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Stavanger, June 14, 2018

Hesham Amin

ABSTRACT

Protein phosphatase 2A (PP2A) is a ubiquitous serine/threonine-specific phosphatase comprising a catalytic subunit C, a scaffolding subunit A and a highly variable regulatory subunit B. The regulatory subunits are essential for substrate specificity and subcellular localization of the PP2A holoenzyme and are classified into B/B55, B', and B" non-related families in higher plants. Previous studies had shown that PP2A B' regulatory subunits have a role in regulation of energy metabolism. An *Arabidopsis* B' θ mutant had shown growth retardation in sucrose free medium, and cytosolic aconitase 3 (ACO3) was shown to interact with B´ζ and B'γ; furthermore, B´ζ had shown interaction with *Arabidopsis* mitochondrial succinate fumarate translocator (AtmSFC). In this thesis, wanted to investigate possibility of interactions between AtmSFC and other PP2A B' regulatory subunits (B'α, B'β and B'θ). Furthermore, wanted to study probability of interaction between B´ζ with ACO1 and truncated ACO1 in which last 192 bp had been removed. Protein-protein interaction were analyzed by bimolecular fluorescence complementation (BiFC) assay. The regulatory subunits were tagged with C-terminal fragment of fluorescent protein Venus while ACO1, truncated ACO1 and AtmSFC were tagged with N-terminal fragment of fluorescent protein Venus. Tagged proteins were transiently expressed in onion epidermal cells. Fluorescence in the cytoplasm was detected in combinations of both ACO1 and B´ζ, whereas no interaction was observed with truncated ACO1. This result indicates that C -terminal end of ACO1 appeared to be necessary for interaction with B´ζ, because the truncated ACO1 that lacked the 64 C-terminal amino acids did not interact with B''ζ. The Fluorescence was also detected between AtmSFC and PP2A B'β. Pervious study has shown the regulatory subunit B´ζ to be targeted to cytoplasm and partly co- localized to mitochondria excluded chloroplast and nucleus, using transient expression of fluorescent tagged protein in tobacco and *Arabidopsis* epidermal cells. Protein subcellular localization programs predicated that B' ζ to be localized in cytoplasm, chloroplast and mitochondria. We wanted to employ another method to study B´ζ subcellular localization. Transgenic plants contain PP2A B' ζ linked to fluorescent protein (YFP) using wild type *Arabidopsis* and knocked out mutants B' ζ were produced followed by subcellular fractionation and western blotting using antibody against YFP. Preliminary results indicate that PP2A B' ζ more localized to cytoplasm and chloroplast than mitochondria. Our results consolidate the prediction of protein subcellular localization programs.

ABBREVIATIONS

ABA	Abscisic acid
ACO	Aconitase
AtmSFC	*Arabidopsis* mitochondrial succinate-fumarate translocator
BiFC	Bimolecular fluorescence complementation
BSA	Bovine serum albumin
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
DSP	Dual specificity phosphatase
DTT	Dithiothreitol
E. coli	*Escherichia Coli*
LB	Luria-Bertani
MCS	Multiple cloning site
MS medium	Murashige and Skoog medium
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PP2A	Protein phosphatase 2A
PPP	Phosphoserine/phosphothreonine specific protein phosphatase
PTM	Metal-ion-dependent protein phosphatase
PTP	Phosphotyrosine phosphatase
PTS	Peroxisomal targeting signal
PVDF	Polyvinylidene difluoride
RCN1	ROOTS CURL IN NAPHTHYLPHTHALAMIC ACID1
RFP	Red fluorescent protein
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
T1	First generation transgenic
T2	Second generation transgenic
T-DNA	Transfer-DNA
Ti-plasmid	Tumor inducing plasmid
WT	Wild Type
YFP	Yellow fluorescent protein

Table of Contents

1 INTRODUCTION

1.1 Protein phosphorylation and dephosphorylation

Reversible protein phosphorylation is a major posttranslational modification of proteins which establishes the phosphorylation state of proteins and regulates a majority of the important signaling pathways in all living organisms. About 30% all eukaryotic proteins are believed to be phosphorylated at any given time (Meimoun et al., 2007).

The process of reversible phosphorylation of proteins controlled by action of protein kinases and phosphatases (Okamura et al., 2017). Protein kinases are enzymes that modifies other proteins by chemically transfer of the terminal phosphate group of adenosine triphosphate (ATP) to a specific amino acid side chain on a target protein, a process called phosphorylation. The hydroxyl group of serine (Ser) , threonine (Thr) and tyrosine (Tyr) amino acid are target sites of protein kinases (Alberts et al., 2013). The reverse reaction of phosphorylation is called, dephosphorylation and is catalyzed by protein phosphatases that remove the phosphate group from target protein (Luan 2003). The reversible phosphorylation of proteins alters the structural conformation of a protein, causing it to become activated, deactivated, or modifying its function (Okamura et al., 2017).

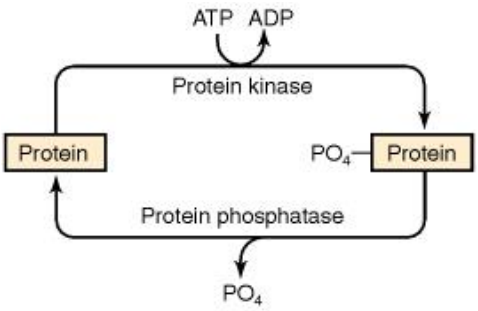

Figure 1.1: Schematic diagram of the conversion of a phosphoprotein to a dephosphoprotein by action of protein kinase and the reversal of this reaction by a protein phosphatase. In the reaction the kinases catalyze the transfer of the terminal (γ) phosphate group of ATP to the hydroxyl moiety of the amino acid , while protein phosphatases dephosphorylate the target protein by removing the phosphate group. The figure is obtained from Siegel (1999).

The reversible phosphorylation of proteins represents a fundamental mechanism used by all eukaryotic organisms and has significant roles in a variety of cellular processes, for example cell differentiation, protein-protein interactions, and apoptosis (Okamura et al., 2017). The genome of Arabidopsis thaliana encodes protein kinases over 1000 and 150 protein phosphatases, which are controlling the phosphorylation status of thousands of proteins (Kerk 2007, Okamura et al., 2017) .

The study of protein phosphorylation and dephosphorylation has a long history, while the large family of protein kinases and their functions has been studied for a long time, the protein phosphatases have only more recently protein phosphatases have only more recently been studied (Luan 2003).

1.2 Protein phosphatases

Eukaryotic protein phosphatases are classified into four distinct gene families each with different active site signatures: (1) PPP (Ser/Thr phospho-protein phosphatases), (2) PPM/PP2C (Mg^{2+}dependtent protein phosphatases), (3) Asp-based protein phosphatases and (4) phospho-tyrosine phosphatases. The PPPs responsible for 80% of the protein phosphatases activity in eukaryotic cells (Lillo et al., 2014).

1.3 Ser/Thr phosphoprotein phosphatases

Ser/Thr phosphoprotein phosphatases (PPPs) are universal enzymes in all eukaryotes and they control the specific dephosphorylation of thousands of phosphoprotein substrates (Farkas et al., 2007). PPPs are classified into subfamilies of PP1, PP2A, PP2B , PP4, PP5, PP6, PP7 and protein phosphatases with kelch-repeat domains. PP1 is a major protein Ser/Thr phosphatase and is ubiquitously expressed in all eukaryotic cells (País et al., 2009b).

The PPP family members contain a highly conserved core catalytic region, with approximately 70% or greater protein sequence identity in any pairwise alignment. Whereas, the non-catalytic N-and C-terminal regions are more variable (Luan 2003). PPPs are multimeric holoenzymes comprise of catalytic subunits and regulatory subunits which modulate substrate specificity, subcellular localizations and catalytic activity (Janssens et al., 2001).

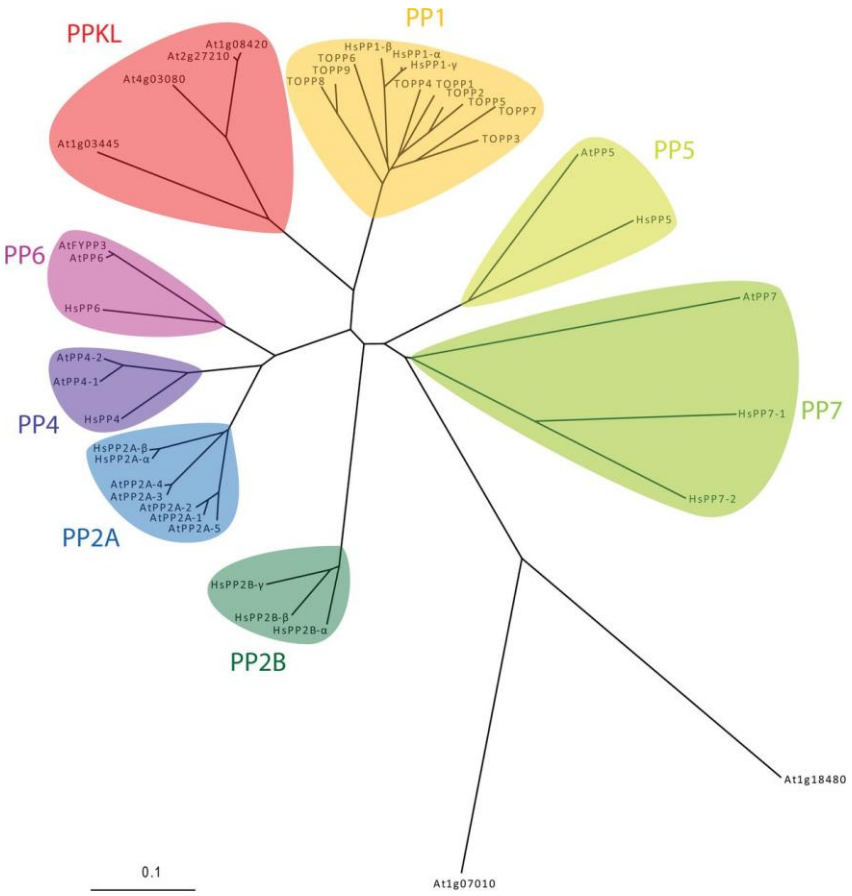

Figure 1.2: Phylogenetic analysis of the PPP family enzymes from *A. thaliana* and *Homo sapiens*. Proteins with an At suffix are from *A. thaliana*, while an Hs suffix denotes human sequences The figure is obtained from Moorhead (2009).

1.4 Protein phosphatase 2A

PP2A is one of the major Ser/Thr phosphatase in eukaryotes. PP2A together with PP1 it is responsible for more than 85% of the total cellular phosphatase activity (País et al., 2009b). Recent studies have identified PP2A as a crucial component that regulates stress responses including biotic stress, heat stress, drought, cold stress and mechanical wounding (Lillo et al., 2014, País et al., 2009b). In plants, PP2A was found to be involved in the signal transduction pathways of several hormones, including abscisic acid, ethylene and auxin (Antolín-Llovera et al., 2011).

1.4.1 PP2A structure

PP2A holoenzymes are heterotrimeric complexes composed of conserved catalytic subunit C, a highly conserved scaffolding subunit A and a regulatory subunit B. The Catalytic (36 kDa) subunits of PP2A phosphatases occurs either in association with scaffolding subunits (65-kDa), or together with a third variable B-subunit in heterotrimeric complexes (Lillo et al., 2014).

The genome of *Arabidopsis thaliana* encodes five PP2A catalytic subunits, three scaffolding subunits and 17 regulatory subunits (Kerk 2007). Association of the PP2A different subunits produces several holoenzymes of (up to 255) with different properties and functions, which indicates the importance of PP2A role in metabolism (Matre et al., 2009).

Whereas C and A subunit sequences are highly conserved across all eukaryotes, regulatory B subunits are more heterogeneous and are thought to be responsible for subcellular localization and substrate specificity of different holoenzymes (Zhou et al., 2004). B subunits are classified into three subfamilies B, B´ and B´´ based on their molecular weight and domains (Terol et al., 2002).

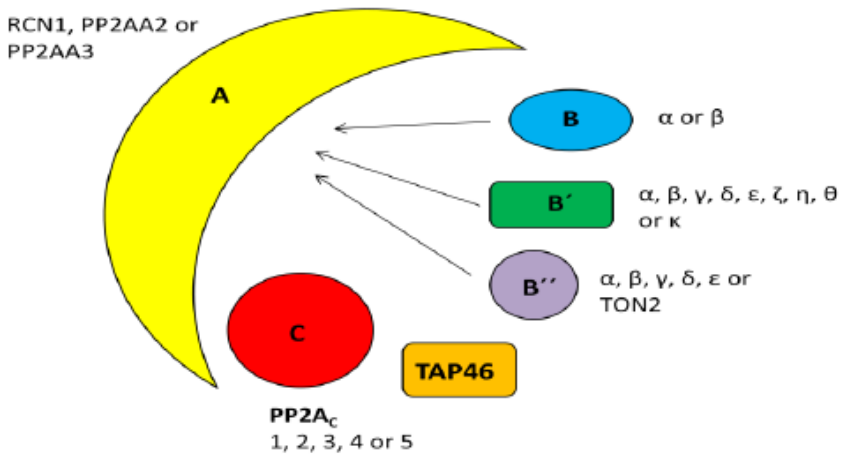

Figure 1.3: Structure of PP2A in *Arabidopsis*. A: scaffolding subunit , B: variable regulatory subunits(B, B´ and B´´), C: catalytic subunit, A subunits are encoded by three genes (RCN1, PP2AA2, PP2AA3); the B subunits are encoded by two related genes (α and β), the B´ subunits are encoded by nine related genes (α, β, γ, δ, ε, ζ, η, θ and κ); the B´´ subunits are encoded by six related genes (α, β, γ, δ, ε and TON2) and TAP46 is an unrelated regulatory subunit. C subunits are encoded by five genes (PP2AC-1, 2, 3, 4 and 5); The figure is based on Janssens and Goris (2001) and Farkas et al., (2007).

1.4.2 PP2A subunits

1.4.2.1 Catalytic subunits

Catalytic subunits are highly conserved throughout all of eukaryotes. In *Arabidopsis,* catalytic subunits shows 79% identity overall (DeLong 2006). in *Arabidopsis* five genes encode for PP2A catalytic subunits (PP2Ac) are divided into two subfamilies based on their amino acid sequence, subfamily I is composed of PP2A-1, PP2A-2, PP2A-5 and subfamily II is composed of PP2A-3 and PP2A-4 (Pérez-Callejón et al., 1998).

Catalytic subunits subfamily I are thought to be involved in defense signaling and plant stress. In *Nicotiana benthamiana* Virus-induced gene silencing of the PP2Ac subfamily I resulted in localized cell death and increase plant defense responses such as increase expression of pathogenesis-related genes and inhibition of growth of a virulent bacterial pathogen indicating a role of PP2Ac subfamily I as negative regulator of plant defense responses (He et al., 2004). Another study found that transcripts of the subfamily I genes are down-regulated by cold in tomato (País et al., 2009a). The mutant of PP2A-2C in *Arabidopsis* was reported to be hypersensitive to NaCl and abscisic acid (ABA) (Pernas et al., 2007). PP2Ac subfamily II Members are thought to be involved in auxin transport (Ballesteros et al., 2013).

1.4.2.2 Scaffolding subunits

The A subunit of PP2A works as a scaffolding molecule to coordinate the assembly of the catalytic subunits and a variable regulatory B subunit. The PP2A scaffolding A subunit is composed of three members, RCN1, PP2AA2 and PP2AA3. The scaffolding subunits are highly conserved, where PP2AA2 and PP2AA3 proteins show 94% amino acid sequence identity with each other and 86% identity with RCN1. The scaffolding subunits are composed of 15 tandem HEAT repeats that form a hook-like architecture for binding the catalytic and regulatory B subunits. The C subunits bind to repeats 11-15, while B subunits bind to repeats 1-10. Each repeat of the 15 repeats consists of 39 amino acids that fold into two antiparallel α-helices. Adjacent α-helices are connected by an intra repeat loop (Farkas et al., 2007, Groves et al., 1999, Mumby 2007).

RCN1 (root curl in naphthylphtalamic acid1) is a regulator for auxin transport and gravitropism as mutant seedlings exhibit altered responses to NPA in root curling and hypocotyl elongation. The mutant of RCN1 result in abscisic acid (ABA) insensitivity at level of seed germination, defects apical hook formation (Garbers et al., 1996) . Plants with mutations in the PP2A subunit A genes PP2AA2 and PP2AA3 exhibit largely normal phenotypes. However, double mutants carrying rcn1 and either PP2AA2 or PP2AA3 show severe deficiencies including defective radial cell expansion, abnormal embryogenesis, sterility and dwarfing. The effects of PP2AA2 and PP2AA3 mutations are unmasked only when RCN1 is absent. This indicates that RCN1 plays a fundamental role in the regulation of PP2A activity (Zhou et al., 2004).

1.4.2.3 Regulatory subunits

The *Arabidopsis thaliana* genome encode for 17 regulatory subunits which determine which determine the substrate specificity and subcellular localization of PP2A holoenzymes(Lillo et al., 2014). Regulatory subunits classified into three non-related families called B (55-kDa), B´(54–74-kDa) and B´´(72–130 kDa) (Terol et al., 2002). B subunits are encoded by two related genes (α and β), B´ are encoded by nine related genes (α, β, γ, δ, ε, ζ, η, θ and κ) and B´´ are encoded by six related genes (α, β, γ, δ, ε and TON2) (Farkas et al., 2007; see Figure 1.3). The 3 D study of structure of PP2A holoenzymes revealed that the B subunits provide an exposed concave surface adjacent to the active site of the C subunit. This concave surface could be a major determinant in substrate recognition (Mumby 2007).

The plant B´ subunits which is objective of study appeared very early in evolution of the eukaryotes, before separation of plants and animals. Protein sequences alignment shows the existence of a high degree of similarity between them, with the central core regions being the most conserved, whereas amino- and carboxyterminal regions the most variable(Terol et al., 2002).Different studies suggest that the highly variable terminal regions B´ subunits the substrate specificity and the cellular localization of PP2A holoenzyme while conserved central region essential for the assembly of the B' regulatory subunits with the other components of the PP2A complex (Janssens & Goris 2001, Zolnierowicz et al., 1994).

In Arabidopsis the B´ subunits (α, β, γ, δ, ε, ζ, η, θ and κ) divided into three subfamilies, α, η and κ (Terol et al., 2002). In this study we focus on PP2AB' subfamily members B' α, B' β, B' ζ

and B’ θ. The B’ α, B’ β subunits belong to B´ α subfamily whereas B’ ζ and B’ θ subunits belong to B’ η subfamily.

Phylogenetic tree of B’ subunits (see Figure 1.4) shows B’ α, B’ β belong to same phylogenetic clade. Sequence alignment of AtB’α and AtB’β show more than 80% similarity on protein level. In *Arabidopsis* genome B’α placed on chromosome V whereas B’β located on chromosome III However, Both B’ α and B’ β targeted to cytoplasm and nucleus (Terol et al., 2002, Wang et al., 2016). It has been reported that B’ α, B’ β subunits positively regulate BR signaling by targeting BZR1 a positive regulator of brassinosteroid responses. The subunits bind to the BZR1 and promote BZR1 dephosphorylation and activation (Tang et al., 2008). Unlike B’ α and B’ β , the B’ ζ and B’ θ negatively regulate BR signaling (Wang et al., 2016).

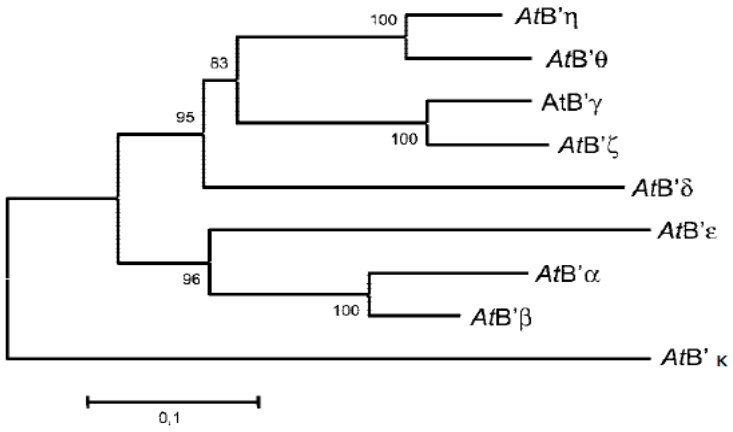

Figure 1.4: Unrooted phylogenetic tree of the PP2A B’ subunit family in *Arabidopsis.* The figure obtained from Terol (2002)

The core region of the B’ ζ and B’ θ comprise of two distinct A subunit-binding domains (ASBD1 and ASBD2) which are believed to be responsible for the interaction with the AC heterodimer (Xu et al., 2006). B’ ζ and B’ θ subunits are up-regulated in senescence stage and negatively regulating plant innate immunity (Kataya et al., 2015b). B’ θ was reported to target to peroxisome as it contains the peroxisomal target signal type 1 (PTS1) SSL at the C-terminal. Whereas B’ ζ with N free terminal showed to be localized to cytoplasm and partly localized to mitochondria excluded nucleus and chloroplast (Matre et al., 2009).

1.5 Energy metabolism

Energy metabolism is the process of generating ATP from nutrients and light through series of interconnected pathways and involvement of different cell organelles. For example, oilseed plants after germinating, convert triglycerides to sucrose which then is used to ATP production. This metabolic pathway involves fatty acid B-oxidation, glyoxylate cycle, and gluconeogenesis. First triglycerides are hydrolyzed to fatty acids, which are then transferred to peroxisomes and B-oxidized to give acetyl-CoA. Acetyl-CoA is converted to succinate through glyoxylate cycle in peroxisomes and transferred to mitochondria through carrier proteins. In mitochondria succinate is converted to malate then transported to cytosol and used for sucrose production (Eastmond et al., 2000)(Figure 1.5).

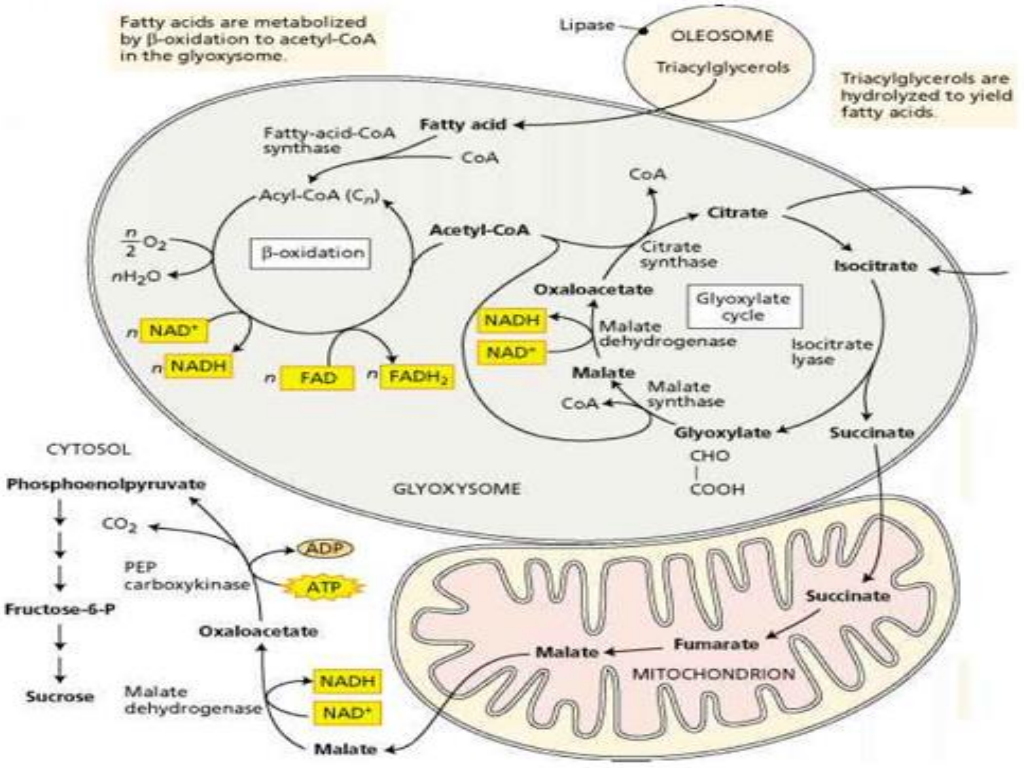

Figure 1.5: Overview of conversion of lipids to sucrose in oilseeds, which begin with hydrolysis of triacylglycerols to free fatty acids. The diagram shows glyoxsome (peroxisome) and mitochondrion with the inner and outer membranes, and the folded cristae. This figure was obtained from www.europeanmedical.info.

It has been revealed that PP2A- B' θ holoenzyme positively affects peroxisomal B-oxidation, where B' θ mutant showed hypocotyl retardation in sucrose free media (Kataya et al., 2015a). Another study found that B' ζ mutant showed developmental arrest of seedlings germinated

without sucrose (Kataya et al., 2015b). These results indicate possible roles of the peroxisomal B’ θ and mitochondrial B’ ζ subunits in energy metabolism.

Recently (at C.Lillo Lab by A.Elshobaky, personal communication) PP2A B’ ζ were shown to interact with two enzymes involved in mitochondrial energy flow, *Arabidopsis* mitochondrial succinate fumarate translocator (AtmSFC) and aconitase 3 (ACO3) using bimolecular fluorescence complementation assays in *onion* epidermis and *Arabidopsis* leaves.

1.5.1 Proteins involved in energy metabolism

Energy metabolism in plant comprises a series of interconnected pathways and different cell organelles are involved, Thus. different proteins contribute in energy metabolism. In this study we focus on two proteins involved in emery metabolism AtmSFC and ACO.

1.5.1.1 Mitochondrial succinate fumarate translocator

Mitochondrial succinate fumarate translocator (SFC) is a carrier protein in the inner mitochondrial membrane which provide a link between glyoxylate cycle in peroxisomes, TCA cycle in mitochondria and gluconeogenesis in the cytosol. SFC imports succinate into mitochondria and transfer fumarate into cytosol. In *Saccharomyces cerevisiae* this transporter encoded by acr1, and acr1 mutantion appeared to be lethal. AtmSFC share same structural features with other carrier proteins in the inner mitochondrial membrane, a molecular mass of 30 kDa, a tripartite structure (three repeats of 100 amino acids) and the presence of two transmembrane α-helices separated by hydrophilic loops in each repeat (Catoni et al., 2003).

1.5.1.2 Aconitase

Aconitase also known as aconitate hydratase is an enzyme that catalyzes reversible isomerization of citrate to isocitrate via the intermediate, cis-aconitate. Aconitase is localized in mitochondria and in cytosol (Hooks et al., 2014). The mitochondrial form of aconitase participates in the tricarboxylic acid cycle, whereas the cytosolic form operates in the reaction of glyoxylate cycle occur outside the glyoxysome. Glyoxysomes are a specialized form of peroxisomes found mainly in the cells of germinating seeds (Eprintsev et al., 2015). It has

been revealed that more than 90% of aconitase activity is cytosolic (Bellis et al., 1993, Hooks et al., 2014).

In *Arabidopsis thaliana*, aconitase is encoded by 3 aconitase genes (ACO1, ACO2 and ACO3). In newly emerged seedling of *Arabidopsis*, expression of ACO1 and ACO2 remained relativity low compared to ACO3 which account for 80% of aconitase activity. ACO3 mutant showed a delay in early seedling growth which was not visible in ACO1 and ACO2 mutants. However, the delayed growth in ACO3 mutant was not visible in older plant, this result indicates ACO1 and ACO2 compensate for lack of ACO3 in older plant, and ACO3 is the main aconitase responsible for citrate metabolism in newly emerged seedlings (Hooks et al., 2014).

It has been found that PP2A B'γ interact with ACO3 and regulate its phosphorylation level in the cytoplasm through using BiFC in the epidermal of *Nicotiana benthamiana* (Konert et al., 2015). B'γ regulatory subunit belong to B' η subfamily which include B' ζ and B' θ regulatory subunits. Phylogenetic tree of B' subunits show that B' γ and B' ζ belong to same phylogenetic clade (Figure 1.4). Sequence alignment shows 81% identity and 88% similarity between two regulatory subunits (Matre et al., 2009).

1.5.2 Organelles involved in energy metabolism

1.5.2.1 Peroxisomes

Peroxisomes are cell organelles found in virtually all eukaryotic cells, involved in diverse metabolic functions including glyoxylate cycle in germinating seeds, fatty acid-β-oxidation, metabolism of ROS and reactive nitrogen species (RNS), and photorespiration (del Río et al., 2002, Hayashi et al., 2003). Peroxisomal proteins are synthesized on free cytosolic ribosome and post-translationally targeted to peroxisomes with help of peroxisomal targeting signals (PTSs). The majority of peroxisomal matrix proteins possess a PTS1 at the C-terminal end consisting of the tripeptide SKL sequence or related variants (Reumann 2004). Only Few matrix proteins are targeted with peroxisomal PTS2 found near the N-terminus or via piggy-backing mechanisms (Corpas et al., 2009, Reumann 2004).

1.5.2.2 Mitochondria

Mitochondria are double-membrane bound organelles found in most eukaryotic organisms , Mitochondria are the main source of energy in non-photosynthetic cell as it produce ATP (adenosine triphosphate) through the process called oxidative phosphorylation. In addition,

the mitochondria play an important role in programmed cell death (Voet et al., 2016). Mitochondria made up of three main parts, the outer membrane, the inner membrane, and the matrix. The outer membrane is smooth and permeable and made up of phospholipid bilayers and proteins, whereas the inner membrane extensively less smooth and folded. The numerous invaginations of the inner membrane are called cristae (Figure 1.5). The inner membrane contains ATP synthase and transport proteins that regulate metabolite passage into and out of the matrix (Alberts et al., 1994).

