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Cloning and Expression of a Tobacco Stearoyl-ACP Desaturase Gene SBIP24 and its Interaction with SABP2 in SA pathway

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by

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ABSTRACT

Cloning and Expression of a Tobacco Stearoyl-ACP Desaturase (SACPD) Gene SBIP24 and its Interaction with SABP2 in SA Pathway

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Salicylic acid binding protein 2 (SABP2) that converts methyl salicylate to salicylic acid (SA) plays an obligatory role in the SA-mediated disease resistance pathway in plants. SABP2 interacts with SBIP24 in a yeast two-hybrid screening. SBIP24 belongs to the stearoyl-acyl carrier protein-desaturase protein family. To biochemically characterize the SBIP24, it was cloned from tobacco leaves using RT-PCR and expressed in *E. coli*. Recombinant SBIP24 was affinity purified using Ni-NTA chromatography. RT-PCR was performed to determine the role of SABP2 in modulating the expression *SBIP24.* TMV infected transgenic C3 (control tobacco plant containing empty silencing vector) and 1-2 (SABP2-silenced) transgenic tobacco plants were used. Preliminary results indicate that SABP2 may regulate the expression of *SBIP24* in tobacco plants. Further studies are needed to confirm these preliminary results.

DEDICATION

To my dear son Zafeer Arefeen

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TABLE OF CONTENT

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

The biological structures and processes that protect an organism from outside invaders comprise its immunity system. In natural habitat, a number of potential enemies surround plants. Because, unlike animals, plants are incapable of simply moving away from these pathogens, plants have evolved a defense system that with the continuous environmental selection pressure has become more diverse and complex (Dong and Fu 2013). Plants have a range of pathogens to deal with such as viruses, bacteria, fungi, nematodes, etc. According to the way pathogens parasitize the plants, pathogens could be grouped as biotrophic or necrotrophic pathogens. Biotrophic pathogens are those that do not kill the host cell because they retrieve nutrients from living host cells during the infection process. In contrast, the necrotrophic pathogen kills the plant tissue by secreting toxins or enzymes (Jackson and Taylor 1996). To compete with these evolving pathogens, plants develop alternative ways of resistance. Plants respond either actively or passively or both to resist pathogens (Karban and Myers 1989) as well as to any foreign molecule termed as an 'elicitor' (pathogen substance) (Dixon 1986). Passive response involves creating physical or chemical barrier mediated by thick cell wall, cuticle, and bark or by secreting toxic chemical compound such as phytoalexins (Dangl and Jones 2001). On the other hand, in active response, plants first recognize the pathogens or defense signal; then induce their internal immune defense responses for local resistance. Local resistance eventually leads to systemic acquired resistance (SAR) by accumulating defense hormone SA and PR (pathogenesis-related) proteins. SAR ensures a long lasting protection against a variety of microorganisms (Dangl and Jones 2001; Dong 2004)

During pathogen attack plant resistance (R) proteins detect the corresponding secreted molecules encoded by the pathogen *Avr* (Avirulence) gene. If the R proteins and Avr

protein directly or indirectly interact with each other, a successful resistance response occurs. If either one is inactive or absent, the virulence of pathogen rules, instigating disease (Dangl and Jones 2001). Among the several different classes of plant R genes, the 2 major classes are pattern recognition receptor (PRR) (Song et al**.** 1995) and nucleotide-binding site leucine-rich repeat NBS-LRR gene (McHale et al**.** 2006).

To establish defense against the pathogens, plants use 2 branches of innate immunity system. In the first branch, the plant recognizes the pathogen/microbial conserved factor (virulence molecules) such as peptidoglycans, bacterial flagellin, lipopolysaccharides, viral proteins, fungal chitin, etc. encoded by *Avr* genes called pathogen associated molecular patterns (PAMPs) or microbe-associated molecular pattern (MAMP) by employing a specific set of transmembrane pattern recognition receptors (PRRs) encoded by '*R'* genes and thus initiate PAMPs triggered immunity (PTI) (Zipfel and Felix 2005; Jones and Dangl 2006). By another branch of immunity system plants ensure resistance against biotrophic pathogen, which is mediated by '*R*' genes encoding intra-cellular polymorphic NB-LRR proteins (Dangl and Jones 2001; Glazebrook 2005). A pathogen also involves its strain-specific avirulent (AVR) protein to interfere with cognate plant 'R' protein and promotes effector-triggered susceptibility (ETS). Finally, for an accelerated and amplified response, plant NB-LRR protein specifically recognizes the respective pathogen effector molecule and induces effector-triggered immunity (ETI) (Jones and Dang 2006).

A successful R-Avr interaction activates a signal transduction pathway that eventually fosters a series of biochemical reactions (Benhamoue 1996) finally leading to the hyper sensitive (HR) response at the infection site (Pontier et al. 1998). This HR response produces reactive oxygen intermediates (ROIs) (Baker and Orlandi 1995) and rapid changes in ion fluxes across the plasma membrane (Baker and Orlandi 1993), which are responsible for inducing hyper sensitive cell death known as programmed cell death (Pontier et al. 1998).

Plants use HR to restrict the spread of infection within the affected region along with sending the signal to the distal uninfected (systemic) part (Ryals et al. 1996). Upon receiving the signal from the pathogen restricted tissue, the distal part produces phytoalexins and PR proteins to induce disease resistance. Expression of the *PR* genes facilitates accumulation of plants' secondary metabolites such as phytoalexins, SA, JA, and ethylene (ET) (Benhamou et al. 1996; Iriti and Faoro 2007).

Depending upon the elicitors and pathways followed by the infection, 2 types of systemic induction have been monitored (Kloepper et al. 1992; Vallad et al. 2004). One is induced systemic resistance (ISR) that is independent of SA and is mediated by JA and ET. It does not include *PR* gene expression and is only applicable for few necrotrophic pathogens (Bostock 2005). The other one is SAR that covers a wide range of pathogens including insect herbivores, nematodes, virus, and bacteria. This long lasting induction is dependent on endogenous SA accumulation and *PR* gene expression (Durrant and Dong 2004)

SA Dependent Defense Pathway

SA dependent signaling pathway for plant defense is solely dependent on accumulation of plant hormone SA. The name salicylic acid is derived from *Salix*, the willow plant that has been in use for SA extraction since the ancient times. SA has been known as an effective therapeutic agent for easing pain and acne and reducing fever since the 18th century (White 1979). Though produced in plant, SA has become the focus of intensive research for some decades (Vlot et al. 2009). In humans the anti-inflammatory drug aspirin (acetyl salicylic acid) has been in use as far back as 1897. The protective role of SA in plant was first explored in 1979 by R. F. White. He and his colleagues showed that injection of aspirin in tobacco leaves before the inoculation of TMV surprisingly reduced lesion number in infected tobacco plants (White 1979; Antoniw and White 1980). The same result was obtained when tobacco plants treated with exogenous SA were infected with alfalfa mosaic virus

(Huijsduijnen et al. 1986). Beyond the role in plant disease resistance, SA also plays a crucial role in plant growth and development, seed germination, cell growth, stomatal closure, respiration, thermogenesis, and fruit yield (Vlot et al. 2009; Vicente and Plasencia 2011)

Role of SA in Local Resistance and SAR.

