trans ?? plant (P3) clearly show *PP4-1* overexpression in comparison to the control. The other plants, including the *pp4-2* overexpression in plants (A2), show similar or even lower expression than control. Although some of these plants showed *PP4-1* overexpression, overall no significant different phenotype was shown compared to wild type and control (vector only).

3.3.2 Expression Studies and Observation of Phenotype for *pp4-2* Overexpression Plants

Expression study of *pp4-2* overexpression in plants were performed on individual mutant plants of *Arabidopsis thaliana* which was analysed the expression of *PP4-2*. Mutant plants transformed using the constitutive pBA002 vector carrying the cDNA of *PP4-2*. Representative plants (A2 and 2A) were selected to perform the expression study and *Arabidopsis thaliana* with pBA002 vector only as well as the wild type were used as control. Figure 3-31 shows the agarose gel electrophoresis of RT-PCR product (*PP4-2*) of *pp4-2* overexpression in plants with vector pBA002, and Figure 3-32 provides the phenotype of some mutant plants.

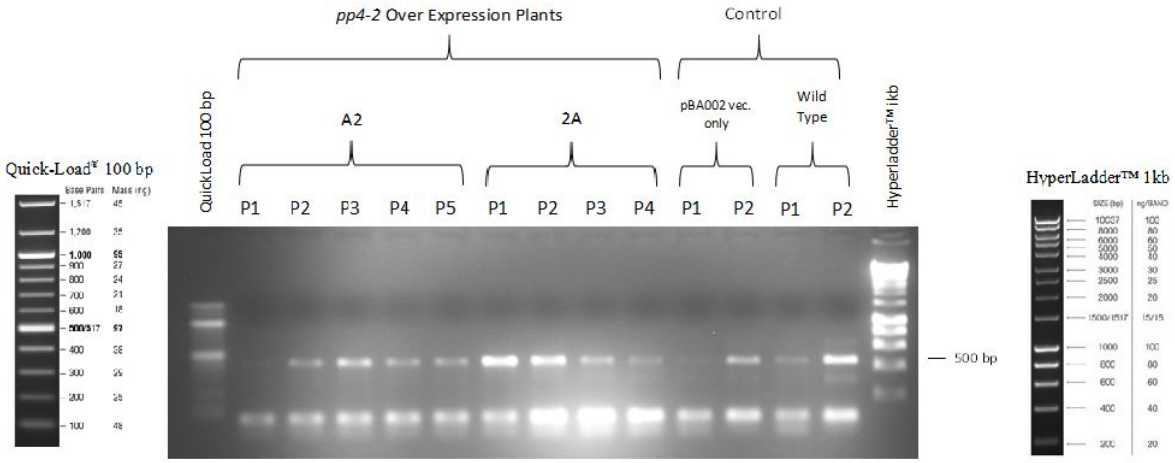


Figure 3-31. The agarose gel electrophoresis of RT-PCR product of *pp4-2* overexpression plants with vector pBA002. 2A and A2 are the same *pp4-2* overexpression in plants. However, 2A plant was planted earlier than A2 plants. *Arabidopsis thaliana* with pBA002 vector only as well as the wild type were used as control. Expected PCR products is about 500 bp. The Primers were AK110f as forward Primer and AK111r as reversed Primer.

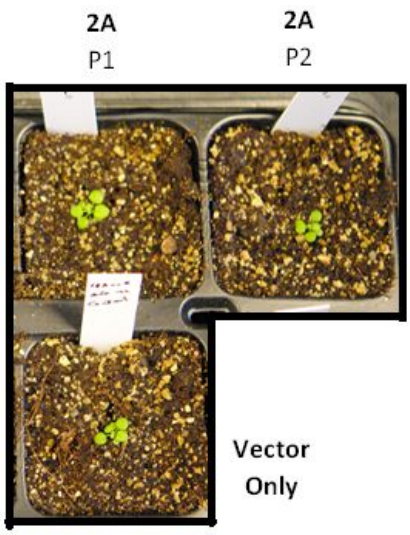


Figure 3-32. The phenotype of some selected *pp4-2* overexpression plants. Showed in this figure, two plants 2A P1 and 2A P2. The age of plants were 2 - 3 week old.

Figure 3-31 shows the expression of *PP4-2* in *PP4-2* overexpression plants. Some of the 2A plants (P1 and P2) are apparently show over expression of *PP4-2* in compare to other *pp4-2* overexpression plants including the controls. The phenotype of overexpression plants (2A P1 and P2) are shown in Figure 3-32. Even though the plants were still young, no significant phenotype was shown compared to control (vector only).

3.3.3 Expression Studies and Observation of Phenotype for *pp4r2l* Overexpression Plants

Expression study of *pp4r2l* overexpression plants were performed on individual mutant plants of *Arabidopsis thaliana* which was focused on analysis expression of *PP4R2L*. Mutant plants transformed using the constitutive pBA002 vector carrying the cDNA of *PP4R2L*. Representative plants (3A and A3) were selected to perform the expression study and *Arabidopsis thaliana* with pBA002 vector only as well as the wild type were used as control. Figure 3-33 shows the agarose gel electrophoresis of RT-PCR product of *pp4r2l* overexpression in plants with vector pBA002.

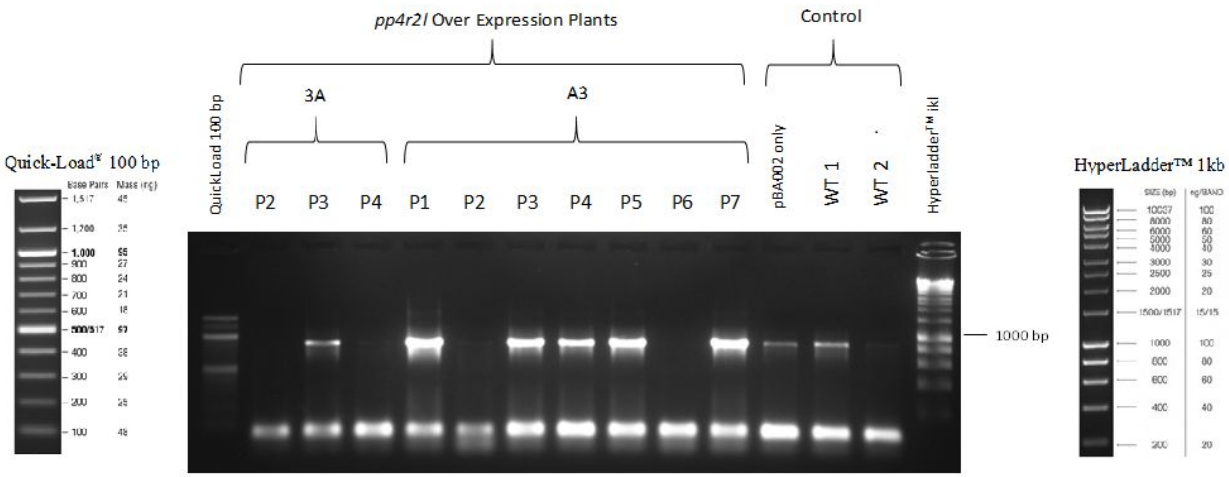


Figure 3-33. The agarose gel electrophoresis of RT-PCR product of *pp4r2l* overexpression plants with vector pBA002. 3A and A3 are the same *pp4r2l* overexpression plants. However, 3A plant was planted earlier than A3 plants. *Arabidopsis thaliana* with pBA002 vector only as well as the wild type were used as control. Expected PCR products for *PP4R2L* is about 1000 bp. Expected PCR products is about 1000 bp. The Primers were PP4R2LEYFPf as forward Primer and PP4R2LEYFPr as reversed Primer.

Figure 3-33 shows that expression of *PP4R2L* in *pp4r2l* overexpression in plants is variable among the selected plants. All plants that we selected were relatively small in comparison to controls. As expected, some of the A3 plants (P1, P3, P4, P5, and P7) are clearly show *PP4R2L* overexpression in comparison to the control. One 3A plant (P3) show slightly higher expression than controls. The other plants show lower expression than controls. Although some of these plants showed very strong *PP4R2L* overexpression, overall no significant different phenotype was shown compared to wild type and control (vector only).

3.3.4 Conclusion of Expression Studies and Observation of Phenotype for Overexpression Plants

We found plants that show higher level expression than wild type for each overexpression lines. However, no significant different phenotype was observed compared to wild type/control for these plants.

3.4 Molecular Cloning of *PSY2L* and *PP4-1*

3.4.1 Molecular Cloning of *PSY2L*

The aim of cloning the *PSY2L* cDNA into vectors is to produce PSY2L protein tagged with fluorescence protein (EYFP). This protein fusion will be used for investigating subcellular localization of PSY2L. The cDNA will be inserted into pCAT-EYFP-1 and pCAT-EYFP-2 vector, respectively. Both pCAT-EYFP-1 and pCAT-EYFP-2 vectors share the same nucleotide sequence with the exception that pCAT-EYFP-1 will ensure that the resulting fusion-protein will carry the EYFP tag on the N–Terminus, whereas the pCAT-EYFP-2 will give the resulting fusion-protein an EYFP tag at the C–terminus.

Template of cDNA is taken from U21916 plasmid from Arabidopsis Biological Resource Center (ABRC). High fidelity PCR was used for amplification of *PSY2L* cDNA. The tubes were labelled as PSY2L_A and PSY2L_B for amplified cDNA that will be inserted into pCAT-EYFP-1 and pCAT-EYFP-2 after digestion, respectively. Figure 3-34 shows the result of gel electrophoresis amplification of PSY2L cDNA. The target band in gel was cut and extracted. The concentration of *PSY2L* cDNA was then measured and the result is shown in Table 3-18.

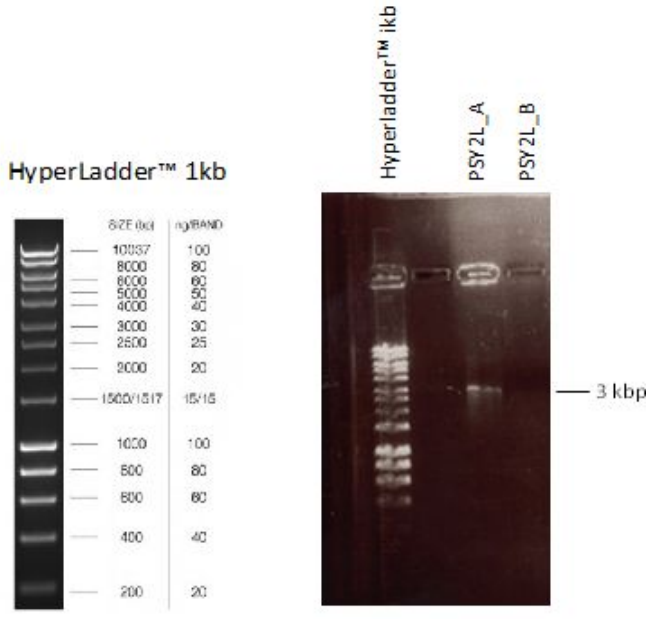


Figure 3-34. Gel electrophoresis shows amplification of *PSY2L* cDNA. Targeted band is about 2.5 – 3.0 kb. Both show positive result, although PSY2L_B has very weak band (almost not appear). Primers for PSY2L_A were EYFPPSY2Lf as forward Primer and EYFPPSY2Lr as reversed Primer. Primers for PSY2L_B were PSY2LEYFPf as forward Primer and PSY2LEYFPr as reversed Primer.

Table 3-18. The concentration of *PSY2L* cDNA after extraction of target band in gel electrophoresis of amplification cDNA

Amplified PSY2L cDNA solution	Concentration (ng/µL)
PSY2L_A	8.2
PSY2L_B	6,7

Both the amplified *psy2l* cDNAs, PSY2L_A and PSY2L_B, were ligated into vector pGEMT easy. For ligating the insert into the vector, the amount of insert and vector was calculated following this equation:

$$amount\ of\ insert\ (ng) = \frac{amount\ of\ vector\ (ng) x\ size\ of\ insert\ (kb)}{size\ of\ vector\ (kb)}\ x\ molar\ ratio\ of\ \frac{insert}{vector}$$

The amount of vector was 16.67 ng/µL (by using 1 µL of 16.67 ng/µL diluted pGEMT easy); size of *psy2l* cDNA is 2,6 kb; and size of pGEMT easy is about 3 kb, the amount of insert (ng) that should be added can be calculated as :

$$amount\ of\ insert\ (ng) = \frac{16{,}67\ ng\ x\ 2.6\ kb}{3\ kb}\ x\ \frac{3}{1}$$

$$amount\ of\ insert\ (ng) = 43.34\ ng$$

According to the concentration of amplified *PSY2L* cDNA solution in Table 3-18 and calculation the amount of insert above, the volume of PSY2L_A and PSY2L_B that should be added to pGEMT easy vector is 43.34 ng / 8.2 ng μL^{-1} ≈ 5 µL and 43.34 ng / 6.4 ng µL-1 ≈ 6.5 µL, respectively. Following the method of ligation (Table 2-18), reagents for each ligation mixture was prepared. The ligation mixture was left at 4°C overnight before it was used in transformation of bacterial cells.

The heat-shock method was used for transformation of ligated plasmids into competent bacterial cells, *Escherichia coli* JM109. The appearance of bacterial colonies in LB agar plates after incubated overnight can be seen in Figure 3-35. The bacteria colonies were selected by adding ampicillin and x-gal onto plate surface. Selection of bacteria with ampicillin resulted survival of cells that have successfully inserted by plasmid. Additionally, blue-white screening with x-gal gave information which colony that the plasmid has successfully inserted with *PSY2L* which shown as white colony (Figure 3-35). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was also added to the

plate to induce protein expression where the inserted cDNA is under the control of the lac operator in pGEMT-easy vector.

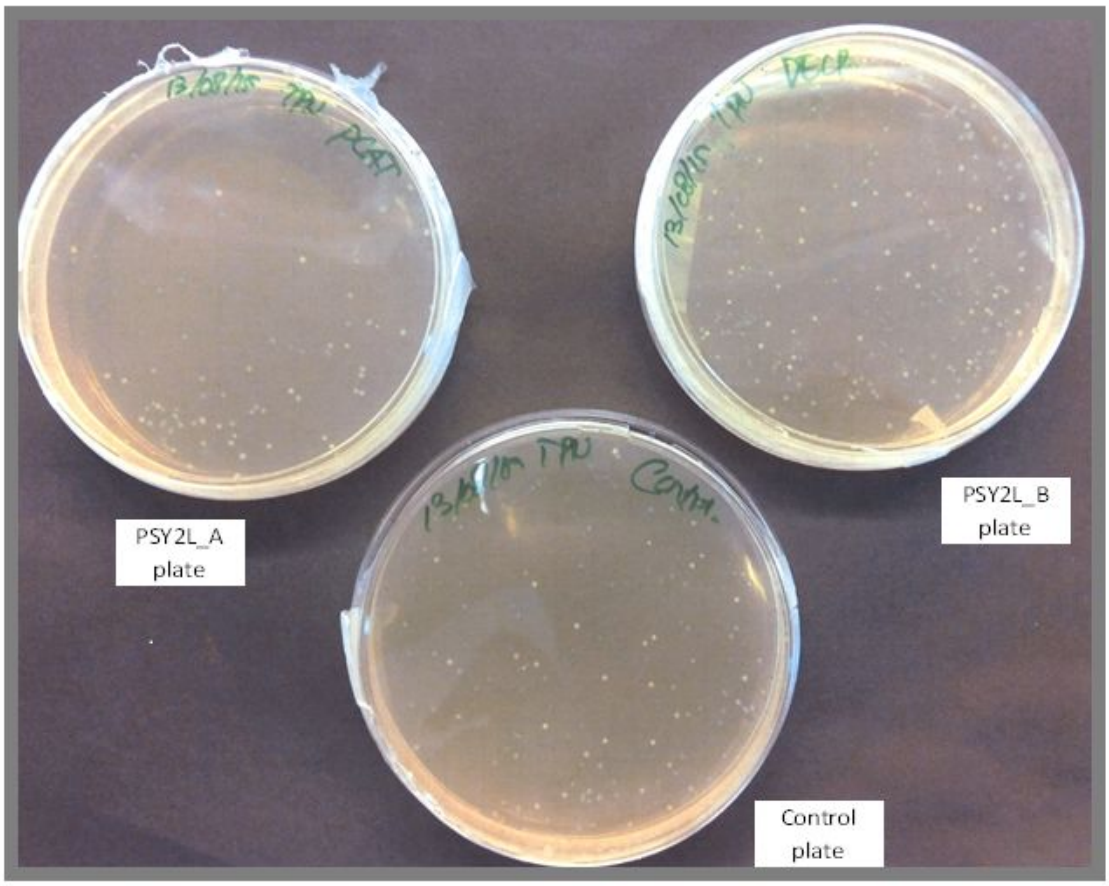


Figure 3-35. The appearance of bacterial colonies in LB agar plates for PSY2L_A and PSY2L_B after incubation overnight. Control plate was helped to verify that the ampicilin resistance in plates are functioning properly during this transformation step.

Ten white colonies from PSY2L_A plate and PSY2L_B plate, respectively, were randomly taken and checked using colony PCR. Lane A and B in Figure 3-36 show the result of gel electrophoresis colony PCR of PSY2L_A plate and PSY2L_B plate, respectively. According to result of colony PCR (Figure 3-37), two expected bands were found for PSY2L_B in lane B, but no positive bands was found for PSY2L_A in lane A. Hence, PSY2L_B was successfully cloned into pGEMT-easy, while failed for cloning of PSY2L_A. Therefore, we repeated ligation and transformation of PSY2L_A with pGEMT-easy. Twelve colonies from this second trial PSY2L_A plate were screened for colony PCR and the result of the gel electrophoresis is provided in Figure 3-36 (lane C). It is shown that six positive bands were found for PSY2L_A.