1.6 Bimolecular fluorescence complementation

Bimolecular fluorescence complementation (BiFC) is a method used to study protein -protein interactions in living organisms (Citovsky et al., 2008). The BiFC assay is based on association of complementary fragments of a fluorescent protein. In the assay two proteins of interest are fused to two non-fluorescent fragments of a fluorescent protein. The interaction of these proteins brings together the non-fluorescent fragments, which may result in the reconstruction of an active fluorescent signal. Since both fused proteins may be directed to subcellular organelles the fluorescent signal is confined to specific subcellular compartments. (Citovsky et al., 2008).

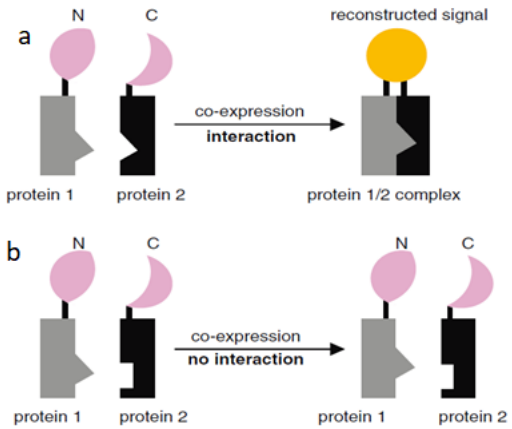

Figure 1.6: Principle of bimolecular fluorescence complementation analysis. A fluorescent protein reporter is split into two, N- and C-terminal, non-fluorescent fragments. Two proteins of interest (protein 1 and 2) are fused to resulting in a reconstructed fluorescent signal **(a)**. In the absence of protein interaction, no fluorescence is produced **(b)**. The figure is obtained from Citovsky et al (2008). these fragments. If the proteins interact with each other the non-fluorescent fragments are brought together

The BiFC assay was originally developed using the yellow fluorescent protein (YFP) (Hu et al., 2002). Venus is an improved version of YFP and has been shown to produce the highest fluorescence intensity of BiFC complexes (Kerppola 2006). Several other fluorescent proteins have been used such as cyan fluorescent protein (CFP) , blue florescent protein (BFP) , and red florescent protein (RFP) , offering BiFC complexes diffrent degrees of brightness and characteristics (Citovsky et al., 2008).

The visualization of protein-protein interactions in living cells with minimal disturbance of cellular environment is a major advantage of BiFC (Kerppola 2009). Another significant advantage is the sensitivity of The BiFC assay and allowing detection of weak and transient interactions (Hu et al., 2002). The BiFC assay requires minimal equipment with relative technical simplicity and does not require information about structure of proteins (Citovsky et al., 2008).

BiFC assay has been used to analyze protein-protein interactions in various plant species. In plant cell research BiFC assay unveiling various structures and mechanisms. Moreover BiFC has been used to image protein– protein interactions in various subcellular compartments for instance, ER, chloroplast, the vacuole and the nucleus (Citovsky et al., 2008).

1.7 Transgenic plants

Transgenic plants are plants that have been genetically modified using genetic engineering processes to introduce a new trait to the plant which does not occur naturally (Rani et al., 2013). Genetically modified plants are used as an important research tool in the hands of molecular biologists to understand the organization and regulation of eukaryotic genes (Bhat et al., 2002)

Transgenic plant offer significant benefits for instance improving production yield, enhancing the nutritional content , lowering transportation cost and production of plants resistant to herbicide pests and virus (Ahmad et al., 2012, Rani & Usha 2013). Transgenic plant that contain genes cod for bacterial enzymes has been used for bioremediation of contaminated soil with Mercury, selenium and organic pollutants, like as polychlorinated biphenyls (PCBs)(Rani & Usha 2013).

1.7.1 Development of transgenic plants

Transgenics plants are generated by altering the genetic-make-up of a plant's genome, where plant-cell's nucleus is the target for the new transgenic DNA. Most of genetically modified are obtained by biolistic delivery of DNA (Micro-Projectile Bombardment) or by *Agrobacterium* mediated transformation (Rani & Usha 2013). Other are being used to obtain transgenic plants include electroporation; and PEG (polyethylene glycol)-mediated uptake of DNA by protoplasts(Ahmad et al., 2012).

In present study we produced transgenic plant that contain PP2A B' ζ linked to YFP to study localization of PP2A B' ζ in the plant cell. The transgenic plant was produced using *Agrobacterium* mediated transformation.

The *Agrobacterium* mediated transformation method involve use of *Agrobacterium tumefaciens* which naturally transform plant cell through breaks and wound (Gelvin 2003) . *Agrobacterium tumefaciens* has the ability to infect the plant cell with a DNA which is called transfer DNA (T-DNA). T-DNA inserted into plant chromosome and altering the genetic-make-up through a tumor inducing plasmid (Ti plasmid) (Gelvin 2003, Rani & Usha 2013). The Ti-plasmid is a large cellular plasmid that replicate independently of bacterial chromosome and has ability to use plant 's cellular machinery make many copies of its own bacterial DNA (Rani & Usha 2013). Ti-plasmid contains virulence genes that code for proteins, this proteins help processing of T-DNA from the Ti-plasmid and transfer of T-DNA to recipient host cells (Gelvin 2003). In order to modify the plant genome, the gene of interest cloned into T-DNA region, then Ti plasmid can be transferred to a plant cell through a process known as the "floral dip". Its involve dipping of flowering plant in suspension of *Agrobacterium* carrying the gene of interest followed by collection of transgenic seeds (Rani & Usha 2013) . The major advantage of use of agrobacterium is transferring large fragments of DNA very efficiently(Chawla 2002).

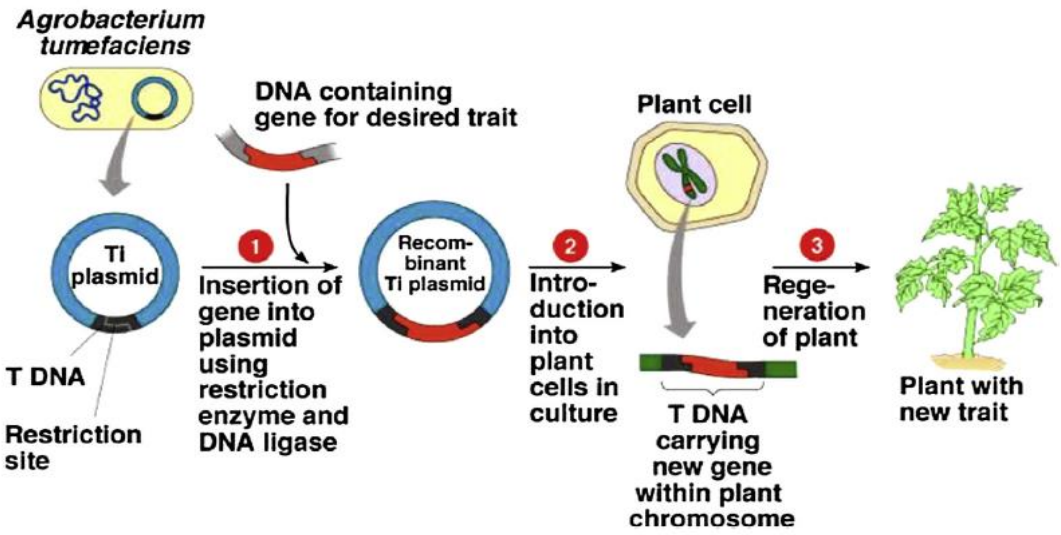

Figure 1.7**:** Agrobacterium mediated transformation. This method used to generate transgenic plant during present study. This figure obtained from Rani (2013)

1.8 Western blotting

Western blot also known as immunoblotting because an antibody is used to specifically detect its antigen. Western blot is an important technique used in immunogenetic and molecular biology to separate , detect and identify specific proteins from a complex mixture of proteins extracted from cells (Jin et al., 2015). A western blot experiment was introduced in 1979 by Towbin (Kurien et al., 2006).

Western blotting involves separation of proteins through SDS-PAGE gel electrophoresis based on molecular weight of proteins. Prior to electrophoresis protein samples boiled with denaturing agent such as DTT. Following electrophoresis the separated molecules are transferred to membrane (nitrocellulose or PVDF). The next step is to block membrane with blocking agent such as milk to avoid non-specific binding of antibody to surface of membrane. The membrane is then incubated with a combination of antibodies, first primary antibody specific to the protein of interest, followed by secondary antibody specific to the primary antibody. The secondary antibody often labelled with enzyme which will produce a detectable signal when combined with substrate (Kurien et al., 2009, Towbin et al., 1979).

1.8.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE separates proteins according to their molecular weight (Westermeier 2016) .Prior to SDS-PAG protein samples are treated with reducing agents such as 2-mercaptoethanol or DTT to reduce disulfide bonds to sulfhydryl groups resulting in denaturation of the secondary and tertiary structure of protein (Laemmli 1970). In addition to that the protein samples are equilibrated in an excess amount of SDS (anionic detergent) that bind to protein at a ratio of about 1.4 g SDS per gram protein (corresponding to one SDS molecule per two amino acid) giving a constant net negative charge per mass unit in all SDS-protein complexes. Thus, denatured proteins become covered in the negatively charged SDS and move to the positively charged electrode through the polyacrylamide gel when a voltage is applied (Reynolds et al., 1970, Westermeier 2016).

The polyacrylamide gel acts as a molecular sieve, larger molecules are more retarded by frictional resistance than smaller molecule (Alberts et al., 2013).The concentration of acrylamide determines the resolution of the gel (Brunelle et al., 2014).The polyacrylamide gel has two phases, the stacking and the separating gel. The Stacking gel usually 4% v/v acrylamide used to concentrate all the proteins into sharp bands prior to the separation. Whereas separating gel with higher concentration of acrylamide (10-12 %v/v) making the gel's pores narrower and used to separate proteins based on size (Westermeier 2016).

1.8.2 Protein transfer

Transfer of protein bands from gel to a membrane to make the proteins accessible to antibody detection done mainly through the electroporation method. Other methods include simple diffusion and vacuum assisted solvent. Protein molecules in gel move toward membrane as the membrane has higher affinity to protein molecules (Kurien & Scofield 2006).

Electroporation method which is method used in present study. Electroporation Involves use of electric current to pull negatively charged proteins from gel to membrane while maintaining the protein bands organization they had within the gel (Towbin et al., 1979). The significant advantages of electroporation are the speed and completeness of transfer compared to simple diffusion or vacuum blotting. Electroelution done by either complete

immersion of a gel-membrane sandwich in a buffer (wet transfer) or gel-membrane sandwich between absorbent paper soaked in transfer buffer (Kurien et al., 2003).

1.8.3 Blocking

Since the membrane has high affinity to proteins, blocking of membrane performed to prevents antibodies from binding to the membrane nonspecifically using blocking agent such as bovine serum albumin (BSA) and non-fat dry milk. Blocking agent bind to all free places in the membrane. So, when antibody is added it will bind only to target protein. eliminates false positive results (Mahmood et al., 2012).

1.8.4 Incubation with primary and secondary antibody

Two methods available to detect target protein on the membrane, direct and indirect method. The direct method, enzyme- or fluorophore-conjugated primary antibody used to detect antigen on the blot. While in the indirect method an unlabeled primary antibody is first used to bind to the antigen, followed by an enzyme- or fluorophore-conjugated secondary antibody specific to primary antibody (see Figure 1.8).The indirect method more preferred by researchers (Kurien & Scofield 2009). The indirect method was method used to detect YFP in present study.

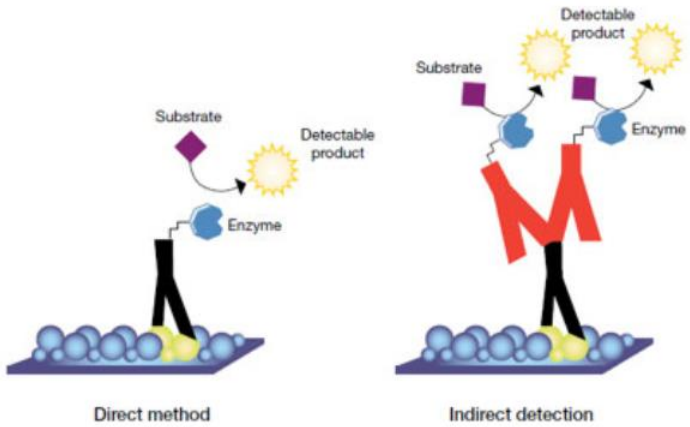

Figure 1.8: The direct and indirect method for detection the target protein on the membrane, this figure obtained from www.thermofisher.com

The choice of primary antibody depends on antigen to be detected. While choice of secondary antibody depends on either the species of animal in which the primary antibody was raised or tag linked to the primary antibody for example biotin, histidine and hemagglutinin (HA) (Kosaka et al.,).

1.8.5 Detection and visualization

The target protein on the blot was detect by aid of antibody labelled with either enzyme or fluorophore. Enzyme-conjugated antibodies are most commonly used for western blotting, because of the variety of substrates available. The Horseradish peroxidase (HRP) and alkaline phosphatase are the two enzymes used most extensively as labels for protein detection. HRP conjugated antibodies are preferred over AP labelled antibody due to smaller size , higher stability and lower cost of HRP enzyme (Gwozdz et al., 2016).

The simplest detection (documentation) method involve use either chromogenic or Chemiluminescent substrates. The chromogenic substrates allow direct visualization of signal development, but signal tend to fade as the blot dries. In the Chemiluminescent substrates the signal persists only as long as the reaction between the enzyme and substrate is occurring. a digital imaging system was used to visualize interaction signal and to obtain quantitative data from western blots (Gwozdz & Dorey 2016). In present study secondary antibody labeled with HRP and a chemiluminescent substrate to detect proteins on the membrane.

1.9 Objectives of study

1.9.1 Role of PP2B' subunits in regulation of metabolism.

PP2A regulatory subunits B' ζ and B' θ have been reported to have a role in energy metabolism, mutants of B' ζ and B' θ have shown growth retardation in sucrose free medium (Kataya et al., 2015b). Recently (at C. Lillo lab by A,Elshobaky, personal communication) PP2A B' ζ was shown to interact with two proteins involved in energy flow to mitochondria, *Arabidopsis* mitochondrial succinate fumarate translocator (AtmSFC) and cytosolic aconitase 3 (ACO3). Another study indicated that cytosolic ACO3 interact with PP2A B' γ (Konert et al., 2015). The main objectives of the present study were:

- To investigate possibility of interaction between AtmSFC and other members of PP2A B' Subunits (B' α, B' β and B' θ) by BiFC.
- To explore the possibility of interaction by ACO1 (isoenzyme of ACO3) and truncated ACO1 with PP2A B' ζ by BiFC. Truncated ACO1 made to explore importance of C-terminal end in interaction with PP2A B' ζ.

1.9.2 Study PP2A B' ζ subcellular localization

In vivo subcellular targeting analysis has shown the *Arabidopsis* PP2A regulatory subunit B´ ζ with a free N-terminus to be targeted to cytoplasm and partly localized to mitochondria and excluded chloroplast and nucleus. However, protein subcellular localization programs predicated that PP2A B' ζ would be localized in cytoplasm, chloroplast and mitochondria (Matre et al., 2009).

In the present study we wanted to employ another method to determine PP2A B' ζ subcellular localization. PP2A B' ζ fused at its C-terminus to YFP, cloned to a binary vector and transformed to wild type and knocked out mutant PP2A B' ζ *Arabidopsis* through *Agrobacterium* mediated transformation to generate transgenic plants. Cell fractionation of transgenic plant followed by western blotting were used to study PP2A B' ζ localization in different cell compartments (Nucleus, chloroplast, mitochondria and cytoplasm).

2 MATERIALS AND METHODS

2.1 Material

2.1.1 Biochemicals

Biochemical used in present study were purchased from Bio-Rad (Hercules, CA, USA), Invitrogen (Carlsbad, CA, USA), Merck (Damstadt, Germany) and Sigma-Aldrich/Fluka (St. Louis, MO, USA).

2.1.2 Kits

Commercial kits:	Source:
GenElute^{TM} Plasmid Miniprep Kit	Sigma-Aldrich (St. Louis, MO, USA)
GenElute^{TM} HP Plasmid Midiprep Kit	Sigma-Aldrich (St. Louis, MO, USA)
GenElute^{TM} PCR Clean-Up Kit	Sigma-Aldrich (St. Louis, MO, USA)
Phire™ Hot Start II DNA Polymerase	Thermo Fisher Scientific (Waltham,MA, USA)
RNeasy®Plant Mini Kit	Qiagen (Hilden,Germany)
High capacity cDNA Reverse Transcription Kit	Applied biosystem, USA
Quick Start™ Bradford protein	Bio-Rad (Hercules, CA, USA)
Mini-PROTEAN® TGX™ precast gels	Bio-Rad (Hercules, CA, USA)
Trans-Blot® Turbo™ transfer system,	Bio-Rad (Hercules, CA, USA)
Clarity ™ western ECL substrate	Bio-Rad (Hercules, CA, USA)

2.1.3 Enzyme

Enzyme:	Source:
Dream Taq DNA polymerase	Thermo Fisher Scientific(Waltham,MA, USA)
Expand High Fidelity DNA polymerase	Invitrogen (Carlsbad, CA, USA)
Restriction enzymes	New England Biolabs (Ipswich, MA, USA)
T4 DNA Ligase	Promega (Madison, Wi, USA)
MultiScribe™ Reverse Transcriptase	Thermo Fisher Scientific (Waltham,MA, USA)
Phire Hot Start II DNA polymerase	Thermo Fisher Scientific (Waltham,MA, USA)

2.1.4 Plant material and growth condition

In the present study *Arabidopsis thaliana* ecotype Columbia was used as wild type, Two *Arabidopsis* T-DNA insertion mutant lines PP2A B’ ζ SALK_ 150586 and SALK_ 107944 in the Columbia genetic background were obtained from European Arabidopsis Stock Centre (NASC, Nottingham, UK). Knockout effects of T-DNA insertions were verified by PCR for SALK_107944 and SALK_150586 using gene-specific primers.

Seeds were sown on soil and transferred to controlled growth room were specific photoperiod condition (long day 16 h light followed by 8 h dark) with temperature varied between 20°C and 22°C. Two times per a week the plants were irrigated with complete Hoagland solution (Hoagland et al., 1950).

Hoagland solution composition:

Chemicals	Concentration
KH2PO4	1 mM
KNO3	5 mM
Ca(NO3)2:4H2O	5 mM
MgSO4:7H2O	2 mM
Fe-EDTA	1 µM
H3BO3	46.23 µM
MnCl2:4H2O	9.2 µM
CuSO4:5H2O	0.36 µM
ZnSO4:7H2O	0.77 µM
Na2MoO4:H2O	0.12 µM

2.1.5 Bacterial strains

Two bacterial strains use in present study, *Escherichia coli* (*E. coli*) *strain* JM109 was used for vector amplification and *Agrobacterium tumefaciens* strain ABI was used for transformation of *Arabidopsis thalian* . Both were provided by Prof. Cathrine Lillo.

2.1.6 DNA material

2.1.6.1 cDNA constructs

Full length cDNA of *Arabidopsis* ACO1 (AT4G35830.1) were obtained from RIKEN BRC (Ibaraki, JAPAN). The cDNA of ACO1 had been inserted to cloning vector pda03083 which carry a ampicillin resistance gene for selection of *E. coli* transformed with vector. Full length cDNA of *Arabidopsis* PP2A B' subunits (B' α, B'β, B' θ and B'ζ) were kindly provided from Dr. Behzad Heidari (university of Stavanger). The list of cDNA constructs is shown in Table 2.1

Table 2.1: list of Genes used in present study and their sources

Gene name	Gene ID	Vector	Source
ACO1	AT4G35830	pda03083	RIKEN BRC(JAPAN)
PP2A B'α	AT5G03470	pWEN18	Dr. Behzad
PP2A B' β	AT3G09880	pWEN25	Dr. Behzad
PP2A B'Θ	AT1G13460	pWEN18	Dr. Behzad
PP2A B'ζ	AT3G21650	pWEN 18	Dr. Behzad
AtmSFC	AT5G01340	pVYNEpUC19	Dr. Ahmed

2.1.6.2 Bimolecular fluorescence complementation vector

Plant compatible Bimolecular fluorescence complementation vectors used in present study were acquired from University of Münster (Münster, Germany). The BiFC vectors used in the study are listed in Table 2.2

Table 2.2: BiFC vectors used in the present study.

Nr.	**Name**	**Vector**	**Fluorophore**	**Multiple cloning site**	**Selection bacteria**
6	pVYCE	pUC19	VenusC155	N of Tag	Ampicillin
7	pVYCE(R)	pUC19	VenusC155	C of Tag	Ampicillin
8	pVYNE	pUC19	VenusN173	N of Tag	Ampicillin
9	pVYNE(R)	pUC19	VenusN173	C of Tag	Ampicillin

The BiFC vectors based on interaction of two parts of yellow fluorescent protein (YFP) obtained from jellyfish *Aequorea victoria.* The BiFC vectors encode either the N-terminal amino acids 1-173 (pVYNE and pVYNE(R); Figure 2.1) of Venus or the C-terminal amino acids 156-239 (pVYCE and pVYCE(R); Figure 2.1) of Venus. These vectors enable expression of protein of interest fused at either their C-terminus to the C- or N-terminal fragments of Venus (pVYNE and pVYCE; Figure 2.1a) or fused at their N-terminus to the C- or N-terminal fragments

of Venus (pVYNE(R) and PVYCE(R); Figure 2.1b).The BiFC expression cassettes have been inserted into the pUC19 vector backbone. The pUC19 vector carries an ampicillin resistance gene for selection of transformed bacterial cells with the vector. The BiFC expression cassettes in pUC19 vector expressed under control of the cauliflower mosaic virus (CaMV) 35S promoter and the terminator of the Nos gene (NosT).

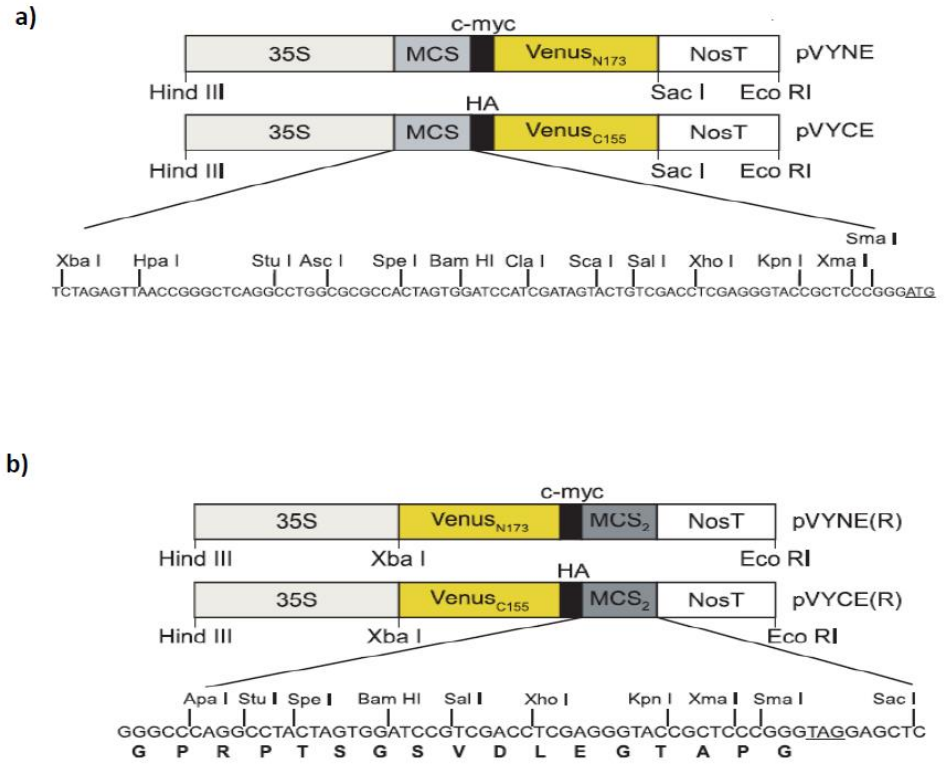

Figure 2.1: Schematic representation of the BiFC vectors a) pVYNE and pVYCE. b) pVYNE(R) and pVYCE(R). 35S, 35S promoter of the cauliflower mosaic virus (CaMV). NosT, terminator of the Nos gene. MCS, multiple cloning site. $Venus_{N173}$, Venus N-terminal fragment reaching from amino acid 1 to 173. $Venus_{C155}$, Venus C-terminal fragment reaching from amino acid 156 to 239. HA, hemagglutinin epitope tag .c-myc, epitope tag. Restriction sites in the expression cassette are indicated. The figure was obtained from (Waadt et al., 2008).

2.1.6.3 T-DNA binary vector

In present study binary vector pBA002 was used. pBA002 vector has plant selectable marker glufosinate ammonium (BASTA), 35S promoter of the cauliflower mosaic virus (CaMV) and bacterial selectable marker (spectinomycin) to enable selection of transformed bacterial colonies (See Figure 2.2).

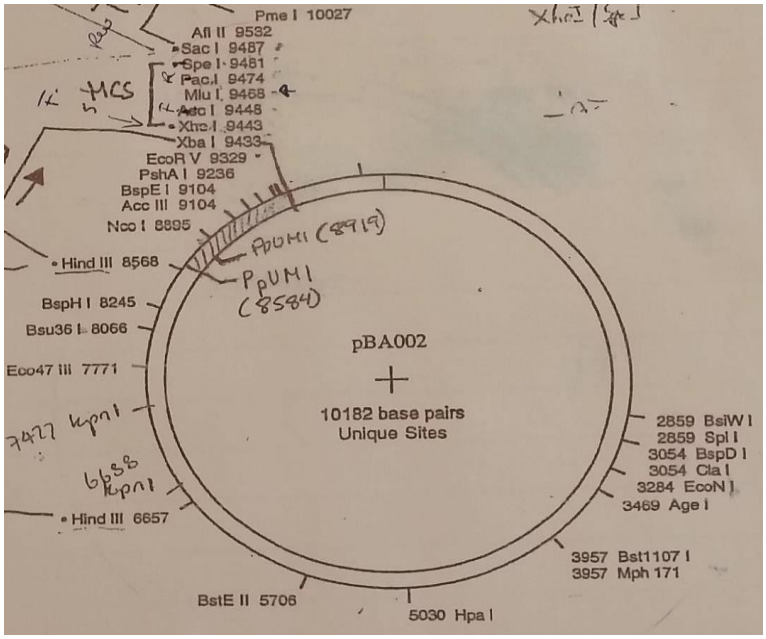

Figure 2.2: Schematic representation of pBA002 vector shows restriction sites. Size of pBA002 vector 10.1Kb

2.1.6.4 Oligonucleotide primers

For amplification of cDNAs by polymerase chain reaction (PCR), a pair of gene-specific primers (forward and reverse) flanked with desired restriction endonuclease sites were designed and ordered from Eurofins Scientific and Invitrogen. List of primers and restriction endonuclease sites used in present study are shown in Table 2.3.

Table 2.3: Primers used in present study. Restriction endonuclease sites are marked in red. ACO1* indicate truncated ACO1.

Name		Primer (5'.....3')	R.E	Destination vector
Primers used for cloning into BiFC vectors				
ACO1	F	GTCGAC ATG GCT TCC GAG AAT CCT TTC	SalI	BifC vector Nr.8
	R	GGTACC TTG TTT GAT CAA GTT CCT GAT	KpnI	
ACO1*	F	GTCGAC GCTTCCGAGAATCCTTTCCG	SalI	BifC vector Nr.8
	R	GGTACC CGTTAGGCCAAGGGTCTCAG	KpnI	
PP2A B'α	F	GTCGAC AGATCATGAAAGGGGCAAATCG	SalI	BifC vectors Nr.7 and Nr.6
	R	GGTACC TCTCGTTCCTCCACTGCTTC	KpnI	
PP2A B' β	F	GTCGAC AATCATGAAAGGTGGGCATCG	SalI	BifC vectors Nr.7 and Nr.6
	R	GGTACC TCTTCTTCTCCTCCTCCTCCA	kpnI	
PP2A B' θ	F	AGTACT TGTGGAAACAGATTCTGAGTAAGC	SacI	BifC vectors Nr.7 and Nr.6
	R	CTCGAG GCTTTTGATTACCAATTTCTTCCAA	XhoI	
Primers used for sequencing of BiFC vectors				
35s	F	CCCACTATCCTTCGCAAGAC		
NosT	R	GACCGGCAACAGGATTCAAT		
Primers used for cloning and sequencing PP2A B'ζ -YFP				
PP2A B'ζ-YFP	F	CTCGAG ATG ATC AAA CAG ATA TTT GGG	XhoI	pBA002
	R	TTAATTAA TTA CTT GTA CAG CTC GTC CAT	PacI	
Primers used for genotyping and gene expression analysis of PP2A B'ζ -YFP				
PP2A B'ζ-YFP	F	GGACATGGCAACGGTTAGAAG		
	R	TTGTAGTTGCCGTCGTCCTT		
Primer used for sequencing PP2A B'ζ -YFP				
PP2A B'ζ	R	CGACCCTGTGGACTCAGAGCTGC		

2.2 Molecular cloning and bimolecular fluorescence complementation analysis

2.2.1 Polymerase chain reaction (PCR)

Polymerase chain reaction was used for genotyping, amplification of cDNA flanked with endonucleases restriction sties and colony PCR. All PCR reaction were carried out using T100™ Thermal Cycler (Bio-Rad). A proofreading DNA polymerase (Expand High Fidelity DNA polymerase) was used for gene cloning while Taq DNA polymerase (Dream Taq DNA polymerase) was used for screening of successful plasmid transformation (colony PCR).

Components of the PCR reactions are presented in Table 2.4 and 2.5. Table 2.4 shows the components needed for a preparative PCR with a proofreading DNA polymerase (Expand High Fidelity DNA polymerase). Table 2.5 shows the components for a colony PCR and genotyping with Taq DNA polymerase (Dream Taq DNA polymerase).