SAR has been described since 1933 (Chester 1933) and is associated with the induction of *PR* genes. PR proteins are responsible for the buildup of resistance in both infected and distal leaves. Expression of PR proteins leads to the activation of secondary defense for enhanced resistance against a broad spectrum of microorganisms (Ryals et al. 1996; Sticher et al. 1997). However, the precise role of PR protein in plant defense is not still understood. PR proteins are highly expressed in infected plants compared to their undetectable levels in the healthy plant**s**. They serve as a molecular marker for SAR development (Durrant and Dong 2004). Involvement of SA in the disease resistance signal transduction by inducing *PR* genes has been described by several studies (Vlot et al. 2009). It has been observed in tobacco plants that, upon TMV inoculations, endogenous SA levels in resistant cultivar increases ~20-fold in infected leaves. This elevated level of SA also corresponds to the increase in *PR1* gene expression levels (Malamy et al. 1990). Studies on cucumber plants treated with *Colletotrichum lagenarium* or *Pseudomonas syringae* suggest that after pathogen inoculation, SA level increases 10- to 100-fold in the phloem exudate leading to SAR development and inducing defense-associated peroxidase activity (Metraux et al. 1990; Rasmussen et al. 1991; Smith et al. 1991). Moreover, transgenic plants of tobacco and *Arabidopsis* expressing the *NahG* gene (that converts SA to catechol by salicylate hydroxylase) exhibit enhanced susceptibility with a defect for SAR induction to viral, fungal, and bacterial pathogens; the phenomenon indicating that SA is essential for SAR development (Gaffney et al. 1993; Delaney et al. 1994). Also, a synthetic analog of SA, benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester (BTH), has been shown to

induce PR gene expression and activation of SAR (Gorlach et al. 1996). So, SA is essential both for the expression of *PR* gene and for biosynthesis of defensive compounds that instigate both local resistance and SAR (Shah 2003). But induction of SAR requires a mobile signal that needs to be transmitted from the infected leaves to systemic leaves through phloem (Kiefer and Slusarenko 2003) . The suggestion that SA could serve as endogenous signal for mediating SAR comes from research indicating SA accumulates in phloem sap of cucumber plant after tobacco necrosis virus or the fungal pathogen *Colletotrichum lagenarium* infection (Metraux et al. 1990). However, another study on cucumber leaves infected with *Pseudomonas syringae* disputes this notion because the detached infected cucumber leaves where the SA has not been accumulated before can also develop SAR in systemic tissue (Rasmussen et al. 1991). This finding is also supported by grafting experiments where tobacco mosaic virus (TMV) infected NahG rootstock is grafted with wild type tobacco scion plant and accumulates SA in spite of its inability to accumulate SA. This study also shows that grafted wild type and NahG rootstock are not able to induce resistance with NahG scion. These results, all together, demonstrate SA is not the mobile signal but its presence is necessary for inducing the unidentified mobile signal in systemic tissues (Vernooij et al. 1994).

Biosynthesis of SA

How SA is synthesized in plants is still not fully elucidated, but research for half a century presents 2 distinct pathways of SA biosynthesis - the isochorismate synthase (ICS) pathway and the phenylalanine ammonia-lyase (PAL) pathway (Fig. 1) (Chen et al. 2009). In the first one, chorismate is derived from shikimic acid pathway and eventually converted into SA via isochorismate. The 2 important enzymes that catalyze the conversion of chorismate to isochorimate and isochorismate to SA are isochorismate synthase 1 (ICS1) and isopyruvate lyase (IPL), respectively (Verberne et al. 2000; Wildermuth et al. 2001; Strawn et al. 2007).

Study on null *ics1* mutants in plants suggests that ICS1 is responsible for catalyzing approximately 90% of pathogen induced SA production (Wildermuth et al. 2001; Garcion et al. 2008), this is essential for both local resistance and SAR (Wildermuth et al. 2001). Another alternate way where SA is synthesized from phenylalanine via benzoic acid has also been studied in tobacco plant. Here in the first stage phenylalanine ammonia lyase (PAL) catalyzes the conversion of phenylalanine into trans-cinnamic acid then trans-cinnamic acid is converted into benzoic acid. Finally benzoic acid is converted into SA by benzoic acid 2-hydroxylase (BA2H) (Ogawa et al. 2005).

As SA is synthesized in chloroplasts upon the biotic and abiotic stress (Fragniere et al. 2011), it needs to be transferred from chloroplast to cytoplasm for further signal transduction and to establish resistance. In order to transmit the downstream signal, SA is first converted into MeSA (lipid mobile) by SA-methyl transferase (Chen et al. 2003) that diffuses through the chloroplast membranes to enter into cytoplasm. In the cytoplasm MeSA is converted back to SA by the esterase activity of SA binding protein 2 (SABP2) (Forouhar et al. 2005).