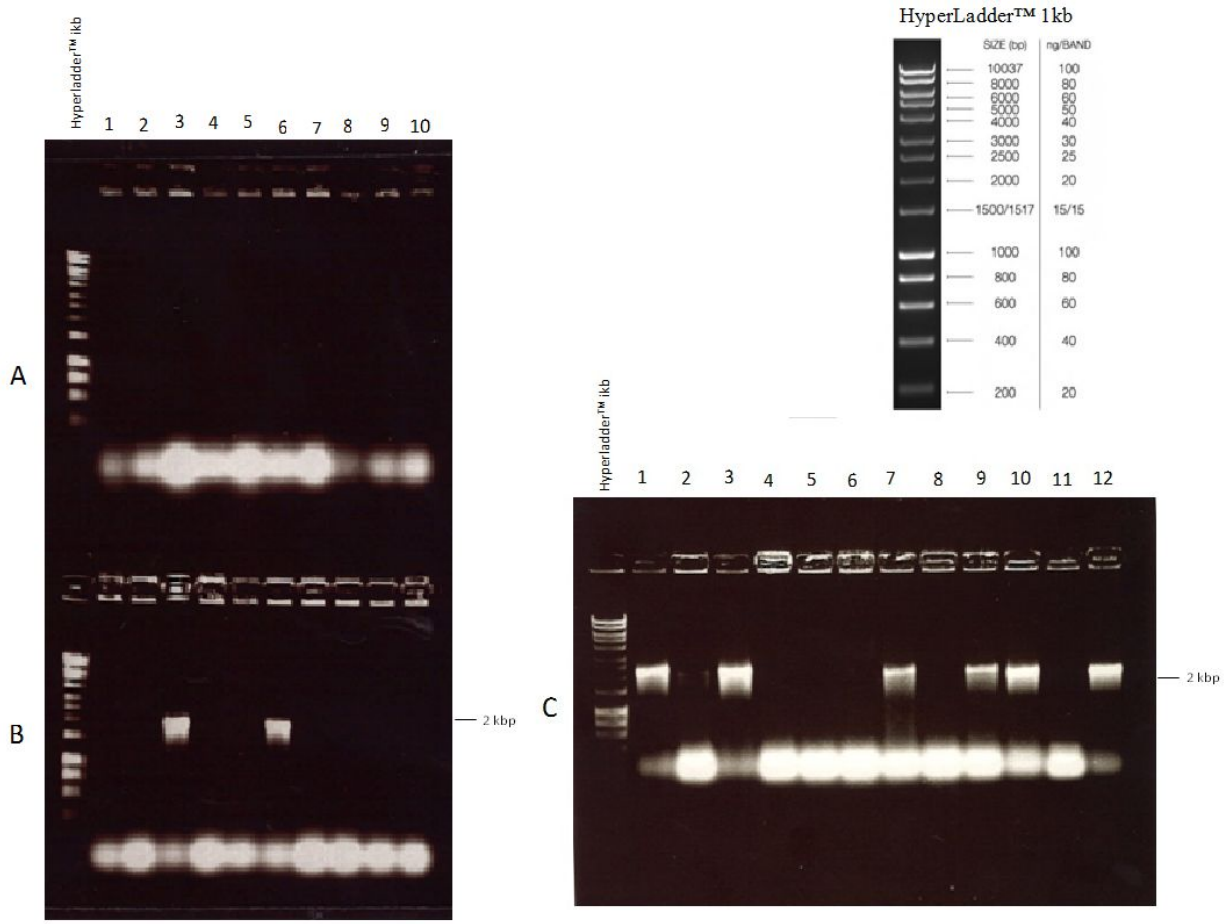


Figure 3-36. The result of gel electrophoresis of colony PCR from PSY2L_A plate (lane A), PSY2L_B plate (lane B), and second trial PSY2L_A plate (lane C).The size of expected PCR product is about 2 kbp. Two positive bands were found for PSY2L_B in lane B (number 3 and 6), but no positive bands was found for PSY2L_A in lane A. After second trial, six positive bands were found for PSY2L_A in lane C (number 1, 3, 7, 9, 10, and 12). Primers for PSY2L_A and PSY2L_B were PSY2L Middle as forward Primer and EYFPPSY2Lr as reversed Primer

According to the positive result from Figure 3-36, two colonies from PSY2L_B plate (number 3 and 6) and two colonies from second trial PSY2L_A plate (number 1, 9, and 10) were selected and cultivated in LB Broth media. Plasmid isolation was then performed using the plasmid miniprep kit, resulting PSY2L_A--pGEMT-easy and PSY2L_B--pGEMT-easy.

To confirm the successful cloning of *PSY2L* cDNA, all isolated plasmids, PSY2L_A--pGEMT-easy (number 1, 9, and 10) and PSY2L_B--pGEMT-easy (number 3 and 6) above were digested with NotI. This restriction enzyme site is repeated twice in the multiple cloning site (MCS) of pGEMT-easy and it should be released the insert (*PSY2L*). As expected, the digestion resulted two different band, correspondent to *psy2l* and vector as shown in Figure 3-32. The top is pGEMT-easy plasmid band (size about 3 kb) and the down band is *PSY2L* cDNA (size about 2.5 kb). Due to the size of pGEMT-easy and *PSY2L* cDNA are very similar, both bands look very close, although they can still be distinguished.

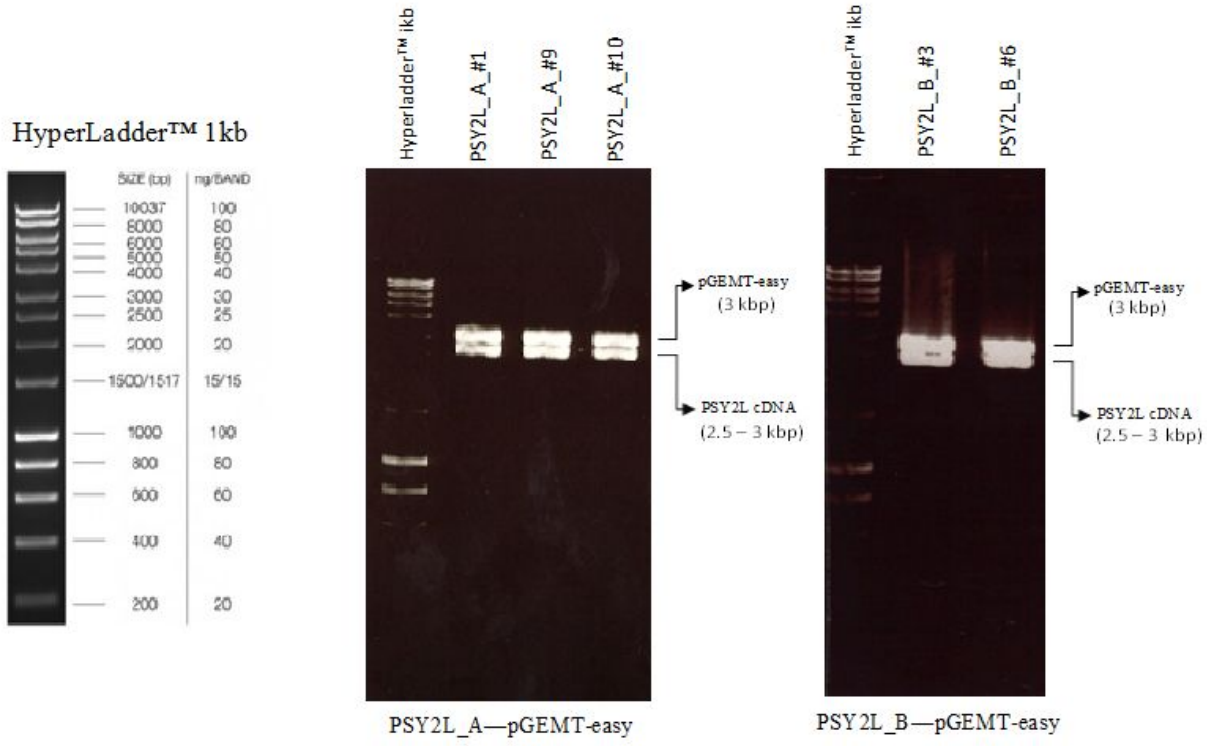


Figure 3-37. The result of gel electrophoresis of PSY2L_A--pGEMT-easy and PSY2L_B--pGEMT-easy after digestion with NotI as cloning confirmation. All digested samples show two distinct bands at size 2.5 – 3 kb, where the top is pGEMT plasmid band (size about 3 kb) and the down band is PSY2L gene (size about 2.5 kb).

Based on result in Figure 3-37, the cloning is successfully confirmed. Then, PSY2L_A and PSY2L_B will be inserted into pCAT-EYFP-1 and pCAT-EYFP-2 after digestion, respectively.

3.4.1.1 Subclone PSY2L_A into pCAT-EYFP-1 vector

We would like to subclone *PSY2L* cDNA from pGEMT-easy to pCAT-EYFP-1 cloning vector in order to produce fusion-protein that will carry the EYFP tag on on the *PSY2L* cDNA N–Terminus end. One of PSY2L_A--pGEMT-easy plasmid (number 9 in Figure 3-36 lane C) was digested using NotI restriction enzyme. The digestion using this enzyme will release *PSY2L* cDNA (insert) from pGEMT-easy, that later can be separated by using gel electrophoresis. The PSY2L_A band in gel was then extracted and the concentration of PSY2L_A was measured.

Meanwhile, the cloning vector, pCAT-EYFP-1, was digested using NotI restriction enzyme as well, continued by dephosphorylation using Shrimp Alkaline Phosphatase (rSAP). NotI will cut specific recognition sites in the vector in order to make it linear. Both PSY2L_A and pCAT-EYFP-1 have to be treated with same restriction enzymes that create compatible ends. Dephosphorylation by rSAP will prevent religation of linearized vector.

The PSY2L_A was ligated into cloning vector, the linear dephosphorylated pCAT-EYFP-1. Following the method of ligation, reagents for each ligation mixture was prepared. The ligation mixture was left at 4°C overnight before it was used in transformation of bacterial cells (*Escherichia coli* JM109) and transferred to LB agar plate containing Ampicillin. Selection of bacteria with ampicillin resulted survival of cells that have successfully inserted by plasmid. Seven colonies from the plate were selected, and PCR colony was performed to check those colonies (Figure 3-38).

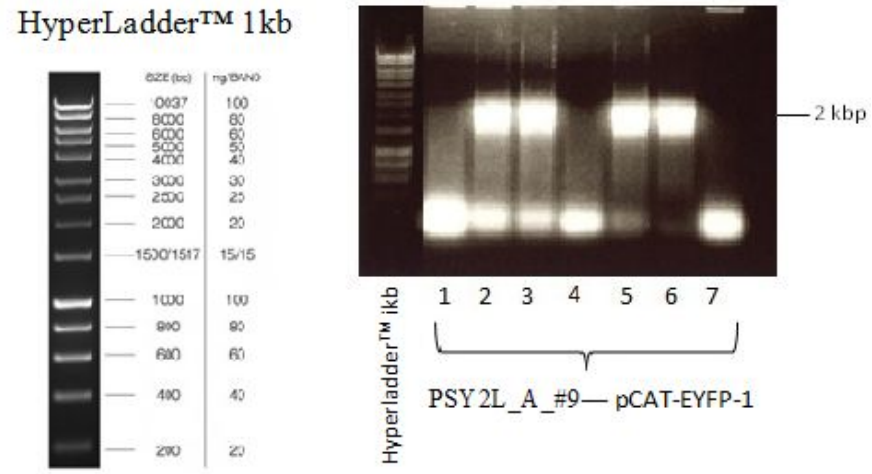


Figure 3-38. The result of gel electrophoresis of colony PCR PSY2L_A_#9 into pCAT-EYFP-1 vector. The size of expected PCR product is about 2 kbp. Colony PCR of PSY2L_A_#9 with vector pCAT-EYFP-1 showed four positive colonies, namely number 2,3,5, and 6. The Primers were PSY2L Middle as forward Primer and EYFPPSY2Lr as reversed Primer.

According to the positive result from Figure 3-38, one colony, number 3, was selected and cultivated overnight in LB Broth media. Plasmid isolation was performed using the plasmid minipreps kit and the concentration was measured. This plasmid was sent for sequencing. Table 3-19 provides the detail of mixture plasmid and selection primers that were used.

Table 3-19. The premixture of PCR PSY2L_A_#9--pCAT-EYFP-1 plasmid for sequencing.

Sample Tube	Plasmid (µL)	Water (µL)	Primer (3 µL)	Sample Sequencing Name
PSY2L_A_#9--pCAT-EYFP-1 number 3	1.0	11.0	c.term.EYFPf	PSY2LYFPM_11ECter_EYFP-C-TERf
	1.0	11.0	Ak92r	PSY2LYFPM_11AK92r_AK92r
	1.0	11.0	PSY2L Middle	PSY2LYFPM_11PSmid_PSY2L middle

According to the result of sequencing, PSY2L_A_#9--pCAT-EYFP-1 number 3 which is PSY2L that carry the EYFP tag on the N–Terminus is correct and can be used for subcellular localization. The sequence is shown in Apendix 3-1.

3.4.1.2 Subclone PSY2L_B into pCAT-EYFP-2 vector

We would like to subclone *PSY2L* cDNA from pGEMT-easy to pCAT-EYFP-2 cloning vector in order to produce fusion-protein that will carry the EYFP tag on the *PSY2L* cDNA C–Terminus end. Two PSY2L_A--pGEMT-easy plasmids (number 3 and 6 in Figure 3-36 lane B) were digested using NotI and NcoI restriction enzyme. The digestion using these enzymes will release *PSY2L* cDNA (insert) from pGEMT-easy, that later can be separated by using gel electrophoresis. The PSY2L_B band in gel was then extracted and the concentration of PSY2L_B was measured.

The vector, pCAT-EYFP-2, was digested using NotI and NcoI restriction enzyme as well. Those enzymes will cut specific recognition sites in the vector in order to make it linear. Both PSY2L_B and pCAT-EYFP-2 have to be treated with same restriction enzymes that create compatible ends.

The PSY2L_B was ligated into cloning vector, linear pCAT-EYFP-2. Following the method of ligation, reagents for each ligation mixture was prepared. The ligation mixture was left at 4^{o}C overnight before it was used in transformation of bacterial cells (*Escherichia coli* JM109) and transferred to LB agar plate containing Ampicillin. Selection of bacteria with ampicillin resulted survival of cells that have successfully inserted by plasmid. Ten colonies from the PSY2L_B_#3--pCAT-EYFP-2 and PSY2L_B_#6--pCAT-EYFP-2 plate were selected, respectively, and PCR colony was performed to check those colonies (Figure 3-39).

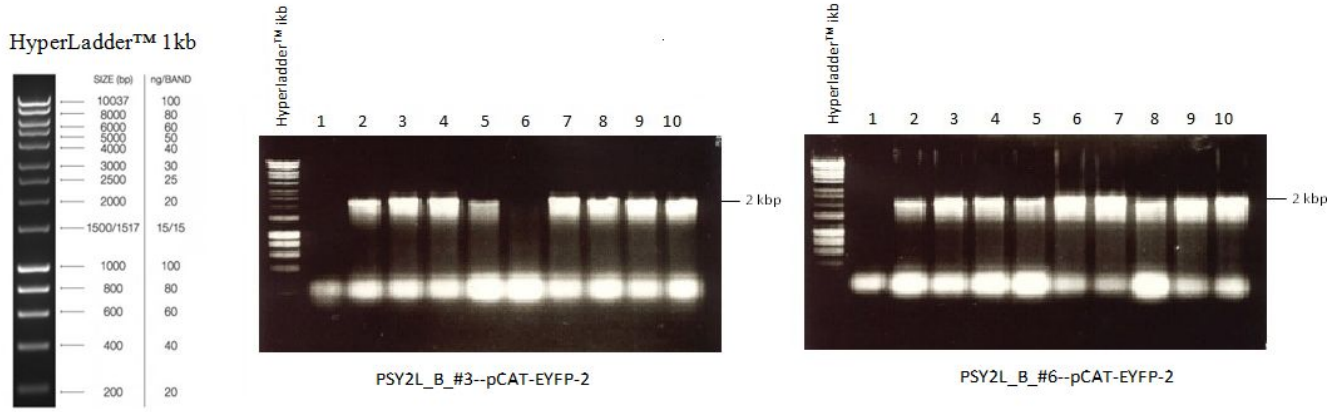


Figure 3-39. The result of gel electrophoresis of colony PCR PSY2L_B_#3 and PSY2L_B_#6 into pCAT-EYFP-1 vector. The size of expected PCR product is about 2 kbp. Colony PCR of PCR PSY2L_B_#3 with vector pCAT-EYFP-2 shows eight positive colonies, namely number 2,3,4,5,7,8,9 and 10. Colony PCR of PCR PSY2L_B_#3 with vector pCAT-EYFP-2 shows eight positive colonies, namely number 2,3,4,5,7,8,9 and 10. Colony PCR of PCR PSY2L_B_#6 with vector pCAT-EYFP-2 shows almost all positive colonies, except number 1. The Primers were PSY2L Middle as forward Primer and EYFPPSY2Lr as reversed Primer.