Table 2.4: Reaction composition in PCR using Expand High Fidelity DNA Polymerase.

Component	**Volume, µl**	**Final conc.**
Nuclease free water	32.5	to 50 µl
10x buffer High Fidelity PCR buffer with MgCl2	5.0	1x
10 mM dNTPs	1	200 µM
Template DNA	1.5	about 200ng
10 µM forward primer	5	1 µM
10 µM reverse primer	5	1 µM
High Fidelity DNA polymerase	0.5	2.5 U

For colony PCR, instead of template DNA, sterile tips were used to touch the isolated *E. coli* colonies on a Luria-Bertani (LB) plate and added directly to PCR mix followed by pipetting up and down to ensure good mixing.

Table 2.5: Reaction composition in PCR using Dream Taq DNA Polymerase.

Component	**Volume, µl**	**Final conc.**
Nuclease free water	19.375	to 25 µl
10x Dream taq buffer	2.5	1x
10 mM dNTPs	0.5	200 µM each
10 mM forward primer	1.25	0.5 mM
10 mM reverse primer	1.25	0.5 mM
Dream Taq DNA polymerase (5 U/µl)	0.125	0.025 U

After mixing either templet DNA or isolated E. coli colonies with components of PCR mix samples placed in thermocycler T100™ Thermal Cycler (BioRad) and subjected PCR programs shown on table depend on type of DNA polymerase.

Table 2.6: PCR programs used for amplification of cDNAs (Expand High Fidelity DNA polymerase) and for colony PCR (Dream Taq DNA Polymerase).

Step	Cycle	Expand High Fidelity DNA polymerase		Dream Taq DNA Polymerase	
		Temperature	Time	Temperature	Time
Initial denaturation	1	94°C	2 min	95°C	3 min
Denaturation	34	94°C	30 sec	95°C	30 sec
Annealing		Ta	30 sec	Ta	30 sec
Extension		72°C	1 min -3 min	72°C	1 min -3 min
Final Extension	1	72°C	10 min	72°C	10 min
Infinitive hold	1	4°C	Unlimited time	4°C	Unlimited time

The annealing temperatures for primer pairs were calculated using New England Biolab calculator (https://tmcalculator.neb.com). Extension time depend on fragment length for Expand High Fidelity DNA polymerase 1 min per 1.5 Kb was used while Dream Taq DNA Polymerase 1 min per 1Kb.

2.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used for determination size and concentration of PCR products, restriction digest and for purification of digested vectors. Agarose gel comprise of 1% (w/v) agarose melted in 1x TAE buffer. Before loading the sample on agarose gel, Crystal 5x DNA Loading Buffer Blue (Bioline) was added (about 1 volume of loading buffer to 4

volumes of sample). GelRed™ (Biotium) fluorescent nucleic acid dye was add previously to loading buffer to make DNA bands visible under UV-light exposure. A Hyper-ladder 10 Kb (Bioline) used to determine size of PCR products (Figure 2.3). Samples separated by electrophoresis which carried out at 90 for 45 min in a Power Pac Basic electrophoresis chamber (Bio-Rad) using 1x TAE buffer as running buffer. Agarose gels were visualized with UV-light using ChemiDoc™ Imaging Systems (Bio-Rad) (Hercules, CA, USA).

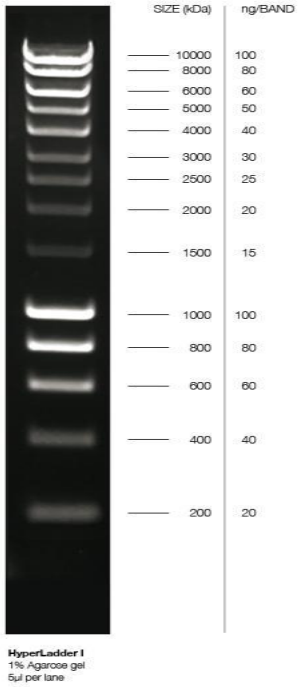

Figure 2.3: Overview of the Hyper-ladder fragments size and concentration (per 5 µl).

TAE (Tris-acetate-EDTA) buffer (50 X)

Tris-acetate, pH 8.3	2 M
EDTA	50 mM

2.2.3 Purification of PCR products

PCR products and restriction endonuclease digested PCR were purified using NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL). Two volumes of Buffer NTI1 mixed with one volume of sample, in case of extraction of DNA from gel, samples Incubated for 5–10 min at 50 °C and subjected for Vortex every 2–3 min until the gel is completely dissolved. Samples transferred the binding column were the DNA is bound to the silica membrane of column. The column

was centrifuged for 30 Sec at 11,000 x g and the eluate discarded. Contaminations were removed by double washing with ethanolic wash buffer NT3 by centrifugation for 30 sec at 11,000 x g and the eluate discarded. Finally, the pure DNA is eluted with warm sterile nuclease free water.

2.2.4 Restriction endonuclease digestion of DNA

Vectors and inserts were digested with restriction endonucleases to produce blunt-ended dsDNA. Restriction site introduced to termini of the cDNAs by PCR. About 500 ng of DNA (insert or vector) mixed with 3 µL of 10x NEBuffer, 0.5 µL of each restriction enzymes, nuclease-free water (to total volume of 20 µl). Suitable restriction buffer for two restriction enzymes determine using NEB double digest finder program (New England biolab) to get highest activity of both restrictions enzymes.

2.2.5 Ligation

Vectors and inserts digested with same restriction enzymes resulting complementary cohesive sticky ends which are joined using T4 DNA ligase (Promega). A stoichiometric ratio of vector-to-insert of approximately 1:3 was used for the ligation of inserts into vectors. The stoichiometric ratio was calculated by:

$$3\times\frac{amount\ of\ vector}{size\ of\ vector}=\frac{amount\ of\ insert}{size\ of\ insert}$$

A ligation reaction contained 1 µl (1 U/µl) T4 DNA Ligase, 1 µl of 10 x Ligase Reaction Buffer, vector and insert in an amount to provide a molar vector: insert ratio of about 1:3 (about 25ng vector was generally used) and water (to a total volume of 20 µl). The ligation reaction was gently mixed placed at room temperature for 1 h, followed by overnight incubation at 4°C.

2.2.6 Preparation of competent *E. coli* JM 109 cells

Cell stock of *E. coli* JM 109 Streaked on LB medium plate to get isolated colonies. The plate incubated at 37 °C for 24 h. Single cell isolated colony incubated with 5 mL of LB broth and incubated at shaker incubator at 37 °C (220 rpm) for 24 h. 1 ml of saturated LB culture transformed to 100 ml of LB broth placed incubator at 37 °C (220 rpm) until OD_{600} reach 0.5. After that 100 LB *E. Coli* culture placed on ice for 20 min and cells collected by centrifugation at 2500 rpm for 10 min. The cells resuspended by in 10 ml of TSS media and divided to small aliquots of 150 µl which is ready for transformation reaction.

TSS media composition (TSS: transformation and storage solution for chemical transformation)

85% LB medium

10% PEG (W/V, MW 8000)

5%DSMO (V/V)

50mM Mgcl2 (pH=6.5).

2.2.7 Transformation of competent *E. coli* JM 109 cells

Competent *E. coli* JM 109 were thawed on ice. Volume of 5 µl ligation reaction or plasmid DNA were added to 150 µl of competent cells and mixed gently by pipetting and incubated on ice for 30 min. The cells were subjected to heat shock at 42 °C for 1 min and immediately placed on ice for 2 min. LB broth (500 µl) added to competent cells and cells incubated on shaker at 37°C for about 2 h to allow cell to express antibiotic resistance gene. Next, cells were distributed on LB agar plates supplemented with suitable antibiotic according to antibiotic resistance marker gene in the vector, plates incubated at 37°C for 24 h. In case of BiFC vectors ampicillin (100µg/mL) was used. While In case pBA002 binary vector spectinomycin (50µg/mL) was used.

LB agar (Merck):		LB broth (Merck):	
Peptone from casein	1%	Peptone from casein	1%
Yeast extract	0.5%	Yeast extract	0.5%
NaCl	1%	NaCl	1%
Agar-agar	1.2%		
pH was adjusted to 7.0		pH was adjusted to 7.0	

2.2.8 Isolation of plasmid DNA

Plasmid DNA was isolated from recombinant bacterial cell using NucleoSpin® plasmid miniprep kits (MACHEREY-NAGEL). of LB broth (5 ml) was supplemented with suitable antibiotic (ampicillin 100 µg/ml or spectinomycin 50 µg/ml) and inoculated with a single recombinant bacterial colony. Followed by overnight incubation at 37°C in a shaker. The bacterial cells were harvested by centrifugation at 11,000 Xg for 30 sec. The bacterial pellet resuspended in 250 µg cell resuspension buffer. The resuspended cells were then lysed by adding 250 µL of lysis solution. The samples were mixed gently by inverting the tube 6-8 times and incubated for 5 min at room temperature after incubation the cell debris was precipitated by adding 300 µL Neutralization buffer. The tube gently inverted 6-8 times and the cell debris collected by centrifugation at 16000Xg for 10 min. The supernatant was transferred to NucleoSpin Plasmid (NoLid) Column which placed into collection tube then samples centrifugated at 11,000 xg for 1 min, flow through discarded and bound DNA washed using 500 µL of preheated (50°C) buffer AW by centrifugation at 11000 xg for 1 min. Another washing buffer supplemented with ethanol used to wash column by centrifugation at 11000 xg for 1 min. the flow-through was discarded and centrifuged at maximum speed for 1 min to remove residual wash solution, the column was transferred to a new tube and plasmid DNA eluted by adding 30-40 µL warm nuclease free water. The plasmid DNA was stored at -20°C.

2.2.9 Sequencing

After cDNA cloned to vectors and verified by colony PCR and restriction digestion. The plasmids were sent for sequencing. The sequencing was done by Microsynth Seqlab (GmbH Germany). The purpose of sequence to ensure that correct nucleotide sequence of cDNA and its deduced amino acid sequence. In case of BiFC vector forward primer specific for 35S promoter gene, reverse primer specific for NoTs terminator gene. while in case of pBA002 binary vector gene specific primer used. The plasmid DNA and primer was mixed and shipped according to the instruction given by Microsynth Seqlab.

2.2.10 DNA precipitation onto gold particles

gold particles (50 mg) were resuspended in 1 ml ethanol and vortexed for 2 min. The gold particles were then sedimented by centrifugation at 10,000 xg for 3 seconds and the supernatant removed. The washing step was repeated twice. After the last washing the gold particles were resuspended in 1ml water, vortexed and aliquoted in 50 µl aliquots. The next steps were performed on the ice. The following components were added one after the another in given order and vortexed for 2 min after each addition. 5 to 7 µl plasmid DNA (1 µg/µl) (final conc.: 40 ng/µl), 50 µl 2.5 M $CaCl_2$ (final conc.: 1 M) and 20 µl 0.1 M Spermidine (final conc.: 10 mM). The DNA was then precipitated onto the gold particles by 10,000×g for 3sec. The particles were resuspended in 250 µl ethanol, vortexed and sedimented by centrifugation at 10,000×g for 3 sec. The supernatant was removed. This washing step was repeated three times. The particles were finally resuspended in 60 µl ethanol and ready for use for the transformation of onion epidermal cell by bombardment.

2.2.11 Transformation of onion epidermal cells by bombardment

The PDS-1000/He system Particle Delivery system (Biorad) was used for transformation of onion epidermal cells (*Allium cepa L*). The PDS-1000/He system accelerate nucleic acid – coated microparticle to velocities necessary to transfect cells. The burst of high-pressure helium gas used to accelerate a plastic macrocarrier disk carrying microparticle toward target cells. A stopping screen retains the plastic microcarrier from reaching target cell while the DNA coated particles continue to the target and transform the target cells.

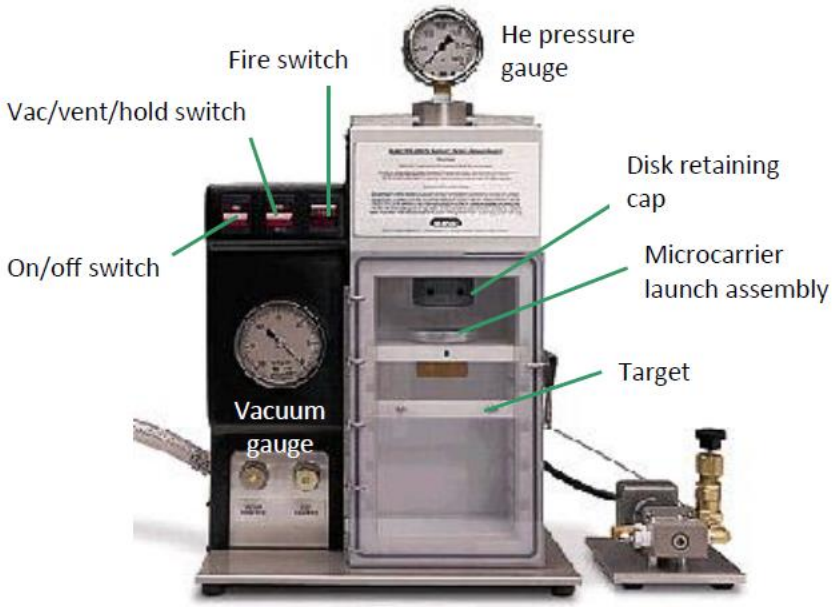

Figure 2.4: Biolistic PDS-1000/He Particle Delivery system.

Prior to bombardment a fresh onion was peeled and cut into appropriate 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 washed with 70% ethanol. The suspension of gold particles coated with the desired plasmids was vortexed thoroughly and 5 µl were loaded onto the macrocarrier holder. The helium bottle was opened, and the pressure adjusted to about 1600 PSI. 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 microcarrier launch assembly. The microcarrier launch assembly was placed into the top shelf and the targeted onion cells placed into the third shelf. The chamber room was closed, the vacuum pump turned on and the power switch on the bombardment device turned on. The vacuum was lowered to about 270 inches Hg and then held. The fire button was pushed until rupture of the rupture disk. The pump was turned off and the vacuum slowly released. The onion was left in Petri dish for about 15-24 h. The epidermal cell layer was peeled off and put on a microscopy slide for analysis by fluorescence microscopy.

2.2.12 Fluorescence microscopy

Microscopy analysis of onion epidermal cells was performed using a Nikon TE-2000U inverted fluorescence microscope. Nikon TE2000 inverted microscope equipped with filters for CFP (cyan fluorescence protein), YFP (yellow fluorescence protein) and RFP (red fluorescence protein). The lens with 20X magnification power was used to examine onion epidermal cells. Images were captured using a Hamamatsu Orca ER 1394 cooled CCD camera and a computer was connected to the microscope to store the pictures.

Figure 2.5: Nikon TE-2000U inverted fluorescence microscope

2.3 Study PP2A B’ ζ subcellular localization

A pair of primer designed to pick up PP2A B’ζ and YFP (forward for PP2A B’ζ and reverse for YFP) from pWEN 18 vector to be directional cloned into binary vector pBA002 (See Table 2.3). The forward primer and reverse primer flanked with desired restriction endonuclease sites XhoI and PacI, respectively. After successful cloning and sequencing results had been revealed. Recombinant plasmid isolated from *E. coli* and transformed to competent *Agrobacterium tumefaciens* ABI. The recombinant *Agrobacterium* were used to transform *Arabidopsis* wild type ecotype Colombia and two knocked out mutants PP2AB’ζ SALK_ 150586 and SALK_107944 through floral dipping protocol. In the study The non-recombinant binary vector pBA002 has been transformed to *Arabidopsis thaliana* wild type and two PP2A B’ ζ mutant lines to be used as controls.

2.3.1 Cultivation of *Arabidopsis* plants

Arabidopsis seeds (5 to 10) were sown directly on soil in each pot and transferred directly into plantroom (16 h light/ 8 h dark) to grow. After approximately 6 weeks, when the plants had grown shoots and started flowering. The inflorescence shoots were cut to encourage plant growth. The plants were ready for transformation after one week. Three days prior to flower dipping plant stopped watering to avoid falling out of pots during dipping.

Figure 2.6: *Arabidopsis* plants 7 to 8 ready for agrobacterium transformation

2.3.2 Preparation of competent *Agrobacterium tumefaciens* ABI

Isolated colony of agrobacterium incubated overnight at 28 °C with 5 ml of LB broth to make start culture. In The next day start culture added to 500 ml LB broth and incubated at 28 °C with vigorous agitation until cells had reached OD550 (0.5-0.8) . The cells pelleted by centrifugation at 4000 xg for 10 min at 4 °C. Ice-cold sterile double distilled water (5-10 ml) and wide bore pipette used to pipette cells gently up and down till no clamps. the pervious step repeated twice but with different volume of ice-cold sterile double distilled water 250 ml and 50 ml respectively. In final step cells resuspend in 5 ml of 10% (v/v) ice-cold sterile glycerol, divide into 100 µl aliquots snap freeze in liquid nitrogen and stored at -70 °C.

2.3.3 Transformation of competent *Agrobacterium tumefaciens* ABI

Competent cells of Agrobacterium tumefaciens ABI were thawed on ice. 5 ml of plasmid DNA (*E. coli* mini prep DNA) were added to 100 µl competent cell and mixed gently. The mixture kept on ice for 5 min then transferred to liquid nitrogen for 5 min. The cells given a heat shock for 5 min in 37 °C water bath. 1 ml of LB broth added to mixture. Next, the mixture incubated at 28 °C at shaker incubator at 200 rpm for 2 hr. The cells collected by centrifugation at 11000 xg for 1 min and disturbed on LB agar plate containing appropriate antibiotic for Agrobacterium (rifamycin 50 µg/ml) and for binary vector pBA002 (spectinomycin 50 µg/ml) was used. The plate incubated for 48 hr at 28 °C.

2.3.4 *Agrobacterium* mediated transformation of *Arabidopsis* by flower dipping

2.3.4.1 Overnight cultures for dipping medium

Isolated colony of *Agrobacterium* carrying suitable binary vector inoculated with 5 ml of LB medium and incubated at 28 °C at 220 rpm for 48 hr. LB medium containing appropriate antibiotic for binary vector pBA002 spectinomycin (50 µg/ml) and agrobacterium (rifamycin 50 µg/ml). After 2 days, 200 ml of LB medium inoculated with 1 ml of preculture and incubated at vigorous agitation for 24 hr at 28 °C. The cells pelleted by centrifugation at 6000 rpm for 10 min and resuspend in 400 ml of infiltration medium (alternativity named dipping medium).

Infiltration medium composition (1 L)

0.5 X Murashiga and Skoog salts

5% (w/v) sucrose

50 µl/L Silwet L-77

2.3.4.2 Dipping of plants

Two months old, flowering *Arabidopsis* plants, containing several flowers were placed upside down in suspension of transformed *Agrobacterium* cells in infiltration medium (Figure 2.7) . The plant were soaked for 10 min then were removed, placed on their side and covered for 24 hr at dark room. The next day the cover were removed and plants rinsed with water and retained to normal growth condition (16 hr light / 8 hr dark). After 3 to 4 weeks seeds were collected.

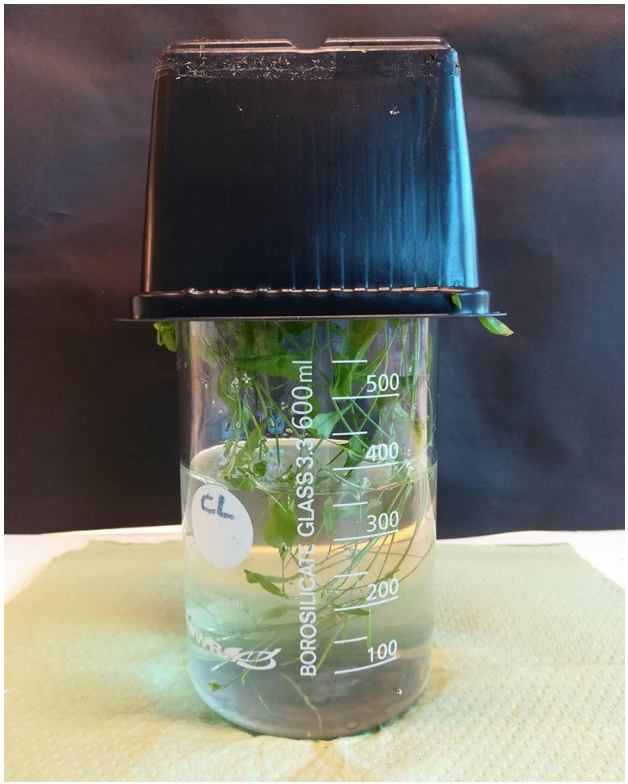

Figure 2.7: Dipping of *Arabidopsis thaliana* in dipping media containing transformed Agrobacterium.

2.3.5 Screening (Selection) of transformed plants

Approximately 1 month after flower dipping. The seeds were harvested for and sterilized according to the following sterilization protocol. The solution of 70% ethanol and 0.01% Triton (1 ml) were added to Eppendorf tube containing seeds and the tubes were placed on a shaker for 10 minutes. The solution of 70% ethanol and 0.01% Triton was removed and replaced with 99.5% ethanol (1 ml). The tubes were then put on a shaker for 10 minutes. The ethanol was removed and replaced with another 1 ml 99.5% ethanol. The tubes were flicked, the ethanol removed, and the seeds were left in the sterile hood for drying.

After the sterilization, the seeds of flower dipped plant were sown on plate containing ½ Murashige and Skoog medium (½ MS medium) (Sigma-Aldrich). The pH of medium was adjusted to pH 5.8. and solidifying agent (0.4%) was added. Plant herbicide glufosinate ammonium (BASTA) with concentration of 10µg/ml was added to medium snice binary vector pBA002 contain plant selectable marker, so only transformed plants will able to grow on medium. Controls of wild type and mutant lines which non-flower dipped (normal seeds) were used on same plate to ensure efficacy of (BASTA).

The plates were then placed in a dark and cold room 4°C for 48 hr before placed in plant room (16 h light/8h dark). After approximately for two weeks. The seedlings showed resistances to BASTA transferred to soil, two weeks after transfer of seedlings, genotyping was performed on seedling.

2.3.6 Genotyping of transformed plants

Genotyping of transformed plants was performed for both T1 and T2 generation plants using Phire™ Plant ™ Direct PCR Kit ,Thermo Fisher Scientific (Waltham,MA, USA). This kit designed to perform PCR directly on plant leaves and seeds without prior DNA isolation steps. For genotyping of transformed plant with pBA002 (PP2A B’ ζ-YFP) new primers designed, the primer designed to anneals to end of PP2A B’ ζ and start of YFP giving product size of 451 bp. Table 2.7 shows components of PCR mix used in genotyping . Table 2.8 illustrate PCR program used.

After one week of transfer seedling to soil. A piece of small leaf approximately 2 mm in diameter was cut using puncher and placed in to 20 µl of dilution buffer. The leaf crushed

against wall of tube using 100 µl pipette tips. The samples centrifuged at low speed to settle plant material down and 0.5 µl of supernatant was used as template for a 20 µl PCR reaction.

Table 2.7: Reaction composition in PCR using Phire™ Hot Start II DNA Polymerase.

Component	**Volume, µl**	**Final conc.**
Nuclease free water	19.375	to 20 µl
2X Phire Plant PCR Buffer	10	1x
Plant tissue (dilution buffer)	0.5	
Forward primer	0.4	0.5 µM
Reverse primer	0.4	0.5 µM
Dream Taq DNA polymerase	0.4	

Table 2.8: PCR program used for Phire™ Hot Start II DNA Polymerase

Cycling step	Temperature	Time	Cycles
Initial denaturation	98 °C	5 min	1
Denaturation	98 °C	5 s	40
Annealing	62°C	5 s	
Extension	72 °C	20s ≤ 1 Kb	
Final Extension	72 °C	1 min	1
Infinitive hold	4°C	∞	1

2.3.7 Gene expression analysis

Gene expression analysis performed on seedlings second generation transgenic plant to verify formation of gene product. First total RNA isolated from plant followed by reverse transpiration reaction to get cDNA finally PCR reaction using Dream Taq DNA polymerase used to test amount of expressed gene.

2.3.7.1 RNA extraction from transformed plants

The total RNA isolated from seedling of T2 generation transgenic plant using RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA, USA) following the protocol provided by manufacturer. This kit isolate DNA free RNA since the silica membrane remove most of the genomic DNA.

2.3.7.2 Reverse transcription (synthesis of cDNA)

The isolated RNAs were used for synthesis cDNAs (complementary DNAs) using The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA, USA). The Generated single-stranded cDNA suitable for quantitative PCR applications. The 2X reverse transcription master mix prepared on ice (Table 2.9) and mixed with the 2µg RNA in 20 µl reaction volume to create the final 1X RT master mix, the sample placed in thermocycler to perform reverse transcription performed according PCR program in Table 2.10

Table 2.9: components of reverse transcription master mix.

Component	Volume/Reaction (µl)
10× RT Buffer	2
25× dNTP Mix (100 mM)	0.8
10× RT Random Primers	2
MultiScribe™ Reverse Transcriptase	1
RNase Inhibitor	1
Nuclease-free H2O	3.2
Total volume	10

Table 2.10: thermal cycler program for reverse transcription.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10	120	5	∞

2.3.7.3 Semiquantitative PCR

Fixed concentration of cDNAs of transgenic plants produced by reverse transcription reaction were used to study amount of gene expression. Dream taq DNA polymerases and primers used for genotyping were used. PCR products were analyzed by gel electrophoresis. Band intensity were measured using ImageJ software.

2.3.8 Cell fractionation

Cell fractionation technique allow study cell organelles in relatively intact form. The cell generally disrupted and separated into several organelle containing fractions by series of centrifugation of increasing speed. Centrifugation separate cell components on basis of size and density, the lager and denser organelle pellet first.

After approximately four weeks of transfer seedling of second generation to soil, 10 g of leaves were collected and cut with a sharp razor blade and homogenized with 30 ml cell fractionation buffer in a Waring blender for 2-3 min . The sample then filtered through two layers of gaz into Erlenmeyer flask on ice. *Arabidopsis thaliana* wild type and two knocked out mutants PP2A B’ζ were used as controls.

Component of cell fractionation buffer:

Competent	Concentration
K-phosphate pH 7.6	50 mM
sucrose	0.4 M
NaCl	50 mM
EDTA	1 mM
Cysteine	14 mM

The suspension of broken cell transferred to 50 ml falcon tubes and serial centrifugation steps performed at 4 ˚C to maintain integrity of cell organelles and proteins , using Avanti j-26S ,Beckman Coulter centrifuge to fractioned cell components in to four main fraction as the following:

1.Tubes centrifuged at 1000 x g for 2 min (nucleus in pellet). And supernatant poured into clean tube.

2.The supernatants centrifuged at 2500 x g for 13 min (chloroplasts and thylakoids are in the pellet). And supernatant poured into clean tube.

3.The supernatants centrifuged at 10 000 x g for 25 min (mitochondria are in pellet). And supernatant (Cytoplasm and soluble part of cell) poured into clean tube.

The Three different pellets obtained from cell fractionation (nucleus, chloroplasts and mitochondria) resuspend in small volume of cell fractionation buffer and last supernatant (Cytoplasm and soluble part of cell) were used for study subcellular localization of PP2A B’ζ-YFP by western blot through use antibody against YFP.

2.3.9 Determination total protein concentration using Bradford assay

The Quick Start™ Bradford protein assay used for determination total protein concentrations in the samples. The Bradford method measure the absorbance shift of the red cationic form of Coomassie Blue G-250 as result of binding to protein to the blue stable anionic.

Standard calibration curve.

Bovine serum albumin (BSA) was used to make standard calibration curve which then used to determine protein concentration. Series of dilution of BSA (2, 1.5, 0.75, 0.5, 0.25, 0.125 mg/ml) in distilled water made and 20 µl of each standard transferred to cuvette mixed with 1 ml of 1 X Bradford dye reagent and sample incubated for 5 min at room temperature. The absorbance measured at 595 nm using SmartSpec™ Plus Spectrophotometer (BioRad). The standard calibration curve created by plotting known concentration of standards against its absorbance readings. The linear equation of this plot used to predict the concentration of protein in samples (See standard calibration curve in Appendix B.3).

2.3.10 Western blotting

Western blot is a technique in which the proteins that are separated by gel electrophoresis, transferred to membrane to be identified and analyzed. Western blotting also called immunoblotting, because an antibody (protein) is used to detect its target protein. Western blot includes five main steps protein gel electrophoresis, protein transfer to membrane, blocking, antibody probing and detection.

2.3.10.1 Protein extraction directly in Laemmli

The four different cell fractions and plant extract contain YFP (control) was homogenized with 4 X Laemmli buffer (Bio-Rad) and 50 mM DTT. 3 parts of sample (37.5 µl) mixed with 1 part of 4 X Laemmli buffer and 50 mM DTT (12.5 µl). The sample was boiled at 95°C for 5 min and centrifuged at room temperature at 15,000 × g for 5 min. The supernatant containing crude total proteins loaded on the gel.

2.3.10.2 SDS gel electrophoresis

Mini-PROTEAN TGX precast gels (4-20%) from Bio-Rad were used. The electrophoresis performed in a Mini-PROTEAN Tetra Cell (Bio-Rad). Protein samples (40 µl) were loaded into the wells. Precision plus protein WasternC Standards (5 µl) was used as marker. Electrophoresis was adjusted at 200 V until the dye reached the bottom of the gel (usually 40 min).