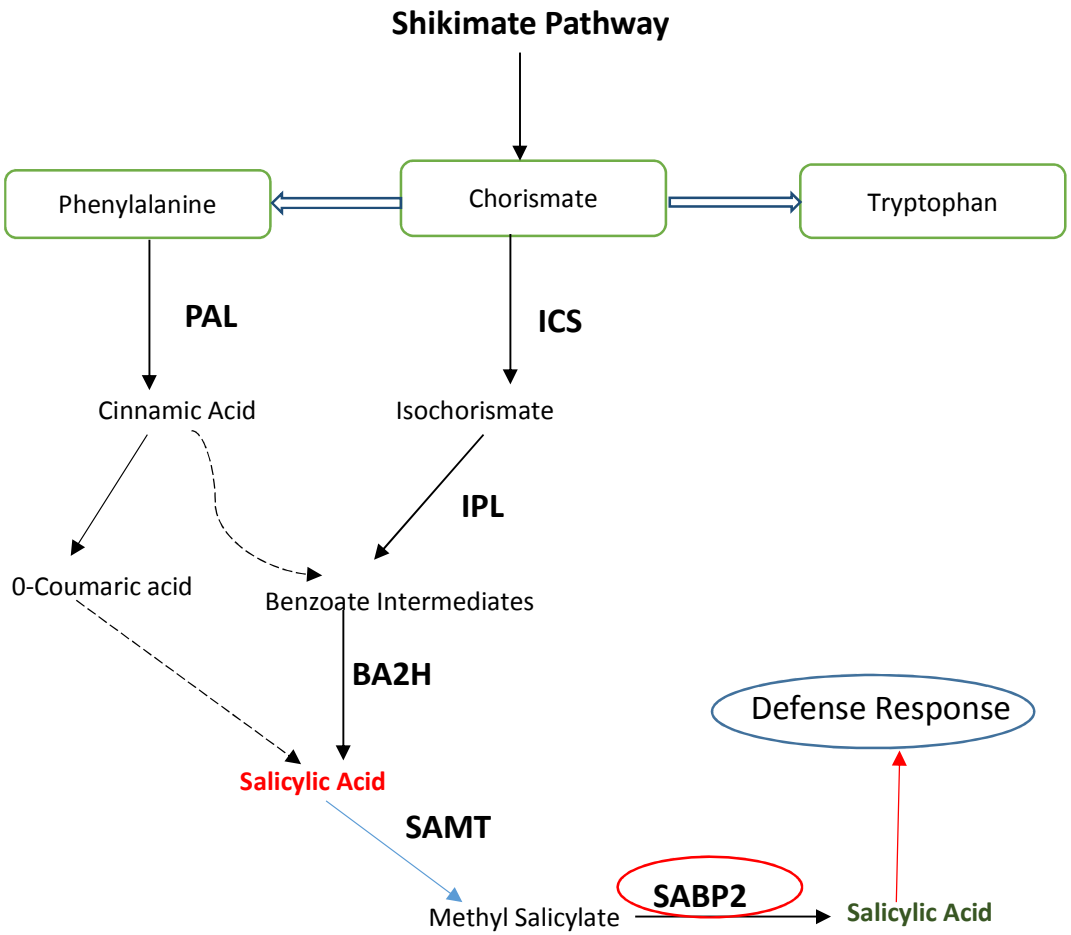

Figure1: Simplified Sketch of SA Biosynthesis and Induction of Defense Response by SA. Enzymes in this figure are shown in bold. Abbreviations: PAL, phenylalanine ammonia lyase; ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; ; BA2H, benzoic acid-2- hydroxylase; SAMT, SA methyltransferase; (Figure adapted from Vlot et al. 2009).

SA Mediated Signaling

Prevailing knowledge as well as studies on constitutive defense mutants indicates that a complex genetic regulatory ne2rk influences both upstream and downstream of SA signaling to confirm a strong defense response (Fig. 2).

Upstream Signaling of SA

Upstream signaling of SA is mediated by either enhanced disease susceptibility 1 (EDS1) and its sequence related interacting partner phytoalexin deficient 4 (PAD4) or nonspecific disease resistance 1 (NDR1). The signals for resistance are initiated by the *R*

gene, that encodes NBS-LRR, TIR-NBS-LRR, CC-NBS-LRR proteins and is transmitted downstream to synthesize SA.

In response to biotrophic pathogens, the lipase like proteins EDS1 and PAD4 act upstream of SA for inducing basal resistance as well as for initiation of ETI (Aarts et al. 1998; Wiermer et al. 2005). Interestingly, research on *eds1* and *pad4* mutants indicates that though they lie upstream of SA, they can be positively regulated by an SA feedback loop. The study shows that exogenous treatments with SA is able to rescue the mutant (*eds1 and pad4)* phenotypes and induce defense response (Zhou et al. 1998; Falk et al. 1999; Feys et al. 2001)

Instead of EDS1, the second major subset of R proteins, CC-NBS-LRR is regulated by NDR1, a glycophosphatidyl-inositol-anchored plasma protein (Century et al. 1997; Aarts et al. 1998; Coppinger et al. 2004). NDR1 is important for transmitting signal for SA accumulation and hence confers disease resistance. In *Arabidopsis thaliana* overexpression of NDR1 ensures enhanced disease resistance against virulent bacteria. On the other hand, mutation in the *NDR1* gene lowers the PTI and ETI expression and results in more susceptibility towards the pathogen (Coppinger et al. 2004; Shapiro and Zhang 2001).

Downstream Signaling of SA

2 types of SA mediated pathways have been observed; one is NPR1 (nonexpressor of pathogenesis-related protein 1) dependent SA pathway in which NPR1 is essential to induce the *PR1* gene expression, the other pathway is NPR1 independent SA signaling pathway where NPR1 is not required for *PR1* gene expression. To activate resistance, the NPR1 independent pathway needs a second signal in addition to SA which could be e.g. cell death, oxidative burst, etc (Shah 2003). NPR1 is one of the major players for spreading downstream signals in the SA-mediated disease resistant pathway (Cao et al. 1997). Mutant plants defective in NPR1 are deficient of (*PR*) gene expression for SAR development and hence

show more susceptibility to infection (Cao et al. 1994). Not only that, it is also found to be involved in jasmonic acid-mediated defense responses (Dong 2004; Pieterse and Von Loon 2004). Mutation in the *NPR1* gene of *Arabidopsis thaliana* results in blocking of SA signaling (Cao et al. 1998). Also a 2 -3 fold increase of *NPR1* transcripts during pathogen infection suggests its critical role in defense signaling. In an uninduced normal condition, NPR1 remains in the cytosol as an oligomer linked by disulphide bridges. But upon pathogen infection, SA accumulation in basal tissue changes the redox potential of cell that eventually converts oligomeric NPR1 into an active monomeric form by reducing its 2 cysteine residues (Cys82 and Cys216). The active monomeric form of NPR1 then shifts from the cytoplasm to the nucleus where it interacts with transcription factor, TGA and assists the binding of TGAs to the promoter regions of SA-responsive genes for *PR* genes expression (Mou et al. 2003). In order to activate SAR, when NPR1 enters the nucleus and binds to transcription factor, it gets phosphorylated by a kinase and eventually gets degraded by ubiquitination. This ubiquitination of phosphorylated NPR1 is critical for initiation of SAR in plants (Spoel et al. 2003). However, besides PR proteins, redox regulators, the mediator complex, WRKY transcription factors, endoplasmic reticulum-resident proteins, and DNA repair proteins are also assumed to play important roles in induction of SAR (Fu and Dong 2013).