According to the positive result from Figure 3-39, one colony from PSY2L_B_#3--pCAT-EYFP-2 plate (number 8) and PSY2L_B_#6--pCAT-EYFP-2 plate (number 5) were selected and cultivated in

LB Broth overnight. Plasmid isolation was performed using the plasmid miniprep kit and the concentration was measured. This plasmid was sent for sequencing. Table 3-20 provides the detail of mixture plasmid and selection primers that were used.

Table 3-20. The premixture of PCR PSY2L_B_#3--pCAT-EYFP-2 and PSY2L_B_#6--pCAT-EYFP-2 plasmid for sequencing.

Sample Tube	Plasmid (µL)	Water (µL)	Primer (3 µL)	Sample Sequencing Name
PSY2L_B_#3--pCAT-EYFP-2 number 8	7.0	5.0	T7	B6PSY2L_T7_T7
	7.0	5.0	PSY2L Middle	B6PSY2LDECR_PSY2LMiddle
	7.0	5.0	SP6	B6PSY2LDECR_SP6
PSY2L_B_#6--pCAT-EYFP-2 number 5	3.0	9.0	T7	-
	3.0	9.0	PSY2L Middle	-
	3.0	9.0	SP6	-

According to the result of sequencing, the sequence of PSY2L_B_#6--pCAT-EYFP-2 number 5 is correct. However, it has a frame shift in the plasmid backbone upstream of the cloned insert. Consequently, the expected protein was not produced properly for subcellular localization investigation. On the other hand, PSY2L_B_#3--pCAT-EYFP-2 number 8 which is PSY2L that carry the EYFP tag on the C–Terminus is correct and can be used for subcellular localization. The sequence is shown in Apendix 3-1.

3.4.2 Molecular Cloning *pp4-1*

The aim of cloning the *PP4-1* cDNA into vectors is to produce PP4-1 tagged with fluorescence protein (EYFP). This protein fusion will be used for investigating subcellular localization of PP4-1. The cDNA will be inserted into pCAT-EYFP-1 and pCAT-EYFP-2 vector, respectively. Both pCAT-EYFP-1 and pCAT-EYFP-2 vectors share the same nucleotide sequence with the exception that pCAT-EYFP-1 will ensure that the resulting fusion-protein will carry the EYFP tag on the N–Terminus, whereas the pCAT-EYFP-2 will give the resulting fusion-protein an EYFP tag at the C–terminus.

Template of cDNA was taken from *PP4-1* in pGEMT-easy vector. This template is Arabidopsis thaliana “Ecotype : Wassilewskija (WS)”, that shares the same sequence of the “Ecotype : Colombia” supplied by Prof. Jose Serrano (CNB in Madrid, Spain). High fidelity PCR was used for amplification of *pp4-1* cDNA. The cDNA was then divided into two parts and labelled as PP4-1_A

and PP4-1_B for amplified cDNA that will be inserted into pCAT-EYFP-1 and pCAT-EYFP-2, respectively. The target band in gel was cut and extracted.

3.4.2.1 Subclone PP4-1_A into pCAT-EYFP-1 vector

We would like to subclone *PP4-1* cDNA to pCAT-EYFP-1 cloning vector in order to produce fusion-protein that will carry the EYFP tag on the N–Terminus. PP4-1_A that already amplified was digested using NotI and SacII restriction enzyme. The vector, pCAT-EYFP-1, was digested using NotI and SacII restriction enzyme as well. Those enzymes will cut specific recognition sites in the vector in order to make it linear. Both PP4-1_A and pCAT-EYFP-1 have to be treated with same restriction enzymes that create compatible ends.

The PP4-1_A was ligated into cloning vector, linear pCAT-EYFP-1. Following the method of ligation, reagents for each ligation mixture was prepared. The ligation mixture was left at 4^{o}C overnight before it was used in transformation of bacterial cells (*Escherichia coli* JM109) and transferred to LB agar plate containing Ampicillin. Selection of bacteria with ampicillin resulted survival of cells that have successfully inserted by plasmid. Five colonies from this plate were selected and PCR colony was performed to check those colonies (Figure 3-40).

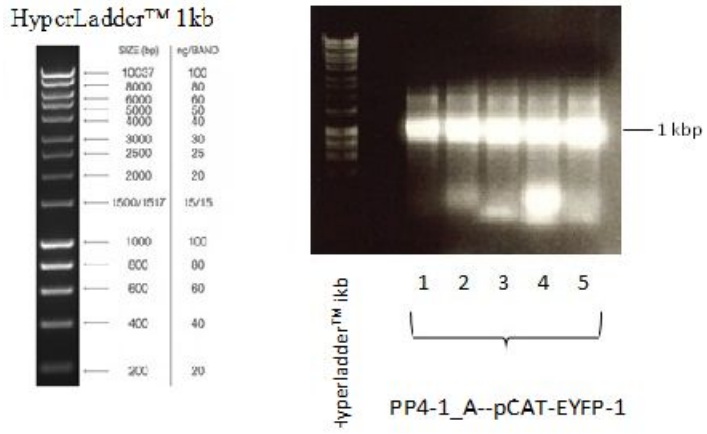


Figure 3-40. The result of gel electrophoresis of colony PCR *PP4-1* into pCAT-EYFP-1 vector. The size of expected PCR product is about 1.0 kbp. Colony PCR of PCR *pp4-1* with vector pCAT-EYFP-1 showed all positive colonies. The Primers were EYFP-C-Terf as forward Primer and AK73r as reversed Primer.

According to the positive result from Figure 3-40, two colonies from PP4-1_A--pCAT-EYFP-1 plate (number 1 and number 3) were selected and cultivated in LB Broth overnight. Plasmid isolation was performed using the plasmid minipreps kit and the concentration was measured. This plasmid was sent for sequencing. Table 3-21 provides the detail of mixture plasmid and selection primers that were used. The sequencing result is shown in Appendix 3-4.

Table 3-21. The premixture of PCR PP4-1_A--pCAT-EYFP-1 plasmid for sequencing.

Sample Plasmid	Plasmid (µL)	Water (µL)	Primer (3 µL)	Sample Sequencing Name
PP4-1_A--pCAT-EYFP-1 number 1	2.5	9.5	EYFP-C-Terf	PP4-1YFPM_1ECter_EYFP-C-TERf
	2.5	9.5	Ak92r	PP4-1YFPM_1AK92r_AK92r
PP4-1_A--pCAT-EYFP-1 number 3	1.0	11.0	EYFP-C-Terf	PP4-1YFPM_3ECter_EYFP-C-TERf
	1.0	11.0	Ak92r	PP4-1YFPM_3AK92r_AK92r

According to the result in Appendix 3-4, all sequences clones of PP4-1_A--pCAT-EYFP-1 are correct can be used for subcellular localization. However, the clone PP4-1_A--pCAT-EYFP-1 number 3 is avoided because it have a single amino acid change.

3.4.2.2 Subclone PP4-1_B into pCAT-EYFP-2 vector

We would like to subclone *PP4-1* cDNA to pCAT-EYFP-2 cloning vector in order to produce fusion-protein that will carry the EYFP tag on the C–Terminus. PP4-1_B that already amplified was digested using NotI and SacI restriction enzyme. The vector, pCAT-EYFP-2, was digested using NotI and SacI restriction enzyme as well. Those enzymes will cut specific recognition sites in the vector in order to make it linear. Both PP4-1_B and pCAT-EYFP-2 have to be treated with same restriction enzymes that create compatible ends.

The PP4-1_B was ligated into cloning vector, linear pCAT-EYFP-2. Following the method of ligation, reagents for each ligation mixture was prepared. The ligation mixture was left at 4^{o}C overnight before it was used in transformation of bacterial cells (*Escherichia coli* JM109) and transferred to LB agar plate containing Ampicillin. Selection of bacteria with ampicillin resulted survival of cells that have successfully inserted by plasmid. Ten colonies from this plate were selected and PCR colony was performed to check those colonies (Figure 3-41).

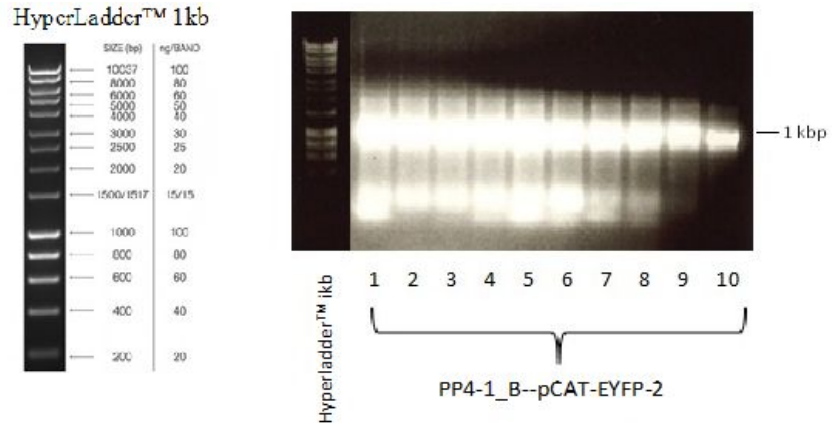


Figure 3-41. The result of gel electrophoresis of colony PCR *PP4-1* into pCAT-EYFP-2 vector. The size of expected PCR product is about 1.0 kbp. Colony PCR of PCR *pp4-1* with vector pCAT-EYFP-1 showed all positive colonies. The Primers were AK74f as forward Primer and AK94r as reversed Primer.

According to the positive result from Figure 3-41, two colonies from PP4-1_B--pCAT-EYFP-2 plate (number 1 and number 3) were selected and cultivated in LB Broth overnight. Plasmid isolation was performed using the plasmid miniprep kit and the concentration was measured. This plasmid was sent for sequencing. Table 3-22 provides the detail of mixture plasmid and selection primers that were used. The sequencing result is shown in Appendix 3-5.

Table 3-22. The premixture of PCR PP4-1_B--pCAT-EYFP-2 plasmid for sequencing.

Sample Plasmid	Plasmid (µL)	Water (µL)	Primer (3 µL)	Sample Sequencing Name
PP4-1_A--pCAT-EYFP-2	1.0	11.0	AK93f	PP4-1DECR_2AK93f_AK93f
number 2	1.0	11.0	Ak94r	PP4-1DECR_2AK94r_AK94r
PP4-1_A--pCAT-EYFP-2	1.7	10.3	AK93f	PP4-1DECR_7AK93f_AK93f
number 7	1.7	10.3	Ak94r	PP4-1DECR_7AK94r_AK94r

According to the result in Appendix 3-5, all sequences clones of PP4-1_B--pCAT-EYFP-2 are correct and can be used for subcellular localization.

3.5 Subcellular Localization Studies of PP4-1, PP4-2, PP4R2L, and PSY2L

To do subcellular localization of protein PP4-1, PP4-2, PP4R2L, and PSY2L, each of their cDNA were fused with fluorescent protein EYFP gene in two different vectors, pCAT-EYFP-1 and pCAT-EYFP-2 through molecular cloning strategy. pCAT-EYFP-1 will ensure that the resulting fusion-protein will carry the EYFP tag on the N–Terminus, whereas the pCAT-EYFP-2 will give the resulting fusion-protein an EYFP tag at the C–terminus. The *PSY2L* and *PP4-1* were successfully cloned into both different vectors (section 3.4.1 and 3.4.2). Fusion of *PP4-2* and *PP4R2L* into these vectors were prepared by Dr. Amr Kataya and previous master student, Sætre (2015). Table 3-23 shows the clones for subcellular localization study.

Table 3-23. The clone preparation for subcellular localization of PP4-1, PP4-2, PP4R2L, and PSY2L.

Gene	pCAT-EYFP-1 Vector	pCAT-EYFP-2 Vector
PP4-1	PSY2L_A_#9--pCAT-EYFP-1 number 3 (see section 3.4.2.1)	PSY2L_B_#3--pCAT-EYFP-2 (see section 3.4.2.2)
PSY2L	PP4-1_A--pCAT-EYFP-1 number 1 (see section 3.4.1.1)	PP4-1_A--pCAT-EYFP-2 number 2 (see section 3.4.1.2)
PP4-2	Sample from Dr. Amr Kataya	Sample from Christine Sætre
PP4R2L	Sample from Christine Sætre	Sample from Christine Sætre

The subcellular localization of the protein from these genes was investigated by using two methods: particle bombardment into onion epidermis cells (gene shooting) and the transient gene expression system using *Arabidopsis* mesophyll protoplasts.

3.5.1 The Cellular Localization Prediction of PP4-1, PP4-2, PP4R2L, and PSY2L using Software Programs.

The subcellular localization of proteins can be predicted by using many bioinformatics programs, online and offline. Even though these prediction might be helpful to indicate the target cell compartment of the protein, the empirical evidence is still needed to give certainty of the actual and precise localization of the protein. Table 3-24 list the result of subcellular localization prediction of these protein by using these programs: TargetP, Cello, LocTree 3, SUBA 3, Yloc, and SubLoc.

Table 3-24. The overview of localization PP4-1, PP4-2, PP4R2L, and PSY2L protein using bioinformatics programs. The used programs were : TargetP (http://www.cbs.dtu.dk/services/TargetP/); Cello (http://cello.life.nctu.edu.tw/); LocTree 3 (https://rostlab.org/services/loctree2/); SUBA 3 (http://suba.plantenergy.uwa.edu.au/); YLoc (http://www-bs.informatik.uni-tuebingen.de/Services/YLoc/webloc.cgi); SubLoc (http://www.bioinfo.tsinghua.edu.cn/SubLoc/eu_predict.htm). Last retrieved for all websites is March 2016.

Protein	Locus	TargetP	Cello	LocTree 3	SUBA 3 (SUBAcon)	YLoc	SubLoc
PP4-1	At4g26720	-	Cytoplasmic	Plastid	Cytosol	Cytoplasm	Cytoplasmic
PP4-2	At5g55260	-	Cytoplasmic	Plastid	Cytosol	Cytoplasm	Cytoplasmic
PP4R2L	At5g17070	-	Cytoplasmic or Nuclear	Nucleus	Nucleus	Nucleus	Nuclear
PSY2L	At3g06670	-	Nuclear	Nucleus	Nucleus	Nucleus	Nuclear

3.5.2 The In Vivo Subcellular Localization of PP4-1, PP4-2, PP4R2L, and PSY2L

All of the clones from Table 3-23 were prepared for subcellular localization study. Two methods were used, namely the biolistic transfection of onion cells (gene shooting) and the transient gene expression system using mesophyll protoplasts. For gene shooting, the plasmid containing interest cDNA with fluorescent protein EYFP gene were precipitated onto gold particles, according to the procedure in Material and Method and will be used for transformation to onion epidermal cells by bombardment. For the transient gene expression system, the plasmids were PEG transfected into isolated protoplasts from *Arabidopsis thaliana* wild type. Confocal microscopy was performed to observe both onion cells and protoplasts. Based on the clone preparation in Table 3-23, eight fusion protein were investigated in this study. Table 3-25 list these fusion proteins.

Table 3-25. The list of fusion proteins for subcellular localization of PP4-1, PP4-2, PP4R2L, and PSY2L.

Gene	Fusion Protein	Vector
PP4-1	PP4-1-EYFP	pCAT-EYFP-1
	EYFP-PP4-1	pCAT-EYFP-2
PSY2L	PSY2L-EYFP	pCAT-EYFP-1
	EYFP-PSY2L	pCAT-EYFP-2
PP4-2	PP4-2-EYFP	pCAT-EYFP-1
	EYFP-PP4-2	pCAT-EYFP-2
PP4R2L	PP4R2L-EYFP	pCAT-EYFP-1
	EYFP-PP4R2L	pCAT-EYFP-2

3.5.2.1 The Subcellular Localization of PP4-1

Figure 3-42 shows the microscopy result for the PP4-1-EYFP and EYFP-PP4-1 fusion protein. The results of two methods, namely the particle bombardment into onion epidermis cells (gene shooting) and the transient gene expression system using *Arabidopsis* mesophyll protoplasts are provided for each fusion protein.

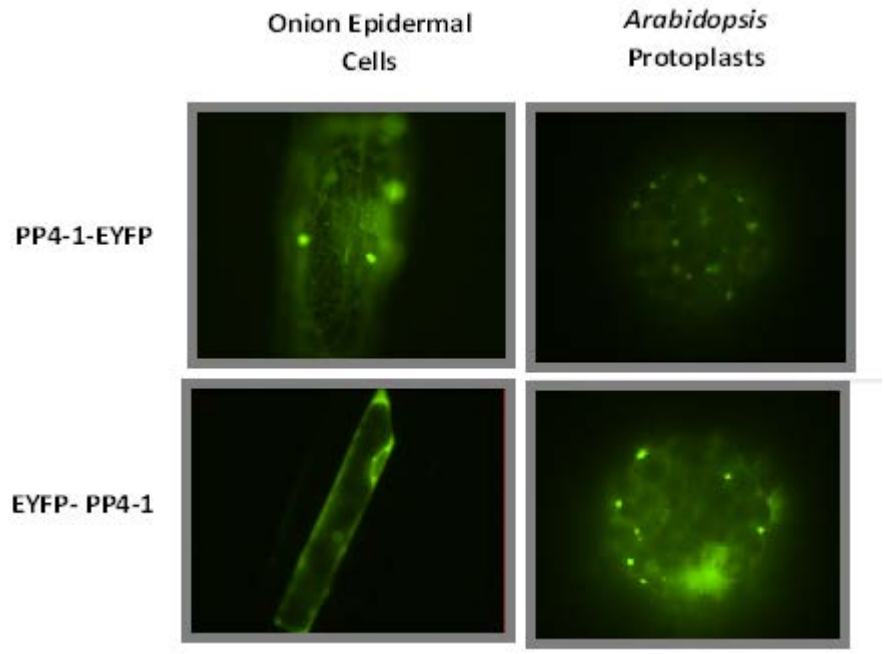


Figure 3-42. Microscopy of the fusion protein PP4-1-EYFP and EYFP-PP4-1 in onion epidermal cell and *Arabidopsis* mesophyll protoplasts. For both, the fusion protein displays aggregation shown by the bright green fluorescence. The pictures were obtained from Dr. Amr Kataya.