1x Tris/ Glycine/SDS electrophoresis buffer (Bio-Rad):

Tris	25 mM
Glycine	192 mM
SDS	0.1% (w/v)
pH 8.3	

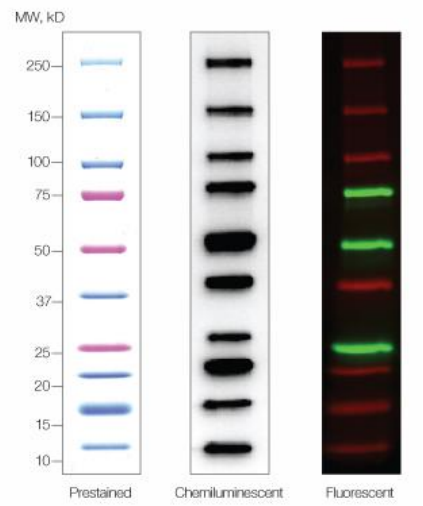

Figure2.8: Overview of the Precision plus protein WasternC Standards fragments size.

2.3.10.3 Transfer proteins onto membrane

After finish of electrophoresis run, gel removed from cassette and washed with distilled water. The proteins in gel transferred to PVDF membrane using Trans-Blot® Turbo™ transfer system (Bio-Rad). The system allowing rapid transfer of protein within 3 min. Trans-Blot Turbo TM Transfer Pack PVDF (0.2 µm pore size) were used. Bottom layer of transfer pack placed at center of cassette base (See Figure 2.9a) followed by the gel and top layer of transfer pack to

form the sandwich. The roller used to get rid of bubbles between gel and membrane. After that cassette closed by cassette lid and placed in Trans-Blot Turbo instrument (See Figure 2.9b).

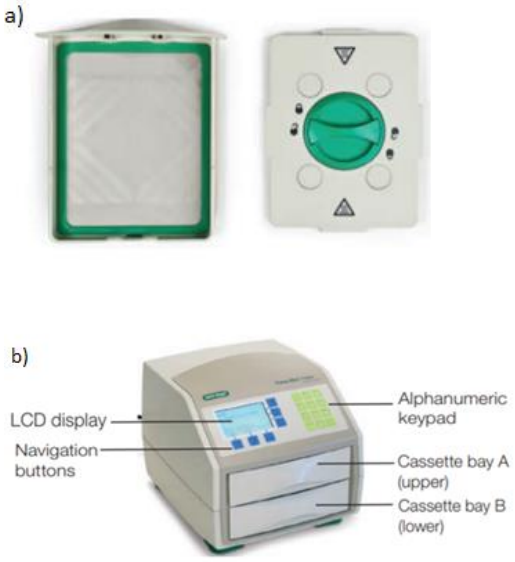

Figure 2.9: a, cassette base lid and cassette base.b, Trans-Blot Turbo instrument. This figure obtained from BioRad

2.3.10.4 Blocking of the membrane

PVDF membrane has high affinity for protein so before proteins detected using antibodies, the membrane must be incubated with blocking agent to block the unoccupied space on the membrane. The membrane blocked by incubation 3% non-fat dry milk powder in 1X PBS buffer for 50 min in orbital shaker at room temperature.

composition of PBS (1X):

Salt	Concentration (mmol/L)	Concentration (g/L)
NaCl	137	8.0
KCl	2.7	0.2
Na2HPO4	10	1.42
KH2PO4	1.8	0.24

2.3.10.5 Antibody probing

After blocking the membrane with non-fat dry milk. The membrane incubated with primary antibody against target protein YFP. The primary antibody used was anti-GFP antibody

produced in rabbit (Sigma Aldrich). The membrane incubated in primary antibody in 1 x PBS buffer with concentration (1:1000) for 50 min on orbital shaker at room temperature. Excess of primary antibody removed by washing three time with 1X PBS buffer each time for 10 min on orbital shaker.

The membrane then incubated with solution of secondary antibody. Labelled secondary antibody Goat anti-rabbit IgG-HRP (ThermoFisher) that target primary antibody was used with concentration of 1:5000 in 1X PBS buffer. Antibody against protein standards, Precision Protein™ Streptactin-HRP conjugate (Bio-Rad) was used with concentration 1:15000 in 1X PBS buffer. The membrane incubated with mixture of antibodies for 50 min on orbital shaker at room temperature. Excess of antibody removed by washing with 1X PBS buffer.

2.3.10.6 Chemiluminescent detection

The secondary antibody and the antibody that target standards protein labelled with Horseradish peroxidase (HRP) enzyme which catalyze oxidation of substrate in presence of oxidizing agent such as hydrogen peroxide resulting characteristic change that could be detected by specific detection methods. Clarity ^{TM} Western ECL Substrate (Bio-Rad) contain chemiluminescence substrate.

The membrane incubated for 5 min with equal volume of peroxidase solution and chemiluminescence substrate (3 ml of each solution) at orbital shaker, after that the membrane washed with distilled water and wrapped with clean plastic envelope. The membrane visualized using ChemiDoc ^{TM} Imaging System.

2.3.10.7 Ponceau S Stain for Western blots

Ponceau staining rapid and reversible staining method produce pink stained proteins band on the western blots. The membrane (blot) incubated with 5 ml of Ponceau S solution (Sigma-Aldrich) for 10 min with gentle agitation then the membrane rinsed in distilled water until the background is clean. the complete remove of stain from protein band possible by continued washing with distilled water.

3 RESULTS

3.1 Role of PP2B' subunits in regulation of metabolism

PP2A regulatory subunit B' ζ and B' θ have been reported to have role in energy metabolism (Kataya et al., 2015b). Recently two proteins involved in energy metabolism in plant, *Arabidopsis* mitochondrial succinate fumarate translocator (AtmSFC) and aconitase3 (ACO3) were shown interaction with PP2A regulatory subunit B' ζ. In the present study we wanted to investigate the possibility of interaction between AtmSFC and PP2A regulatory subunit B' α, B' β and B' θ. The three B' regulatory subunits were cloned into suitable BiFC vectors to be expressed as N free and C free tagged proteins. Furthermore, we wanted to study possibility of interaction between aconitase1 (ACO1) and PP2A regulatory subunit B' ζ. Truncated ACO1 made to through designed primers to pick up first 2505 bp of full length ACO1 giving PCR product lacked 192 bp (See Appendix A1), to explore importance of C-terminal end in interaction with PP2A B' ζ. Protein-protein interactions investigated in onion epidermal cells by fluorescence microscopy.

3.1.1 Generation of BiFC constructs

The first goal was to generate constructs expressing BiFC-tagged version of PP2A regulatory subunits (B' α , B' β and B' θ) and ACO1. Full length cDNA of PP2A regulatory subunits had been obtained from Dr, Behzad, whereas full length cDNA of ACO1 had been from RIKEN BRC (Ibaraki, JAPAN) (See Table 2.1 in material and method section). The BiFC vectors (described in section 2.1.6.2 in Materials and methods) were obtained from The University of Münster.

PP2A regulatory subunits (B' α, B' β and B' θ) were cloned into BiFC vector number 6 pVYCEpUC19 ($Venus_{C155}$) and BiFC vector number 7 pVYCEpUC19 ($Venus_{C155}$) to be expressed as N free tagged and C free tagged protein, respectively. Full length cDNA of *Arabidopsis* ACO1 and truncated ACO1 cloned into BiFC vector number 8 to be expressed as N free tagged protein pVYNEpUC19 ($Venus_{N173}$).

To prepare the full-length cDNA for directional cloning, restriction recognition sites were introduced to the termins of the cDNAs using PCR. In PP2A B' α, PP2A B' β and ACO1 the forward primer introduced a SalI site at 5' end and the reverse primer introduced a KpnI site at the 3' end, whereas in PP2A B' θ the forward primer introduced a SacI site at 5'end and the reverse

primer a XhoI at 3'end (See Table 2.3 material and method section). Thus, resulting PCR products contain additional DNA sequence corresponding to restriction sites in multiple cloning sites in BiFC vectors. In case of ACO1 two primer pairs were used. The first primer pair used to pick up full length ACO1 (2697 bp), while a second primer pair was used to amplify first 2505 bp of ACO1 resulting ACO1 truncated, that lack last 192 bp of CDS. 192 bp code for 64 C-terminal amino acids (See Table2.3 material and method section, see Appendix A.1 for more details).

The cDNAs amplified with primers (See Table2.3 in material and method section) and small amount of PCR product were analyzed by gel electrophoresis to verify successful amplification the size of PCR products was correct (theoretical size of the cDNAs: B'α 1488bp, B' β 1500bp, B' θ 1480 bp, ACO1 2697 bp and truncated ACO1 2505bp.The concentration of PCR product obtained was enough for subsequent sub-cloning into BiFC vectors. Figure 3.1 shows PCR products of regulatory subunit around 1.5kb, truncated ACO1 2.5 kb and 2.7 for ACO1.

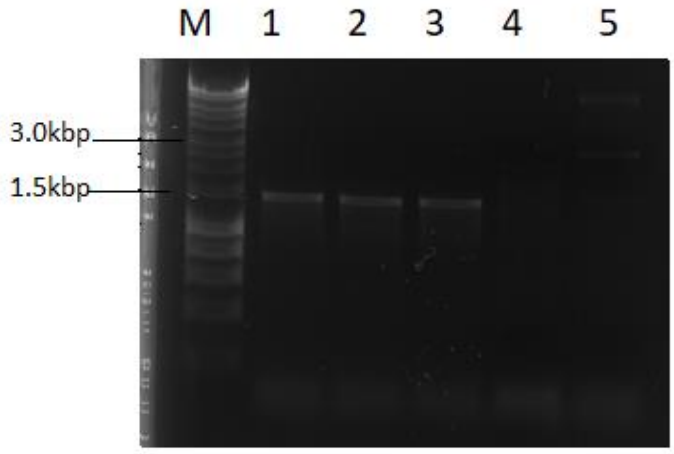

Figure 3.1: PCR product analysis of preparative PCR for B'α, B' β and B' θ and ACO1. Small aliquots (3 µl) of the PCR products were run on a 1% agarose gel (90 V, 45 min). Lane M: Size standard; Hyper-ladder I. Lane1: B'α. Lane2: B'β. Lane3 B' θ. Lane4: ACO1truncated. Lane5:ACO1.

The PCR product then purified using NucleoSpin® Gel and PCR Clean-up kit (See section 2.2.3 in material and method). The resulting DNA concentration was between 50-60 ng/µl. After that the PCR products and BiFC vectors were double digested with same restriction enzymes. The digested PCR product and digested BiFC vectors were purified using NucleoSpin® Gel and PCR Clean-up kit and verified by agarose gel electrophoresis. The concentration of digested PCR products was between 30-40 ng/µl, while concentration of digested BiFC vectors were between 15-20 ng/µl.

The purified cDNAs were ligated into BiFC vector using a stoichiometric ratio vector to insert of about 1:3. The obtained BiFC vectors gave the ability to fuse cDNA to both 5' and 3' end of N-terminal and C-terminal fragment of Venus. Since ACO1 localized in cytoplasm, so it was decided that cDNA of ACO1 and truncated ACO1 to be ligated into pVYNE were they expressed as N free tagged proteins. Whereas B'α, B'β and B'θ were ligated into pVYCE and PVYCE(C)to be expressed as N free tagged and C free tagged proteins, respectively.

After ligation of cDNA into BiFC vectors. The recombinant plasmid was transformed into *E. coli* for replication. Colony PCR test was performed to direct screening of transformed *E. coli* cells where 5 colonies were tested using gene specific primers. Colony PCR was followed by analysis on agarose gel electrophoresis. Figure 3.2 shows colony PCR of regulatory subunits the positive colony gave PCR product around 1.5 kb, while Figure 3.3 shows colony PCR of full length and truncated ACO1.

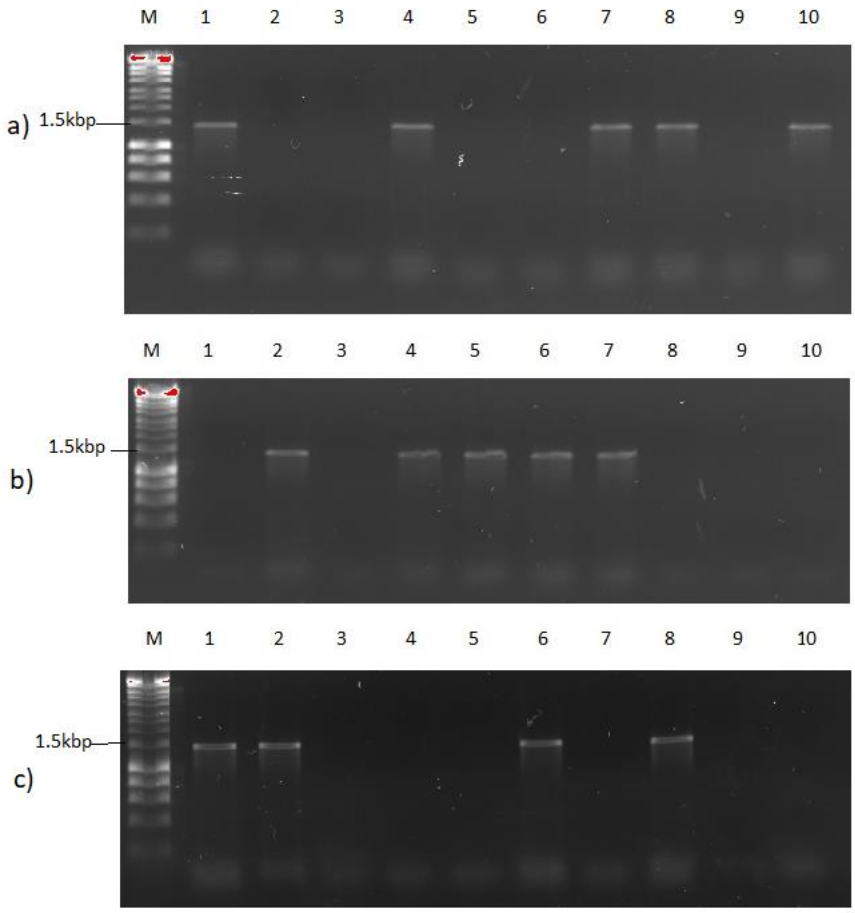

Figure 3.2: Analysis of colony PCR by agarose gel electrophoresis. The PCR was performed using gene specific primers. PCR products were run on 1% agarose gel (90V, 45min). A: colony PCR of B' α. Lane 1-5: pVYCE- B'α and lane 6-10: pVYCE(C)- B'α. B: colony PCR of B' β. Lane 1-5: pVYCE- B'β and lane 6-10: pVYCE(C)- B' β. C: colony PCR of B' θ. Lane 1-5: pVYCE- B' θ and lane 6-10: pVYCE(C)- B' θ.

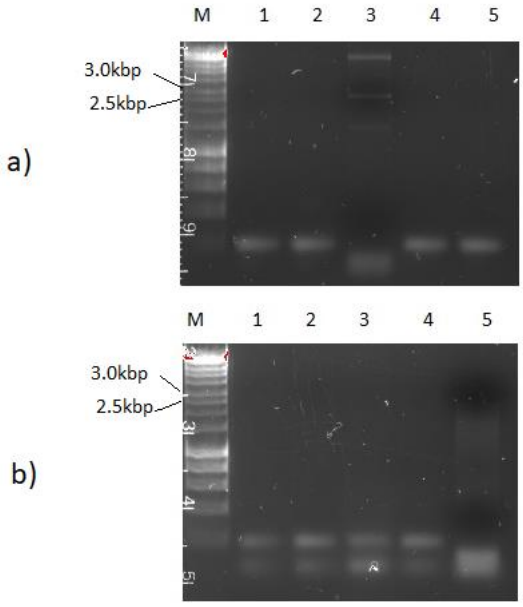

Figure 3.3: Analysis of colony PCR by agarose gel electrophoresis. The PCR was performed using gene specific primers. PCR products were run on 1% agarose gel (90V, 45min). A: colony PCR of pVYNE-ACO1.The lane number 3 shows PCR product between 2.5 kbp and 3 kbp. B: colony PCR of pVYNE-Truncated ACO1. The lane number 5 shows very faint band at 2.5kbp which is size of truncated ACO1.

The transformation efficiency was varied between 20 to 50%, the number of transformed cell decrease with increase the size of the insert, where in case of full length ACO1 cloning was repeated in order to be successfully cloned into pVYNE vector. The recombinant plasmid is then isolated using NucleoSpin® plasmid miniprep kits which has capacity to isolate up to up to 50 µg of plasmid DNA.

To verify that the insert was present in the vector, double restriction digestion was performed to recombinant plasmids using same restriction enzymes used for cloning the insert to the vector (Figure3.4). The presence of insert was verified, and recombinant plasmid were sent to sequencing.

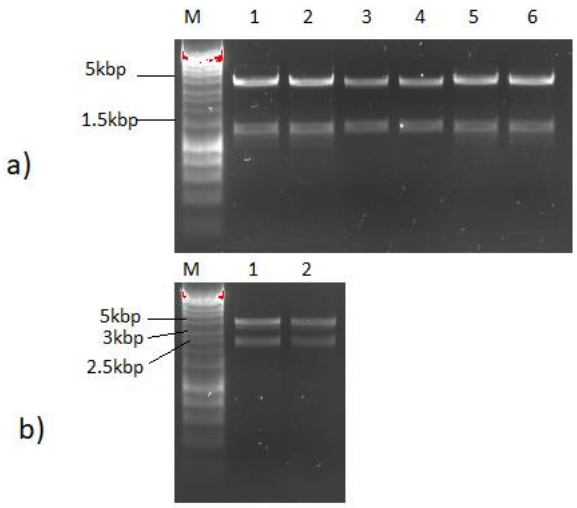

Figure 3.4: Analysis of restriction digestion on agarose gel electrophoresis. A: restrication digestion of regulatory subunits in BiFC vectors gave size of BiFC vector around 5 kpb and size of regulatory subunits around 1.5 kbp , lane 1: pVYCE- B' α, lane 2: pVYCE(C)- B' α, lane 3: pVYCE- B' β, lane 4: pVYCE(C)- B' β, lane 5: pVYCE- B' θ , lane 6: pVYCE(C)- B' θ. B: restriction digestion of ACO1 in BiFC vector, lane 1: pVYNE-Truncated ACO1 and lane 2 pVYNE-ACO1.

The inserts were sequenced using the 35S forward primer and the Nots reverse primer (see Table 2.3 in Material and methods; see Appendix A.2 for sequencing analysis data). PP2A regulatory subunit B' α, β and θ were fully covered in both vector (pVYCE and pVYCE(C)). ACO1 and truncated ACO1 in pVYNE vector were not fully covered and internal primer probably needed to cover whole sequence. All sequence of inserts shows very good alignment to their cDNA with very low mutation, so it was decided to use recombinant plasmids for study protein-protein interaction.

3.1.2 visualization of protein-protein interactions

After generation of BiFC vectors for regulatory subunits (PP2A B' α, β and θ), ACO1 and truncated ACO1, two BiFC vectors were obtained from Dr, Ahmed at Lillo lab. The two BiFC vector for regulatory subunit PP2A B' ζ and *Arabidopsis* mitochondrial Succinate/fumarate transporter (AtmSFC) . ACO1 and AtmSFC were designed to be fused with N-terminal fragment of Venus, whereas regulatory subunits PP2A B' were fused with C-terminal fragment of Venus (Figure3.5).

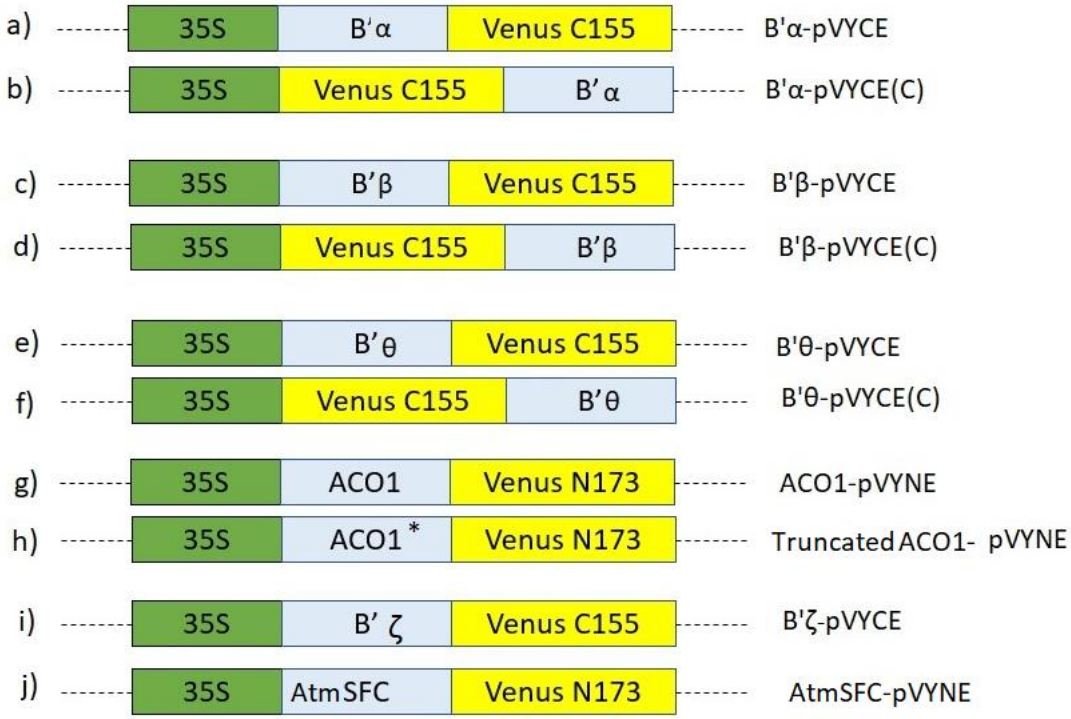

Figure3.5: schematic representation of generated and obtained BiFC constructs, from a to h generated during the study while I and j obtained from Dr Ahmed. The regulatory subunits PP2A B' α, β and θ were fused at C-terminus in pVYCE vector, while fused at N-terminus in pVYCE(C) vector. ACO1 were fused at its C-terminus in pVYNE vector. All regulatory subunits were fused to C-terminal fragment of Venus(C155). While AtmSFC and ACO1 were fused to N-terminal fragment of Venus (N173). The expression of all the fusion protein is controlled by the CaMV 35S promoter.

ACO1 and truncated ACO1 were combined with PP2A B' ζ, whereas regulatory subunits PP2A B'α, B'β and B'θ were combined with AtmSFC. The combination of plasmids were transformed into onion epidermis (See Table3.1). Peroxisomal marker as well as mitochondrial marker were used in the experiment to determine localization of positive interaction. Protein-protein interaction were analyzed by fluorescence microscopy.

The mitochondrial marker harbored the mitochondrial import peptide fused with red fluorescent protein (RFP). The import peptide target RFP to mitochondrial matrix. While the peroxisomal marker harbored the peroxisomal targeting signal 1 (PTS1) sequence SKL fused to RFP. SKL targets RFP to peroxisomal matrix (Matre et al., 2009).

Table 3.1: Combination of BiFC vectors used to study protein-protein interaction and results of interaction

Plasmid A	Plasmid B	Results of interaction
ACO1-pVYNE	PP2AB' ζ-pVYCE	Positive interaction
Truncated ACO1-pVYNE	PP2AB' ζ-pVYCE	No interaction
AtmSFC- pVYNE	PP2AB' α -pVYCE	No interaction
AtmSFC- pVYNE	PP2AB' α -PVYCE(C)	No interaction
AtmSFC- pVYNE	PP2AB' β -pVYCE	Positive interaction
AtmSFC- pVYNE	PP2AB' β -PVYCE(C)	No interaction
AtmSFC- pVYNE	PP2AB' θ -pVYCE	No interaction
AtmSFC- pVYNE	PP2AB' θ -PVYCE(C)	No interaction

Fluorescence microscopy analysis of ACO1-pVYNE combined with PP2AB'ζ-pVYCE revealed fluorescence in cytoplasm (Figure 3.6), whereas no fluorescence was detected when truncated ACO1-pVYNE combined with PP2AB' ζ-pVYCE. Alignment of last 192 bp that are truncated from ACO 1 with last 192 bp of ACO2 and ACO3 revealed this part of gene conserved between three genes despite the difference in gene size ACO1 (2697 bp), ACO2 (2988bp), ACO3(2973bp) (See alignment in Appendix A.1)

Fluorescence was observed in combination of AtmSFC-pVYNE and PP2AB'β -pVYCE (N free tagged B'β). However, no fluorescence was observed when AtmSFC- pVYNE combined with PP2AB'β -pVYCE(C) (C free tagged B'β). The interaction between AtmSFC- pVYNE and PP2AB' β-pVYCE shows fluorescence in cytoplasm and in form of speckles within onion epidermal

cells (Figure 3.7). The mitochondrial and peroxisomal marker were used to study localization of interaction between SFC-pVYNE and PP2AB' β-pVYCE. The expression of both markers was quite low therefore no date was obtained about localization of interaction.

When SFC- pVYNE was combined with PP2A B' α and B'θ non-specific background fluorescence was obtained. The background fluorescence was also observed in the negative control experiment between empty vectors pVYNE and pVYCE (Figure 3.8).

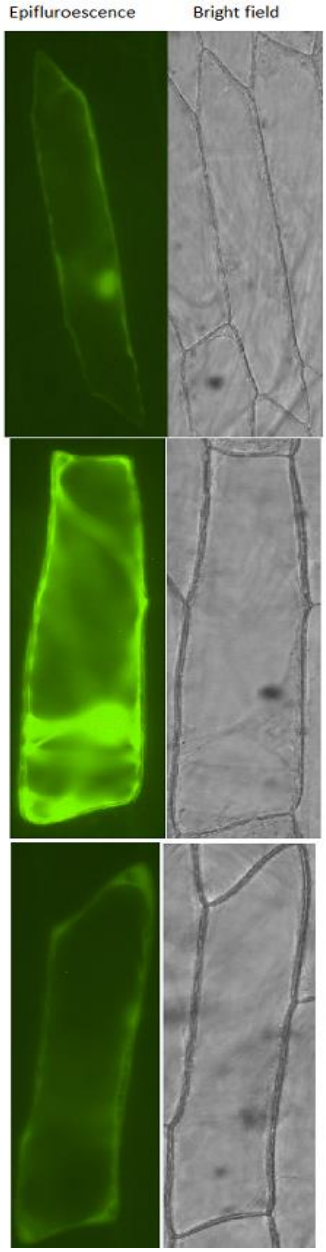

Figure 3.6: Interactions of ACO1 with PP2AB' ζ-examined by BiFC in onion epidermal cells. The interaction between ACO1 and PP2AB' ζ it seems to be cytosolic as fluorescence diffused throughout cell.

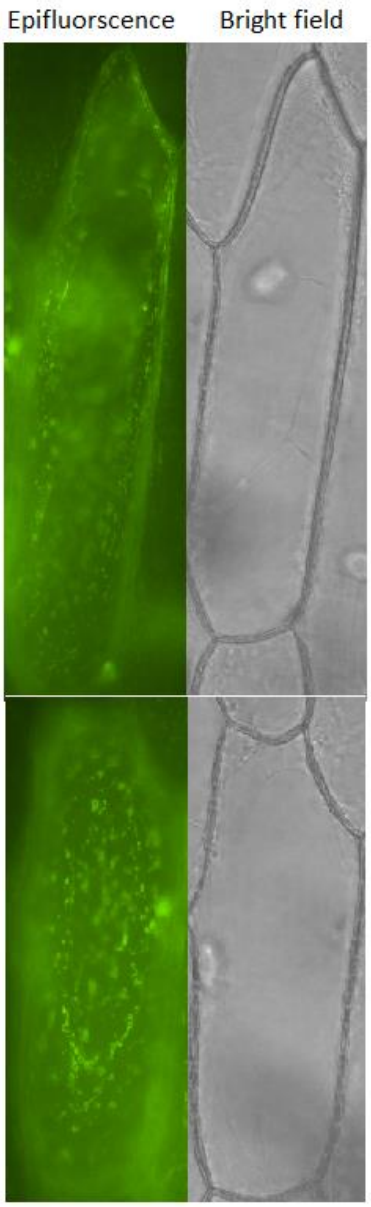

Figure 3.7: Interactions of AtmSFC-pVYNE and PP2AB' β -pVYCE in onion epidermal cells. The interaction between AtmSFC and PP2AB' β it seems to be in one of cell organelles (speckles). Co-expression of AtmSFC and PP2AB' β with mitochondrial and peroxisomal marker was very low. The use of organelles markers did not provide more evidence about localization of interaction.

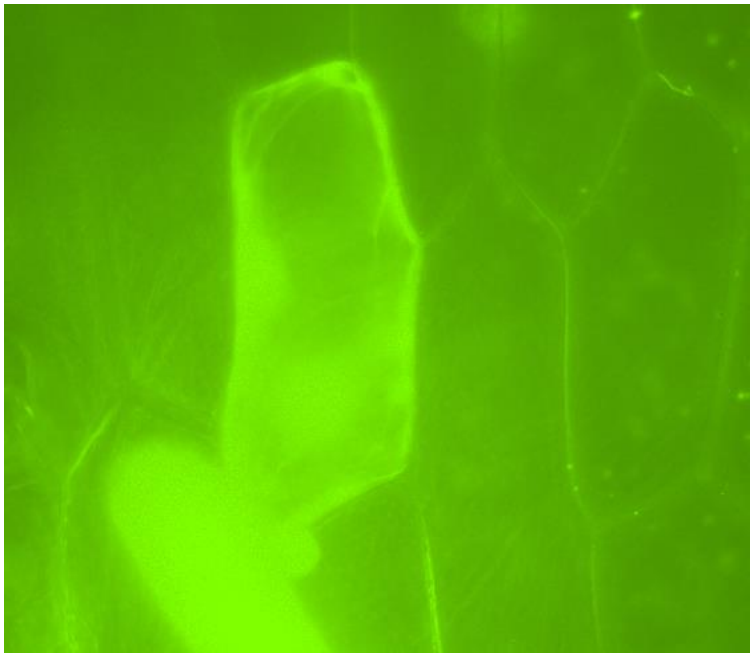

Figure 3.8: nonspecific background fluorescence when SFC- pVYNE was combined with PP2A B’ α and B’θ , The background fluorescence was also observed in the negative control experiment between empty vectors pVYNE and pVYCE

3.2 Study of PP2A B’ ζ localization

It has been reported when N-free terminal PP2A regulatory subunit B’ ζ linked to fluorescent tag and transiently expressed in onion epidermal cells to be localized the cytoplasm and partly co-localized with mitochondria (Matre et al., 2009). In the present study we wanted to use another method to investigate PP2A B’ ζ localization through production of new transgenic line, where N free terminal PP2A B’ ζ was linked fluorescent tag (YFP) and cloned into *Agrobacterium* vector (pBA002) then PP2A B’ ζ-YFP pBA002 vector was transformed into both wild type *Arabidopsis* and two mutant lines of PP2A B’ ζ through flower dipping. The total proteins from the leaves of second generation transgenic plant were used to study localization by western blot through the use of an antibody against YFP protein in four cell fraction, nucleus, chloroplast, mitochondria and cytoplasm (soluble part of cell).