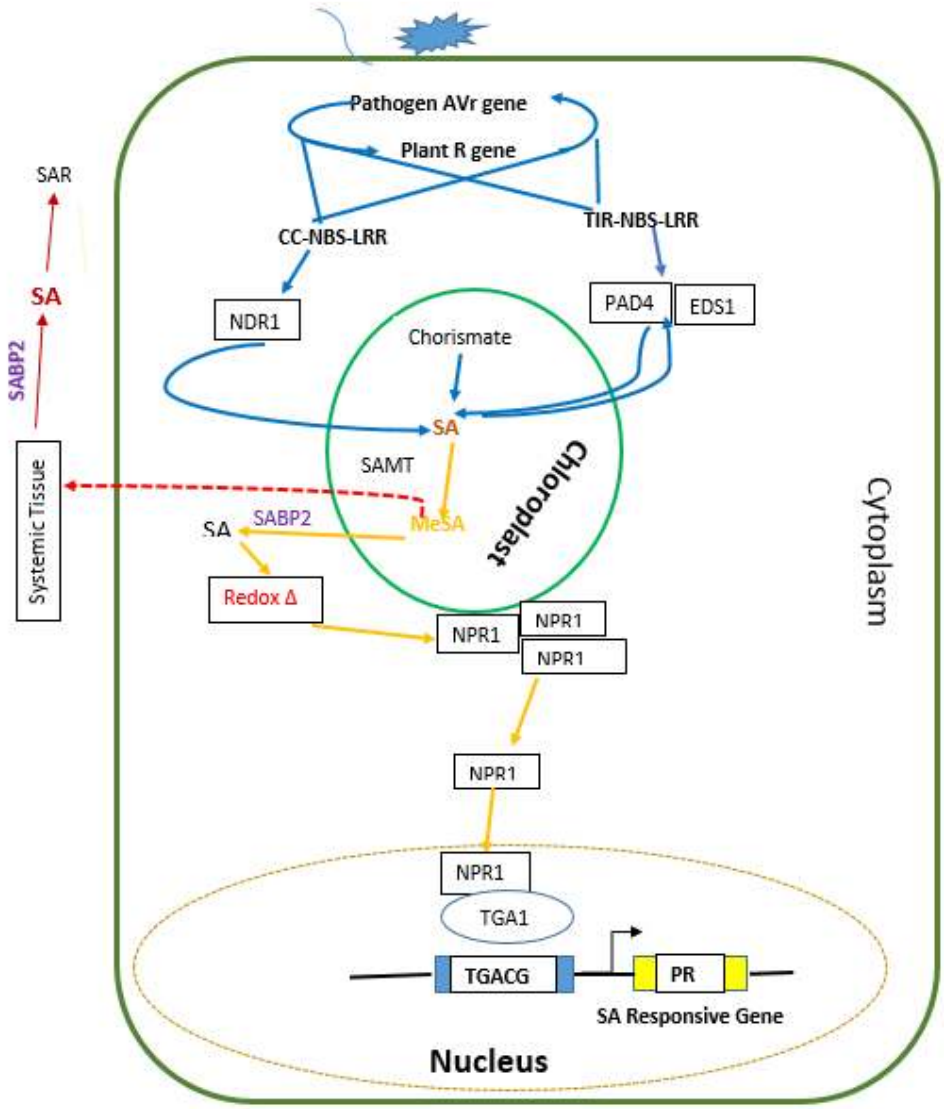

Figure 2: Upstream and Downstream Signaling of SA. Interaction of Pathogen *AvR* and Plant *R* gene upon pathogen attack, transmits 2 possible downstream signals for SA accumulation either via NDR1 or via PAD4 and EDS1; Accumulated SA in chloroplast converts to MeSA by SA methyl transferase (SAMT); MeSA diffuses through chloroplast membrane and migrates into the cytoplasm where it again converts back to SA by the esterase activity of SABP2. As a result SA levels increase in cytoplasm and eventually changes the redox potential which leads to the disruption of oligomer NPR1 into a monomer. Then monomeric form of NPR1 shifts to the nucleus and initiates the transcription of SA responsive defense genes including *PR1* to confer resistance. At the same time MeSA diffuses to the distal part of plant and spreads the signal in systemic tissues for ensuring SAR. (Figure adapted from Kumar 2014).

SA Independent Signaling

Though SA plays a critical role in initiating disease resistance response in plants, many studies on plants defective in SA dependent disease response also suggest SA is not indispensable for triggering the defense response against all kinds of pathogens (Thomma et al. 1998; Thomma et al. 1999). These studies show defects in JA signaling or ethylene sensing causes enhanced susceptibility towards some fungi such as *Botrytis cinerea* (Thomma et al. 1998; Thomma et al. 1999). These kinds of pathogens rapidly kill the plant cell to obtain nutrients and thus escapes from HR triggered resistance response (Jackson and Taylor 1996). Actually these (SA, JA, ET) phytohormones the major players of plant defense signaling pathways also interact synergistically or antagonistically to tune up the intricate signaling ne2rks of disease resistance (Glazebrook 2001; Hammond- Kosack and Parker 2003; Kachroo and Kachroo 2007).

JA Mediated Defense Response

JA is a lipid based hormone that is involved in regulating many physiological processes. The synthesis of JA starts with the conversion of linolenic acid to 12-oxo-phytodienoic acid (OPDA) then, after reduction and oxidations, it forms jasmonic acid. Other than the conversion of linolenic acid to OPDA (occurs in chloroplast) all subsequent reactions take place in the peroxisome (Katsir et al. 2008). JA plays an important role in inducing defense responses against insect herbivores (Reymond et al. 2000) and other abiotic and biotic stresses (Farmer and Ryan 1990). Though the biosynthesis of JA is already known, a major part of JA mediated disease resistance signal transduction is still obscure. Instead of *PR-1*, *PR-2*, and *PR-5* plant defensing gene, *PDF1.2* along with *PR-3* and *PR-4* genes are required for inducing an SA independent but JA-dependent signaling pathway (Reymond and Farmer 1998). Some evidence also suggests jasmonates are essential for systemic defense and

initiating signal for SAR. They show SAR could be achieved by foliar application of jasmonic acid, where SAR is blocked in mutants impaired in JA synthesis or JA responses (Truman et al. 2007). However, the role of jasmonates in initiating SAR is controversial. Exogenous application of JA or its derivatives fail to confer enhance of resistance against an avirulent (Avr) *P. syringae* strain either in the infected or distal untreated *Arabidopsis* leaves (Chaturvedi et al. 2008).

ET Mediated Defense Response

The plant hormone ET is known for playing an important roles in multiple plant processes such as seed germination, leaf and flower senescence, fruit ripening, organ abscission, and seedling emergence (Abeles et al. 1992; Bleecker and Kende 2000). Besides SA and JA, ET is also found to be play a role in plant defense (Feys and Parker 2000; McDowell and Dangl 2000; Glazebrook 2001). Evidence shows plant pathogen interaction increases ET biosynthesis and also induces a set of genes called ET response genes that are regarded as markers of host reaction to pathogenic invasion (de Laat and van Loon 1982; Lotan et al. 1990; Flach et al. 1993). But the role of ET in plant defense signaling is ambiguous. In *Arabidopsis*, ethylene-insensitive mutant *ein2-1* fails to exhibit resistance to infection caused by gray mold fungus *Botrytis cinerea* like the wild type plant (Thomma et al. 1999). But ethylene-insensitive *ein1* and *ein2* mutants exhibit more resistant to *Pseudomonas syringae* (Bent et al. 1992). It has been found that in *Arabidopsis,* in response to pathogen, activation of the defense gene, *PDF1.2* (required for inducing JA response) is blocked in the ET response mutant *ein2-1* (Penninckx et al. 1998) supporting the hypothesis that both ET and JA signaling pathways are interlinked. Both ET and JA pathways also have been demonstrated to be required for induction of induced systemic resistance (ISR) triggered by the root colonizing bacterium, *P. fluorescens* (Pieterse et al. 1999).

Cross Talk between Signaling Pathways

Depending upon the elicitors, 2 types of pathways (SA dependent and SA independent) are followed (Kloepper et al. 1992; Vallad et al. 2004). Recent studies indicate they actually do not function independently. They work synergistically or antagonistically with each other to orchestrate the complex ne2rk of disease signal transduction and ensure better regulation towards disease resistance in plants. How they modulate each other is poorly understood.