According to the result in Figure 3-42, both fusion protein seem to be widespread throughout the cytoplasmic cell with few in nucleus, giving the impression of PP4-1 as a cytosolic protein and probably nucleus targeting protein.

3.5.2.2 The Subcellular Localization of PSY2L

Figure 3-43 shows the microscopy result for the PSY2L-EYFP and EYFP-PSY2L fusion protein. The results of two methods namely the particle bombardment into onion epidermis cells (gene shooting) and the transient gene expression system using *Arabidopsis* mesophyll protoplasts are provided for each fusion protein.

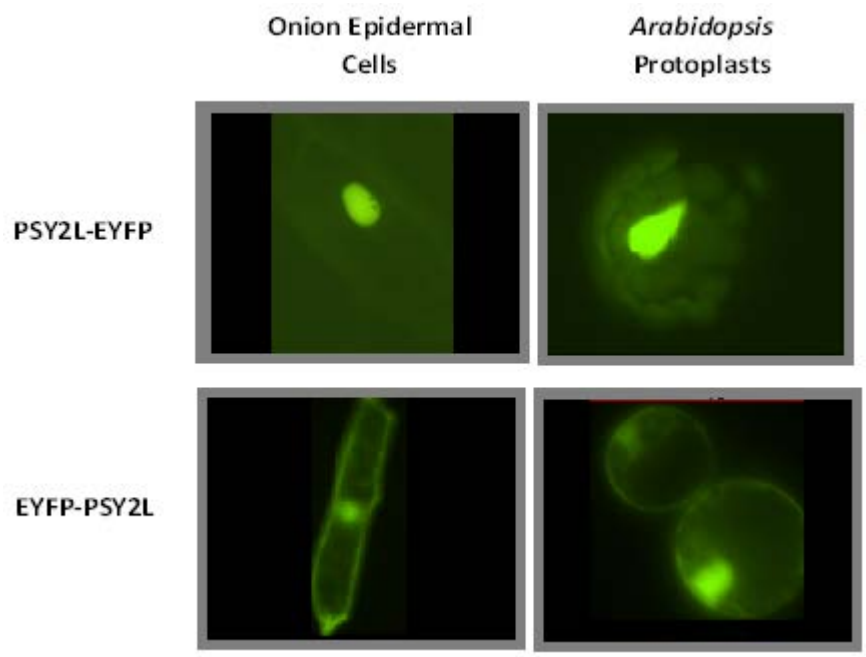


Figure 3-43. Microscopy of the fusion protein PSY2L-EYFP and EYFP-PSY2L in onion epidermal cell and *Arabidopsis* mesophyll protoplasts. For both, the fusion protein displays aggregation shown by the bright green fluorescence. The pictures were obtained from Dr. Amr Kataya.

According to the result in Figure 3-43, both fusion protein seem to be localized in nucleus, giving the impression of PSY2L as a nuclear protein.

3.5.2.3 The Subcellular Localization of PP4R2L

Figure 3-44 shows the microscopy result for the PP4R2L-EYFP and EYFP-PP4R2L fusion protein. The results of two methods namely the particle bombardment into onion epidermis cells (gene shooting) and the transient gene expression system using Arabidopsis mesophyll protoplasts are provided for each fusion protein.

According to the result in Figure 3-44, no PP4R2L-EYFP protein aggression is appeared in onion epidermal cell and Arabidopsis mesophyll protoplasts. The fusion protein is seemingly widespread throughout the cytoplasmic cells. However, for EYFP-PP4R2L, the fusion protein is localized in nucleus of onion cell, but it widespread throughout cytoplasmic in Arabidopsis mesophyll protoplasts or network-linked structures (Endoplasmic Reticulum).

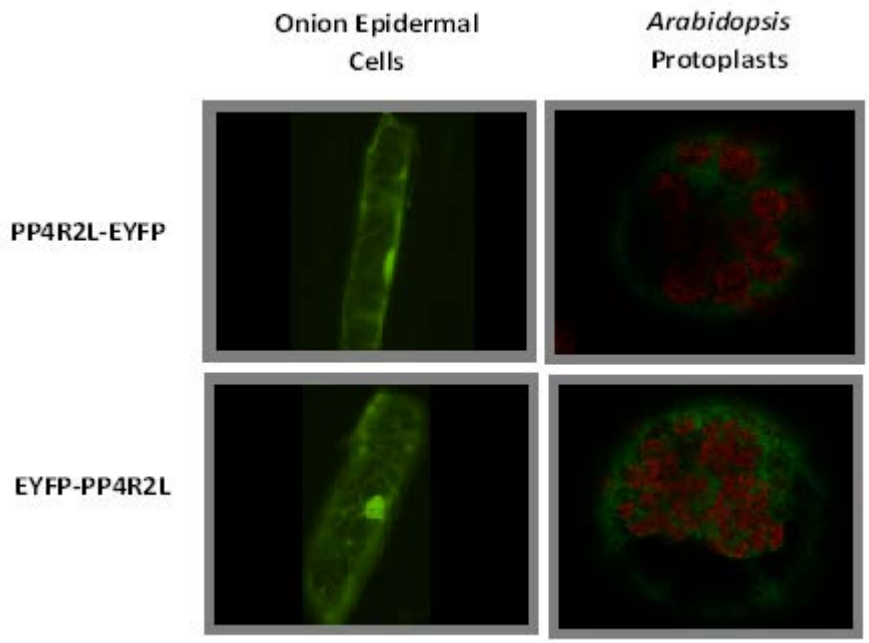


Figure 3-44. Microscopy of the fusion protein PP4R2L-EYFP and EYFP-PP4R2L in onion epidermal cell and *Arabidopsis* mesophyll protoplasts. The image in protoplasts for both fusion protein were taken from Sætre (2015).

3.5.2.4 The Subcellular Localization of PP4-2

Figure 3-45 shows the microscopy result for the PP4-2-EYFP and EYFP-PP4-2 fusion protein. The results of two methods namely the particle bombardment into onion epidermis cells (gene shooting) and the transient gene expression system using *Arabidopsis* mesophyll protoplasts are provided for each fusion protein.

According to the result in Figure 3-45, both fusion protein seem to be widespread throughout the cytoplasmic cell with few in nucleus, giving the impression of PP4-2 as a cytosolic protein and probably nucleus targeting protein.

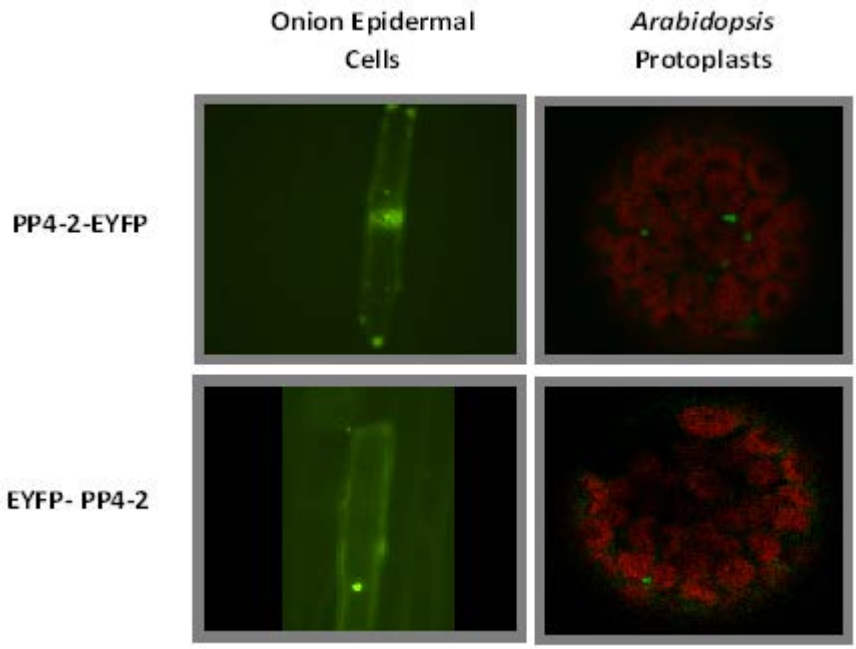


Figure 3-45. Microscopy of the fusion protein PP4-2-EYFP and EYFP-PP4-2 in onion epidermal cell and *Arabidopsis* mesophyll protoplasts. The image in protoplasts for both fusion protein were taken from Sætre (2015).

3.5.3 Conclusion of the Subcellular Localization

Based on the results from section 3.5.2.1; 3.5.2.2; 3.5.2.3; and 3.5.2.4; the cellular localization of PP4-1, PP4-2, PP4R2L, and PSY2L can be implicated in Table 3-26.

Table 3-26. The conclusion of *in vivo* subcellular localization of PP4-1, PP4-2, PP4R2L, and PSY2L.

Gene	Fusion Protein	Prediction of cellular localization
PP4-1	PP4-1-EYFP	Cytoplasmic, Nucleus (?)
	EYFP-PP4-1	Cytoplasmic, Nucleus (?)
PSY2L	PSY2L-EYFP	Nucleus
	EYFP-PSY2L	Nucleus
PP4-2	PP4-2-EYFP	Cytoplasmic, Nucleus (?)
	EYFP-PP4-2	Cytoplasmic, Nucleus (?)
PP4R2L	PP4R2L-EYFP	Cytoplasmic, Nucleus
	EYFP-PP4R2L	Nucleus, Endoplasmic Reticulum (?)

4. DISCUSSION AND OUTLOOK

4.1 Transgenic Line Plants

4.1.1 T-DNA Insertional Mutagenesis Plants

T-DNA insertional mutagenesis is a loss of function approach resulting null function of the inserting gene, but the insertion of T-DNA element into an Arabidopsis chromosome can lead to many different outcomes. Krysan et al. (1999) describes several possibilities and propose a standard nomenclature to describe them, which is called “knockology”. Therefore, the consequences of inserting a T-DNA element into the Arabidopsis genome depends on the nature of the T-DNA as well as the precise site of insertion.

To initiate expression studies for catalytic and regulatory subunits of PP4, T-DNA insertion lines were obtained and homozygous mutants were successfully isolated for all lines, *psy2l* SALK_125872, *psy2l* SALK_048064, *pp4r2l* SALK_093041, *pp4-1* GK_651B07, *pp4-1* SALK_070977, *pp4-2* SAIL_569_H09, and *pp4-2* SALK_049725. Overall, except for the *psy2l* SALK_048064 lines, all homozygous mutants lines resembles to wild type phenotype as control. When it is said resemble to phenotype, the observations of the appearance of these mutants were emphasized on general outlook such as height, growth rate, the ability to produce seeds, seedling shape, and leaf shape which are, in this case, similar to wild type.

On the other hand, *psy2l* SALK_048064 mutant line gives a very unique and interesting phenotype. The homozygous mutants of *psy2l* SALK_048064 line appear to have so called dwarf phenotype strongly. Moreover, the heterozygous mutants of this line have slightly smaller size than wild type but bigger than homozygous mutants, so we termed them semi-dwarf. Due to the phenotype of this heterozygous mutant, which is different from wild type and homozygous mutant, it is indicated that the two alleles, inserted and not inserted, are said to be co-dominant. The *psy2l* SALK_048064 has T-DNA inserted in exon 3 of total 25 in *PSY2L* gene. Expression study by RT-PCR toward the homozygous mutants of this line showed that the *PSY2L* is knocked out. Additionally, the expression in heterozygous mutants is lower than wild type, but higher than the homozygous mutants, indicated that the *PSY2L* is knocked down. T-DNA insertion into exon *PSY2L* is equally effective in knocking down/out the target gene. Moreover, the insertion is located near to start codon or 5’ end of coding region, make it more effectively to result null function of target gene.

Not just the size is way smaller than wild type which is an indication of slower growth, but the colour of leaf were also vulnerable to turn slightly pink, particularly during first month of growth. This was more obvious when conduct the isolation of RNA step for expression study, the dwarf plants gave pink to red liquid after crushing and addition of cell lysis solution. Although no chemical analysis was perform to conduct this allegation, it might be indication of accumulation or precipitation of anthocyanins. Production of anthocyanin in *Arabidopsis thaliana* is a clear visible marker of plant response to unfavourable growth conditions (Chalker-Scott, 1999).

In addition to being dwarf, the homozygous SALK_048064 mutants showed longer life span than wild type, with delayed flowering. The mutants started flowering approximately 4 months after germination, and still live after 5 – 6 months. Meanwhile, *Arabidopsis thaliana* wild type itself has a very rapid cycle, about 6 – 7 weeks from germination to mature seed. In *Caenorhabditis elegans,* putative homologs of *PSY2L* (*At3g06670*), *SMK-1,* acts specifically with DAF-16 (FOXO transcription factor) to promote longevity. Reduced expression of SMK-1, by RNAi, shortened the life span of wild type animals (Wolff et al., 2006). Moreover, Wolff reported that loss of SMK-1 function suppressed some forms of stress resistance, such as oxidative, UV, and innate immune response, but not heat-stress response. The case in Arabidopsis, knock out/down PSY2L resulting longer life span, is seemingly opposite observation compared to reduction of *SMK-1* in *C. elegans.* Along with the dwarfism phenotype*,* this interesting trait is needed more investigation to reveal the possibility presence of additional factor in this mutant plants and to more understand the role of *PSY2L* in the bigger realm in plants. However, the ability of mutant that loss of SMK-1 function toward stress resistance might be explain the easier accumulation of anthocyanin in the plant mutants.

Unlike SALK_048064, the other *psy2l* mutant line, SALK_125872, resembles to wild type phenotype. The T-DNA of *psy2l* SALK_125872 is inserted after stop codon, make it flank-tagged downstream of translation. T-DNA insertion after stop codon is least effective compared to insertion in other parts of gene. The expression study for the homozygous mutants was conducted twice for this mutant. The result is seemingly inconsistent, one showed that the level of expression is slightly higher than wild type, on the other is slightly lower. However, unlike homozygous SALK_048064, no drastic reduction in level of expression was detected for both results.

For investigation of *PP4R2L* gene, one SALK line war examined, SALK_093041. The T-DNA of *pp4r2l* SALK_093041 mutants is inserted in exon 7 of total 8 in *PP4R2L* gene. The insertion is located near to stop codon or 3’ end of coding region. RT-PCR of the two homozygous mutants using pair of

primers that amplified full cDNA showed various result, one knocked-up and another slightly knocked-down. It was decided to repeat the expression analysis by using two sets of primers, “Front1” primers (amplified from near 5’ to middle cDNA) and “Back2” primers (amplified from near middle cDNA to near 5’). On gel RT-PCR, the band is stronger in mutants than wild type by using “Front1” primers but slightly lower by using “Back2” primers. To explain this result, it needs to refer the location of insertion which is near stop codon. The “Back2” primers amplified the interrupted part of cDNA, while the “Front1” primers amplified the uninterrupted part of cDNA. By observing the amplification of full cDNA and half cDNA from near 5’ to middle, the insertion in this line is tend to increase the level of expression, instead of decrease it as the expectation. It is not clear, however, why insertion into the exon did not knock out/down the target gene. Because it is still unclear, the study of expression in this line need more investigation to be understood. Additionally, no significant change of phenotype was observed.

Two lines of PP4-2 gene were investigated, *pp4-2* SAIL_569_H09, and *pp4-2* SALK_049725. The T-DNA of *pp4-2* SAIL_569_H09 mutants is inserted in intron 1 of total 7 in *PP4-2* gene. However, the location of insertion is very close to border with exon 2 of total 8 in *PP4-2* gene. If insertion is into an intron, wild type transcript may be produces in such mutants because intron can be spliced out together with inserted T-DNA sequence. However, the expression analysis by RT-PCR toward two sample mutants showed various result. One mutant showed the abovementioned expectation, which is the level of expression is similar to wild type. The other one was apparently knock out. Sometimes, not all T-DNA insertions into an intron are spliced out (Hurtado et al., 2006), even only a small percentage of insertions (about 4 %) in introns produce a reduced level of wild type, the rest are knockouts (Wang, 2008). Alternative splicing of pre-mRNA can be the reason of the knockout, given that in this case, location of insertion is very close to border between intron and exon of targeted gene. The phenotype of this mutant line was similar to wild type.

The T-DNA of the other *pp4-2* mutant line, SALK_049725, is inserted in exon 8 of total 8 in *PP4-2* gene, very close to stop codon. The expression analysis by RT-PCR toward two sample mutants showed various result. One mutant showed the over-expression, which is the level of expression is higher than wild type. The other one was apparently knock out. Although the T-DNA inserted in exon, it does not have to result in the null function of gene. Hurtado et al. (2006) reported that insertion at 39 bp before stop codon did not affect transcript level. The phenotype of this mutant line was also similar to wild type. The higher number of sample mutants is needed to get more

reliable conclusion along with more repetition of RT-PCR and or performing more advanced expression study.