3.2.1 Generation of pBA002-PP2A B’ ζ/YFP

pWEN 18- PP2A B’ ζ vector contains N free terminal PP2A B’ ζ linked to fluorescent tag (YFP) A pair of primer designed to pick up PP2A B’ζ and YFP (forward for PP2A B’ζ and reverse for YFP) (See Table 2.3 in material and method section). Restriction enzyme recognition sites were added to primer XhoI for the forward and PacI for the reverse. Thus, the resulting PCR product contain restriction sites corresponding to restriction endonuclease sites in the multiple cloning site of the binary vector (pBA002). The estimated PCR product size is about 2.3 Kb (PP2A B’ ζ 1641 bp and YFP 724bp). PCR using High fidelity polymerase performed and small amount of PCR product were analyzed using agarose gel electrophoresis (Figure 3.9).

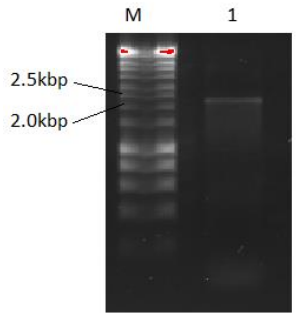

Figure 3.9: PCR product analysis of P2A B’ ζ/YFP. Small aliquots (3 µl) of the PCR products were run on a 1% agarose gel (90 V, 45 min). Lane M: Size standard; Hyperladder I. Lane1: P2A B’ ζ/YFP with product size of 2.3 kb (1.6 kb P2A B’ ζ+0.7 kb YFP).

PCR product was purified using NucleoSpin® Gel and PCR Clean-up kit (see section 2.2.3 in material and method). After that pBA002 vector and PP2A B' ζ/YFP were double digested with XhoI and PacI. The digested PCR product and digested pBA002 vector were purified using NucleoSpin® Gel and PCR Clean-up kit.

The digested PP2A B' ζ/YFP and pBA002 vector were ligated and then transformed to *E. coli* for replication. Colony PCR was performed using gene specific primer followed by analysis using agarose gel electrophoresis (Figure 3.10). The number of positive transformed cell was quite low as only positive colony was obtained after second trial(PCR product size about 2.3 Kb), maybe because the large size of binary vector 10.1 Kb.

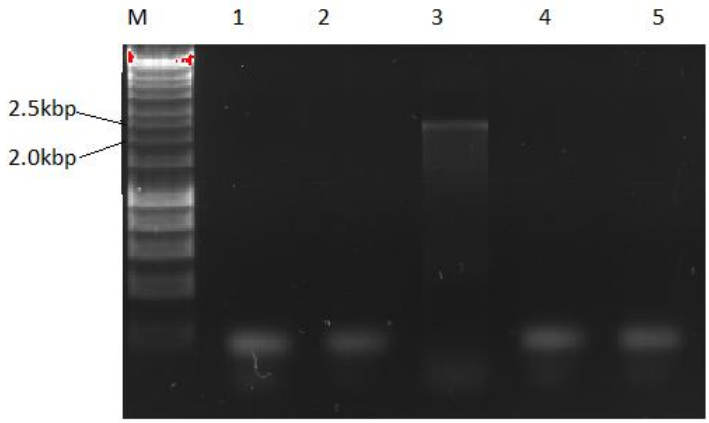

Figure 3.10: Analysis of colony PCR by agarose gel electrophoresis. The PCR was performed using gene specific primers. PCR products were run on 1% agarose gel (90V,45min). The lane number 3 shows band with product size is about 2.3 Kb between 2.0 kbp and 2.5 kbp.

The recombinant plasmid was pBA002-PP2A B' ζ/YFP isolated using NucleoSpin® plasmid miniprep kits. To verify that the insert (PP2A B' ζ/YFP) in the vector, double restriction digestion using XhoI and PacI was performed followed by analysis on agarose gel electrophoresis (Figure 3.11). The presence of insert was verified.

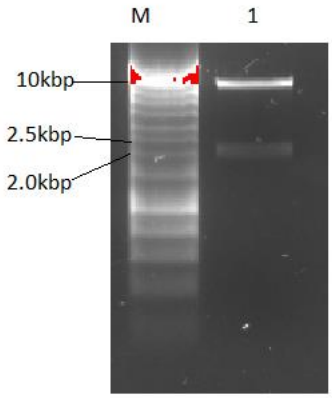

Figure 3.11: Analysis of restriction digestion on agarose gel electrophoresis. In lane number 1 two bands were obtained first band about 10 kb equivalent to size of pBA002 vector (10.1 kb) the second band shows product size between 2.0 kbp and 2.5 kbp which is approximately same size of PP2A B' ζ/YFP (2.3 Kb).

The recombinant plasmid was sent for sequencing using three primers, forward and reverse primer for PP2A B' ζ, and reverse for YFP. (see Table 2.3 in Material and methods; see Appendix B.1 for sequencing analysis data). PP2A B' ζ was fully covered and had very good alignment with cDNA. The sequence data obtained showed that YFP was linked to PP2A B' ζ. After generation of construct (Figure 3.12), the recombinant plasmid transformed to *Agrobacterium*.

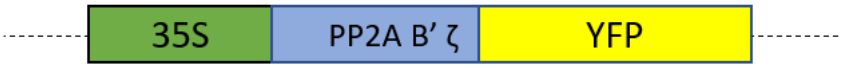

Figure 3.12: schematic representation of generated pBA002-PP2A B' ζ/YFP. PP2A B' ζ was fused with its N-terminal to YFP. The expression of fusion protein controlled by CaMV promoter.

3.2.2 transformation of *Agrobacterium*

The recombinant plasmid transformed to *Agrobacterium tumefacien* ABI (See material and method section 2.3.3). Colony PCR test and analysis on agarose gel electrophoresis revealed that the transformation efficiency was good (Figure 3.13).

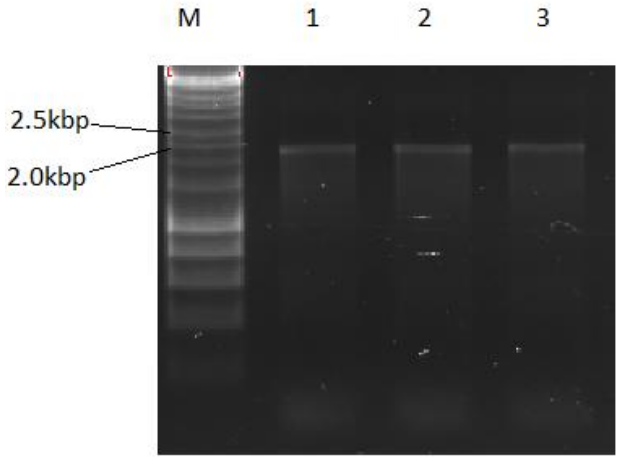

Figure 3.13**:** Analysis of colony PCR by agarose gel electrophoresis. The PCR was performed using gene specific primers. All the colonies selected was successfully transformed. Positive colonies show PCR product size 2.3 Kb.

3.2.3 Screening of transformed *Arabidopsis* plant

Using successfully transformed *Agrobacterium* cells *Arabidopsis* plants (Wild type and two mutant lines for PP2A B' ζ SALK_ 150586 and SALK_ 107944) were transformed by floral dipping (See material and method section 2.3.5).

3.2.3.1 Screening of T1 generation from transformed *Arabidopsis* plants

Seeds were collected from transformed plants approximately after one month and screened by sowing them on ½ MS plates containing BASTA (10 µg/ml), since pBA002 contain BASTA resistance the transformed seedlings should show growth with more green, bigger leaves and longer roots than non-transformed seedling. The non-transformed wild type and two mutant line for PP2A B' ζ were used as control to compare the growth of seedling.

Seeds were sown on big square plates which divide into four equal parts. The first part equipped was with non-transformed *Arabidopsis* plants which was used as controls, the second part was used to sow seeds collected from plants transformed with empty pBA002 (no insert) and the remaining two parts were used to sow transformed plant with pBA002-PP2A B' ζ/YFP.

Most of seed collected from transformed plants showed inhibited growth. Approximately 2-7 seedling showed ability to grow with longer roots and big green cotyledons unaffected by

presence of BASTA in the medium. The wild type *Arabidopsis* showed more transformed seedlings than two mutant lines for PP2A B’ ζ (SALK_ 150586 and SALK_ 107944) (Figure 3.14). In the mutant PP2A B’ ζ SALK_ 107944 the number of positive transformed seedlings was very low when compared to wild type *Arabidopsis*. The seedling that were unaffected by herbicide BASTA and passed screening were transferred to soil. All the seedlings survived the transfer. Approximately two weeks after transfer seedlings was subjected to genotyping test.

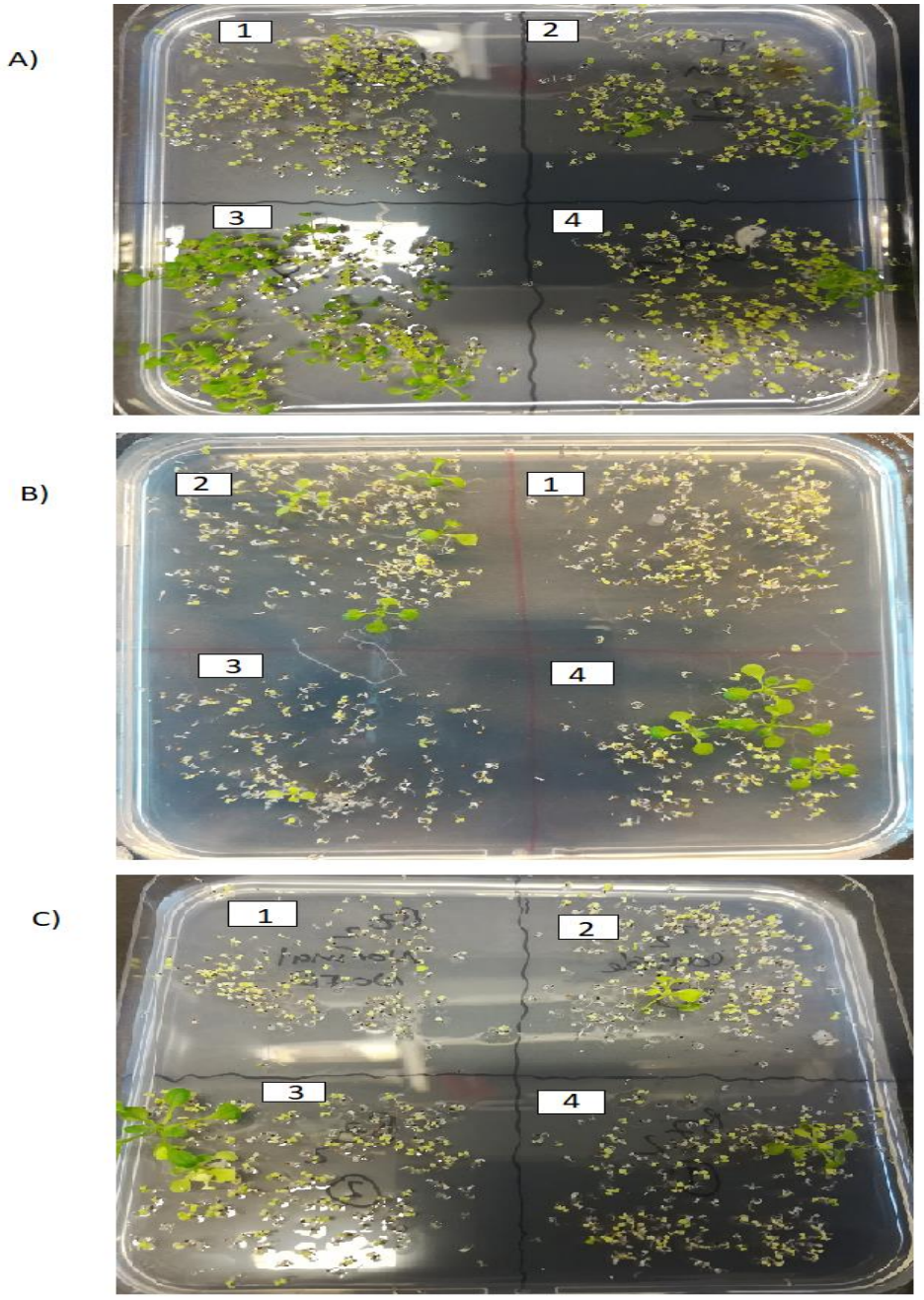

Figure 3.14: two weeks old seedlings harvested from *Arabidopsis* plants. The seeds were sown on ½ MS plates containing BASTA (10 µg/ml). A: seedlings of wild type *Arabidopsis*, B: seedling of mutant PP2A B' ζ SALK_ 150586, C seedling of mutant PP2A B' ζ SALK_ 107944. The number indicate 1: non-transformed seedling (Controls) ,2: seedling from plant transformed with pBA002 (no insert), 3 and 4: seedling from plant transformed with pBA002-PP2A B' ζ/YFP. All three Controls A1, B1 and C1 showed inability to resist herbicide BASTA and showed inhibited growth. In A2 wild type *Arabidopsis* transformed with pBA002 (no insert), three seedlings were unaffected by BASTA and had big green leaves and longer roots, same phenotype was obtained in A3 and A4 where wild type *Arabidopsis* transformed with pBA002-PP2A B' ζ/YFP. Only 5 transformed seedlings obtained from mutant PP2A B' ζ SALK_ 150586 in B3 and B4. Lowest transformation efficacy was obtained with mutant PP2A B' ζ SALK_ 107944.

3.2.3.2 Screening of from T2 generation transformed *Arabidopsis* plants

After two month of transfer T1 seedling to soil, the seeds were collected from each transformed plant separately and screened by sowing them on ½ MS plates containing BASTA (10 µg/ml) (as described in section 3.2.2.1). Approximately all the transformed T2 generation seedlings of wild type *Arabidopsis* and two mutant lines for PP2A B' ζ (SALK_ 150586 and SALK_ 107944) were shown ability to grow with longer roots and big cotyledons unaffected by presence of BASTA in the medium (Figure 3.15 B, C and D). Non-transformed wild type *Arabidopsis* and non-transformed mutant PP2A B' ζ SALK_ 150586 and SALK_107944 showed inhibited growth (Figure 3.15 A). Some of T2 seedling that passed screening were transferred to soil to harvest the leaves and later used to investigate PP2A B' ζ localization through western blot. The remaining of T2 seedling were used in gene expression study.

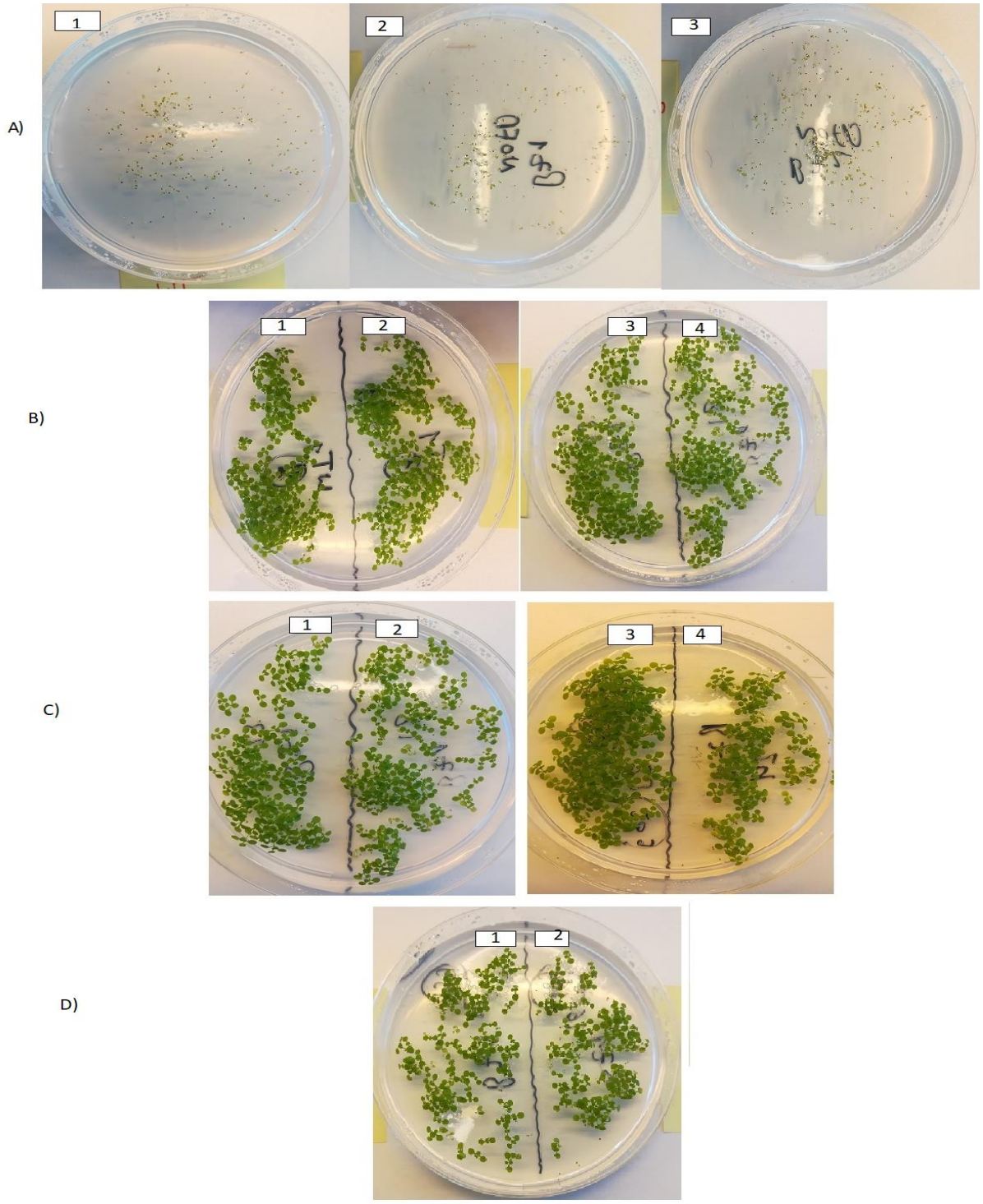

Figure 3.15: Two weeks old seedlings harvested from *Arabidopsis* plants. A: controls, A1: seedlings of non-transformed wild type *Arabidopsis*, A2: seedlings of non-transformed mutant PP2A B' ζ SALK_150586, A3: seedling of mutant PP2A B' ζ SALK_ 107944. B: Seedlings of T2 transformed wild type, B 1-3 transformed with pBA002-PP2A B' ζ/YFP, B4 transformed with Empty pBA002 (No insert). C Seedlings of T2 transformed mutant seedling of mutant PP2A B' ζ SALK_ 150586, C1-3 transformed with pBA002-PP2A B' ζ/YFP, C4 transformed with Empty pBA002 (No insert). D: Seedlings of T2 transformed mutant seedling of mutant PP2A B' ζ SALK_ 107944, D1 transformed with pBA002-PP2A B' ζ/YFP and D2 transformed with Empty pBA002 (No insert).

3.2.4 Genotyping of Transformed plants

Genotyping was performed on both T1 and T2 generation seedling transformed with pBA002-PP2A B' ζ/YFP, to verify presence (insertion) of PP2A B' ζ/YFP in plant genome using Phire™ Plant ™ Direct PCR Kit (See material and method section 2.3.6). In genotyping experiment new primers were designed to give PCR product that contain part of PP2A B' ζ and part of YFP to verify PP2A B' ζ fused with YFP in plant genome, PCR product size 451 bp (see Appendix B.2 for more details). The non-transformed wild type *Arabidopsis* and non-transformed two mutant lines PP2A B' ζ were used as control to ensure the band is specific for transformed plant. Genotyping was performed followed by analysis by agarose gel electrophoresis (Figure 3.16 for T1 generation and figure 3.17 for T2 generation). In genotyping of T1 transformed plants, the plants that did not show band of 451 bp were cancelled from study. The transformed plants that gave positive results in genotyping of T1 transformed plants showed same results (451 bp) but with higher band intensity.

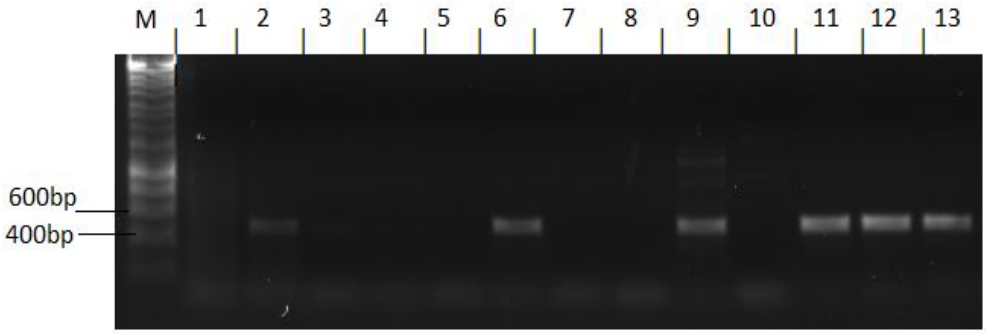

Figure 3:16: Analysis of genotyping of T1 generation transformed plants by agarose gel electrophoresis. M: 10 kb hyper ladder, lane 1: non-transformed wildtype *Arabidopsis*(control), lane 2-7: transformed wild type *Arabidopsis* plants, lane 8: non-transformed mutant PP2A B' ζ SALK_ 107944 (control), lane 9: transformed mutant PP2A B' ζ SALK_ 107944, lane 10: non-transformed mutant PP2A B' ζ SALK_150586 (control), lane 11-13: transformed mutant PP2A B' ζ SALK_150586. All non-transformed plants (controls lane 1, 8 and 10) gave negative results indicating that band of 451 bp specific to PP2A B' ζ/YFP and not found plant genome. In the lane number 4,5 and 7 no band obtained. The plants did not show specific band were cancelled from the study. While in the lane number 3 faint band was obtained.

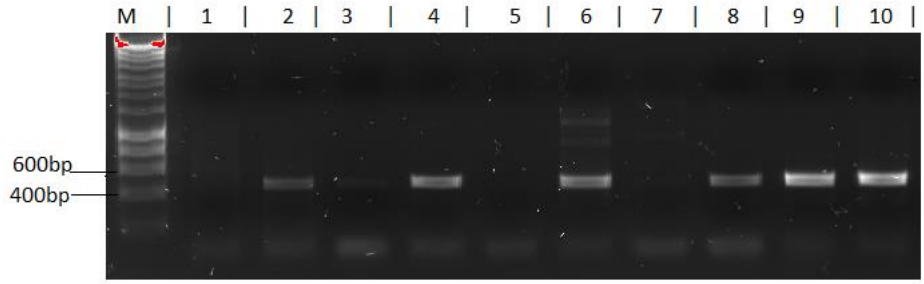

Figure 3.17: Analysis of genotyping of T2 generation transformed plants by agarose gel electrophoresis. M: 10 kb hyper ladder, lane 1: non-transformed wildtype *Arabidopsis* (control), lane 2-4: T2 generation transformed wild type *Arabidopsis* plants, lane 5: non-transformed mutant PP2A B' ζ SALK_ 107944 (control), lane 6: T2 generation transformed mutant PP2A B' ζ SALK_ 107944, lane 7: non-transformed mutant PP2A B' ζ SALK_150586(control), lane 8-10 :T2 generation transformed mutant PP2A B' ζ SALK_150586. All three controls gave negative results. All the transformed plants showed specific band (451bp) in genotyping of T1 generation plants gave same PCR product size but with higher band intensity.

3.2.5 Gene expression analysis

Genotyping was performed to ensure that insert (PP2A B' ζ-YFP) was present in plant genome, while gene expression study was performed to verify formation of gene product from its encoding gene PP2A B' ζ-YFP.The first step involve isolation of total transcribed RNA from seedlings of second generation transformed plants using RNeasy Plant Mini Kit (See material and method section 2.3.7.1) which remove the genomic DNA, the isolated RNA quantified by NanoDrop 2000 and fixed concentration were used for synthesis of complementary DNA (cDNA) (See Material and method section 2.3.7.2). Conventional PCR were then used to synthesis of dsDNA from fixed concentration of cDNA using Dream Taq DNA polymerase and same primers used for genotyping to study expression of fused gene (See Appendix B.2 for more details). PCR product were analyzed by agarose gel electrophoresis (Figure 3.18).

The agarose gel electrophoresis showed that two transformed wild type *Arabidopsis* plants and three transformed mutant PP2A B' ζ SALK_150586 had specific band of 451 bp. The gel image was then analyzed by ImageJ software (Schindelin et al., 2012) to measure density of the band thus converted into quantitative data (area under the curve) (See Table3.2.)

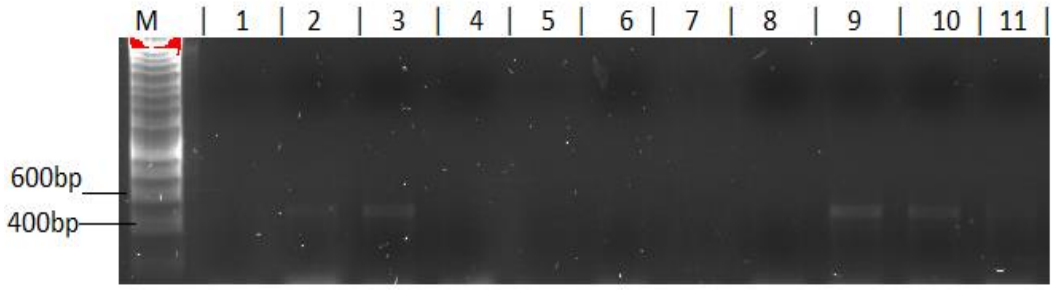

Figure 3.18: Analysis of gene expression study of transformed plants by agarose gel electrophoresis. M: 10 kb hyper-ladder, lane 1: non-transformed wildtype *Arabidopsis* (control), lane 2-5 transformed wild type *Arabidopsis* plants, lane 6 :non-transformed mutant PP2A B' ζ SALK_ 107944 (control), lane 7: transformed mutant PP2A B' ζ SALK_ 107944, lane 8: non-transformed mutant PP2A B' ζ SALK_ 150586 (control), lane 9-11 : transformed mutant PP2A B' ζ SALK_150586. The lane number 2 and 3 from wild type transformed gave specific band 451bp , Three lane(9,10 and 11) from transformed mutant line PP2A B' ζ SALK_150586 gave same band, while in Lane number 7 (transformed mutant PP2A B' ζ SALK_ 107944) no band was obtained.

Table 3.2: Quantitative data obtained by analysis of agarose gel of gene expression study (Figure3.18) using ImageJ.

Lane number	Quantitative data
2	2814.6
3	30783.9
9	6500.3
10	4380.7
11	690

3.2.6 Western blot

Approximately four weeks after transfer of T2 seedlings to soil, the leaves were collected, and cell fractionation was performed (See Material and method section 2.3.10). Four cell fractions were obtained nucleus, chloroplast, mitochondria and soluble part of cell. Bradford assay was used for determination the total amount of protein extracted from each fraction before loading SDSPAGE. The western blot experiment was performed as described in material and method section X. The Blot incubated with antibody against YFP, YFP molecular weight about 27 kDa whereas PP2A B' ζ 61.7 kDa. Thus, the fusion protein around 88.7 kDa. The blot was

stained with ponceau stain before blocking step and antibody incubation to visualize total protein in membrane.