The interactions between SA and JA are found to be both positive and negative (Pena-Cortes et al. 1993; Schenk et al. 2000). More evidence supports the antagonistic interaction of these 2 pathways rather than synergistic interaction (Mur et al. 2006). SA plays a critical role in defense against biotrophic pathogens where JA plays a key role to induce defense against necrotrophic pathogens (Glazebrook 2005). *Arabidopsis eds4* and *pad4* mutants are unable to induce SA mediated disease resistance, but these mutants show heightened responses to inducers of JA-dependent gene expression (Gupta et al. 2000). Studies on tobacco and tomato plants also reveal the antagonistic effect of JA and SA (Doares et al. 1995; Niki et al. 1998).

SA and ET signaling pathways have been found to interact in both positive and negative ways. Tomato plants infected with *X. campestris* pv. *Vesicatoria* requires ET synthesis to activate the defense signaling via SA accumulation (O'Donnell et al. 2001). On the other hand, genetic data indicates that the ET signaling pathway negatively affects SA-dependent responses (Lawton et al. 1994).

Unlike with SA, ET, and JA signaling always seems to interact synergistically (Kachroo and Kachroo 2007). Microarray analysis of *Arabidopsis thaliana* suggests that ethylene treatment induces almost half of the genes which also get induced upon JA treatment (Schenk et al. 2000).

SA Binding Proteins (SABPs)

Over the years, remarkable progress has been made in understanding the SA signaling in plants that contributes to the development of SAR (Vlot et al. 2009). Comprehensive studies have been conducted to investigate the proteins that bind to SA in the defense pathway. Among the SA binding proteins discovered, SABP is the first one (a tetramer, of 240 kDa). It is a catalase and reversibly binds with SA (Chen et al. 19933a; Chen et al. 19933b). Pathogen infection increases the SA level in cells and this increased level of SA inhibits the H_2O_2 degrading activity of SABP. Consequently, reactive oxygen species such as H_2O_2 accumulate in the cells. This increased level of H_2O_2 activates the hypersensitive response and eventually leads to apoptotic cell death to limit pathogen growth (Conrath et al. 1995; Chen et al. 2003). Another SA-binding protein, SABP2 (SA binding protein 2) has higher affinity for SA (Du and Klessig 1997; Kumar and Klessig 2003). SABP2 has been found to play an indispensable role in both local resistance and SAR following TMV infection (Kumar and Klessig 2003). In addition to SABP and SABP2 another SA binding protein, a chloroplast carbonic anhydrase (SABP3) has been identified that has antioxidant properties and may possibly play a role in hypersensitive response in tobacco (Slaymaker et al. 2002).

Tobacco SABP2 and its Interacting Proteins (SBIPs)

SABP2 a 29 KDa, soluble protein expresses in very low levels in plants (Forouhar et al. 2005). It plays a vital role in synthesis of SA from MeSA (Kumar and Klessig 2003). MeSA is the inactive form of SA but it works as a phloem-mobile signal for SAR development (Park et al. 2007; Vlot et al. 2008). The esterase activity of SABP2 is required for activating both local and systemic resistance by converting MeSA to SA. SABP2 silenced plants are suppressed in local resistance and fail to develop SAR (Kumar and Klessig 2008). The discovery of SABP2 has opened a new possibility of understanding the intriguing SA

pathways. In an attempt to further reveal the protein cascade that is playing an important role, directly or indirectly in SABP2 mediated SA pathway, a yeast two-hybrid screening was performed using SABP2 as bait and tobacco leaf proteins as prey proteins. Several proteins have been identified that show physical interaction with SABP2. SBIP24 is one such SABP2 interacting protein. Bioinformatic analysis revealed that SBIP24 is a putative stearoyl-CoA-desaturase like protein (delta 9 desaturase). Interestingly, previous studies on stearoyl-CoA-desaturase from different plant species indicate that it plays a significant role in defense signaling. In animal systems it has been recognized as a critical enzyme that plays an important role in obesity, cancer, cardio vascular diseases and diabetes (Cohen et al. 2003; Flowers 2009; Igal 2010; Wan et al. 2010).

Stearoyl-CoA-Desaturase Protein

Fatty acid desaturases are nonheme iron containing, oxygen dependent enzymes that catalyze the desaturation process and introduce double bonds into the hydrocarbon chain (Meesapyodsuk et al. 2000). These proteins existas 2 distinct evolutionary families: (i) The acyl carrier protein (ACP) desaturases that are plastid localized soluble plant or cyanobacterial enzymes and use acyl-ACPs as substrate. These enzymes need NADPH, oxygen, and an electron transport system with ferredoxin-NADPH reductase and ferredoxin to perform their activities (Shanklin and Cahoon, 1998) They also employ 2 atoms of iron and there are 2 D/EXXH motifs of amino acid sequences that help in binding the di-iron complex (Fox et al. 1993; Shanklin and Cahoon, 1998; Sperling et al. 2003) (ii) On the other hand, the membrane-bound insoluble desaturases are found in wide range of taxa including cyanobacteria, plants, animals, bacteria, yeast, etc. They are localized in the membranes of the cyanobacterial thylakoid, plant endoplasmic reticulum (ER), and plant plastid. They use acyl-CoA or acyl lipid as substrate. These membrane bound desaturases also use either

ferredoxin (in cyanobacteria and plant plastids) or cytochrome *b*5 (in plant ER) as an electron donor (Shanklin et al. 1994; Murata and Wada 1995; Shanklin and Cahoon 1998; Tocher et al. 1998). Interestingly, the first double bond produced in the saturated fatty acids in plants is always created by the soluble stearoyl- ACP desaturase that is unique to the plant kingdom (Shanklin and Cahoon, 1998).

Stearoyl- CoA-Desaturase (*SCD*) in Animal System

Unlike in plants, animals get monounsaturated fatty acids either by de-novo synthesis or directly from their diet. In animals the membrane bound endoplasmic reticulum resident enzyme stearoyl CoA desaturase (*SCD*) catalyzes the D^9-cis desaturation of a range of fatty acyl-CoA substrates. But SCD prefers palmitoyl- and stearoyl-CoA as substrates and converts them to palmitoleoyl (16:1) and oleoyl-CoA (18:1), respectively. Once the monounsaturated fatty acids are formed, they are used as precursors for the synthesis of triacylglycerols (TAG), membrane phospholipids, and sphingolipids. Stearoyl CoA desaturases are the key and rate limiting enzymes that determine the membrane function and fat storage in animals. To study the metabolic and physiological role of *SCD* more intensively, it has been cloned from several different species of animals including human, mouse, rat, *Drosophila* and *Caenorhabditis elegans*. Also, several isoforms of *SCD* (*SCD1*, *SCD2*, *SCD3, SCD4*, *SCD5*) in mouse, rat, and human have been identified (Paton and Ntambi, 2009). Mice with targeted disruption of *SCD1* (delta 9 stearoyl CoA desaturase) show increased insulin sensitivity, reduced body adiposity, and diet induced weight gain compared to wild type mice (Ntambi and Miyazaki 2002) suggesting an important role for *SCD1* in diabetes and obesity. *SCD1*, the rate limiting enzyme of lipid biosynthesis, is also found to be repressed during leptin mediated weight loss (Zhang et al. 1994). Leptin is a hormone that mediates specific

metabolic affects and depletes triglycerides from liver and other peripheral tissues (Cohen et al. 2003).