For the PP4-1 gene, two lines were observed, *pp4-1* GK_651B07 and *pp4-1* SALK_070977. Both mutant lines showed similar phenotype compared to wild type. The T-DNA of *pp4-1* GK_651B07 mutant is inserted before start codon, make it flank-tagged upstream of translation. On the other hand, the T-DNA of *pp4-1* SALK_070977 mutant is inserted after stop codon, make it flank-tagged downstream of translation. For the expression analysis by RT-PCR, two sample mutants for each lines were analysed. The level of transcription in *pp4-1* GK_651B07 mutant line is slightly higher or similar compared to wild type, and in *pp4-1* SALK_070977 line is similar to wild type. For *pp4-1* GK_651B07 mutant line, the insertion before start codon can cause elevated expression if it located in promoter. For *pp4-1* SALK_070977 mutant lines, it can be said that T-DNA insertion does not affect the level of expression, since T-DNA insertion after stop codon is least effective compared to insertion in other parts of gene.

4.1.2 amiRNA Plants

The amiRNA lines for gene *PP4-1* (applied also for *PP4-2*), *PP4R2L*, and *PSY2L*. The amiRNA will reduce the expression of the genes. Besides monitoring the level of their expressions, we also observed the phenotype of the individual plant.

Laborious works have done for searching the knocked down mutant *pp4-1* amiRNA plants. The level of expression is seemingly various and unstable between sample in the same generation and even between different generations. Not only that, their phenotype is also similar to the control. These observations proposed an allegation, whether the plant has some endogenous mechanism against the amiRNA that targeted *PP4-1* and *PP4-2*. amiRNA is small RNA based gene silencing. Silencing is normally achieved by post-transcriptional down regulation of transcript accumulation via small RNAs that act in a sequence specific manner by base pairing to complementary mRNA molecules (Bolle et al., 2011). This explain the variable level of expression in the sample plants. The knock down lines, such amiRNA lines, are not null mutants and show a diverse pattern of phenotypes due to difference in the level of activity remaining. No solid conclusion can be drawn regarding the function of *PP4-1* and *PP4-2* in plants for this case. However, further investigations might be needed to study the partial or conditional inactivation of *PP4-1* and *PP4-2* against these amiRNA.

The knocked down *psy2l* amiRNA plants show some interesting phenotype, such as reducing size and twisting leaf. This is correlated to the loss of function mutant (*psy2l* SALK_048064 line), which also showed a very strong phenotype, such as dwarfism and delay of growth. While, no significant different phenotype was shown by the knocked down plant of *pp4r2l* amiRNA plants compared to wild type.

4.1.3 Overexpression Plants

We found plants that show higher level expression than wild type for each overexpression lines. However, no significant different phenotype was observed compared to wild type/control for these plants. The effect of increasing transcription level of these catalytic or regulatory subunits might be not clearly visible by observing phenotype only.

4.2 Subcellular Localization

The function of protein is usually related to its subcellular localization. The prediction of subcellular localization of PP4-1, PP4-2, PP4R2L, and PSY2L protein were analyzed using several bioinformatics programs. Overall, PP4-1 and PP4-2 catalytic subunits were predicted as cytoplasmic protein, and the regulatory subunits, PP4R2L and PSY2L, were nuclear proteins.

To establish the subcellular localization of aforementioned proteins experimentally, we chose two different transformations: transient expression system of *Arabidopsis* mesophyll protoplasts and particle bombardment into onion epidermal cells. Although the isolation procedure of mesophyll protoplasts by Yoo et al. (2007) is relatively simple, experimental handling procedure should be considered carefully, given that protoplasts without cell walls are vulnerable and easy to disrupt. The isolation usually results in homogenous protoplasts and shows physiological perceptions and responses similar to cell-autonomous. The transformation of the protoplast was achieved by using polyethylene glycol (PEG), which has been extensively used to induce DNA uptake in protoplasts. The transformation efficiency of Arabidopsis mesophyll protoplasts is high (up to 90%). The onion epidermal cells have simple structure, uniform size and shape, and also transparent. The cells are relatively easy to transform by bombardment with gold particle coated with the plasmid DNA using “gene gun”, and have been successfully used for this subcellular localization studies. In contrast to mesophyll protoplasts method, the particle bombardment method shows relatively poor efficiency. It also indulges significant mechanical stress to the onion epidermal cells. Using

both methods, the genes are quickly expressed and within overnight, the cells were ready to be analyzed by confocal microscopy.

PSY2L fusion proteins (both N and C tagging) are strongly aggregated in nucleus. PP4R2L-EYFP fusion protein is seemingly widespread throughout the cytoplasmic cells. However, for EYFP-PP4R2L, the fusion protein is localized in nucleus of onion cell, but it was widespread throughout cytoplasma in protoplasts or in network-linked structures (Figure 3-44). The different localization of PP4R2L may be a consequence of different location tagging with fluorescent protein, since it can abrogate many targeting signals.

In onion epidermal cells and protoplast, localization for PP4-1 with both N and C tagging, are scattered mostly in cytoplasm, give impression that the protein is cytoplasmic. However, they are also found in nucleus. In the same pattern, PP4-2, the other catalytic subunit, also shows similar localization. Whether they are active there or are transported to different location at different times remain to be examined. However, giving the impression of both proteins being widespread in cytosol and nucleus their regulatory subunits are mostly found in nucleus, raises a hint that they are probably nucleus targeting proteins. Moreover, the blastp analysis shows that PP4-1 has 94 % identity with PP4-2, also hint an overlapping role between these catalytic subunits. Further study is also needed to reveal the distinguish role between them. The discrepancy found between the results obtained using subcellular localization prediction software and the results from the *in vivo*, emphasizes the fact that the predictions of actual biology events, such as subcellular localization and protein function, are more challenging.

Performing more subcellular localization studies at different times for many more protoplasts leaves and onion epidermal cells are needed to optimize the localization of these proteins, whether the proteins are localized at different places in different time and treatment. It is also important to investigate the localization of these proteins in different part of plants, for instance, using different source of protoplasts instead of leaves. Another possibility is cell bombardment to *Arabidopsis* roots, since Pujol et al. (2000) reported that PP4 localized in root plastid by immunolocalization analysis.

After subcellular localization of the catalytic and regulatory subunits is established, the next logical step includes the study of interaction of the subunits. It can be between catalytic subunits, regulatory subunits, or between catalytic and regulatory subunits. This is called protein-protein interaction studies. Protein-protein interaction analysis available today are: techniques such as

bimolecular fluorescence complementation, protein complex immunoprecipitation, label transfer, or tandem affinity purification. When proteins interact or assemble, new functions and specificity become available. Protein interactions allow new binding sites at the assembly interface, as well as providing multifunctional activity and specificity, such as found in polymerases and signal transduction.

4.3 Future Research

The understanding of physiological functions of the PP4 in plant is still in its infancy. The result from this thesis and previous thesis by Sætre (2015) are still categorized as introduction and more explorations are widely open to fully understand this topic. Screening of several T-DNA lines, amiRNA lines, and overexpression lines of PP4 subunits in this thesis through expression studies (RT-PCR) and observation of their phenotype can be treated as gate for further investigation. Although knocking out one of the regulatory subunit gene, *PSY2L*, in SALK_048064, has been captured to show a very interesting phenotype, intensive observation of phenotype of certain parts of this mutant is still needed. Undoubtedly, reverse genetics technique is still needed to identify the function of other subunits. Besides that, the possibility of involvement of plant PP4 complex in the DNA damage response is still relevant to be tested by pharmacological assays using cisplatin (O'Neill et al., 2004, Gingras et al., 2005, Hastie et al., 2006).

Although the subcellular localization of PP4 subunits has been defined, verification of experiments can be done in order to strengthen the conclusion, followed by protein-protein interaction studies. Additionally, other molecular analysis such as expression pattern of these subunit genes in *Arabidopsis* is opened to be considered.

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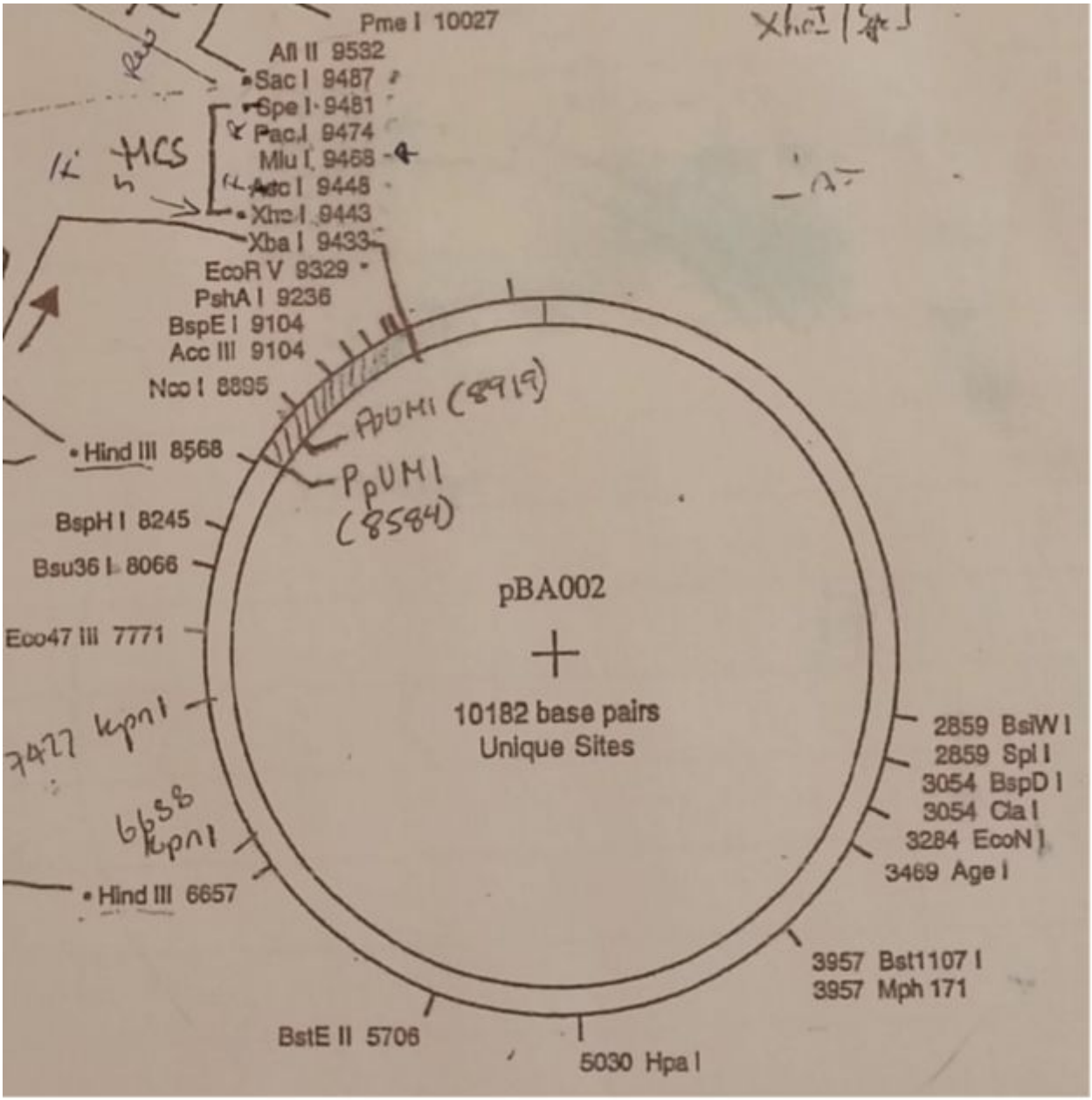
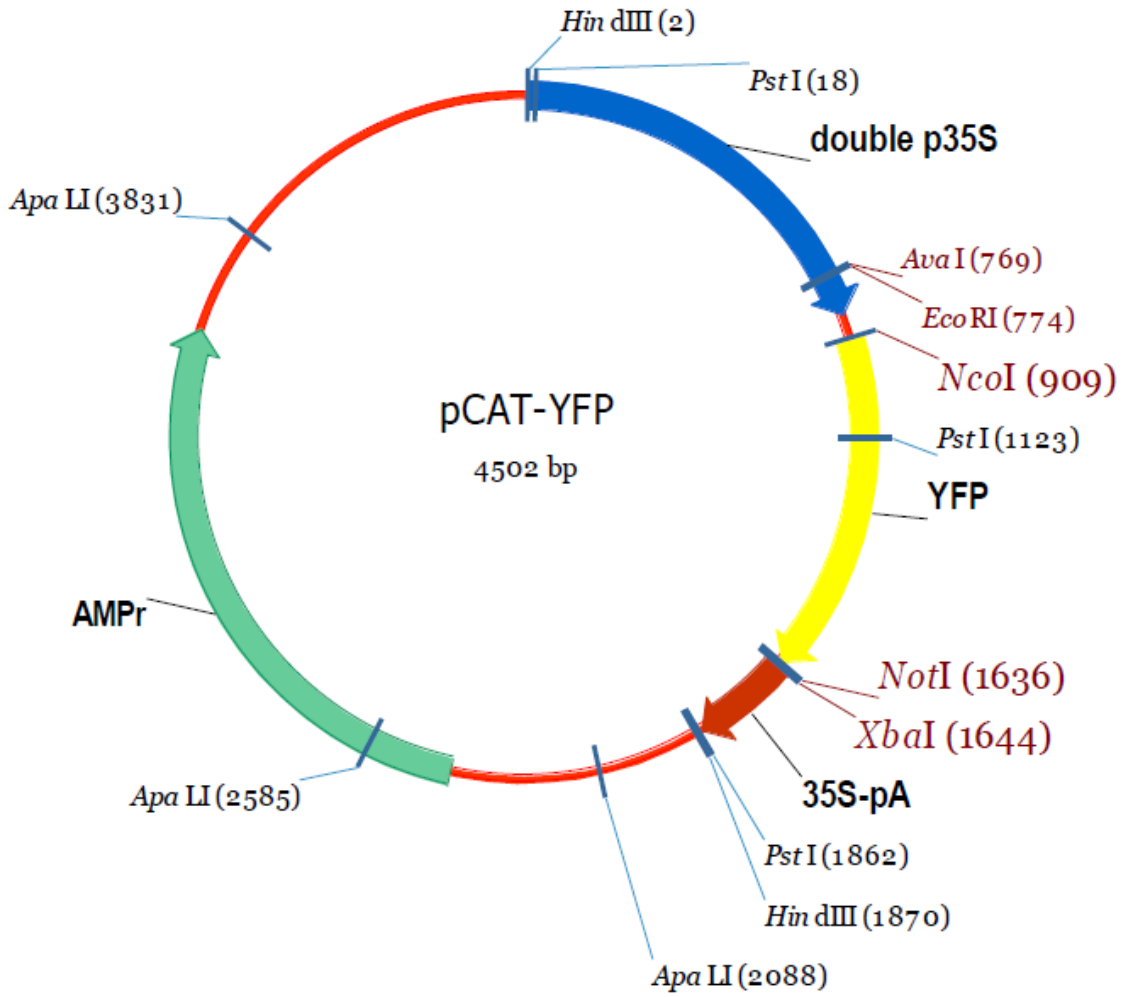
APPENDICES

APPENDIX 2-1 Vector Map of pBA002



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

APPENDIX 2-2 Vector Map of pCAT-EYFP-1



This is the image of vector map of pCAT-EYFP-1. Both vectors pCAT-EYFP-1 and pCAT-EYFP-2 share the same nucleotide sequence. The difference is pCAT-EYFP-1 will ensure that the resulting fusion-protein will carry the EYFP tag on the N–Terminus, whereas the pCAT-EYFP-2 will give the resulting fusion-protein an EYFP tag at the C–terminus. The vectors were used for molecular cloning and this vector map was designed by Dr. Amr Kataya (Ma et al., 2006)