Two western blot experiments were performed, in the first trial, one of transformed mutant PP2A B’ ζ SALK_ 150586 was selected. The concentration of total protein loaded on SDS-PAGE average was 0.4 ng/µL. A plant extract that contain YFP was used as control to ensure that antibody used is working against YFP (antigen). The visualization of the blot showed that a specific band of YFP (27 kDa) was obtained in control, whereas no bands were observed in the transformed plant (Figure 3.19)

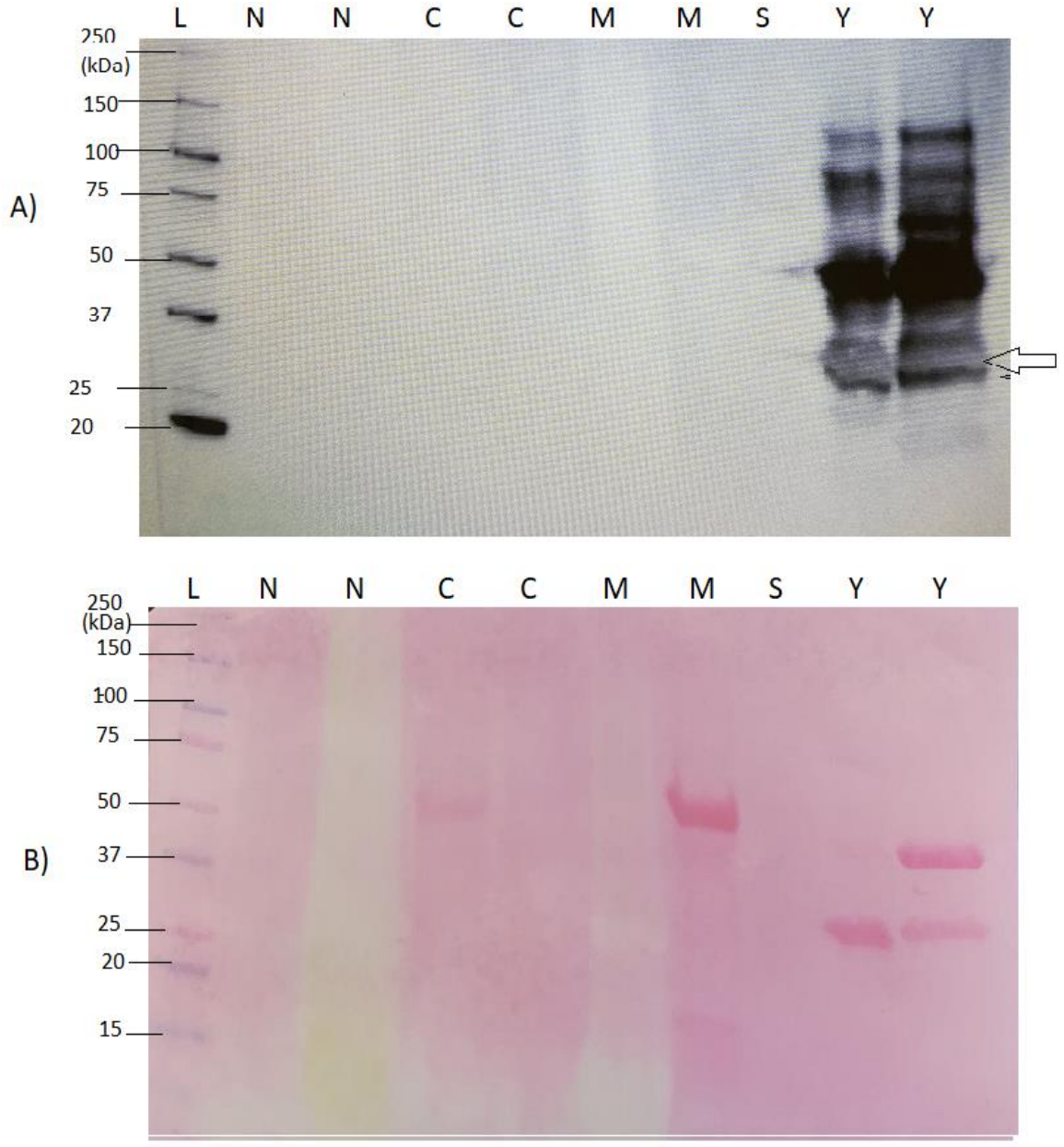

Figure 3.19: Western blot experiment of transformed mutant PP2A B' ζ SALK_ 150586, A: The blot visualized by ChemiDoc Imaging System (BioRad) B: Ponceau staining of membrane. N: nucleus fraction, C: chloroplast fraction of cell, M: mitochondrial fraction, S: soluble fraction of cell, Y plant extract contain YFP (control). In the experiment each cell fraction was loaded twice. In A) YFP specific band at 27 kDa (positioned with arrow on the right side of membrane) was only observed in controls and no band was observed in any fraction of transformed plant.

In the second trial the transformed wild type *Arabidopsis* plant that showed highest band intensity in gene expression analysis (See table 3.2) were selected for western blot experiment and the non-transformed wild type *Arabidopsis* was used as control. Higher concentration of extracted total proteins were loaded on SDS-PAGE approximately 1.3 ng/ µL. The plant extract that contain YFP used in second trial as control.

Visualization of the second western experiment showed that the transformed wild type plant has two bands in three cell fractions (chloroplast fraction, mitochondrial fraction and soluble fraction of cell). The first band around 27 kDa (YFP band) and second band around 88 kDa (YFP- PP2A B’ ζ fused protein band). The highest band intensity was obtained in the chloroplast fraction. However, for control plant extract that contain YFP no bands were observed. This could be due to incomplete transfer of control protein to the membrane as control was on edge of SDS gel. In The cell fractions of non-transformed wild type *Arabidopsis,* no bands were shown (Figure 3.20).

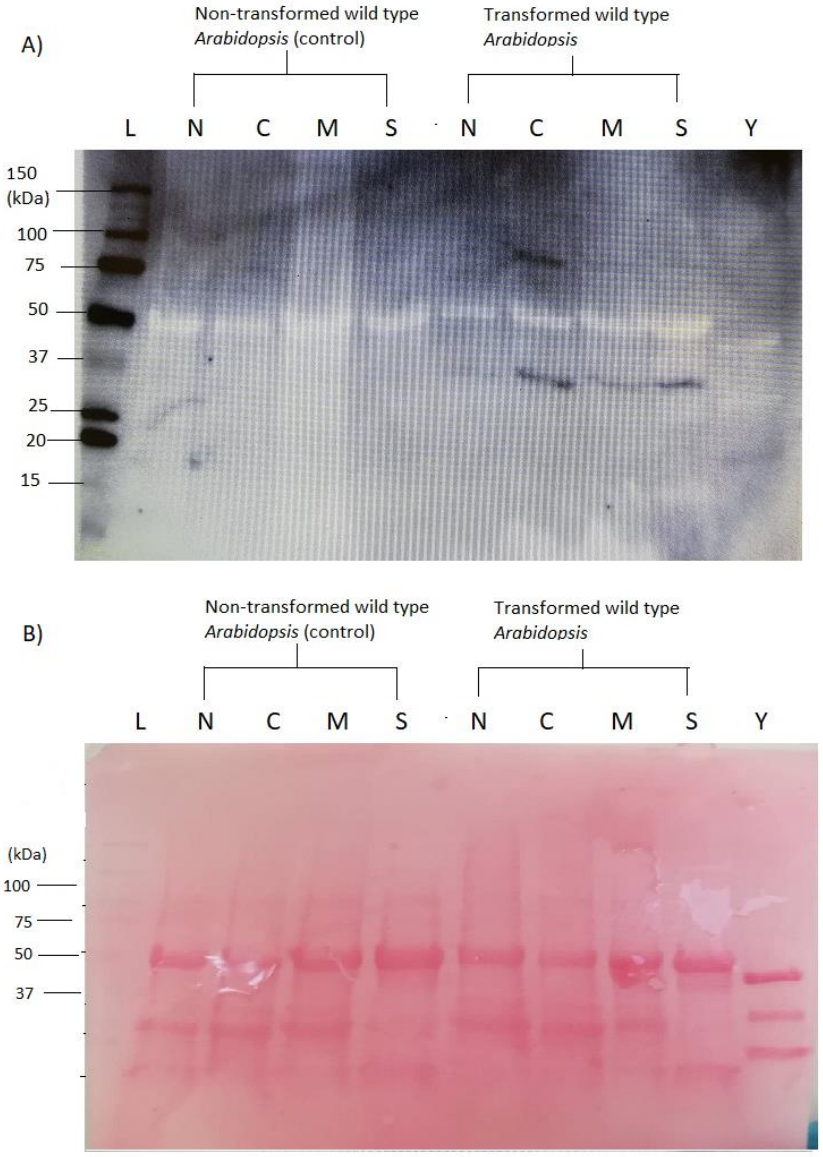

Figure 3.20: Western blot experiment of transformed wild type *Arabidopsis*. Non-transformed wild type was used as control. A: The blot visualized by ChemiDoc Imaging System (BioRad) B: Ponceau stanning of membrane. N: nucleus fraction, C: chloroplast fraction of cell, M: mitochondrial fraction, S: soluble fraction of cell, Y plant extract contain YFP (control). In A the transformed wild type plant two bands in three cell fractions (chloroplast fraction, mitochondrial fraction and soluble fraction of cell), yellow dotted line with drawn to simplify determination of molecular weight of band. First band approximately 27 kDa (YFP band) second band between 100-75 kDa (88 kDa YFP- PP2A B' ζ fused protein band). Highest band intensity was obtained in chloroplast fraction of cell, specific band of YFP not observed in non-transformed wild type and unexpectedly plant extract contains YFP.

4 DISCUSSION AND OUTLOOK

4.1 Bimolecular fluorescence complementation analysis

BiFC has become a widely used approach to study protein interactions in normal cell environment and is well established in various plant species (Ohad et al., 2007). In the present study the BiFC vectors were transiently expressed using onion (*Allium cepa*) epidermal cells. The epidermal layer of onions contains large living transparent cells that have uniform size and shape (Scott et al., 1999). For plant researchers, onion epidermal cells are an ideal experimental system for conducting the BiFC assay because of the ease in obtaining, preparing of onion tissues and the direct visualization of fluorescence with minimal auto fluorescence (Hollender et al., 2010). The cells are easy transformed by cell bombardment with gold particles coated with the plasmid DNA using a biolistic gun. The genes are quickly expressed within 16-24 hr, and the cell can be analyzed by epifluorescence or confocal microscopy (Citovsky et al., 2008).

In onion epidermal cells, fluorescence in cytoplasm was detected in combinations of ACO1 and B'ζ (See figure 3.5 in Results). It has been reported that both ACO1 and B'ζ localized to the cytoplasm (Eprintsev et al., 2015, Matre et al., 2009). Recently (at C. Lillo lab by Ahmed Elshobaky, personal communication) B' ζ was shown to interact with cytosolic ACO3 (isoenzyme of ACO1). The cellular localization of both protein and interaction between ACO3 and B' ζ consolidate our finding. No fluorescence was observed between truncated ACO1 and B' ζ This indicates that the C-terminal end of ACO1 appeared to be necessary for interaction with B' ζ because the truncated that lacked the 64 C-terminal amino acids did not interact with B' ζ. The C-terminal end of ACO1 seem to be conserved as high degree of alignment obtained with C-terminal of ACO2 and ACO3.

Fluorescence was observed in combination AtmSFC and B'β, despite that AtmSFC is known to be a transporter protein in the inner mitochondrial membrane and B'β to be localized in cytoplasm and nucleus (Catoni et al., 2003, Wang et al., 2016). The observed fluorescence was in cytoplasm and in form of speckles (See figure 3.6 in Results). The interaction between AtmSFC and B'β was repeated and same results were obtained. Peroxisomal and mitochondrial markers were included in BiFC experiments to study localization of AtmSFC and B'β interaction. The fluorescence signal did not localize to peroxisomes or mitochondria in any experiments and was rather evenly distributed in the cell. This could be because addition

of internal marker increases complexity of transformation snice three vectors have to be taken by single cell.

Non-specific fluorescence represents a challenge of BiFC assays. High expression of proteins tagged with fluorescent fragments may results in fluorescent complex formation in absence of specific interaction between candidate proteins fused to the fragments (Kerppola 2009). The expression of proteins from a constitutive and strong promotor can result in nonspecific BiFC signals (Caplan et al., 2008). The fusion proteins in present study were expressed under the CaMV 35S promoter which is a strong promotor. High concentration of vector DNA transformed to the cell may also result in unspecific fluorescence. Avoiding overexpression may reduce nonspecific signal by using a less active or native promoter of candidate protein (Ozalp et al., 2005).

Combination of AtmSFC with regulatory subunits B' α and B'θ gave nonspecific background fluorescence. The background fluorescence was also observed in negative control experiment using empty BiFC vectors (See Figure3.7 in Results). Thus, the negative control experiment allowed us to distinguish between specific and non-specific interactions.

The BiFC vectors used in the present study are based on the fluorescent protein Venus. Venus is a modified version of YFP and is the brightest fluorescent protein. It has been reported that using fragments of Venus generate the highest fluorescence intensity of BiFC complexes (Nagai et al., 2002, Shyu et al., 2006). However, it has also been reported to result in a higher background signal. This might be the reason for nonspecific background fluorescence obtained in negative control experiment and in combination of AtmSFC with regulatory subunits B' α and B'θ.

4.2 Study of PP2A B' ζ localization

Transgenic plants are used as an important research tool to study the organization and regulation of genes in plant biology (Bhat, 2002). Transgenic plants are generated through altering the genetic makeup of plant genome to introducing new trait to plant. The most widely used and the most successful method in generation of transgenic plant for research purpose is *Agrobacterium*-mediated transformation. *Agrobacterium* has ability infects the plant through Ti plasmid (Tumor inducing plasmid) The Ti plasmid integrates a segment of its DNA, known as T-DNA into the plant genome (Bhat, 2002).

In present study transgenic plants generated to study localization of Arabidopsis PP2AB' ζ using *Agrobacterium*-mediated transformation and pBA002 binary vector. PP2AB' ζ linked to YFP cloned in the T-DNA region of binary vector. Virulence genes in the binary vector code for a series of proteins cut T-DNA and facilitate transduction of PP2AB' ζ YFP to plant genome. In present study the wild type *Arabidopsis* and two Knocked out mutants PP2AB' ζ have been used to study localization of PP2AB' ζ. Knocked out mutants could provide more advantage over the wild type, because expressed PP2AB' ζ could interfere with PP2AB' ζ YFP. After generation of recombinant plasmid pBA002-PP2AB' ζ /YFP had been confirmed by colony PCR and sequencing. The recombinant plasmid transformed to Agrobacterium ABI. transformed Agrobacterium used to insert PP2AB' ζ YFP into plant genome of wild type Arabidopsis and two Knocked out mutants PP2AB' ζ.

4.2.1 Screening of transformed plant

In first generation screening, plant seeds were collected from transformed plants and screened by sowing them on ½ MS plates containing plant herbicide BASTA. Since pBA002 vector contained BASTA resistance, only seedlings from seeds which had integrated the plasmid into their genome would be able to grow uninhibited. Only few seedlings from transformed plants were able to grow on medium. This indicate either low transformation efficiency of *Agrobacterium* or DNA repair mechanism in plant were able to remove the integrated DNA. More seedlings were obtained from transformed wild type *Arabidopsis* than transformed knocked out mutants PP2AB'ζ (See Figure 3.13 in Results). This was probably due to genome of knocked out mutants PP2AB' ζ already modified and could be more resistant to

genetic alteration. Approximately 10-15 transformed seedlings from each transformation were transferred to soil to grow plant.

In second generation screening. seeds were collected after two months and screened by sowing them on ½ MS plates containing plant herbicide BASTA. Most of seeds of second generation plant were able to grow on medium unaffected by herbicide. This indicate that pBA002 plasmid integrated in genome of second generation plants and stable transgenic plant had been obtained (See Figure 3.13 in Results).

4.2.2 Genotyping of transformed plant

Genotyping of transformed plant was performed to verify the presence of PP2AB' ζ -YFP inserted in to plant genome. It has been reported the the interrupted transformation process could result in plant expressing selectable marker but contain no T-DNA giving a false positive result. New primers were designed to give PCR product that contains part of PP2A B' ζ and part of YFP to verify PP2A B' ζ fused with YFP in the plant genome. In genotyping of first generation plants, the specific band of 451 bp were obtained in transformed wild type and two knocked out mutants PP2AB' B' ζ. However, some of the plants that passed the BASTA screening did not show PCR product on agarose gel (See Figure 3.15). This indicates some plants have selectable marker in their genome but do not contain T-DNA, so this plant cancelled from study.

In genotyping of second generation plants, the specific band of 451 bp was obtained in all transformed plants. This indicates permanent integration of PP2AB'ζ-YFP in the plant genome of transformed plants.

4.2.3 Gene expression analysis

A gene expression study was carried out to verify formation of gene product from its encoding gene. PP2AB' ζ-YFP in pBA002 vector is expressed under the CaMV 35S promoter which is a strong promotor. The total RNA extracted from second generation seedling. After that cDNA was synthesized by reverse transcription of RNA. Fixed concentration cDNA was used with conventional PCR to . Different band intensity was obtained between transformed plants this might be because variation in transformation efficiency. Some of the transformed plants that contain PP2AB' ζ-YFP in their genome did not show PCR product this indicate that PP2AB' ζ-

YFP was not transcribed to RNA. The plants that showed highest band intensity were used in western blot due to high level of expression obtained.

4.2.4 western blotting

In present study transgenic plant generated to express PP2A B' ζ fused to YFP. The fusion protein around 88.7kDa. In first trial experiment, one of the transformed mutant PP2A B' ζ SALK_ 150586 was selected. No band were obtained in four cell compartments (See Figure 3.17). Low concentration of total protein had been used this might be reason. Also, Ponceau stanning of membrane showed few bands.

In second trial experiment transformed wild type *Arabidopsis* were selected and higher concentration of total extracted protein. highest band intensity was obtained in chloroplast followed by cytoplasm and mitochondria. This was preliminary result and need further study.

4.2.5 Comparison our finding with the previous study and subcellular predication programs

It has been reported that the *Arabidopsis* PP2A regulatory subunit B´ ζ with a free N-terminus was targeted to cytoplasm and partly localized to mitochondria and excluded chloroplast and nucleus. However, protein subcellular localization programs predicated that PP2A B' ζ to be localized in cytoplasm, chloroplast and mitochondria (Matre et al., 2009).In the present study we have found PP2A B' ζ to be localized more in chloroplast and cytoplasm but it also confirms PP2A B' ζ do not target to nucleus. Since transformed plants have YFP fused to PP2A B' ζ examination of leaves using confocal microscope could be useful to study localization PP2A B' ζ.

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APPENDIX

A. Role of PP2B' subunits in regulation of metabolism.

A1. Full length ACO1 and truncated ACO1

Alignments of full of full length ACO1 against truncated ACO1

In present study two primer pairs were designed, first primer pair used to pick up full CDS ACO1 (2697 bp), a second primer pair was used to pick first 2505 bp giving truncated version of ACO1 where last 192 bp has been truncated, 192 bp code for 62 amino acids at C-terminal end(See Table 2.3 in Material and method section).

Alignments of full length ACO1 against truncated ACO1 using clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). f. ACO1 stand for full while t. ACO1 stand for truncated ACO1

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f.ACO1      ATGGCTTCCGAGAATCCTTTCCGAAGCATATTGAAGGCGTTAGAGAAGCCTGATGGTGGT    60
t.ACO1      ATGGCTTCCGAGAATCCTTTCCGAAGCATATTGAAGGCGTTAGAGAAGCCTGATGGTGGT    60
            ************************************************************

f.ACO1      GAATTCGGTAACTACTACAGCTTACCTGCTTTGAACGATCCCAGGATCGATAAACTACCT    120
t.ACO1      GAATTCGGTAACTACTACAGCTTACCTGCTTTGAACGATCCCAGGATCGATAAACTACCT    120
            ************************************************************

f.ACO1      TATTCCATTAGGATACTTCTTGAATCGGCCATACGTAACTGTGATGAGTTCCAAGTTAAG    180
t.ACO1      TATTCCATTAGGATACTTCTTGAATCGGCCATACGTAACTGTGATGAGTTCCAAGTTAAG    180
            ************************************************************

f.ACO1      AGCAAAGATGTTGAGAAGATTCTTGATTGGGAGAATACTTCTCCCAAGCAGGTTGAGATT    240
t.ACO1      AGCAAAGATGTTGAGAAGATTCTTGATTGGGAGAATACTTCTCCCAAGCAGGTTGAGATT    240
            ************************************************************

f.ACO1      CCGTTCAAGCCTGCTCGGGTTCTTCTTCAGGACTTTACTGGTGTTCCTGCTGTTGTTGAT    300
t.ACO1      CCGTTCAAGCCTGCTCGGGTTCTTCTTCAGGACTTTACTGGTGTTCCTGCTGTTGTTGAT    300
            ************************************************************

f.ACO1      CTTGCTTGCATGAGAGATGCCATGAATAATCTCGGTGGTGATTCTAATAAAATTAATCCG    360
t.ACO1      CTTGCTTGCATGAGAGATGCCATGAATAATCTCGGTGGTGATTCTAATAAAATTAATCCG    360
            ************************************************************

f.ACO1      CTGGTCCCTGTAGATCTTGTCATTGATCACTCCGTTCAGGTGGATGTGGCGAGATCAGAG    420
t.ACO1      CTGGTCCCTGTAGATCTTGTCATTGATCACTCCGTTCAGGTGGATGTGGCGAGATCAGAG    420
            ************************************************************

f.ACO1      AACGCAGTGCAGGCAAACATGGAGCTTGAGTTCCAGCGTAACAAGGAAAGATTTGCTTTT    480
t.ACO1      AACGCAGTGCAGGCAAACATGGAGCTTGAGTTCCAGCGTAACAAGGAAAGATTTGCTTTT    480
            ************************************************************

f.ACO1      CTTAAGTGGGGATCCAACGCCTTTCACAACATGCTTGTCGTACCTCCTGGATCTGGAATA    540
t.ACO1      CTTAAGTGGGGATCCAACGCCTTTCACAACATGCTTGTCGTACCTCCTGGATCTGGAATA    540
            ************************************************************

f.ACO1      GTTCATCAAGTCAACCTAGAATACCTTGCCAGAGTTGTTTTCAACACAAATGGACTTCTT    600
t.ACO1      GTTCATCAAGTCAACCTAGAATACCTTGCCAGAGTTGTTTTCAACACAAATGGACTTCTT    600
            ************************************************************

f.ACO1      TACCCAGACAGTGTTGTTGGCACAGACTCTCACACCACTATGATTGATGGACTGGGTGTT    660
t.ACO1      TACCCAGACAGTGTTGTTGGCACAGACTCTCACACCACTATGATTGATGGACTGGGTGTT    660
            ************************************************************

f.ACO1      GCTGGATGGGGAGTTGGCGGTATAGAAGCGGAAGCTACCATGCTTGGTCAGCCAATGAGC    720
t.ACO1      GCTGGATGGGGAGTTGGCGGTATAGAAGCGGAAGCTACCATGCTTGGTCAGCCAATGAGC    720
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                ************************************************************

f.ACO1          ATGGTCCTACCCGGTGTTGTGGGTTTCAAGCTAACGGGAAAGTTAAGAGATGGAATGACA    780
t.ACO1          ATGGTCCTACCCGGTGTTGTGGGTTTCAAGCTAACGGGAAAGTTAAGAGATGGAATGACA    780
                ************************************************************

f.ACO1          GCTACTGATTTGGTCTTAACAGTGACTCAGATGTTGAGGAAACATGGAGTAGTTGGAAAG    840
t.ACO1          GCTACTGATTTGGTCTTAACAGTGACTCAGATGTTGAGGAAACATGGAGTAGTTGGAAAG    840
                ************************************************************

f.ACO1          TTTGTTGAATTCCACGGGGAAGGGATGAGAGAATTGTCTTTAGCTGACCGTGCTACAATT    900
t.ACO1          TTTGTTGAATTCCACGGGGAAGGGATGAGAGAATTGTCTTTAGCTGACCGTGCTACAATT    900
                ************************************************************

f.ACO1          GCCAATATGTCTCCTGAGTACGGTGCGACCATGGGATTCTTCCCAGTCGATCATGTCACT    960
t.ACO1          GCCAATATGTCTCCTGAGTACGGTGCGACCATGGGATTCTTCCCAGTCGATCATGTCACT    960
                ************************************************************

f.ACO1          TTGCAGTATCTAAGGTTGACAGGCAGGAGCGATGACACTGTCTCCATGATAGAGGCGTAT   1020
t.ACO1          TTGCAGTATCTAAGGTTGACAGGCAGGAGCGATGACACTGTCTCCATGATAGAGGCGTAT   1020
                ************************************************************

f.ACO1          TTACGAGCAAACAAGATGTTTGTGGATTACAGTGAGCCGGAGAGTAAGACAGTTTATTCC   1080
t.ACO1          TTACGAGCAAACAAGATGTTTGTGGATTACAGTGAGCCGGAGAGTAAGACAGTTTATTCC   1080
                ************************************************************

f.ACO1          TCATGTCTGGAATTGAATCTCGAGGATGTGGAACCTTGTGTTTCTGGTCCCAAGAGGCCT   1140
t.ACO1          TCATGTCTGGAATTGAATCTCGAGGATGTGGAACCTTGTGTTTCTGGTCCCAAGAGGCCT   1140
                ************************************************************

f.ACO1          CATGATCGTGTTCCTTTGAAGGAAATGAAAGCGGACTGGCATTCTTGCTTGGACAATAGA   1200
t.ACO1          CATGATCGTGTTCCTTTGAAGGAAATGAAAGCGGACTGGCATTCTTGCTTGGACAATAGA   1200
                ************************************************************

f.ACO1          GTAGGATTCAAGGGTTTCGCTGTACCTAAAGAAGCACAGAGTAAGGCTGTAGAGTTCAAT   1260
t.ACO1          GTAGGATTCAAGGGTTTCGCTGTACCTAAAGAAGCACAGAGTAAGGCTGTAGAGTTCAAT   1260
                ************************************************************

f.ACO1          TTTAACGGGACCACAGCACAGCTTAGACATGGAGATGTTGTTATAGCAGCAATCACCAGT   1320
t.ACO1          TTTAACGGGACCACAGCACAGCTTAGACATGGAGATGTTGTTATAGCAGCAATCACCAGT   1320
                ************************************************************

f.ACO1          TGCACAAATACTTCAAACCCTAGTGTAATGCTTGGCGCTGCCTTAGTTGCAAAAAAGGCC   1380
t.ACO1          TGCACAAATACTTCAAACCCTAGTGTAATGCTTGGCGCTGCCTTAGTTGCAAAAAAGGCC   1380
                ************************************************************

f.ACO1          TGCGACCTAGGACTGGAGGTTAAGCCATGGATCAAAACTAGTCTTGCTCCAGGCTCTGGA   1440
t.ACO1          TGCGACCTAGGACTGGAGGTTAAGCCATGGATCAAAACTAGTCTTGCTCCAGGCTCTGGA   1440
                ************************************************************

f.ACO1          GTTGTAACAAAGTACTTGGCAAAGAGTGGCTTGCAGAAGTACTTGAATCAGCTCGGCTTC   1500
t.ACO1          GTTGTAACAAAGTACTTGGCAAAGAGTGGCTTGCAGAAGTACTTGAATCAGCTCGGCTTC   1500
                ************************************************************

f.ACO1          AGTATCGTTGGTTATGGGTGCACCACATGCATTGGAAACTCGGGGGATATCCATGAAGCT   1560
t.ACO1          AGTATCGTTGGTTATGGGTGCACCACATGCATTGGAAACTCGGGGGATATCCATGAAGCT   1560
                ************************************************************

f.ACO1          GTGGCTTCAGCAATAGTTGATAATGACTTGGTGGCATCCGCTGTGTTGTCTGGGAACAGA   1620
t.ACO1          GTGGCTTCAGCAATAGTTGATAATGACTTGGTGGCATCCGCTGTGTTGTCTGGGAACAGA   1620
                ************************************************************

f.ACO1          AATTTTGAGGGACGTGTTCACCCGTTAACAAGAGCTAACTATCTAGCTTCCCCACCGCTT   1680
t.ACO1          AATTTTGAGGGACGTGTTCACCCGTTAACAAGAGCTAACTATCTAGCTTCCCCACCGCTT   1680
                ************************************************************

f.ACO1          GTTGTAGCCTATGCTCTGGCTGGAACTGTTGACATTGATTTTGAGACACAGCCCATTGGA   1740
t.ACO1          GTTGTAGCCTATGCTCTGGCTGGAACTGTTGACATTGATTTTGAGACACAGCCCATTGGA   1740
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                ************************************************************

f.ACO1          ACTGGGAAAGATGGAAAACAGATATTTTTCAGGGACATTTGGCCCTCTAACAAAGAAGTT    1800
t.ACO1          ACTGGGAAAGATGGAAAACAGATATTTTTCAGGGACATTTGGCCCTCTAACAAAGAAGTT    1800
                ************************************************************

f.ACO1          GCTGAGGTTGTTCAATCTAGTGTCCTTCCTGATATGTTCAAAGCTACATATGAAGCAATC    1860
t.ACO1          GCTGAGGTTGTTCAATCTAGTGTCCTTCCTGATATGTTCAAAGCTACATATGAAGCAATC    1860
                ************************************************************

f.ACO1          ACCAAAGGAAATTCCATGTGGAATCAGTTATCTGTGGCGTCAGGTACTCTCTATGAGTGG    1920
t.ACO1          ACCAAAGGAAATTCCATGTGGAATCAGTTATCTGTGGCGTCAGGTACTCTCTATGAGTGG    1920
                ************************************************************

f.ACO1          GACCCGAAATCAACTTACATTCACGAGCCGCCTTATTTCAAGGGCATGACCATGTCTCCA    1980
t.ACO1          GACCCGAAATCAACTTACATTCACGAGCCGCCTTATTTCAAGGGCATGACCATGTCTCCA    1980
                ************************************************************

f.ACO1          CCCGGTCCACATGGTGTGAAAGACGCATACTGTTTACTCAATTTTGGAGACAGTATTACC    2040
t.ACO1          CCCGGTCCACATGGTGTGAAAGACGCATACTGTTTACTCAATTTTGGAGACAGTATTACC    2040
                ************************************************************

f.ACO1          ACTGATCACATCTCACCAGCTGGTAGCATCCACAAGGACAGTCCTGCGGCTAAGTACTTG    2100
t.ACO1          ACTGATCACATCTCACCAGCTGGTAGCATCCACAAGGACAGTCCTGCGGCTAAGTACTTG    2100
                ************************************************************

f.ACO1          ATGGAACGAGGTGTGGATAGAAGAGACTTCAACTCATACGGGAGTCGCCGTGGTAATGAT    2160
t.ACO1          ATGGAACGAGGTGTGGATAGAAGAGACTTCAACTCATACGGGAGTCGCCGTGGTAATGAT    2160
                ************************************************************

f.ACO1          GAGATTATGGCGAGAGGCACTTTTGCAAATATCCGTATTGTCAACAAACACTTGAAAGGA    2220
t.ACO1          GAGATTATGGCGAGAGGCACTTTTGCAAATATCCGTATTGTCAACAAACACTTGAAAGGA    2220
                ************************************************************

f.ACO1          GAAGTTGGTCCCAAAACAGTTCACATTCCCACTGGAGAGAAGCTTTCTGTTTTCGATGCT    2280
t.ACO1          GAAGTTGGTCCCAAAACAGTTCACATTCCCACTGGAGAGAAGCTTTCTGTTTTCGATGCT    2280
                ************************************************************

f.ACO1          GCCATGAAATATAGGAACGAGGGACGCGACACAATCATTTTGGCTGGTGCTGAATACGGT    2340
t.ACO1          GCCATGAAATATAGGAACGAGGGACGCGACACAATCATTTTGGCTGGTGCTGAATACGGT    2340
                ************************************************************

f.ACO1          AGTGGAAGTTCTCGTGATTGGGCTGCCAAGGGTCCAATGCTTCTGGGTGTGAAAGCTGTG    2400
t.ACO1          AGTGGAAGTTCTCGTGATTGGGCTGCCAAGGGTCCAATGCTTCTGGGTGTGAAAGCTGTG    2400
                ************************************************************

f.ACO1          ATTTCAAAGAGCTTCGAGCGAATTCACCGAAGCAATTTGGTGGGAATGGGAATCATACCT    2460
t.ACO1          ATTTCAAAGAGCTTCGAGCGAATTCACCGAAGCAATTTGGTGGGAATGGGAATCATACCT    2460
                ************************************************************

f.ACO1          TTGTGCTTCAAGGCGGGAGAAGATGCTGAGACCCTTGGCCTAACGGGTCAGGAGCTTTAC    2520
t.ACO1          TTGTGCTTCAAGGCGGGAGAAGATGCTGAGACCCTTGGCCTAACG---------------    2505
                *******************************************

f.ACO1          ACCATTGAGCTCCCAAACAATGTTAGTGAGATCAAACCAGGACAAGATGTAACAGTCGTC    2580
t.ACO1          ------------------------------------------------------------    2505

f.ACO1          ACAAACAATGGCAAATCTTTCACATGTACACTCCGATTTGACACAGAGGTGGAGTTGGCT    2640
t.ACO1          ------------------------------------------------------------    2505

f.ACO1          TATTTCGATCACGGAGGGATTTTGCAATACGTTATCAGGAACTTGATCAAACAATAA       2697
t.ACO1          ---------------------------------------------------------       2505
```

last 192 bp truncated from ACO1

GGTCAGGAGCTTTACACCATTGAGCTCCCAAACAATGTTAGTGAGATCAAACCAGGACAAGATGTAACAGTCGTC
ACAAACAATGGCAAATCTTTCACATGTACACTCCGATTTGACACAGAGGTGGAGTTGGCTTATTTCGATCACGGA
GGGATTTTGCAATACGTTATCAGGAACTTGATCAAACAATAA

By performing alignment of last 192 bp of ACO 1 with last 192 bp of ACO2 and ACO3, we have found this part of gene conserved between three genes despite the difference in gene size ACO1 (2697 bp), ACO2 (2988), ACO3(2973).