SCD is also found to be associated with atherosclerosis, a disease where arteries become thick by the accumulation of fatty materials such as cholesterols. By using the fat-1 transgenic mouse model, it has been shown that decreased n-6/n-3 fatty acid ratio reduces atherosclerotic lesions in mice. Interestingly, the ratio of n-6/n-3 is determined by the activity of stearoyl-CoA- desaturase (Wan et al. 2010)

A recent study reveals that *SCD1* index can be used as a biomarker for the early detection of metabolic diseases such as diabetes and cardiovascular diseases. The new test based on the delta 9 fatty acid desaturase index would enable patients to identify the disease earlier and thus help them to maintain an appropriate lifestyle (Flowers 2009).

Most interestingly, there are some recent findings that suggest *SCD1* could be a central target of growth factors and hormones that have a key role in cell cycle events during cell proliferation in cancer. Studies on mono unsaturated fatty acid (MUFA) synthesis in cell during replicative senescence further strengthen this fact. In senescence, the aging cells do not lose metabolic activity but lose their division ability after several rounds of mitosis. Additionally, the expression of *SCD1* is reduced drastically in a normal fibroblast when it reaches the senescent state. This reduction is also evident in the fatty acid synthase (FAS), the main substrate for *SCD1*. So, when cells stop proliferation, they also repress fatty acid synthesis and desaturation (Igal 2010).

Stearoyl-CoA-Desaturase in Plant

In plants fatty acid biosynthesis occurs in chloroplasts/plastids with the aid of acetyl CoA carboxylase and fatty acid synthase complex. The initial product of fatty acid synthesis, acetyl CoA, goes through a series of elongation, condensation, dehydration, and reduction

reaction before it forms palmitate (16:0) and stearate (18:0) (Harwood 1988). Once 16:0 or 18:0 is produced, acyl carrier protein desaturase from the soluble stearoyl-acyl carrier protein-desaturase family introduces a double bond between carbon 9 and 10 of stearoyl-ACP and makes it unsaturated (McKeon and Stumpf 1982; Shanklin and Somerville 1991; Knutzon et al. 1992) Though SACPD can use both 16:0 and 18:0 as a substrate, it preferentially uses 18:0 as substrate. After the desaturation of 18:0 to 18:1 (Δ9), the product 18:1 (Δ9) ACP enters either the plastidial glycerolipid synthesis pathway known as prokaryotic pathway or eukaryotic cytoplasmic pathway (Browse and Somerville 1991). If 18:1 ACP stays in the chloroplast, it initiates the formation of phosphatidic acid (PA) and other chloroplastic lipids by the acylation of Glycerol-3-Phosphate (G3P).The enzyme that catalyzes the acylation reaction is *ACT1*-encoded G3P acyl transferase. This is the first step of plastidial glycerolipid synthesis. Alternatively, 18:1 ACP can also be exported out to plastids as a CoA-thioester and enter into the eukaryotic lipid biosynthesis pathway to produce PA, the precursor of other phospholipids and glycerolipids. Therefore, stearoyl-acyl-carrier-protein-desaturase-mediated conversion of stearic acid (18:0) to oleic acid (18:1) is regarded as the key step in regulating the levels of unsaturated fatty acids in cells (Kachroo et al. 2001).

Along with SA and JA-mediated pathways, fatty acid derived signals especially 18:0 and 18:1 levels in plants plays an important role in regulation of plant defenses. They are associated with multiple responses including abiotic and biotic responses (Kachroo and Kachroo 2009; Savchenko et al. 2010). In *Arabidopsis* a stearoyl coA desaturase *SSI2* (Suppressor of SA Insensitive 2) that regulates the 18:0 and 18:1 levels in plants has earned a lot of attention for altering defense signals and exhibiting resistance against multiple pathogens (Kachroo et al 2001; Kachroo et al 2007) . The *Arabidopsis ssi2* mutant plants that are defective in 18:1 FA level are dwarfed in size compared to their wild type (Col 0) plants.

In response to bacterial and oomycete pathogens they show enhanced resistance by exhibiting spontaneous cell death lesions on their leaves, over expressing pathogenesis related (*PR-1*) genes, and eventually accumulating high level of SA (Kachroo et al. 2001; Shah et al. 2001). The *ssi2* mutant plants are defective in JA mediated defense responses. They are more susceptible to necrotrophic pathogens and are repressed in JA mediated induction of the *PDF1.2* gene (Kachroo et al. 2001). Studies on *ssi2* suppressor mutants suggest this altered disease response is due to reduction of 18:1 levels rather than an increased level of 18:0 levels. Though the *Arabidopsis* genome possess 7 highly conserved isoforms (*SSI2, S-ACP-DES 1, S-ACP-DES 2, S-ACP-DES 3, S-ACP-DES 4, S-ACP-DES 5, S-ACP-DES 6)* of stearoyl-ACP-desaturases, the major portion of 18:1 pool in plant is mainly contributed by SSI2. These studies also demonstrate that plants always maintain a threshold level of 18:1 level by both transcriptional and posttranslational regulation (Chandra-Shekara et al. 2007; Kachroo and Kachroo 2007; Kachroo et al. 2008; Kachroo et al. 2003). The 18:1 mediated defense response is observed in diverse groups of plants regardless of the biosynthesis pathway they follow. In soybean silencing of SACPD causes elevated level of resistance against bacterial and oomycete pathogens by accumulating high level of 18:0 and low levels of 18:1 levels (Kachroo et al. 2001). Also, silencing induces an *ssi2* like defense phenotype in mutant plants (Kachroo et al. 2008). Similar results were also found in rice and wheat (Jiang et al. 2009; Song et al. 2013). To get further insight into how 18:1 levels are maintained in plants, a study performing a mutation in chloroplastic enzyme G3P acyl transferase (*ACT1*) that disrupts the acylation of 18:1 with G3P reveals that *ssi2act1* mutant plants accumulate higher amounts of 18:1 and thus rescues *ssi2* mutant plants from SA and JA mediated phenotypes (Kachroo et al. 2003). Interestingly, the exogenous application of benzo-(1,2,3)-thiadiazole-7-carbothioic acid (BTH) on *act1* and *ssi2act1* plant induces high levels of *PR-1* expression like that seen in wild type plant suggesting *act1* mutant plants are SA responsive

and do not alter SA signaling. So the *act1* mutation recovers *ssi2* related defense response either by suppressing SA/SAG levels or by increasing the level of 18:1. The first possibility is refuted because *ssi2NahG* transgenic plants also exhibit stunted growth like *ssi2* and also show spontaneous cell death, *PR* gene expression, and partial resistance to bacteria and oomycetes (Shah et al. 2001). The second possibility that *act1* mutation works indirectly by increasing the level of 18:1 and this increase eventually rescues the *ssi2* mutation is supported by the evidence that exogenous spray of 18:1 rescues the responsiveness of *ssi2* to JA (Kachroo and Kachroo 2009).