APPENDIX 3-1 The Sequencing of Successful Cloning of *PSY2L*

```
                                          1                                                  50
                    AT3G06670.1    (1) -------------------------MGAPEKSQSNTNSMQRVKVYHLNED
>PSY2LYFPM_11PSmid_PSY2L middle    (1) --------------------------------------------------
       >PSY2LYFPM_11AK92r_AK92r    (1) --------------------------------------------------
 >PSY2LYFPM_11ECter_EYFP-C-TERf    (1) HMVLLEFVTAAGITLGMDELYKAAAMGAPEKSQSNTNSMQRVKVYHLNED
                  >B6PSY2L_T7_T7    (1) ----------------------SMGMGAPEKSQSNTNSMQRVKVYHLNED
       >B6PSY2LDECR_PSY2LMiddle    (1) --------------------------------------------------
               >B6PSY2LDECR_SP6    (1) --------------------------------------------------
         >LigB6_B-5_AK93f_Ak93f    (1) ---------------FHHLRTIAMGMGAPEKSQSNTNSMQRVKVYHLNED
                    AT3G06670.2    (1) -------------------------MGAPEKSQSNTNSMQRVKVYHLNED
                                         51                                                 100
                    AT3G06670.1   (26) GKWDDRGTGHVSIDFVERSEELSLCVIDEEDNETLLVHPINPEDIYRKQE
>PSY2LYFPM_11PSmid_PSY2L middle    (1) --------------------------------------------------
       >PSY2LYFPM_11AK92r_AK92r    (1) --------------------------------------------------
 >PSY2LYFPM_11ECter_EYFP-C-TERf   (51) GKWDDRGTGHVSIDFVERSEELSLCVIDEEDNETLLVHPINPEDIYRKQE
                  >B6PSY2L_T7_T7   (29) GKWDDRGTGHVSIDFVERSEELSLCVIDEEDNETLLVHPINPEDIYRKQE
       >B6PSY2LDECR_PSY2LMiddle    (1) --------------------------------------------------
               >B6PSY2LDECR_SP6    (1) --------------------------------------------------
         >LigB6_B-5_AK93f_Ak93f   (36) GKWDDRGTGHVSIDFVERSEELSLCVIDEEDNETLLVHPINPEDIYRKQE
                    AT3G06670.2   (26) GKWDDRGTGHVSIDFVERSEELSLCVIDEEDNETLLVHPINPEDIYRKQE
                                        101                                                 150
                    AT3G06670.1   (76) DTIISWRDPERSTELALSFQETAGCSYVWDQICTMQRNLHFSSLNSETFH
>PSY2LYFPM_11PSmid_PSY2L middle    (1) --------------------------------------------------
       >PSY2LYFPM_11AK92r_AK92r    (1) --------------------------------------------------
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (101) DTIISWRDPERSTELALSFQETAGCSYVWDQICTMQRNLHFSSLNSETFH
                  >B6PSY2L_T7_T7   (79) DTIISWRDPERSTELALSFQETAGCSYVWDQICTMQRNLHFSSLNSETFH
       >B6PSY2LDECR_PSY2LMiddle    (1) --------------------------------------------------
               >B6PSY2LDECR_SP6    (1) --------------------------------------------------
         >LigB6_B-5_AK93f_Ak93f   (86) DTIISWRDPERSTELALSFQETAGCSYVWDQICTMQRNLHFSSLNSETFH
                    AT3G06670.2   (76) DTIISWRDPERSTELALSFQETAGCSYVWDQICTMQRNLHFSSLNSETFH
                                        151                                                 200
                    AT3G06670.1  (126) SLNSELRELPAVELTTLPLILKIVTESGITDQMRLTELILKDHDFFRNLM
>PSY2LYFPM_11PSmid_PSY2L middle    (1) --------------------------------------------------
       >PSY2LYFPM_11AK92r_AK92r    (1) --------------------------------------------------
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (151) SLNSELRELPAVELTTLPLILKIVTESGITDQMRLTELILKDHDFFRNLM
                  >B6PSY2L_T7_T7  (129) SLNSELRELPAVELTTLPLILKIVTESGITDQMRLTELILKDHDFFRNLM
       >B6PSY2LDECR_PSY2LMiddle    (1) --------------------------------------------------
               >B6PSY2LDECR_SP6    (1) --------------------------------------------------
         >LigB6_B-5_AK93f_Ak93f  (136) SLNSELRELPAVELTTLPLILKIVTESGITDQMRLTELILKDHDFFRNLM
                    AT3G06670.2  (126) SLNSELRELPAVELTTLPLILKIVTESGITDQMRLTELILKDHDFFRNLM
                                        201                                                 250
                    AT3G06670.1  (176) GVFKICEDLENVDGLHMIFNIVKGIILLNSSQILEKIFGDELIMEIIGCL
>PSY2LYFPM_11PSmid_PSY2L middle    (1) --------------------------------------------------
       >PSY2LYFPM_11AK92r_AK92r    (1) --------------------------------------------------
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (201) GVFKICEDLENVDGLHMIFNIVKGIILLNSSQILEKIFGDELIMEIIGCL
                  >B6PSY2L_T7_T7  (179) GVFKICEDLENVDGLHMIFNIVKGIILLNSSQILEKIFGDELIMEIIGCL
       >B6PSY2LDECR_PSY2LMiddle    (1) --------------------------------------------------
               >B6PSY2LDECR_SP6    (1) --------------------------------------------------
         >LigB6_B-5_AK93f_Ak93f  (186) GVFKICEDLENVDGLHMIFNIVKGIILLNSSQILEKIFGDELIMEIIGCL
                    AT3G06670.2  (176) GVFKICEDLENVDGLHMIFNIVKGIILLNSSQILEKIFGDELIMEIIGCL
                                        251                                                 300
                    AT3G06670.1  (226) EYDPGVPHSQHHRNFLKEHVVFKE--------------------AIPIK
>PSY2LYFPM_11PSmid_PSY2L middle    (1) --------------------------------------------------
       >PSY2LYFPM_11AK92r_AK92r    (1) --------------------------------------------------
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (251) EYDPGVPHSQHHRNFLKEHVVFKE--------------------AIPIK
                  >B6PSY2L_T7_T7  (229) EYDPGVPHSQHHRNFLKEHVVFKE--------------------AIPIK
       >B6PSY2LDECR_PSY2LMiddle    (1) --------------------------------------------------
               >B6PSY2LDECR_SP6    (1) --------------------------------------------------
         >LigB6_B-5_AK93f_Ak93f  (236) EYDPGVPHSQHHRNFLKEHVVFKE--------------------AIPIK
                    AT3G06670.2  (226) EYDPGVPHSQHHRNFLKEHVVFKERQSHVFFVRKEHAHYGCFGISAIPIK
                                        301                                                 350
                    AT3G06670.1  (255) DPLVLSKIHQTYRIGYLKDVVLARVLDDAIVANLNSVIHANNAIVVSLLK
>PSY2LYFPM_11PSmid_PSY2L middle    (1) ----------------------------DAIVANLNSVIHANNAIVVSLLK
       >PSY2LYFPM_11AK92r_AK92r    (1) --------------------------------------------------
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (280) DPLVLSKIHQTYRIGYLKDVVLARVLDDAIVANLNSVIHANNAIVVSLLK
                  >B6PSY2L_T7_T7  (258) DPLVLSKIHQTYRIGYLKDVVLARVLDDAIVANLNSVIHANNAIVVSLLK
       >B6PSY2LDECR_PSY2LMiddle    (1) ----------------------------DAIVANLNSVIHANNAIVVSLLK
               >B6PSY2LDECR_SP6    (1) --------------------------------------------------
         >LigB6_B-5_AK93f_Ak93f  (265) DPLVLSKIHQTYRIGYLKDVVLARVLDDAIVANLNSVIHANNAIVVSLLK
                    AT3G06670.2  (276) DPLVLSKIHQTYRIGYLKDVVLARVLDDAIVANLNSVIHANNAIVVSLLK
                                        351                                                 400
                    AT3G06670.1  (305) DDSTFIQELFARLRSPSTSMESKKNLVYFLHEFCSLSKSLQVVQQLRLFR
>PSY2LYFPM_11PSmid_PSY2L middle   (24) DDSTFIQELFARLRSPSTSMESKKNLVYFLHEFCSLSKSLQVVQQLRLFR
       >PSY2LYFPM_11AK92r_AK92r    (1) --------------------------------------------------
```

```
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (330) DDSTFIQELFARLRSPSTSMESKKNLVXFLXEFCSLS------------
                 >B6PSY2L_T7_T7  (308) DDSTFIQELFARLRSPSTSMESKKNLVYFLHEFCSLSKSLQVVXQLRLFR
       >B6PSY2LDECR_PSY2LMiddle   (24) DDSTFIQELFARLRSPSTSMESKKNLVYFLHEFCSLSKSLQVVQQLRLFR
               >B6PSY2LDECR_SP6    (1) ------------------------------------------------
         >LigB6_B-5_AK93f_Ak93f  (315) DDSTFIQELFARLRSPSTSMESKKNLVYFLHEFCSLSKSLQVVQQLRLFR
                     AT3G06670.2  (326) DDSTFIQELFARLRSPSTSMESKKNLVYFLHEFCSLSKSLQVVQQLRLFR
                                        401                                             450
                     AT3G06670.1  (355) DLINEGIFHVIEEVLQIPDKKLVLTGTDILILFLTQDPNLLRSYVVRTEG
>PSY2LYFPM_11PSmid_PSY2L middle   (74) DLINEGIFHVIEEVLQIPDKKLVLTGTDILILFLTQDPNLLRSYVVRTEG
       >PSY2LYFPM_11AK92r_AK92r    (1) ------------------------------------------------
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (367) ------------------------------------------------
                 >B6PSY2L_T7_T7  (358) DLIN--------------------------------------------
       >B6PSY2LDECR_PSY2LMiddle   (74) DLINEGIFHVIEEVLQIPDKKLVLTGTDILILFLTQDPNLLRSYVVRTEG
               >B6PSY2LDECR_SP6    (1) ------------------------------------------------
         >LigB6_B-5_AK93f_Ak93f  (365) DLINEG--------------------------------------------
                     AT3G06670.2  (376) DLINEGIFHVIEEVLQIPDKKLVLTGTDILILFLTQDPNLLRSYVVRTEG
                                        451                                             500
                     AT3G06670.1  (405) NPLLGLLVKGMMEDFGDKMHCQFLEIIRTLLDANALSGGAQRANIMDIFY
>PSY2LYFPM_11PSmid_PSY2L middle  (124) NPLLGLLVKGMMEDFGDKMHCQFLEIIRTLLDANALSGGAQRANIMDIFY
       >PSY2LYFPM_11AK92r_AK92r    (1) ------------------------------------------------
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (367) ------------------------------------------------
                 >B6PSY2L_T7_T7  (362) ------------------------------------------------
       >B6PSY2LDECR_PSY2LMiddle  (124) NPLLGLLVKGMMEDFGDKMHCQFLEIIRTLLDANALSGGAQRANIMDIFY
               >B6PSY2LDECR_SP6    (1) ------------------------------------------------
         >LigB6_B-5_AK93f_Ak93f  (371) ------------------------------------------------
                     AT3G06670.2  (426) NPLLGLLVKGMMEDFGDKMHCQFLEIIRTLLDANALSGGAQRANIMDIFY
                                        501                                             550
                     AT3G06670.1  (455) EKHLPELVDVITASCPEKSSNASEGAARRIFTKPEVLLNICELLCFCIMQ
>PSY2LYFPM_11PSmid_PSY2L middle  (174) EKHLPELVDVITASCPEKSSNASEGAARRIFTKPEVLLNICELLCFCIMQ
       >PSY2LYFPM_11AK92r_AK92r    (1) ------------------------------------------------
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (367) ------------------------------------------------
                 >B6PSY2L_T7_T7  (362) ------------------------------------------------
       >B6PSY2LDECR_PSY2LMiddle  (174) EKHLPELVDVITASCPEKSSNASEGAARRIFTKPEVLLNICELLCFCIMQ
               >B6PSY2LDECR_SP6    (1) ----------------------------------------------FCIXQ
         >LigB6_B-5_AK93f_Ak93f  (371) ------------------------------------------------
                     AT3G06670.2  (476) EKHLPELVDVITASCPEKSSNASEGAARRIFTKPEVLLNICELLCFCIMQ
                                        551                                             600
                     AT3G06670.1  (505) DASRTKCSFLQNNVTEKVLHLTRRKEKYLVVAAIRFVRTLLSVHDDYVQN
>PSY2LYFPM_11PSmid_PSY2L middle  (224) DASRTKCSFLQNNVTEKVLHLTRRKEKYLVVAAIRFVRTLLSVHDDYVQN
       >PSY2LYFPM_11AK92r_AK92r    (1) ----------------------XKEKYLVVAAIRFVRTLLSVHDDYVQN
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (367) ------------------------------------------------
                 >B6PSY2L_T7_T7  (362) ------------------------------------------------
       >B6PSY2LDECR_PSY2LMiddle  (224) DASRTKCSFLQNNVTEKVLHLTRRKEKYLVVAAIRFVRTLLSVHDDYVQN