```
ACO2      GGTCATGAACGCTACACAGTCCACCTTCCTACAAAAGTTAGTGACATTAGACCCGGTCAA  60
ACO1      GGTCAGGAGCTTTACACCATTGAGCTCCCAAACAATGTTAGTGAGATCAAACCAGGACAA  60
ACO3      GGTCACGAACGCTACACGATCCATCTCCCAACCGATATCTCAGAGATAAGACCTGGCCAA  60
          ***** **.*  ***** .*  * ** **:*...*:.* : :** ** *.*** ** ***

ACO2      GACGTCACTGTAACCACTGACAGTGGCAAATCCTTTGTCTGCACCCTGCGTTTTGATACA  120
ACO1      GATGTAACAGTCGTCACAAACAATGGCAAATCTTTCACATGTACACTCCGATTTGACACA  120
ACO3      GATGTTACCGTCACTACCGACAACGGAAAATCTTTCACTTGCACAGTCCGCTTCGACACA  120
          ** ** ** **..  ** .***. **.***** ** .  ** **. * ** ** ** ***

ACO2      GAGGTGGAATTGGCATACTATGATCACGGCGGTATTCTACCATACGTCATCCGGAGTTTG  180
ACO1      GAGGTGGAGTTGGCTTATTTCGATCACGGAGGGATTTTGCAATACGTTATCAGGAACTTG  180
ACO3      GAGGTGGAATTGGCATACTTTAACCATGGAGGCATACTTCCATATGTTATCAGAAACTTG  180
          ********.*****:** *: .* ** **.** **: * *.*** ** ***.*.*. ***

ACO2      AGCGCCAAGTGA  192
ACO1      ATCAAACAATAA  192
ACO3      AGCAAGCAATAG  192
          * *.. .*.*..
```

A2. Sequence analysis of BIFC vectors

All of insert give high degree of identity when blasted against NCBI. Screenshot of first result obtained on Blast N NCBI program website (https://blast.ncbi.nlm.nih.gov/Blast.cgi), then sequence obtained aligned against its CDS using Clustal Omega website (https://www.ebi.ac.uk/Tools/msa/clustalo/) , very low mutation in sequence was founded ,Alignment not supplied here.

Sequence analysis of PP2AB' α in pVYCE vector

cDNA: PP2A B' α AT5G03470

insert was sequenced from both side using the primers forward 35S and reverse Nots.

Nucleotide sequence obtained by forward 35S.

ATGTTTAAGAAGATCATGAAAGGGGCAAATCGAAAAGCCTCTAAAGCTGAGGCGAATGATTCGTCTATGTATGGG
TTTGATCCACCAGGTAGGTCAGGTCCTGGATCCAATATGATTGTAAATCACGCATCTCGTGGTTCGCTTGTTCCT
TCTTCTCCAAATTCAATGGCGGCGGCCACCACACAACCGCCTCCTATGTATTCAGTCGAGCCTCTTCCCTTGTTC
AGAGACGTCTCTGTCTCCGAGCGTCAATCCTTGTTCTTGAGGAAACTCCAGATTTGTTGTTTTCAATTTGATTTT
ACGGATACGTTGAAGAATGCGAGAGAGAAGGAGATTAAGAGGCAAACGTTATTGGAGCTAGTTGATTTTATACAG
TCTGGAGCAGGGAAGCTCACTGAAGTTTGTCAAGAAGAAATGGTAAAGATGATTTCGGTTAATATATTCCGGTGT
CTTCCTCCGGCTTCACACGAGAATACAGGTCAAGAGCCTGCTGATCTTGAGGAAGAGGAGCCTTATTTGGAACCA
TCTTGGCCTCATTTACAGCTAATTTATGAGTTGCTGCTGAGATACATTGTTCCTTCTGACACAGATACAAAAGTT
GCAAAACGGTATATTGATCATTCTTTTGTGTTGAGGTTGCTTGAATTGTTTGAAACTGAAGATCCTAGAGAAAGG
GAGTACTTGAAAACGATTCTTCATAGGATTTATGGCAAGTTTATGGTTCACAGGCCCTTTATTAGGAAAGCAATG
AACCATATTTTCTATAGGTTTATTTATGAGACAGAGAGACATAGTGGGATTGGGGAGCTTTTGGAGATTCTCGGT
AGTATTATTAATGGGTTTGCCTTGCCAATGAAGGAGGAGCACAAGCTCTTTTTGATTAGGGCGTTGATACCGCTG
CATAAGCCAAAACCAATCGCTATGTATCACCAACAATTGTCTTACTGCATCGTTCAGTTTGTGGAGAAAGAT

972 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein (ATB' ALPHA), mRNA	1796	1796	100%	0.0	100%	NM_120427.3

The sequence 100% match with first result in BlastN NCBI

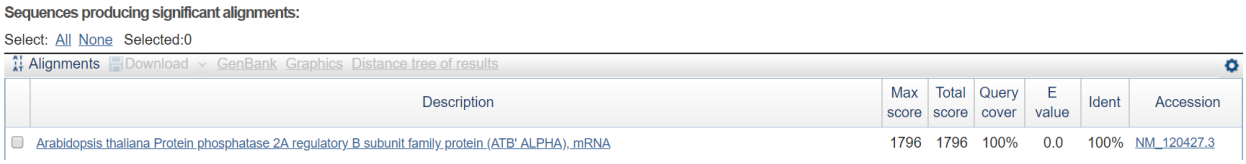
Nucleotide sequence obtained by reverse Nots

CTAAGAAGTGATCATAGGATCTTCTCTTTCTCGTTCCTCCACTGCTTCATCTAATCTCCTCCACCTATTTTGGCG
TTGCTCTTCCACTTGTTTAGATTTGGCTTGTTTTTCTTCGTACTGTTGCTGACATTCTTCAAAGAGCTCAGGGTC
CATCTCCATGAACATTCTCTTTATATTCGCACTTAGACCGTGTACCGCTTGGTTCCAATGAGACTCTATGTTTTT
CTCCAGGGATGCAAATATGATGGGAAGGATCACTCCTCGGTTCTGAGCTATTAGACCCACCACATGCTCGTTGTT
CCACAAGAACAGAGCTCGTTCTGCAACCTGAAAATTAGAGCTACTGAGGCATCGAGCAATCTGTTGGAACAGAGG
GACCATACAGCGTTGGAACTCAACAGTTTGTGTTGCCTCAAGAACCTCTTCAAGTTCACCAAGAAAGAGAACCTC
TTTTGTACAATTTGTCACAGGCCAAAACTTTAACAAGCCCCTGATCACCGTATCCGCAAGCTTATAATCTTTCTC
CACAAACTGAACGATGCAGTAAGACAATTGTTGGTGATACATAGCGATTGGTTTTGGCTTATGCAGCGGTATCAA
CGCCCTAATCAAAAAGAGCTTGTGCTCCTCCTTCATTGGCAAGGCAAACCCATTAATAATACTACCGAGAATCTC
CAAAAGCTCCCCAATCCCACTATGTCTCTCTGTCTCATAAATAAACCTATAGAAAATATGGTTCATTGCTTTCCT
AATAAAGGGCCTGTGAACCATAAACTTGCCATAAATCCTATGAAGAATCGTTTTCAAGTACTCCCTTTCTCTAGG

ATCTTCAGTTTCAAACAATTCAAGCAACCTCAACACAAAAGAATGATCAATATACCGTTTTGCAACTTTTGTATC
TGTGTCAGAAGGAACAATGTATCTCAGCAGCAACTCATAAATTAGCTGTAAATGAG

956 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)



The sequence 100% match with first result in BlastN NCBI

Sequence analysis of PP2AB' α in pVYCE(C) vector

cDNA: PP2A B' α AT5G03470

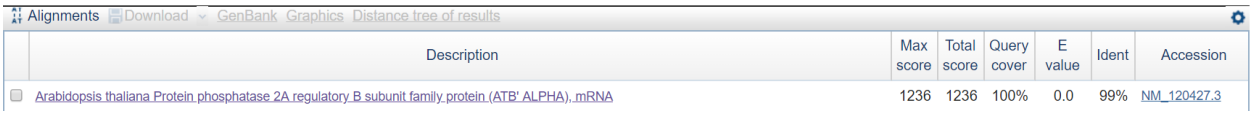
insert was sequenced from both side using the primers forward 35S and reverse Nots.

Nucleotide sequence obtained by forward 35S.

ATGTTTAAGAAGATCATGAAAGGGGCAAATCGAAAAGCCTCTAAAGCTGAGGCGAATGATTCGTCTATGTATGGG
TTTGATCCACCAGGTAGGTCAGGTCCTGGATCCAATATGATTGTAAATCACGCATCTCGTGGTTCGCTTGTTCCT
TCTTCTCCAAATTCAATGGCGGCGGCCACCACACAACCGCCTCCTATGTATTCAGTCGAGCCTCTTCCCTTGTTC
AGAGACGTCTCTGTCTCCGAGCGTCAATCCTTGTTCTTGAGGAAACTCCAGATTTGTTGTTTTCAATTTGATTTT
ACGGATACGTTGAAGAATGCGAGAGAGAAGGAGATTAAGAGGCAAACGTTATTGGAGCTAGTTGATTTTATACAG
TCTGGAGCAGGGAAGCTCACTGAAGTTTGTCAAGAAGAAATGGTAAAGATGATTTCGGTTAATATATTCCGGTGT
CTTCCTCCGGCTTCACACGAGAATACAGGTCAAGAGCCTGCTGATCTTGAGTTGCGGAGGAGCCTTATTTGGAAC
CATCTTGGCCTCATTTACAGCTAATTTATGAGTTGCTGCTGAGATACATTGTTCCTTCTGACACAGATACAAAAG
TTGCAAAACGGTATATTGATCATTCTTTTGTGTTGAGGTTGCTTGAATTGTTTGAAACTGAAGATCCTAGAGAAA
GGGAGTA

682 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)



The sequence 99% match with first result in BlastN NCBI

Nucleotide sequence obtained by reverse Nots

CTAAGAAGTGATCATAGGATCTTCTCTTTCTCGTTCCTCCACTGCTTCATCTAATCTCCTCCACCTATTTTGGCG
TTGCTCTTCCACTTGTTTAGATTTGGCTTGTTTTTCTTCGTACTGTTGCTGACATTCTTCAAAGAGCTCAGGGTC
CATCTCCATGAACATTCTCTTTATATTCGCACTTAGACCGTGTACCGCTTGGTTCCAATGAGACTCTATGTTTTT
CTCCAGGGATGCAAATATGATGGGAAGGATCACTCCTCGGTTCTGAGCTATTAGACCCACCACATGCTCGTTGTT
CCACAAGAACAGAGCTCGTTCTGCAACCTGAAAATTAGAGCTACTGAGGCATCGAGCAATCTGTTGGAACAGAGG
GACCATACAGCGTTGGAACTCAACAGTTTGTGTTGCCTCAAGAACCTCTTCAAGTTCACCAAGAAAGAGAACCTC
TTTTGTACAATTTGTCACAGGCCAAAACTTTAACAAGCCCCTGATCACCGTATCCGCAAGCTTATAATCTTTCTC
CACAAACTGAACGATGCAGTAAGACAATTGTTGGTGATACATAGCGATTGGTTTTGGCTTATGCAGCGGTATCAA
CGCCCTAATCAAAAAGAGCTTGTGCTCCTCCTTCATTGGCAAGGCAAACCCATTAATAATACTACCGAGAATCTC
CAAAAGCTCCCCAATCCCACTATGTCTCTCTGTCTCATAAATAAACCTATAGAAAATATGGTTCATTGCTTTCCT
AATAAAGGGCCTGTGAACCATAAACTTGCCATAAATCCTATGAAGAATCGTTTTCAAGTACTCCCTTTCTCTAGG
ATCTTCAGTTTCAAACAATTCAAGCAACCTCAACACAAAAGAATGATCAATATACCGTTTTGCAACTTTTGTATC
TGTGTCAGAAGGAACAATGTATCTCAGCAGCAACTCATAAATTAGCTGTAAATGAGGC

958 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein (ATB' ALPHA), mRNA	1236	1236	100%	0.0	99%	NM_120427.3

The sequence 99% match with first result in BlastN NCBI

Sequence analysis of PP2AB' β in pVYCE vector

cDNA: PP2A B' β AT3G09880

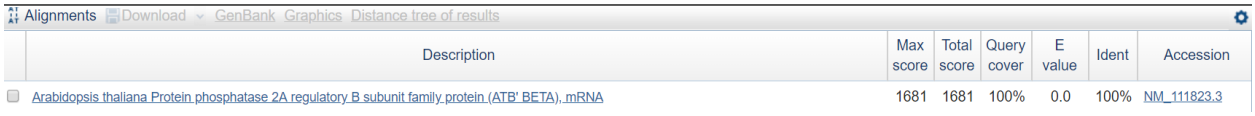
insert was sequenced from both side using the primers forward 35S and reverse Nots.

Nucleotide sequence obtained by forward 35S.

ATGTTTAAGAAAATCATGAAAGGTGGGCATCGAAAGCCCTCTAAATCCGAAGCTAATGAACCGTCTAGTT
ATGGGATTGGTCTACCTGATAATAGGTCAGGTCCTGGCTCCAATGTGGTCGTTAGTCACGCTTCTCGTGG
TGCATTGGTTAATTCTTCTCCGTCGCCAGTGACTGCAACTCCACCTCCTCCGCCTTTGGGCTCGGTGGAG
CCACTCCCGTTGTTTAGAGATGTGCCGGTTTCAGAGAGGCAAACTTTGTTCTTGAGGAAACTTCAGAATT
GCTGTTTCCTTTTCGATTTCACTGACACGATTAAGAATGCTAGAGACAAGGAGATTAAGAGGCAAACGTT
GTTGGAGCTGGTGGATTTTATACAGTCTGGATCTAGTAAGATCTCAGAGTCGTGTCAGGAAGAAATGATT
AAGATGATTTCTGTTAATATTTTCCGGTCTCTCCCACCAGCATCACACGAGAATACGGGTCAAGAGCCTG
CAGATCCTGAGGAAGAAGAACCTTATTTGGAACCTTCTTGGCCTCACTTACAGCTAGTTTACGAGTTGCT
CCTGAGATATGTTGTTTCGACTGACACTGATACAAAGGTGGCCAAACGGTATATCGACCATTCTTTTGTG
TTAAAGTTGCTCGATTTGTTTGATTCTGAGGACCCAAGAGAGAGGGAGTATTTGAAAACGATTCTTCATA
GGATATATGGGAAGTTTATGGTCCACAGACCCTTTATTAGGAAAGCAATCAACAACATATTCTATAGGTT
TATTTATGAGACAGAGAGACACAGTGGGATTGGGGAACTCTTGGAGATTCTAGGCAGTATCATAAACGGG
TTTGCATTGCCTATGAAGGAGGAACACAAGCTATTCTTAATCAGGGTGTTGATACCATTGCATAAGCCAA

925 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)



Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein (ATB' BETA), mRNA	1681	1681	100%	0.0	100%	NM_111823.3

The sequence 100% match with first result in BlastN NCBI

Nucleotide sequence obtained by reverse Nots

AAGTGAAAGAATTATACCCTTTTTGATCGATCTAGGAAGTGATCATATGATCTTCTTCTCCTCCTCCTCCATCTC
GCTCCGCCGCTGCTTCTGCTAATCTCTTCCATGTATATTGCCGTTGTTCTTCTACTTCTTTGGATTTCGCTTGTT
TCTCTTCATACTGTCTCTGACATTCTTCAAACAGTTCAGGATCCATTTCCATGAACATTTTCTTGATATTTGTGG
TTAGACCATGAACTGCTTGGTTCCAGTGAGACTGGATGTTCTTCTCCAGTGTGGGATATATGATTGGAAGGATCA
CGCTTCGGTTTTGCGCGATTAGACCCACAATGTGCTCGTTGTTCCACAAGAACAGTGCTCGTTCTGCAACCTGAA
AGTGGGAGCTAGTGAGACAGCGACCAATTTGTTGGAATAATGGAACCATGCAACGCTGGAACTCGACAGGCTGTG
TTGCCTCAAGAACTTCTTCAAGTTCTCCAAGGAAGAGATTCTCCTTTGAGCAGTTTGTCACGGGCCAATACTTTA
ACAATCCCCTGATTACTGTATCCGCAAGCTTATAATCTTTTTCCACAAACTGAACGATGCAATAAGATAACTGCT
GATGATATACAACGATTGGTTTTGGCTTATGCAATGGTATCAACACCCTGATTAAGAATAGCTTGTGTTCCTCCT
TCATAGGCAATGCAAACCCGTTTATGATACTGCCTAGAATCTCCAAGAGTTCCCCAATCCCACTGTGTCTCTCTG
TCTCATAAATAAACCTATAGAATATGTTGTTGATTGCTTTCCTAATAAAGGGTCTGTGGACCATAAACTTCCCAT
ATATCCTATGAAGAATCGTTTTCAAATACTCCCTCTCTCTTGGGTCCTCAGAATCAAACAAATCGAGCAACTTTA
ACACAAAAGAATGGTCGATATACCGTTTGGCCACCTTTGTATCAGTGTCAGTCGAAACAACATATCTCAGGAGCA
ACTCGTAAACTAGCTGTAAGTGAGGCCAAGAAGGTTCCAAATAAGGTTCTTCTTCCTCAGGATCTGCAGGCTCTT
GACCCGTATTCTCGTGTGATGCTGGTGGGAGAGACCGGAAAATATTAACAGAAATCATCTT

1112 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein (ATB' BETA), mRNA	2052	2052	100%	0.0	100%	NM_111823.3

The sequence 100% match with first result in BlastN NCBI

Sequence analysis of PP2AB' β in pVYCE(C) vector

cDNA: PP2A B' β AT3G09880

insert was sequenced from both side using the primers forward 35S and reverse Nots.

Nucleotide sequence obtained by forward 35S.

ATGTTTAAGAAAATCATGAAAGGTGGGCATCGAAAGCCCTCTAAATCCGAAGCTAATGAACCGTCTAGTT
ATGGGATTGGTCTACCTGATAATAGGTCAGGTCCTGGCTCCAATGTGGTCGTTAGTCACGCTTCTCGTGG
TGCATTGGTTAATTCTTCTCCGTCGCCAGTGACTGCAACTCCACCTCCTCCGCCTTTGGGCTCGGTGGAG
CCACTCCCGTTGTTTAGAGATGTGCCGGTTTCAGAGAGGCAAACTTTGTTCTTGAGGAAACTTCAGAATT
GCTGTTTCCTTTTCGATTTCACTGACACGATTAAGAATGCTAGAGACAAGGAGATTAAGAGGCAAACGTT
GTTGGAGCTGGTGGATTTTATACAGTCTGGATCTAGTAAGATCTCAGAGTCGTGTCAGGAAGAAATGATT
AAGATGATTTCTGTTAATATTTTCCGGTCTCTCCCACCAGCATCACACGAGAATACGGGTCAAGAGCCTG
CAGATCCTGAGGAAGAAGAACCTTATTTGGAACCTTCTTGGCCTCACTTACAGCTAGTTTACGAGTTGCT
CCTGAGATATGTTGTTTCGACTGACACTGATACAAAGGTGGCCAAACGGTATATCGACCATTCTTTTGTG
TTAAAGTTGCTCGATTTGTTTGATTCTGAGGACCCAAGAGAGAGGGAGTATTTGAAAACGATTCTTCATA
GGATATATGGGAAGTTTATGGTCCACAGACCCTTTATTAGGAAAGCAATCAACAACATATTCTATAGGTT

782 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein (ATB' BETA), mRNA	1423	1423	100%	0.0	100%	NM_111823.3

The sequence 100% match with first result in BlastN NCBI

Nucleotide sequence obtained by reverse Nots

GTTCCACAAGAACAGTGCTCGTTCTGCAACCTGAAAGTGGGAGCTAGTGAGACAGCGACCAATTTGTTGGAATAA
TGGAACCATGCAACGCTGGAACTCGACAGGCTGTGTTGCCTCAAGAACTTCTTCAAGTTCTCCAAGGAAGAGATT
CTCCTTTGAGCAGTTTGTCACGGGCCAATACTTTAACAATCCCCTGATTACTGTATCCGCAAGCTTATAATCTTT
TTCCACAAACTGAACGATGCAATAAGATAACTGCTGATGATATACAACGATTGGTTTTGGCTTATGCAATGGTAT
CAACACCCTGATTAAGAATAGCTTGTGTTCCTCCTTCATAGGCAATGCAAACCCGTTTATGATACTGCCTAGAAT
CTCCAAGAGTTCCCCAATCCCACTGTGTCTCTCTGTCTCATAAATAAACCTATAGAATATGTTGTTGATTGCTTT
CCTAATAAAGGGTCTGTGGACCATAAACTTCCCATATATCCTATGAAGAATCGTTTTCAAATACTCCCTCTCTCT
TGGGTCCTCAGAATCAAACAAATCGAGCAACTTTAACACAAAAGAATGGTCGATATACCGTTTGGCCACCTTTGT
ATCAGTGTCAGTCGAAACAACATATCTCAGGAGCAACTCGTAAACTAGCTGTAAGTGAGGCCAAGAAGGTTCCAA
ATAAGGTTCTTCTTCCTCAGGATCTGCAGGCTCTTGACCCGTATTCTCGTGTGATGCTGGTGGGAGAGACCGGAA
AATATTAACAGAAATCATCTT

771 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein (ATB' BETA), mRNA	1384	1384	100%	0.0	100%	NM_111823.3

The sequence 100% match with first result in BlastN NCBI

Sequence analysis of PP2AB' θ in pVYCE vector

cDNA: PP2A B' θ AT1G13460

insert was sequenced from both side using the primers forward 35S and reverse Nots.

Nucleotide sequence obtained by forward 35S.

ATGTGGAAACAGATTCTGAGTAAGCTTCCTAAGAAGTCTTCTTCTAAGAACCATTCTTCTTCATCTAGTT
CCACTTCAAAAAGTAGTGATAATGGCGCCAGTAAATCGGGTAATTCTCAAACCCAGAACGCTCCTCCTGT
AAAACCTTCTGCTGATTCAGGGTTTAAAGAAGGGAATTTGAAAGGAAATGGTAACGGTTTCACTCCATAT
GAAGCATTGCCAGGTTTCAAAGATGTTCCTAATGCAGAGAAACAGAACTTGTTTGTGAGAAAACTCAGCT
TGTGCTGTGTTGTTTTCGATTTCTCGGATCCAACGAAGAACGTTAAGGAAAAGGATATTAAGCGGCAGAC
ATTGCTTGAGCTTGTGGATTATGTAGCTTCTCCTAATGGAAAGTTTAGTGAAACTGTGATTCAAGAAGTG
GTTAGAATGGTTTCTGTTAACATATTCAGAACCTTGAATCCTCAACCGCGCGAGAATAAAGTTATTGATG
CGTTAGACTTGGAGGAAGAAGAGCCTTCTATGGATCCTACTTGGCCTCACTTGCAGCTTGTCTATGAAAT
TCTCCTGCGGCTTATTGCTTCACCAGAGACAGACACCAAGCTGGCTAAGAAATACATTGACCAATCTTTT
GTCTCAAGGTTACTTGATTTATTTGATTCAGAGGATCCTAGAGAAAGAGATTGTCTTAAGACCGTTCTAC
ATCGCATCTACGGTAAATTTATGGTTCATCGTCCTTTCATAAGGAAATCCATTAATAACATTTTCTATCG
GTTTGTTTTCGAGACTGAGAAACACAATGGGATTGCTGAGTTTCTAGAGATTTTGGGAAGTATCATCAAT

851 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein mRNA	1552	1552	100%	0.0	100%	NM_202087.2
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein mRNA	1552	1552	100%	0.0	100%	NM_101216.2
Arabidopsis thaliana chromosome 1 sequence	1552	1552	100%	0.0	100%	CP002684.1
Arabidopsis thaliana At1g13460 mRNA, complete cds	1552	1552	100%	0.0	100%	BT026384.1

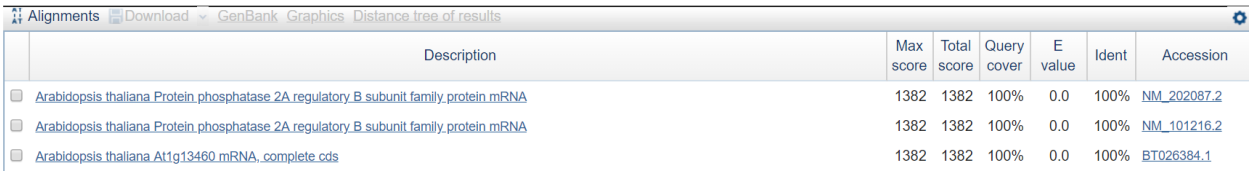
The sequence 100% match with fourth result in BlastN NCBI, which is PP2A B' θ AT1G13460

Nucleotide sequence obtained by reverse Nots

CTTTTGATTACCAATTTCTTCCAACCGTTTCCACGTTGCCTCACGTTTTGCGCCAATTTCAGCTTCTTTTGATTC
ATCTTCTCTAAACTTAGCAAGACAATCCTTAAACAACTCTGCATCAATGTCATTAAAGATCTTCTGCACATTCAA
CGTCAAGCTATGAACAGCTTGGTTCCAATGCTTCTGAGTGTTTCTCTCCAATGCAGGGAATATGATTGGAAGAAT
AACTTTCCGGTTCTGCATTATTAGATTCTCGATATGATCGTTGTTCCATAAGAACAAAGCTCTTTCTGCAACCTG
AAAATGGAGACTGTTCAAACACCGAGCAACTTGGCGAAACAATGGGACCATACACCGTTGGAATTCAGGTGGTTG
AGTTGCTTCTAATACTTCCTCTAGCTCGTTTAGAAACATGACTTCTTTGGAACTATTTGTGACAGGCCAAGACTT
GAGTAGTCCCCTTATAACCGTATCAGCGAGTTTGCAATCTTTTTCCACAAACTGTGTGATACAATAAGACAATTG
CTGATGATACATTTGCAAGCTTTTCGGTTTGTGAAGAGGTACCAAAGCTCTAACCAAAAACACTTTATGCTCGTC
TTTAAGCGGCAGAGCAAATCCATTGATGATACTTCCCAAAATCTCTAGAAACTCAGCAATCCCATTGTGTTTCTC
AGTCTCGAAAACAAACCGATAGAAAATGTTATTAATGGATTTCCTTATGAAAGGACGATGAACCATAAATTTA

784 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein mRNA	1382	1382	100%	0.0	100%	NM_202087.2
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein mRNA	1382	1382	100%	0.0	100%	NM_101216.2
Arabidopsis thaliana At1g13460 mRNA, complete cds	1382	1382	100%	0.0	100%	BT026384.1

The sequence 100% match with fourth result in BlastN NCBI which is PP2A B' θ AT1G13460

Sequence analysis of PP2AB' θ in pVYCE(C) vector

cDNA: PP2A B' θ AT1G13460

insert was sequenced from both side using the primers forward 35S and reverse Nots.

Nucleotide sequence obtained by forward 35S.

ATGTGGAAACAGATTCTGAGTAAGCTTCCTAAGAAGTCTTCTTCTAAGAACCATTCTTCTTCATCTAGTT
CCACTTCAAAAAGTAGTGATAATGGCGCCAGTAAATCGGGTAATTCTCAAACCCAGAACGCTCCTCCTGT
AAAACCTTCTGCTGATTCAGGGTTTAAAGAAGGGAATTTGAAAGGAAATGGTAACGGTTTCACTCCATAT
GAAGCATTGCCAGGTTTCAAAGATGTTCCTAATGCAGAGAAACAGAACTTGTTTGTGAGAAAACTCAGCT
TGTGCTGTGTTGTTTTCGATTTCTCGGATCCAACGAAGAACGTTAAGGAAAAGGATATTAAGCGGCAGAC
ATTGCTTGAGCTTGTGGATTATGTAGCTTCTCCTAATGGAAAGTTTAGTGAAACTGTGATTCAAGAAGTG
GTTAGAATGGTTTCTGTTAACATATTCAGAACCTTGAATCCTCAACCGCGCGAGAATAAAGTTATTGATG
CGTTAGACTTGGAGGAAGAAGAGCCTTCTATGGATCCTACTTGGCCTCACTTGCAGCTTGTCTATGAAAT
TCTCCTGCGGCTTATTGCTTCACCAGAGACAGACACCAAGCTGGCTAAGAAATACATTGACCAATCTTTT
GTCTCAAGGTTACTTGATTTATTTGATTCAGAGGATCCTAGAGAAAGAGATTGTCTTAAGACCGTTCTAC
ATCGCATCTACGGTAAATTTATGGTTCATCGTCCTTTCATAAGGAAATCCATTAATAACATTTTCTATCG

780 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein mRNA	1423	1423	100%	0.0	100%	NM_202087.2
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein mRNA	1423	1423	100%	0.0	100%	NM_101216.2
Arabidopsis thaliana chromosome 1 sequence	1423	1423	100%	0.0	100%	CP002684.1
Arabidopsis thaliana At1g13460 mRNA, complete cds	1423	1423	100%	0.0	100%	BT026384.1

The sequence 100% match with fourth result in BlastN NCBI which is PP2A B' θ AT1G13460

Nucleotide sequence obtained by reverse Nots

TTACAATGAACTCTTTTGCTTTTGATTACCAATTTCTTCCAACCGTTTCCACGTTGCCTCACGTTTTGCGCCAAT
TTCAGCTTCTTTTGATTCATCTTCTCTAAACTTAGCAAGACAATCCTTAAACAACTCTGCATCAATGTCATTAAA
GATCTTCTGCACATTCAACGTCAAGCTATGAACAGCTTGGTTCCAATGCTTCTGAGTGTTTCTCTCCAATGCAGG
GAATATGATTGGAAGAATAACTTTCCGGTTCTGCATTATTAGATTCTCGATATGATCGTTGTTCCATAAGAACAA
AGCTCTTTCTGCAACCTGAAAATGGAGACTGTTCAAACACCGAGCAACTTGGCGAAACAATGGGACCATACACCG
TTGGAATTCAGGTGGTTGAGTTGCTTCTAATACTTCCTCTAGCTCGTTTAGAAACATGACTTCTTTGGAACTATT
TGTGACAGGCCAAGACTTGAGTAGTCCCCTTATAACCGTATCAGCGAGTTTGCAATCTTTTTCCACAAACTGTGT
GATACAATAAGACAATTGCTGATGATACATTTGCAAGCTTTTCGGTTTGTGAAGAGGTACCAAAGCTCTAACCAA
AAACACTTTATGCTCGTCTTTAAGCGGCAGAGCAAATCCATTGATGATACTTCCCAAAATCTCTAGAAACTCAGC
AATCCCATTGTGTTTCTCAGTCTCGAAAACAAAC

709 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein mRNA	1290	1290	100%	0.0	99%	NM_202087.2
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein mRNA	1290	1290	100%	0.0	99%	NM_101216.2
Arabidopsis thaliana At1g13460 mRNA, complete cds	1290	1290	100%	0.0	99%	BT026384.1

The sequence 99% match with third result in BlastN NCBI which is PP2A B' θ AT1G13460.

Sequence analysis of ACO1-pVYNE vector (Full length ACO1)

cDNA: ACO1 AT4G35830.1

Insert was sequenced from both side using the primers forward 35S and reverse Nots.

Nucleotide sequence obtained by forward 35S.

GCTATCCTTTCCGAAGCATATTGAAGGCGTTAGAGAAGCCTGATGGTGGTGAATTCGGTAACTACTACAGCTTAC
CTGCTTTGAACGATCCCAGGATCGATAAACTACCTTATTCCATTAGGATACTTCTTGAATCGGCCATACGTAACT
GTGATGAGTTCCAAGTTAAGAGCAAAGATGTTGAGAAGATTCTTGATTGGGAGAATACTTCTCCCAAGCAGGTTG
AGATTCCGTTCAAGCCTGCTCGGGTTCTTCTTCAGGACTTTACTGGTGTTCCTGCTGTTGTTGATCTTGCTTGCA
TGAGAGATGCCATGAATAATCTCGGTGGTGATTCTAATAAAATTAATCCGCTGGTCCCTGTAGATCTTGTCATTG
ATCACTCCGTTCAGGTGGATGTGGCGAGATCAGAGAACGCAGTGCAGGCAAACATGGAGCTTGAGTTCCAGCGTA
ACAAGGAAAGATTTGCTTTTCTTAAGTGGGGATCCAACGCCTTTCACAACATGCTTGTCGTACCTCCTGGATCTG
GAATAGTTCATCAAGTCAACCTAGAATACCTTGCCAGAGTTGTTTTCAACACAAATGGACTTCTTTACCCAGACA
GTGTTGTTGGCACAGACTCTCACACCACTATGATTGATGGACTGGGTGTTGCTGGATGGGGAGTTGGCGGTATAG
AAGCGGAAGCTACCATGCTTGGTCAGCCAATGAGCATGGTCCTACCCGGTGTTGTGGGTTTCAAGCTAACGGGAA
AGTTAAGAGATGGAATGACAGCTACTGATTTGGTCTTAACAGTGACTCAGATGTTGAGGAAACATGGAGTAGTTG
GAAAGTTTGTTGAATTCCACGGGGAAGGGATGAGAGAATTGTCTTTAGCTGACCGTGCTACAATTGCCAATATGT
CTCCTGAGTACGGTGCGACCATGGGATTCTTAGTCGATCTCAC

943 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana aconitase 1 (ACO1), mRNA	1714	1714	99%	0.0	99%	NM_119749.4

The sequence 99% match with first result in BlastN NCBI which is PP2A B' θ AT1G13460.

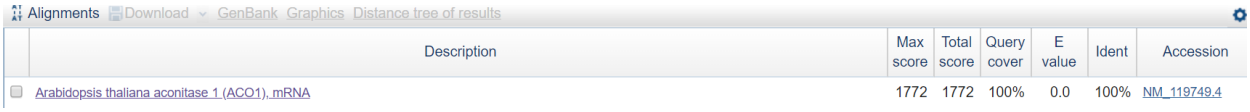
Nucleotide sequence obtained by reverse Nots

TTATTGTTTGATCAAGTTCCTGATAACGTATTGCAAAATCCCTCCGTGATCGAAATAAGCCAACTCCACCTCTGT
GTCAAATCGGAGTGTACATGTGAAAGATTTGCCATTGTTTGTGACGACTGTTACATCTTGTCCTGGTTTGATCTC
ACTAACATTGTTTGGGAGCTCAATGGTGTAAAGCTCCTGACCCGTTAGGCCAAGGGTCTCAGCATCTTCTCCCGC
CTTGAAGCACAAAGGTATGATTCCCATTCCCACCAAATTGCTTCGGTGAATTCGCTCGAAGCTCTTTGAAATCAC
AGCTTTCACACCCAGAAGCATTGGACCCTTGGCAGCCCAATCACGAGAACTTCCACTACCGTATTCAGCACCAGC
CAAAATGATTGTGTCGCGTCCCTCGTTCCTATATTTCATGGCAGCATCGAAAACAGAAAGCTTCTCTCCAGTGGG
AATGTGAACTGTTTTGGGACCAACTTCTCCTTTCAAGTGTTTGTTGACAATACGGATATTTGCAAAAGTGCCTCT
CGCCATAATCTCATCATTACCACGGCGACTCCCGTATGAGTTGAAGTCTCTTCTATCCACACCTCGTTCCATCAA
GTACTTAGCCGCAGGACTGTCCTTGTGGATGCTACCAGCTGGTGAGATGTGATCAGTGGTAATACTGTCTCCAAA
ATTGAGTAAACAGTATGCGTCTTTCACACCATGTGGACCGGGTGGAGACATGGTCATGCCCTTGAAATAAGGCGG

CTCGTGAATGTAAGTTGATTTCGGGTCCCACTCATAGAGAGTACCTGACGCCACAGATAACTGATTCCACATGGA
ATTTCCTTTGGTGATTGCTTCATATGTAGCTTTGAACATATCAGGAAGGACACTAGATTGAACAACCTCAGCAAC
TTCTTTGTTAGAGGGCCAAATGTCCCTGAAAAATATCTGTTTTCCATCTTTCCCAGTTC

959 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana aconitase 1 (ACO1), mRNA	1772	1772	100%	0.0	100%	NM_119749.4

The sequence 100% match with first result in BlastN NCBI

Sequence analysis of truncated ACO1-pVYNE vector (partial length ACO1)

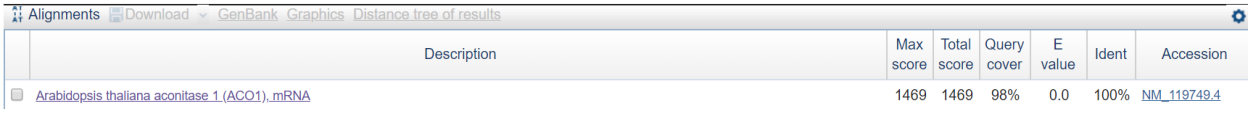
Insert was sequenced from both side using the primers forward 35S and reverse Nots.

Nucleotide sequence obtained by forward 35S.

ATGGCGAGAATTCCGAAGCATATTGAAGGCGTTAGAGAAGCCTGATGGTGGTGAATTCGGTAACTACTACAGCTT
ACCTGCTTTGAACGATCCCAGGATCGATAAACTACCTTATTCCATTAGGATACTTCTTGAATCGGCCATACGTAA
CTGTGATGAGTTCCAAGTTAAGAGCAAAGATGTTGAGAAGATTCTTGATTGGGAGAATACTTCTCCCAAGCAGGT
TGAGATTCCGTTCAAGCCTGCTCGGGTTCTTCTTCAGGACTTTACTGGTGTTCCTGCTGTTGTTGATCTTGCTTG
CATGAGAGATGCCATGAATAATCTCGGTGGTGATTCTAATAAAATTAATCCGCTGGTCCCTGTAGATCTTGTCAT
TGATCACTCCGTTCAGGTGGATGTGGCGAGATCAGAGAACGCAGTGCAGGCAAACATGGAGCTTGAGTTCCAGCG
TAACAAGGAAAGATTTGCTTTTCTTAAGTGGGGATCCAACGCCTTTCACAACATGCTTGTCGTACCTCCTGGATC
TGGAATAGTTCATCAAGTCAACCTAGAATACCTTGCCAGAGTTGTTTTCAACACAAATGGACTTCTTTACCCAGA
CAGTGTTGTTGGCACAGACTCTCACACCACTATGATTGATGGACTGGGTGTTGCTGGATGGGGAGTTGGCGGTAT
AGAAGCGGAAGCTACCATGCTTGGTCAGCCAATGAGCATGGTCCTACCCGGTGTTGTGGGTTTCAAGCTAACGGG
AAAGTTAAGAGATGGAATGACAGCTACTGATTTGGTCTTAACAGTGACTCAGATG

807 Nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana aconitase 1 (ACO1), mRNA	1469	1469	98%	0.0	100%	NM_119749.4

The sequence 100% match with first result in BlastN NCBI and 98% query coverage.

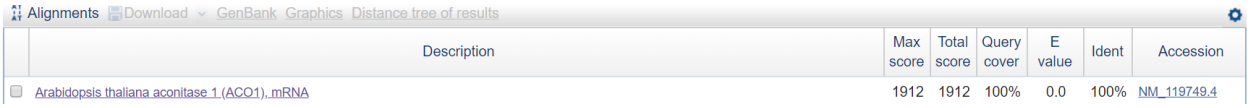
Nucleotide sequence obtained by reverse Nots

CGTTAGGCCAAGGGTCTCAGCATCTTCTCCCGCCTTGAAGCACAAAGGTATGATTCCCATTCCCACCAAATTGCT
TCGGTGAATTCGCTCGAAGCTCTTTGAAATCACAGCTTTCACACCCAGAAGCATTGGACCCTTGGCAGCCCAATC
ACGAGAACTTCCACTACCGTATTCAGCACCAGCCAAAATGATTGTGTCGCGTCCCTCGTTCCTATATTTCATGGC
AGCATCGAAAACAGAAAGCTTCTCTCCAGTGGGAATGTGAACTGTTTTGGGACCAACTTCTCCTTTCAAGTGTTT
GTTGACAATACGGATATTTGCAAAAGTGCCTCTCGCCATAATCTCATCATTACCACGGCGACTCCCGTATGAGTT
GAAGTCTCTTCTATCCACACCTCGTTCCATCAAGTACTTAGCCGCAGGACTGTCCTTGTGGATGCTACCAGCTGG
TGAGATGTGATCAGTGGTAATACTGTCTCCAAAATTGAGTAAACAGTATGCGTCTTTCACACCATGTGGACCGGG
TGGAGACATGGTCATGCCCTTGAAATAAGGCGGCTCGTGAATGTAAGTTGATTTCGGGTCCCACTCATAGAGAGT
ACCTGACGCCACAGATAACTGATTCCACATGGAATTTCCTTTGGTGATTGCTTCATATGTAGCTTTGAACATATC
AGGAAGGACACTAGATTGAACAACCTCAGCAACTTCTTTGTTAGAGGGCCAAATGTCCCTGAAAAATATCTGTTT
TCCATCTTTCCCAGTTCCAATGGGCTGTGTCTCAAAATCAATGTCAACAGTTCCAGCCAGAGCATAGGCTACAAC

AAGCGGTGGGGAAGCTAGATAGTTAGCTCTTGTTAACGGGTGAACACGTCCCTCAAAATTTCTGTTCCCAGACAA
CACAGCGGATGCCACCAAGTCATTATCAACTATTGCTGAAGCCACAGCTTCATGGATATCCCCCGAGTTTCCAAT
GCATGTGGTGCACCCATAACCAACGATACTGAAGCCGAGCTGATTCAAGTACTTCTGCAA

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

1034 nucleotides



Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana aconitase 1 (ACO1), mRNA	1912	1912	100%	0.0	100%	NM_119749.4

The sequence 100% match with first result in BlastN NCBI.

B. Study PP2A B’ ζ subcellular localization

B1. Sequence analysis of PP2AB’ ζ-YFP in pBA002 vector

The insert PP2AB’ ζ-YFP sequenced using forward and reverse primers for PP2AB’ ζ and reverse primer for YFP (See Table 2.3 in material and method section). All the sequence results are 99-100% identical to cDNA. The sequence data obtained from reverse primer YFP showed the PP2AB’ ζ fused to YFP.

1. Nucleotide sequence obtained by forward PP2AB’ ζ