Similarly, a mutation in the *GLY1* gene that encodes G3P dehydrogenase stops G3P formation from dihydroxy acetone phosphate via ACT1-derived step also causes restoration of 18:1 levels in *ssi2gly1* plants (Kachroo et al. 2004; Nandi et al. 2004). Additionally, exogenous application of glycerol that lowers the 18:1 level by increasing endogenous G3P levels is found to promote *ssi2* like phenotype in wild type plant (Kachroo et al. 2004; Kachroo et al. 2005). Glycerol cannot induce the *ssi2* like phenotype in *act1* or *ssi2act1* plant that is defective in phosphorylation of glycerol to G3P (Kachroo et al. 2005).

All the studies together indicate that the 18:1 level is involved in SA mediated plant defense signaling and reduction of 18:1 levels is responsible for enhanced resistance (Kachroo et al. 2004). Therefore the importance of stearoyl-CoA desaturase that regulates the 18:1 level in plant is imperative in SA mediated defense signaling.

Hypotheses

Hypothesis I: SBIP-24 is a stearoyl ACP desaturase of tobacco plant.

Characterization of SBIP24 is a prerequisite for uncovering the interaction between SABP2 and SBIP24 in SA mediated disease resistant pathway. After the yeast two-hybrid screening, primary BLAST analysis of partial SBIP24 sequences suggests that SBIP24 is a putative stearol ACP desaturase in tobacco plant. So, before studying the molecular and the biological function of this unknown protein, it is crucial to verify if SBIP24 is indeed a stearoyl ACP desaturase.

Hypothesis II: SABP2 regulates the gene expression of SBIP24.

Interestingly, *Arabidopsis* ssi2 mutant plants that are impaired in oleic acid synthesis exhibit enhanced resistance against biotrophic pathogen by accumulating high level of SA, they spontaneously develop cell lesion (Kachroo et al. 2001). These phenomenon have been found not only in *Arabidopsis* but also in diverse groups of plants including rice, soybean, parsley (Kachroo and Kachroo 2009). On the other hand, SABP2 that converts MeSA to SA acid works as a critical component for ensuring local resistance as well as SAR. Local resistance and SAR are found to be blocked in SABP2 silenced plants (Kumar and Klessig 2003). Result of a yeast two-hybrid screening together with all these findings, led to hypothesize that in response to biotrophic pathogen infection, SABP2 could regulate the expression SBIP24 in tobacco plant.

CHAPTER 2

MATERIALS AND METHODS

Plant Materials

For cloning cDNA was synthesized from *Nicotiana tabacum* cv. Xanthi nc (XNN) and expression analysis was conducted with cDNA prepared from 2 transgenic lines of tobacco plants; The C3 plant line containing empty silencing vector (pHANNIBAL) and *Nicotiana tabacum* cv. Xanthi nc, (1-2) in which *SABP2* gene expression is silenced by RNA interference (Kumar and Klessig 2003). Soil containing peat moss (Fafard Canadian growing mix F-15, Agawam, MA) was autoclaved for 20 minutes prior to growing the plants. Seedlings were transferred to 4 x 4 inch flats after 14 days. After 3-4 weeks, young plants were transferred individually to 8" pots. The experiments were performed with 6- to 8-week old plants. All stages of plants were grown in a controlled growth chamber (PGW 36, Conviron, Canada) set at 16-h day cycle maintained at 22°C.

Chemicals and Reagents

Sodium dodecyl sulfate (SDS), ß-mercaptoethanol (ß-ME), tetramethylethylenediamine (TEMED), ammonium persulfate (APS), bovine serum albumin (BSA), bovine thrombin, coomassie brilliant blue R-250, coomassie brilliant blue G-250, ponceau-S, ethylene diamine tetra acetic acid (EDTA), TRIS base, phenylmethylsulfonyl fluoride (PMSF), glycine, glycerol, methanol, imidazole, Tween-20, Triton X-100, N,N-Bis (2-hydroxyethyl) glycine (Bicine), magnesium chloride ($MgCl_2$), sodium chloride (NaCl), sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), benzamidine-HCl, ammonium sulfate $(NH_4)_2SO_4$), and all other standard chemicals were purchased from Fisher Scientific, Pittsburgh, PA. The 30% acrylamide solution, Bradford's

reagent, prestained low molecular weight marker, 10x SDS loading buffer, SDS dye were purchased from Bio-Rad, Hercules, CA. Polyvinylidene fluoride (PVDF) membranes were purchased from Millipore, Billerica, MA. Bicinchoninic acid (BCA) Pierce ECL western blotting substrate was purchased from Thermo Scientific, Rockford, IL. Kodak developer and fixer replenisher were purchased from Sigma-Aldrich, St. Louis, MO. Mouse monoclonal anti poly-Histidine antibody and Goat anti-Mouse HRP conjugate for Western blotting were purchased from Sigma, Oligo dT-20, Taq DNA polymerase (Invitrogen, CA), dithiothreitol (DTT), DNA ladder (New England Biolabs), MMLV reverse transcriptase, RNAse free DNAse, recombinant RNAsin (Promega), and gel loading dye (Bio-Rad).

Vectors and Kits

pDONR221, pDEST17 vectors, and pertinent reagents for BP and LR reactions were purchased from Invitrogen (Carlsbad, CA). QIAprep Spin Miniprep and QIAGEN gel extraction kits were purchased from Qiagen (Valencia, CA). Advantage HF 2 PCR Kit was purchased from Clontech.

Apparatus

French press (Thermo Electron corporation), Ultrasonicator (Fisher Scientific), Thermocycler (Eppendorf), NanoDrop Spectrophotometer, Gel electrophoresis apparatus for agarose (Fisher Biotech) and protein (BIO-RAD), Western blot apparatus (Bio-Rad), gel doc (UVP) system, pH meter, (Beckman) etc.

Oligonucleotides

The primers were custom synthesized by Eurofins MWG Operon. Listed below (Table. 1) are the primers used in this study for cloning and gene-expression. Lyophilized oligonucleotides were resuspended in nuclease-free water and finally diluted to 10 pmol/µl (=10 µM).