               >B6PSY2LDECR_SP6    (6) DASRSKCSFLQNNVTEKVLHLTRRKEKYLVVAAIRFVRTLLSVHDDYVQN
         >LigB6_B-5_AK93f_Ak93f  (371) ------------------------------------------------
                     AT3G06670.2  (526) DASRTKCSFLQNNVTEKVLHLTRRKEKYLVVAAIRFVRTLLSVHDDYVQN
                                        601                                             650
                     AT3G06670.1  (555) YVVKNNLLKPIIDVFIANGTRYNLLNSAVLDLLEHIRKGNATLLLKYIVD
>PSY2LYFPM_11PSmid_PSY2L middle  (274) YVVKNNLLKPIIDVFIANGTRYNLLNSAVLDLLEHIRKGNATLLLKYIVD
       >PSY2LYFPM_11AK92r_AK92r   (28) YVVKNNLLKPIIDVFIANGTRYNLLNSAVLDLLEHIRKGNATLLLKYIVD
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (367) ------------------------------------------------
                 >B6PSY2L_T7_T7  (362) ------------------------------------------------
       >B6PSY2LDECR_PSY2LMiddle  (274) YVVKNNLLKPIIDVFIANGTRYNLLNSAVLDLLEHIRKGNATLLLKYIVD
               >B6PSY2LDECR_SP6   (56) YVVKNNLLKPIIDVFIANGTRYNLLNSAVLDLLEHIRKGNATLLLKYIVD
         >LigB6_B-5_AK93f_Ak93f  (371) ------------------------------------------------
                     AT3G06670.2  (576) YVVKNNLLKPIIDVFIANGTRYNLLNSAVLDLLEHIRKGNATLLLKYIVD
                                        651                                             700
                     AT3G06670.1  (605) TFWDQLAPFQCLTSIQAFKVKYEQCLESAGPKSTSDAVDPRRRVDERALE
>PSY2LYFPM_11PSmid_PSY2L middle  (324) TFWDQLAPFQCLTSIQAFKVKYEQWFRKCRTKKHF---------------
       >PSY2LYFPM_11AK92r_AK92r   (78) TFWDQLAPFQCLTSIQAFKVKYEQCLESAGPKSTSDAVDPRRRVDERALE
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (367) ------------------------------------------------
                 >B6PSY2L_T7_T7  (362) ------------------------------------------------
       >B6PSY2LDECR_PSY2LMiddle  (324) TFWDQLAPFQCLTSIQAFKVKYEQCLESAGPKSTSDAVDPRR--------
               >B6PSY2LDECR_SP6  (106) TFWDQLAPFQCLTSIQAFKVKYEQCLESAGPKSTSDAVDPRRRVDERALE
         >LigB6_B-5_AK93f_Ak93f  (371) ------------------------------------------------
                     AT3G06670.2  (626) TFWDQLAPFQCLTSIQAFKVKYEQCLESAGPKSTSDAVDPRRRVDERALE
                                        701                                             750
                     AT3G06670.1  (655) KEEEDYFNEDSDEEDSASASNTQKEKPASNIQKEQPKPHLSNGVAASPTS
>PSY2LYFPM_11PSmid_PSY2L middle  (359) ------------------------------------------------
       >PSY2LYFPM_11AK92r_AK92r  (128) KEEEDYFNEDSDEEDSASASNTQKEKPASNIQKEQPKPHLSNGVAASPTS
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (367) ------------------------------------------------
                 >B6PSY2L_T7_T7  (362) ------------------------------------------------
       >B6PSY2LDECR_PSY2LMiddle  (366) ------------------------------------------------
               >B6PSY2LDECR_SP6  (156) KEEEDYFNEDSDEEDSASASNTQKEKPASNIQKEQPKPHLSNGVAASPTS
         >LigB6_B-5_AK93f_Ak93f  (371) ------------------------------------------------
                     AT3G06670.2  (676) KEEEDYFNEDSDEEDSASASNTQKEKPASNIQKEQPKPHLSNGVAASPTS
                                        751                                             800
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                    AT3G06670.1  (705) SSPRSGGLVDYEDDEDDEDYKPPPRKQPEASEDEEGELLRLKRKSALVER
>PSY2LYFPM_11PSmid_PSY2L middle  (359) ----------------------------------------------------
        >PSY2LYFPM_11AK92r_AK92r  (178) SSPRSGGLVDYEDDEDDEDYKPPPRKQPEASEDEEGELLRLKRKSALVER
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (367) ----------------------------------------------------
                  >B6PSY2L_T7_T7  (362) ----------------------------------------------------
      >B6PSY2LDECR_PSY2LMiddle  (366) ----------------------------------------------------
              >B6PSY2LDECR_SP6  (206) SSPRSGGLVDYEDDEDDEDYKPPPRKQPEASEDEEGELLRLKRKSALVER
         >LigB6_B-5_AK93f_Ak93f  (371) ----------------------------------------------------
                    AT3G06670.2  (726) SSPRSGGLVDYEDDEDDEDYKPPPRKQPEASEDEEGELLRLKRKSALVER
                                       801                                              850
                    AT3G06670.1  (755) EQEPSKKPRLGKSSKRENVFAVLCSTLSHAVLTGKKSPGPAGSAARSIVA
>PSY2LYFPM_11PSmid_PSY2L middle  (359) ----------------------------------------------------
        >PSY2LYFPM_11AK92r_AK92r  (228) EQEPSKKPRLGKSSKRENVFAVLCSTLSHAVLTGKKSPGPAGSAARSIVA
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (367) ----------------------------------------------------
                  >B6PSY2L_T7_T7  (362) ----------------------------------------------------
        >B6PSY2LDECR_PSY2LMiddle  (366) ----------------------------------------------------
              >B6PSY2LDECR_SP6  (256) EQEPSKKPRLGKSSKRENVFAVLCSTLSHAVLTGKKSPGPAGSAARSIVA
         >LigB6_B-5_AK93f_Ak93f  (371) ----------------------------------------------------
                    AT3G06670.2  (776) EQEPSKKPRLGKSSKRENVFAVLCSTLSHAVLTGKKSPGPAGSAARSIVA
                                       851                                              900
                    AT3G06670.1  (805) KGAEDSKSSEENNSSSSDDENHKDDGVSSSEHETSDNGKLNGEESLVVAP
>PSY2LYFPM_11PSmid_PSY2L middle  (359) ----------------------------------------------------
        >PSY2LYFPM_11AK92r_AK92r  (278) KGAEDSKSSEENNSSSSDDENHKDDGVSSSEHETSDNGKLNGEESLVVAP
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (367) ----------------------------------------------------
                  >B6PSY2L_T7_T7  (362) ----------------------------------------------------
        >B6PSY2LDECR_PSY2LMiddle  (366) ----------------------------------------------------
              >B6PSY2LDECR_SP6  (306) KGAEDSKSSEENNSSSSDDENHKDDGVSSSEHETSDNGKLNGEESLVVAP
         >LigB6_B-5_AK93f_Ak93f  (371) ----------------------------------------------------
                    AT3G06670.2  (826) KGAEDSKSSEENNSSSSDDENHKDDGVSSSEHETSDNGKLNGEESLVVAP
                                       901                         930
                    AT3G06670.1  (855) KSSPEMAVNGS-------------------
>PSY2LYFPM_11PSmid_PSY2L middle  (359) ----------------------------
        >PSY2LYFPM_11AK92r_AK92r  (328) KSSPEMAVNGS-------------------
 >PSY2LYFPM_11ECter_EYFP-C-TERf  (367) ----------------------------
                  >B6PSY2L_T7_T7  (362) ----------------------------
        >B6PSY2LDECR_PSY2LMiddle  (366) ----------------------------
              >B6PSY2LDECR_SP6  (356) KSSPEMAVNGSAAAIITSEFAAACRSTIWE
         >LigB6_B-5_AK93f_Ak93f  (371) ----------------------------
                    AT3G06670.2  (876) KSSPEMAVNGS-------------------
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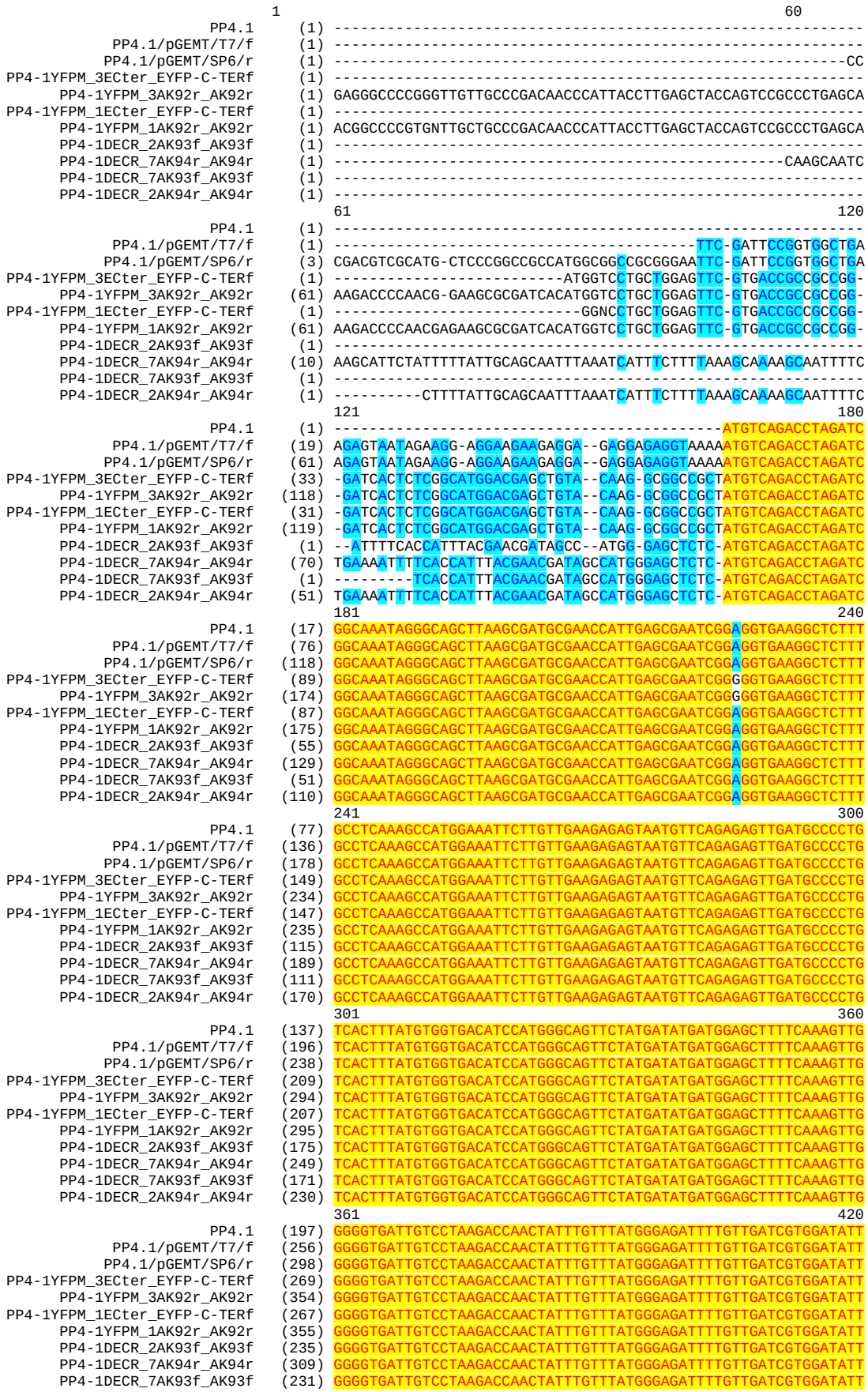
APPENDIX 3-2 The Sequencing of Successful Cloning of *PP4-1*



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      PP4-1DECR_2AK94r_AK94r   (290) GGGGTGATTGTCCTAAGACCAACTATTTGTTTATGGGAGATTTTGTTGATCGTGGATATT
                                     421                                                       480
                     PP4.1     (257) ATTCGGTTGAGACATTTCTACTTCTACTCGCACTCAAGGTTAGATATCCAGACCGCATAA
           PP4.1/pGEMT/T7/f    (316) ATTCGGTTGAGACATTTCTACTTCTACTCGCACTCAAGGTTAGATATCCAGACCGCATAA
          PP4.1/pGEMT/SP6/r    (358) ATTCGGTTGAGACATTTCTACTTCTACTCGCACTCAAGGTTAGATATCCAGACCGCATAA
PP4-1YFPM_3ECter_EYFP-C-TERf   (329) ATTCGGTTGAGACATTTCTACTTCTACTCGCACTCAAGGTTAGATATCCAGACCGCATAA
      PP4-1YFPM_3AK92r_AK92r   (414) ATTCGGTTGAGACATTTCTACTTCTACTCGCACTCAAGGTTAGATATCCAGACCGCATAA
PP4-1YFPM_1ECter_EYFP-C-TERf   (327) ATTCGGTTGAGACATTTCTACTTCTACTCGCACTCAAGGTTAGATATCCAGACCGCATAA
      PP4-1YFPM_1AK92r_AK92r   (415) ATTCGGTTGAGACATTTCTACTTCTACTCGCACTCAAGGTTAGATATCCAGACCGCATAA
      PP4-1DECR_2AK93f_AK93f   (295) ATTCGGTTGAGACATTTCTACTTCTACTCGCACTCAAGGTTAGATATCCAGACCGCATAA
      PP4-1DECR_7AK94r_AK94r   (369) ATTCGGTTGAGACATTTCTACTTCTACTCGCACTCAAGGTTAGATATCCAGACCGCATAA
      PP4-1DECR_7AK93f_AK93f   (291) ATTCGGTTGAGACATTTCTACTTCTACTCGCACTCAAGGTTAGATATCCAGACCGCATAA
      PP4-1DECR_2AK94r_AK94r   (350) ATTCGGTTGAGACATTTCTACTTCTACTCGCACTCAAGGTTAGATATCCAGACCGCATAA
                                     481                                                       540
                     PP4.1     (317) CTCTCATCAGAGGAAACCATGAAAGCAGGCAAATCACACAGGTTTATGGATTTTATGATG
           PP4.1/pGEMT/T7/f    (376) CTCTCATCAGAGGAAACCATGAAAGCAGGCAAATCACACAGGTTTATGGATTTTATGATG
          PP4.1/pGEMT/SP6/r    (418) CTCTCATCAGAGGAAACCATGAAAGCAGGCAAATCACACAGGTTTATGGATTTTATGATG
PP4-1YFPM_3ECter_EYFP-C-TERf   (389) CTCTCATCAGAGGAAACCATGAAAGCAGGCAAATCACACAGGTTTATGGATTTTATGATG
      PP4-1YFPM_3AK92r_AK92r   (474) CTCTCATCAGAGGAAACCATGAAAGCAGGCAAATCACACAGGTTTATGGATTTTATGATG
PP4-1YFPM_1ECter_EYFP-C-TERf   (387) CTCTCATCAGAGGAAACCATGAAAGCAGGCAAATCACACAGGTTTATGGATTTTATGATG
      PP4-1YFPM_1AK92r_AK92r   (475) CTCTCATCAGAGGAAACCATGAAAGCAGGCAAATCACACAGGTTTATGGATTTTATGATG
      PP4-1DECR_2AK93f_AK93f   (355) CTCTCATCAGAGGAAACCATGAAAGCAGGCAAATCACACAGGTTTATGGATTTTATGATG
      PP4-1DECR_7AK94r_AK94r   (429) CTCTCATCAGAGGAAACCATGAAAGCAGGCAAATCACACAGGTTTATGGATTTTATGATG
      PP4-1DECR_7AK93f_AK93f   (351) CTCTCATCAGAGGAAACCATGAAAGCAGGCAAATCACACAGGTTTATGGATTTTATGATG
      PP4-1DECR_2AK94r_AK94r   (410) CTCTCATCAGAGGAAACCATGAAAGCAGGCAAATCACACAGGTTTATGGATTTTATGATG
                                     541                                                       600
                     PP4.1     (377) AGTGTTTGCGTAAATATGGCTCTTCAAATGTCTGGAGATACTGCACCGACATTTTTGACT
           PP4.1/pGEMT/T7/f    (436) AGTGTTTGCGTAAATATGGCTCTTCAAATGTCTGGAGATACTGCACCGACATTTTTGACT
          PP4.1/pGEMT/SP6/r    (478) AGTGTTTGCGTAAATATGGCTCTTCAAATGTCTGGAGATACTGCACCGACATTTTTGACT
PP4-1YFPM_3ECter_EYFP-C-TERf   (449) AGTGTTTGCGTAAATATGGCTCTTCAAATGTCTGGAGATACTGCACCGACATTTTTGACT
      PP4-1YFPM_3AK92r_AK92r   (534) AGTGTTTGCGTAAATATGGCTCTTCAAATGTCTGGAGATACTGCACCGACATTTTTGACT
PP4-1YFPM_1ECter_EYFP-C-TERf   (447) AGTGTTTGCGTAAATATGGCTCTTCAAATGTCTGGAGATACTGCACCGACATTTTTGACT
      PP4-1YFPM_1AK92r_AK92r   (535) AGTGTTTGCGTAAATATGGCTCTTCAAATGTCTGGAGATACTGCACCGACATTTTTGACT
      PP4-1DECR_2AK93f_AK93f   (415) AGTGTTTGCGTAAATATGGCTCTTCAAATGTCTGGAGATACTGCACCGACATTTTTGACT
      PP4-1DECR_7AK94r_AK94r   (489) AGTGTTTGCGTAAATATGGCTCTTCAAATGTCTGGAGATACTGCACCGACATTTTTGACT
      PP4-1DECR_7AK93f_AK93f   (411) AGTGTTTGCGTAAATATGGCTCTTCAAATGTCTGGAGATACTGCACCGACATTTTTGACT
      PP4-1DECR_2AK94r_AK94r   (470) AGTGTTTGCGTAAATATGGCTCTTCAAATGTCTGGAGATACTGCACCGACATTTTTGACT
                                     601                                                       660
                     PP4.1     (437) ACATGAGTCTTTCAGCTGTTGTGGAGAACAAGATATTCTGTGTTCATGGTGGTCTTTCTC
           PP4.1/pGEMT/T7/f    (496) ACATGAGTCTTTCAGCTGTTGTGGAGAACAAGATATTCTGTGTTCATGGTGGTCTTTCTC
          PP4.1/pGEMT/SP6/r    (538) ACATGAGTCTTTCAGCTGTTGTGGAGAACAAGATATTCTGTGTTCATGGTGGTCTTTCTC