```
ATGATCAAACAGATATTTGGGAAATTACCTAGAAAGCCTTCCAAATCATTGCAGAATGACTCCAATGGTGAAGGA
GGTGTTAATAATTCTTATTATGCTTCGAATTCGAGTACTACTAGTATCTCTAAACCTTCTTCAACATCTTCGAAA
TCATCATCAGCCTCGGGTTCGCGTGTAGCTAATGGAACTCTTGCTCCTAACTCAATGAGCTCTAATAGGAATACT
AATCAAGGGAAGAAACCATTGGGAGGAGATGCTGTTGTACAAGCTGGACCGTTTCCGTCTTCTGGTGGGGTTTAT
GAGGCTTTGCCTAGCTTTAGAGATGTTCCTATATCGGAGAAACCGAATCTCTTTATCGGGAAGTTGAGTATGTGC
TGTGTTGTTTTTGACTTTAGTGATCCGTCTAAGAACCTTAAGGAGAAAGAGATTAAGAGGCAGACATTGCTTGAG
CTTGTTGATTATGTTGCATCAGTTGGTTTTAAGTTTAACGATGTTTCGATGCAAGAGTTAACGAAGATGGTAGCG
GTTAATCTGTTTAGAACTTTTCCTTCTGCGAATCACGAGAGTAAAATTCTTGAAATACATGATATGGATGATGAA
GAACCTTCTTTGGAGCCAGCTTGGCCTCATGTTCAAGTTGTGTATGAGATTCTTCTCAGATTCGTGGCTTCTCCC
ATGACTGATGCAAAGCTTGCCAAGAGATATATTGACCATTCTTTTGTCTTGAAGCTCTTAGACTTGTTTGATTCT
GAAGATCAAAGAGAGAGGGAATATCTAAAAACTATTCTGCATCGGGTGTACGGGAAGTTCATGGTGCATCGACCT
TACATCAGAAAGGCGATAAACAATATCTTCTACAGATTCATATCCGAGACTGAAAA
```

881 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein mRNA	1628	1628	100%	0.0	100%	NM_113060.5
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein mRNA	1628	1628	100%	0.0	100%	NM_001338546.1
Arabidopsis thaliana chromosome 3 sequence	1628	1628	100%	0.0	100%	CP002686.1
Arabidopsis thaliana mRNA for putative B' regulatory subunit of PP2A, complete cds, clone: RAFL09-56-F08	1628	1628	100%	0.0	100%	AK227037.1

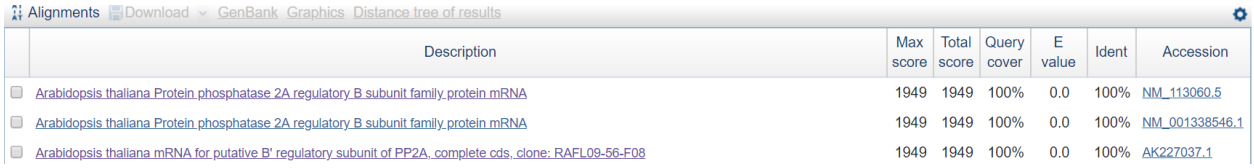
The sequence 100% match with results in BlastN NCBI. The result show 100% query cover

2. Nucleotide sequence obtained by reverse PP2AB’ ζ

```
CTACGACCCTGTGGACTCAGAGCTGCTTGTAGTAAGATTGACTGAGGACACAAATCTTGGAACCAGTACTGCCTC
GTTGGTTACAACGGTCTTTGAAGTAGCTAAATCTTCTAACCGTTGCCATGTCCTTTCCCGTTTCGCTTTAACCTC
TGTTTTATTCACTTCTTCTACTTGGAATTTGGCTAAACACTCGTCGAAAAGAACTTGGTCAATCTCGCATAATAC
TTTCCTCACGTTTATAGTCAGACTTTGAACTGCTTGGTTCCAATGTCCACGCGTGTTTCTCTCAAGAGCTGGGAA
GACTATAGGCATTATCACTTTATGGTTCTGAGTGATCAGGTTTCTTATGTGATCGTTGTTCCATAGAAACAATGC
TCTTTCAGCAACCTGGAAATGTGAACTGTTGAGGCATCGAGCAATTTGTCGGGATAATGGAACCATACAACGTTG
AAACTCAGCGGCTTGAGTTGCTTCCAAGACTTCTTCTAACTCTCCAAGAAACATAACTTCCTTTGAGCTGTTAGT
CACAGGCCAATATTTTAAAAGACCTCTAATAACGGTATCAGCGAGCTTGAAGTCCTTTTCTACAAACTGAACAAT
GCAATACGAAAGCTGTTGGTGATAGACTGATGAACATTTAGGCTTGTGGAGAGGAATCAAGGCTCGCAAAAGGAA
GAGCTTGTGCTCTTCTTTTAAAGGCAAAGCAAAACCATTAATTATACTTCCAAGAATCTCTAGCAACTCCGCAAT
GCCATTATGCTTTTCAGTCTCGGATATGAATCTGTAGAAGATATTGTTTATCGCCTTTCTGATGTAAGGTCGATG
CACCATGAACTTCCCGTACACCCGATGCAGAATAGTTTTTAGATATTCCCTCTCTCTTTGATCTTCAGAATCAAA
CAAGTCTAAGAGCTTCAAGACAAAAGAATGGTCAATATATCTCTTGGCAAGCTTTGCATCAGTCATGGGAGAAGC
CACGAATCTGAGAAGAATCTCATACACAACTTGAACATGAGGCCAAGCTGGCTCCAAAGAAGGTTCTTCATCATC
CATAT
```

1055 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)



Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein mRNA	1949	1949	100%	0.0	100%	NM_113060.5
Arabidopsis thaliana Protein phosphatase 2A regulatory B subunit family protein mRNA	1949	1949	100%	0.0	100%	NM_001338546.1
Arabidopsis thaliana mRNA for putative B' regulatory subunit of PP2A, complete cds, clone: RAFL09-56-F08	1949	1949	100%	0.0	100%	AK227037.1

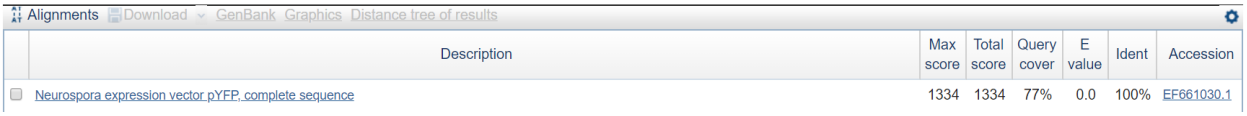
The sequence 100% match with results in BlastN NCBI. The result show 100% query cover

3. Nucleotide sequence obtained by reverse YFP

TTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAGGACCATGTGATCGCG
CTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGTAGCTCAGGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTC
GCCGATGGGGGTGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGATCTTGAA
GTTCACCTTGATGCCGTTCTTCTGCTTGTCGGCCATGATATAGACGTTGTGGCTGTTGTAGTTGTACTCCAGCTT
GTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCACCAGGGTGTCGCCCTC
GAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAAGAAGATGGTGCGCTCCTGGACGTAGCCTTC
GGGCATGGCGGACTTGAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGGCGAAGCACTGCAGGCCGTAGCC
GAAGGTGGTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGTCAGCTTGCC
GTAGGTGGCATCGCCCTCGCCCTCGCCGGACACGCTGAACTTGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAG
GATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATCGACCCTGTGGACTCAGAGCTGCTTGTAGT
AAGATTGACTGAGGACACAAATCTTGGAACCAGTACTGCCTCGTTGGTTACAACGGTCTTTGAAGTAGCTAAATC
TTCTAACCGTTGCCATGTCCTTTCCCGTTTCGCTTTAACCTCTGTTTTATTCACTTCTTCTACTTGGAATTTGGC
TAAACACTCGTCGAAAAGAACTTGGTCAATCT

932 nucleotides

Blast against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)



Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Neurospora expression vector pYFP, complete sequence	1334	1334	77%	0.0	100%	EF661030.1

The sequence 100% match with first result in BlastN NCBI with 100% identity.

B2. Primers used in genotyping and gene expression analysis.

In genotyping and gene expression analysis experiment new primers was designed to give PCR product that contain part of PP2A B’ ζ and part of YFP to verify PP2A B’ ζ fused with YFP in plant genome, PCR product size 451 bp (See Table 2.3 in Material and method section).

Full length PP2A B’ ζ-YFP 2.3 kb. PP2A B’ ζ (gray color) and YFP in (yellow color). Amplified PCR product 451 bp highlighted with red color

ATGATCAAACAGATATTTGGGAAATTACCTAGAAAGCCTTCCAAATCATTGCAGAATGACTCCAATGGTGAAG
GAGGTGTTAATAATTCTTATTATGCTTCGAATTCGAGTACTACTAGTATCTCTAAACCTTCTTCAACATCTTCGA
AATCATCATCAGCCTCGGGTTCGCGTGTAGCTAATGGAACTCTTGCTCCTAACTCAATGAGCTCTAATAGGAAT
ACTAATCAAGGGAAGAAACCATTGGGAGGAGATGCTGTTGTACAAGCTGGACCGTTTCCGTCTTCTGGTGGG
GTTTATGAGGCTTTGCCTAGCTTTAGAGATGTTCCTATATCGGAGAAACCGAATCTCTTTATCGGGAAGTTGAG
TATGTGCTGTGTTGTTTTTGACTTTAGTGATCCGTCTAAGAACCTTAAGGAGAAAGAGATTAAGAGGCAGACA
TTGCTTGAGCTTGTTGATTATGTTGCATCAGTTGGTTTTAAGTTTAACGATGTTTCGATGCAAGAGTTAACGAA
GATGGTAGCGGTTAATCTGTTTAGAACTTTTCCTTCTGCGAATCACGAGAGTAAAATTCTTGAAATACATGATA
TGGATGATGAAGAACCTTCTTTGGAGCCAGCTTGGCCTCATGTTCAAGTTGTGTATGAGATTCTTCTCAGATTC
GTGGCTTCTCCCATGACTGATGCAAAGCTTGCCAAGAGATATATTGACCATTCTTTTGTCTTGAAGCTCTTAGA
CTTGTTTGATTCTGAAGATCAAAGAGAGAGGGAATATCTAAAAACTATTCTGCATCGGGTGTACGGGAAGTTC
ATGGTGCATCGACCTTACATCAGAAAGGCGATAAACAATATCTTCTACAGATTCATATCCGAGACTGAAAAGC
ATAATGGCATTGCGGAGTTGCTAGAGATTCTTGGAAGTATAATTAATGGTTTTGCTTTGCCTTTAAAAGAAGA
GCACAAGCTCTTCCTTTTGCGAGCCTTGATTCCTCTCCACAAGCCTAAATGTTCATCAGTCTATCACCAACAGCT
TTCGTATTGCATTGTTCAGTTTGTAGAAAAGGACTTCAAGCTCGCTGATACCGTTATTAGAGGTCTTTTAAAAT
ATTGGCCTGTGACTAACAGCTCAAAGGAAGTTATGTTTCTTGGAGAGTTAGAAGAAGTCTTGGAAGCAACTCA
AGCCGCTGAGTTTCAACGTTGTATGGTTCCATTATCCCGACAAATTGCTCGATGCCTCAACAGTTCACATTTCCA
GGTTGCTGAAAGAGCATTGTTTCTATGGAACAACGATCACATAAGAAACCTGATCACTCAGAACCATAAAGTG
ATAATGCCTATAGTCTTCCCAGCTCTTGAGAGAAACACGCGTGGACATTGGAACCAAGCAGTTCAAAGTCTGA
CTATAAACGTGAGGAAAGTATTATGCGAGATTGACCAAGTTCTTTTCGACGAGTGTTTAGCCAAATTCCAAGT
AGAAGAAGTGAATAAAACAGAGGTTAAAGCGAAACGGGAAA**GGACATGGCAACGGTTAGAAGATTTAGCT**
ACTTCAAAGACCGTTGTAACCAACGAGGCAGTACTGGTTCCAAGATTTGTGTCCTCAGTCAATCTTACTACA
AGCAGCTCTGAGTCCACAGGGTCGATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCC
TGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCAC
CTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGAC
CACCTTCGGCTACGGCCTGCAGTGCTTCGCCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCC
GCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGC
CGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACG
GCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGA
AGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACT
ACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCTACCAGTCCGC
CCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCAC
TCTCGGCATGGACGAGCTGTACAAGTAA

B3. Standard curve for protein determination

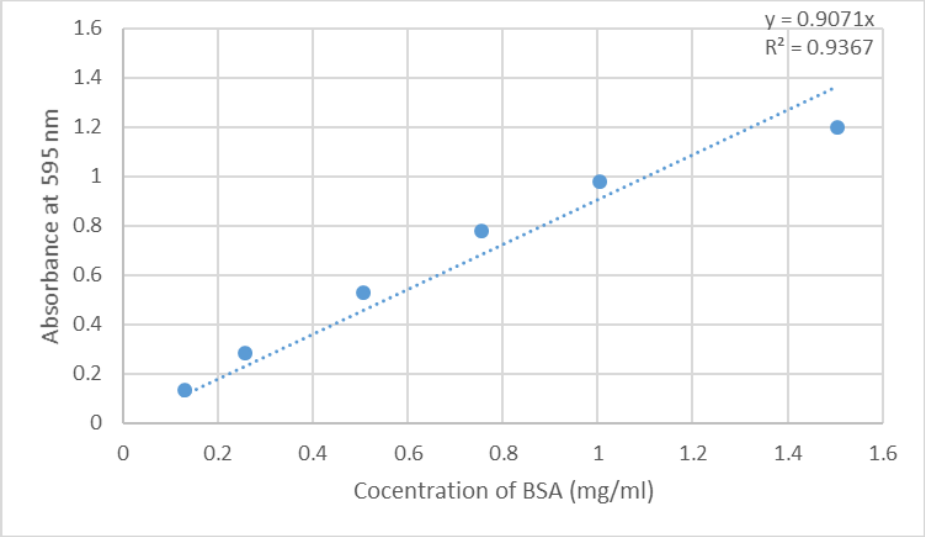


Figure B3: Standard curve used for protein determination of protein samples prior SDSPAGE (see section 2.3.9 in Materials and methods). Protein concentrations were quantified using a Bradford assay with BSA as standard.