PP4-1YFPM_3ECter_EYFP-C-TERf   (509) ACATGAGTCTTTCAGCTGTTGTGGAGAACAAGATATTCTGTGTTCATGGTGGTCTTTCTC
      PP4-1YFPM_3AK92r_AK92r   (594) ACATGAGTCTTTCAGCTGTTGTGGAGAACAAGATATTCTGTGTTCATGGTGGTCTTTCTC
PP4-1YFPM_1ECter_EYFP-C-TERf   (507) ACATGAGTCTTTCAGCTGTTGTGGAGAACAAGATATTCTGTGTTCATGGTGGTCTTTCTC
      PP4-1YFPM_1AK92r_AK92r   (595) ACATGAGTCTTTCAGCTGTTGTGGAGAACAAGATATTCTGTGTTCATGGTGGTCTTTCTC
      PP4-1DECR_2AK93f_AK93f   (475) ACATGAGTCTTTCAGCTGTTGTGGAGAACAAGATATTCTGTGTTCATGGTGGTCTTTCTC
      PP4-1DECR_7AK94r_AK94r   (549) ACATGAGTCTTTCAGCTGTTGTGGAGAACAAGATATTCTGTGTTCATGGTGGTCTTTCTC
      PP4-1DECR_7AK93f_AK93f   (471) ACATGAGTCTTTCAGCTGTTGTGGAGAACAAGATATTCTGTGTTCATGGTGGTCTTTCTC
      PP4-1DECR_2AK94r_AK94r   (530) ACATGAGTCTTTCAGCTGTTGTGGAGAACAAGATATTCTGTGTTCATGGTGGTCTTTCTC
                                     661                                                       720
                     PP4.1     (497) CAGCTATTATGACTCTTGATCAGATTAGGACAATTGACCGGAAGCAAGAAGTACCACATG
           PP4.1/pGEMT/T7/f    (556) CAGCTATTATGACTCTTGATCAGATTAGGACAATTGACCGGAAGCAAGAAGTACCACATG
          PP4.1/pGEMT/SP6/r    (598) CAGCTATTATGACTCTTGATCAGATTAGGACAATTGACCGGAAGCAAGAAGTACCACATG
PP4-1YFPM_3ECter_EYFP-C-TERf   (569) CAGCTATTATGACTCTTGATCAGATTAGGACAATTGACCGGAAGCAAGAAGTACCACATG
      PP4-1YFPM_3AK92r_AK92r   (654) CAGCTATTATGACTCTTGATCAGATTAGGACAATTGACCGGAAGCAAGAAGTACCACATG
PP4-1YFPM_1ECter_EYFP-C-TERf   (567) CAGCTATTATGACTCTTGATCAGATTAGGACAATTGACCGGAAGCAAGAAGTACCACATG
      PP4-1YFPM_1AK92r_AK92r   (655) CAGCTATTATGACTCTTGATCAGATTAGGACAATTGACCGGAAGCAAGAAGTACCACATG
      PP4-1DECR_2AK93f_AK93f   (535) CAGCTATTATGACTCTTGATCAGATTAGGACAATTGACCGGAAGCAAGAAGTACCACATG
      PP4-1DECR_7AK94r_AK94r   (609) CAGCTATTATGACTCTTGATCAGATTAGGACAATTGACCGGAAGCAAGAAGTACCACATG
      PP4-1DECR_7AK93f_AK93f   (531) CAGCTATTATGACTCTTGATCAGATTAGGACAATTGACCGGAAGCAAGAAGTACCACATG
      PP4-1DECR_2AK94r_AK94r   (590) CAGCTATTATGACTCTTGATCAGATTAGGACAATTGACCGGAAGCAAGAAGTACCACATG
                                     721                                                       780
                     PP4.1     (557) ATGGTGCCATGTGTGATCTCCTATGGTCTGATCCTGAAGATATTGTTGATGGCTGGGGAT
           PP4.1/pGEMT/T7/f    (616) ATGGTGCCATGTGTGATCTCCTATGGTCTGATCCTGAAGATATTGTTGATGGCTGGGGAT
          PP4.1/pGEMT/SP6/r    (658) ATGGTGCCATGTGTGATCTCCTATGGTCTGATCCTGAAGATATTGTTGATGGCTGGGGAT
PP4-1YFPM_3ECter_EYFP-C-TERf   (629) ATGGTGCCATGTGTGATCTCCTATGGTCTGATCCTGAAGATATTGTTGATGGCTGGGGAT
      PP4-1YFPM_3AK92r_AK92r   (714) ATGGTGCCATGTGTGATCTCCTATGGTCTGATCCTGAAGATATTGTTGATGGCTGGGGAT
PP4-1YFPM_1ECter_EYFP-C-TERf   (627) ATGGTGCCATGTGTGATCTCCTATGGTCTGATCCTGAAGATATTGTTGATGGCTGGGGAT
      PP4-1YFPM_1AK92r_AK92r   (715) ATGGTGCCATGTGTGATCTCCTATGGTCTGATCCTGAAGATATTGTTGATGGCTGGGGAT
      PP4-1DECR_2AK93f_AK93f   (595) ATGGTGCCATGTGTGATCTCCTATGGTCTGATCCTGAAGATATTGTTGATGGCTGGGGAT
      PP4-1DECR_7AK94r_AK94r   (669) ATGGTGCCATGTGTGATCTCCTATGGTCTGATCCTGAAGATATTGTTGATGGCTGGGGAT
      PP4-1DECR_7AK93f_AK93f   (591) ATGGTGCCATGTGTGATCTCCTATGGTCTGATCCTGAAGATATTGTTGATGGCTGGGGAT
      PP4-1DECR_2AK94r_AK94r   (650) ATGGTGCCATGTGTGATCTCCTATGGTCTGATCCTGAAGATATTGTTGATGGCTGGGGAT
                                     781                                                       840
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           PP4.1/pGEMT/T7/f    (676) TGAGCCCTCGTGGTGCCGGATTCCTTTTTGGTGGCAGTGTTGTCACGTCTTTTAACCACT
          PP4.1/pGEMT/SP6/r    (718) TGAGCCCTCGTGGTGCCGGATTCCTTTTTGGTGGCAGTGTTGTCACGTCTTTTAACCACT
PP4-1YFPM_3ECter_EYFP-C-TERf   (689) TGAGCCCTCGTGGTGCCGGATTCCTTTTTGGTGGCAGTGTTGTCACGTCTTTTAACCACT
      PP4-1YFPM_3AK92r_AK92r   (774) TGAGCCCTCGTGGTGCCGGATTCCTTTTTGGTGGCAGTGTTGTCACGTCTTTTAACCACT
PP4-1YFPM_1ECter_EYFP-C-TERf   (687) TGAGCCCTCGTGGTGCCGGATTCCTTTTTGGTGGCAGTGTTGTCACGTCTTTTAACCACT
      PP4-1YFPM_1AK92r_AK92r   (775) TGAGCCCTCGTGGTGCCGGATTCCTTTTTGGTGGCAGTGTTGTCACGTCTTTTAACCACT
      PP4-1DECR_2AK93f_AK93f   (655) TGAGCCCTCGTGGTGCCGGATTCCTTTTTGGTGGCAGTGTTGTCACGTCTTTTAACCACT
      PP4-1DECR_7AK94r_AK94r   (729) TGAGCCCTCGTGGTGCCGGATTCCTTTTTGGTGGCAGTGTTGTCACGTCTTTTAACCACT
      PP4-1DECR_7AK93f_AK93f   (651) TGAGCCCTCGTGGTGCCGGATTCCTTTTTGGTGGCAGTGTTGTCACGTCTTTTAACCACT
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                                     841                                                       900
                     PP4.1     (677) CAAACAACATAGACTACATAGCCCGTGCCCATCAACTAGTTATGGAGGGTTACAAATGGA
           PP4.1/pGEMT/T7/f    (736) CAAACAACATAGACTACATAGCCCGTGCCCATCAACTAGTTATGGAGGGTTACAAATGGA
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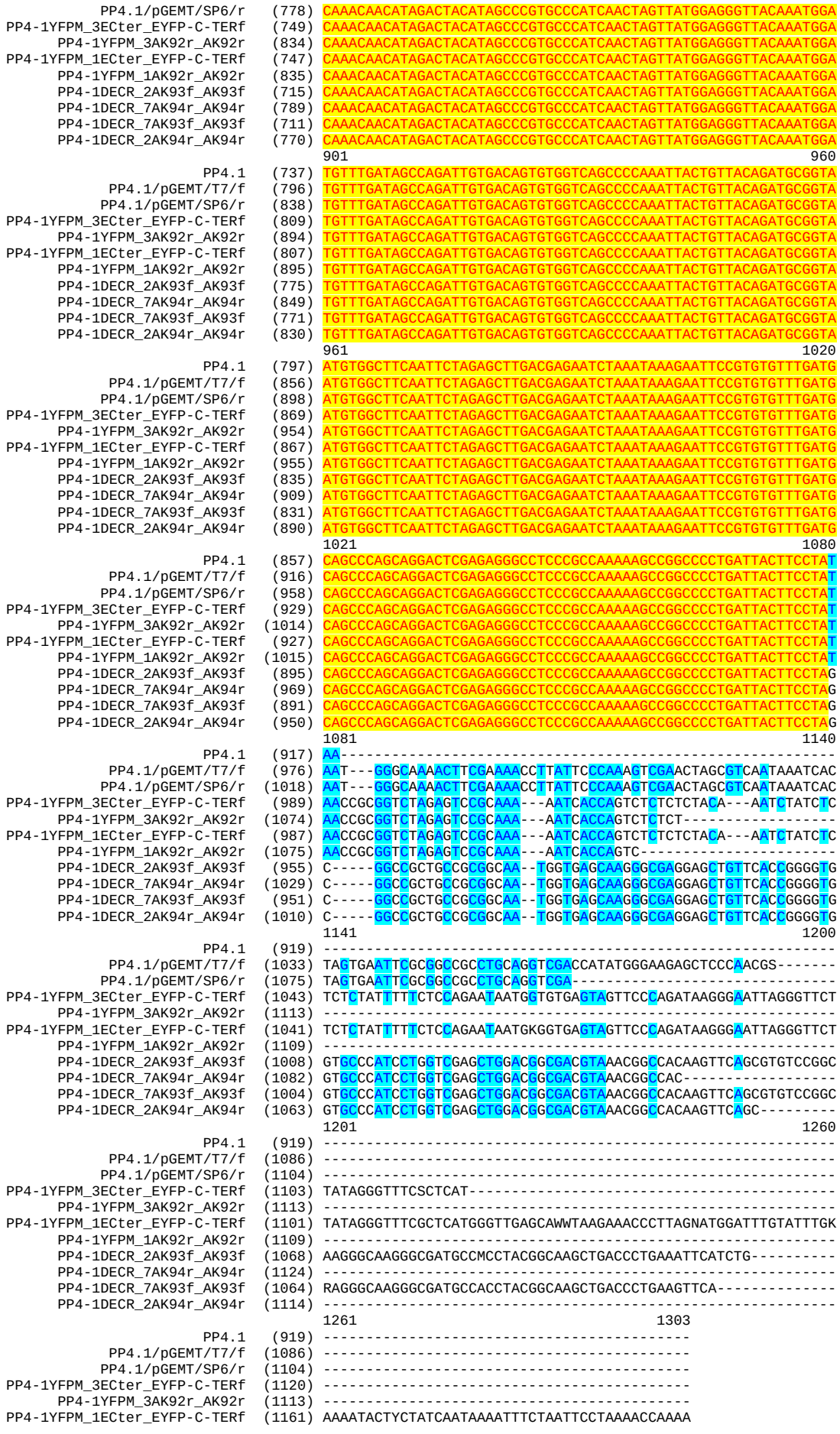
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PP4-1YFPM_3AK92r_AK92r (834) CAAACAACATAGACTACATAGCCCGTGCCCATCAACTAGTTATGGAGGGTTACAAATGGA
PP4-1YFPM_1ECter_EYFP-C-TERf (747) CAAACAACATAGACTACATAGCCCGTGCCCATCAACTAGTTATGGAGGGTTACAAATGGA
PP4-1YFPM_1AK92r_AK92r (835) CAAACAACATAGACTACATAGCCCGTGCCCATCAACTAGTTATGGAGGGTTACAAATGGA
PP4-1DECR_2AK93f_AK93f (715) CAAACAACATAGACTACATAGCCCGTGCCCATCAACTAGTTATGGAGGGTTACAAATGGA
PP4-1DECR_7AK94r_AK94r (789) CAAACAACATAGACTACATAGCCCGTGCCCATCAACTAGTTATGGAGGGTTACAAATGGA
PP4-1DECR_7AK93f_AK93f (711) CAAACAACATAGACTACATAGCCCGTGCCCATCAACTAGTTATGGAGGGTTACAAATGGA
PP4-1DECR_2AK94r_AK94r (770) CAAACAACATAGACTACATAGCCCGTGCCCATCAACTAGTTATGGAGGGTTACAAATGGA
901 960
PP4.1 (737) TGTTTGATAGCCAGATTGTGACAGTGTGGTCAGCCCCAAATTACTGTTACAGATGCGGTA
PP4.1/pGEMT/T7/f (796) TGTTTGATAGCCAGATTGTGACAGTGTGGTCAGCCCCAAATTACTGTTACAGATGCGGTA
PP4.1/pGEMT/SP6/r (838) TGTTTGATAGCCAGATTGTGACAGTGTGGTCAGCCCCAAATTACTGTTACAGATGCGGTA
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PP4-1YFPM_3AK92r_AK92r (894) TGTTTGATAGCCAGATTGTGACAGTGTGGTCAGCCCCAAATTACTGTTACAGATGCGGTA
PP4-1YFPM_1ECter_EYFP-C-TERf (807) TGTTTGATAGCCAGATTGTGACAGTGTGGTCAGCCCCAAATTACTGTTACAGATGCGGTA
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PP4-1DECR_2AK94r_AK94r (830) TGTTTGATAGCCAGATTGTGACAGTGTGGTCAGCCCCAAATTACTGTTACAGATGCGGTA
961 1020
PP4.1 (797) ATGTGGCTTCAATTCTAGAGCTTGACGAGAATCTAAATAAAGAATTCCGTGTGTTTGATG
PP4.1/pGEMT/T7/f (856) ATGTGGCTTCAATTCTAGAGCTTGACGAGAATCTAAATAAAGAATTCCGTGTGTTTGATG
PP4.1/pGEMT/SP6/r (898) ATGTGGCTTCAATTCTAGAGCTTGACGAGAATCTAAATAAAGAATTCCGTGTGTTTGATG
PP4-1YFPM_3ECter_EYFP-C-TERf (869) ATGTGGCTTCAATTCTAGAGCTTGACGAGAATCTAAATAAAGAATTCCGTGTGTTTGATG
PP4-1YFPM_3AK92r_AK92r (954) ATGTGGCTTCAATTCTAGAGCTTGACGAGAATCTAAATAAAGAATTCCGTGTGTTTGATG
PP4-1YFPM_1ECter_EYFP-C-TERf (867) ATGTGGCTTCAATTCTAGAGCTTGACGAGAATCTAAATAAAGAATTCCGTGTGTTTGATG
PP4-1YFPM_1AK92r_AK92r (955) ATGTGGCTTCAATTCTAGAGCTTGACGAGAATCTAAATAAAGAATTCCGTGTGTTTGATG
PP4-1DECR_2AK93f_AK93f (835) ATGTGGCTTCAATTCTAGAGCTTGACGAGAATCTAAATAAAGAATTCCGTGTGTTTGATG
PP4-1DECR_7AK94r_AK94r (909) ATGTGGCTTCAATTCTAGAGCTTGACGAGAATCTAAATAAAGAATTCCGTGTGTTTGATG
PP4-1DECR_7AK93f_AK93f (831) ATGTGGCTTCAATTCTAGAGCTTGACGAGAATCTAAATAAAGAATTCCGTGTGTTTGATG
PP4-1DECR_2AK94r_AK94r (890) ATGTGGCTTCAATTCTAGAGCTTGACGAGAATCTAAATAAAGAATTCCGTGTGTTTGATG
1021 1080
PP4.1 (857) CAGCCCAGCAGGACTCGAGAGGGCCTCCCGCCAAAAAGCCGGCCCCCTGATTACTTCCTAT
PP4.1/pGEMT/T7/f (916) CAGCCCAGCAGGACTCGAGAGGGCCTCCCGCCAAAAAGCCGGCCCCCTGATTACTTCCTAT
PP4.1/pGEMT/SP6/r (958) CAGCCCAGCAGGACTCGAGAGGGCCTCCCGCCAAAAAGCCGGCCCCCTGATTACTTCCTAT
PP4-1YFPM_3ECter_EYFP-C-TERf (929) CAGCCCAGCAGGACTCGAGAGGGCCTCCCGCCAAAAAGCCGGCCCCCTGATTACTTCCTAT
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PP4-1YFPM_1ECter_EYFP-C-TERf (927) CAGCCCAGCAGGACTCGAGAGGGCCTCCCGCCAAAAAGCCGGCCCCCTGATTACTTCCTAT
PP4-1YFPM_1AK92r_AK92r (1015) CAGCCCAGCAGGACTCGAGAGGGCCTCCCGCCAAAAAGCCGGCCCCCTGATTACTTCCTAT
PP4-1DECR_2AK93f_AK93f (895) CAGCCCAGCAGGACTCGAGAGGGCCTCCCGCCAAAAAGCCGGCCCCCTGATTACTTCCTAG
PP4-1DECR_7AK94r_AK94r (969) CAGCCCAGCAGGACTCGAGAGGGCCTCCCGCCAAAAAGCCGGCCCCCTGATTACTTCCTAG
PP4-1DECR_7AK93f_AK93f (891) CAGCCCAGCAGGACTCGAGAGGGCCTCCCGCCAAAAAGCCGGCCCCCTGATTACTTCCTAG
PP4-1DECR_2AK94r_AK94r (950) CAGCCCAGCAGGACTCGAGAGGGCCTCCCGCCAAAAAGCCGGCCCCCTGATTACTTCCTAG
1081 1140
PP4.1 (917) AA----------
PP4.1/pGEMT/T7/f (976) AAT---GGGCAAAACTTCGAAAACCTTATTCCCAAAGTCGAACTAGCGTCAATAAATCAC
PP4.1/pGEMT/SP6/r (1018) AAT---GGGCAAAACTTCGAAAACCTTATTCCCAAAGTCGAACTAGCGTCAATAAATCAC
PP4-1YFPM_3ECter_EYFP-C-TERf (989) AACCGCGGTCTAGAGTCCGCAAA---AATCACCAGTCTCTCTCTACA---AATCTATCTC
PP4-1YFPM_3AK92r_AK92r (1074) AACCGCGGTCTAGAGTCCGCAAA---AATCACCAGTCTCTCT----------
PP4-1YFPM_1ECter_EYFP-C-TERf (987) AACCGCGGTCTAGAGTCCGCAAA---AATCACCAGTCTCTCTCTACA---AATCTATCTC
PP4-1YFPM_1AK92r_AK92r (1075) AACCGCGGTCTAGAGTCCGCAAA---AATCACCAGTC----------
PP4-1DECR_2AK93f_AK93f (955) C-----GGCCGCTGCCGCGGCAA--TGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTG
PP4-1DECR_7AK94r_AK94r (1029) C-----GGCCGCTGCCGCGGCAA--TGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTG
PP4-1DECR_7AK93f_AK93f (951) C-----GGCCGCTGCCGCGGCAA--TGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTG
PP4-1DECR_2AK94r_AK94r (1010) C-----GGCCGCTGCCGCGGCAA--TGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTG
1141 1200
PP4.1 (919) ----------
PP4.1/pGEMT/T7/f (1033) TAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAAGAGCTCCCAACGS-------
PP4.1/pGEMT/SP6/r (1075) TAGTGAATTCGCGGCCGCCTGCAGGTCGA----------
PP4-1YFPM_3ECter_EYFP-C-TERf (1043) TCTCTATTTTTCTCCAGAATAATGGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCT
PP4-1YFPM_3AK92r_AK92r (1113) ----------
PP4-1YFPM_1ECter_EYFP-C-TERf (1041) TCTCTATTTTTCTCCAGAATAATGKGGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCT
PP4-1YFPM_1AK92r_AK92r (1109) ----------
PP4-1DECR_2AK93f_AK93f (1008) GTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGC
PP4-1DECR_7AK94r_AK94r (1082) GTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCAC----------
PP4-1DECR_7AK93f_AK93f (1004) GTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGC
PP4-1DECR_2AK94r_AK94r (1063) GTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGC---------
1201 1260
PP4.1 (919) ----------
PP4.1/pGEMT/T7/f (1086) ----------
PP4.1/pGEMT/SP6/r (1104) ----------
PP4-1YFPM_3ECter_EYFP-C-TERf (1103) TATAGGGTTTCSCTCAT----------
PP4-1YFPM_3AK92r_AK92r (1113) ----------
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PP4-1DECR_7AK93f_AK93f (1064) RAGGGCAAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCA----------
PP4-1DECR_2AK94r_AK94r (1114) ----------
1261 1303
PP4.1 (919) ----------
PP4.1/pGEMT/T7/f (1086) ----------
PP4.1/pGEMT/SP6/r (1104) ----------
PP4-1YFPM_3ECter_EYFP-C-TERf (1120) ----------
PP4-1YFPM_3AK92r_AK92r (1113) ----------
PP4-1YFPM_1ECter_EYFP-C-TERf (1161) AAAATACTYCTATCAATAAAATTTCTAATTCCTAAAACCAAAA

```
PP4-1YFPM_1AK92r_AK92r  (1109) ----------------------------------------
PP4-1DECR_2AK93f_AK93f  (1118) ----------------------------------------
PP4-1DECR_7AK94r_AK94r  (1124) ----------------------------------------
PP4-1DECR_7AK93f_AK93f  (1110) ----------------------------------------
PP4-1DECR_2AK94r_AK94r  (1114) ----------------------------------------
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