Table 1: List of Primers Used in This Study

Primer	**Sequence (5′ → 3′)**	**Purpose**
1. Gateway attB forward primer	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGC TCTGAAACTCAATCCG	Cloning with signal peptide
2. Gateway attB reverse primer	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAGAG CTTAATCTCTCTACC	Cloning with and without signal peptide
3. DK637: Gateway attB forward primer	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGCTTC AACACTTCGTCCC	Cloning without signal peptide
4. SABP2 forward primer	TTAGCAGCTTCTGGCACTGA	Gene expression analysis
5. SABP2 reverse primer	AGCCAAGAAAACAGCAGCAT	Gene expression analysis
6. DK558: SBIP24 forward primer	ATG CAGACATTCTTGAAT	Gene expression analysis
7. DK559: SBIP24 reverse primer	GAGCTTAATCTC TCT ACC	Gene expression analysis

Methods

Cloning and Expression of SBIP24 (Hypothesis I)

Bioinformatics Analysis

The partial DNA sequence of SBIP24 obtained by sequencing yeast two-hybrid clone was used to search for full length sequence of SBIP24 from the SOL Genomics Network database (SGN), which is a Clade Oriented Database (COD) containing genomic, genetic, phenotypic and taxonomic information for plant genomes of Solanaceae family including *Nicotiana tabaccum* (Bombarely et al. 2010) The nucleotide sequence from SGN (unigenes) for *Nicotiana tabacum* was then converted into the ORF using Expasy translate tool (Wilkins et al. 1999). Further BLAST analysis was conducted to search for the homologus gene sequences from other plant species using NCBI BLAST (Camacho et al. 2009). Nucleotide and protein sequences of SBIP24 were aligned with other known delta 9 fatty acid desaturases using clustalw2 (McWilliam et al. 2013). Conserved amino acids of stearoyl ACP desaturase was identified through the use of conserved domain database (Marchler et al. 2011). To predict the presence of putative signal sequences in SBIP24, the ChloroP 1.1 Server was used (Emanuelsson et al. 1999; Bendtsen et al. 2005).

Cloning and Heterologous Expression of SBIP24 into pDEST17 with Signal Peptide

Instead of traditional restriction endonucleases and ligase cloning, a gateway cloning system was used in order to clone SBIP24 into pDEST17 plasmid. This cloning technology involves 2 reactions- The BP reaction and the LR reaction. In the BP reaction, BP clonase initiates the recombination reaction that takes place between an attB-flanked PCR product (or an expression clone) and a donor (pDONR). A vector containing attP site is used to create an entry clone, where the gene of interest becomes flanked by attL sites. On the other hand, LR clonase mediates the recombination reaction that takes place between an entry clone containing attL sites and a destination vector containing attR sites to create an expression

clone carrying attB sites. Gateway cloning has a dual selection system. Along with antibiotic resistance selection, this system also uses the lethal *ccdB* gene to identify positive clones.

mRNA Isolation from Tobacco Plant. Total RNA was isolated from wild type tobacco XNN plant leaves by using Trizol reagent following manufacturer's instructions. The leaf tissues were homogenized into powder using the mechanical grinder with the aid of liquid nitrogen; 1 ml of Trizol was added to the powder and kept for 5 minutes before adding 200 µl of chloroform. Then the mixture was centrifuged at 12,000x g for 10 minutes at 4 °C. This process separated the mixture into 3 phases, a phenol-chloroform lower phase, an interphase, and a colorless upper aqueous phase. The RNA-containing aqueous phase was taken in a clean eppendorf tube and the RNA was precipitated with 500 µl isopropyl alcohol followed by incubation at 28 °C for 10min. Another centrifugation was performed at 12000x g for 10min at 4 °C to obtain the pellet; the supernatant was discarded and 1 ml of 75% ethanol was added to the pellet. Pellet with the ethanol was centrifuged at 7500x g for 5 min; the supernatant was decanted and the pellet was air dried for 5-10 min and then resuspended in 43 µl diethylpyrocarbonate (DEPC) treated sterile water followed by adding 5 µl of 10x DNAse buffer and 2 µl of DNase. Trizol (500 µl) and 100 µl of chloroform were added to the DNAse treated RNA sample. To separate the aqueous phase, a centrifugation at 12000x g was performed for 15 minutes at 4 °C. The supernatant was transferred to another eppendorf tube and 250 µl of isopropanol was added to the sample. The mixture was incubated at 28 °C for 10 minutes and again centrifuged at 12000x g for 10 minutes at 4 °C. Finally the pellet was washed with 0.5 ml cold 75% ethanol followed by another centrifugation at 7500x g for 5 minutes at 4 °C. The pellet was air dried and resuspended in 20 µl of DEPC treated water. Sample was heated for 10 minutes at 55-60 °C. The concentration and purity of RNA were measured by using the Nanodrop spectrophotometer.

cDNA Synthesis. To produce the first strand cDNA, 2 µl (0.5 µg/µl) of oligo-dT was added to 8 µl (1µg) of total RNA, mixed and incubated at 75 °C for 10 minutes in a thermocycler. Then the sample was cooled to 4 °C. A mixture containing 1 µl reverse transcriptase (RT) (M-MLV), 4 µl 5x RT buffer, 1 µl RNAsin (RNAse inhibitor), 1 µl 10 mM dNTP, and 3 µl depc treated water was added to the RNA and oligo-dT mix. For cDNA synthesis, the mixture was incubated at 42 °C for 60 minutes followed by 70 °C for 10 minutes in a thermocycler. The newly synthesized cDNA was stored in -20 °C for future use. The integrity of cDNA was checked by PCR amplifying the tobacco housekeeping gene *EF1α*.

Polymerase Chain Reaction (PCR). Forward, 5′-GGGGACAAGTTTGTACAAAAA AGCAGGCTTAATGGCTCTGA AACTCAATCCG- 3′ (Primer no.1 in Table. 1) and reverse, 5 ′GGGGACCACTTTGTACAA GAAAGCTGGGTTTCAGAG CTTAAT CTCTCTACC- 3′ primers (Primer no. 2 in Table. 1) were designed for cloning SBIP24 using a gateway attB primer designing protocol (Hartley et al. 2000). The SBIP24 gene was amplified using cDNA prepared from tobacco leaves as template and with primers containing both the start and stop codons. Advantage HF 2 PCR Kit (Clontech) was used for the PCR. 2 microliter of tobacco cDNA was mixed with 5 µl of 10x HF2 PCR Buffer, 1 µl of 50X HF dNTP mix, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 1 µl of 50 X Advantage HF polymerase mix, 38 µl of PCR-Grade H_2O. The PCR reaction was carried out with an initial denaturing step for 1 min at 94 °C followed by 35 cycles (94 °C for 30 seconds, 65 °C for 3 minutes, 68 °C for 1.5 minutes), and a final extension step at 68 °C for 5 minutes.

Agarose Gel Electrophoresis. The amplified PCR products were analyzed by 1.2 % agarose gel electrophoresis. To make a 1.2 % agarose gel, 0.60 gm of agar was melted in 49.40 ml 1X TAE buffer and cooled down in a 55 °C water bath; the gel running tray was placed into the chamber perpendicular to the running direction; a comb was inserted in the tray; after cooling down the agarose mixture to 55 °C, 2.5 µl of ethidium bromide (10 mg/ml) was added to the mixture; gel was poured into the running tray and was allowed to solidify; the comb was gently removed and 1X TAE buffer was poured until the agarose gel was covered fully; In the first well 1 Kb (8 µl) DNA ladder (25ng/ µl) was loaded as a marker; 10 µl of 6X DNA dye was mixed with 50 µl of PCR product and loaded into 3 other wells. Finally, the gel was run at 80 volts for ~90 minutes. The ethidium bromide stained gel was visualized under UV light.

Purification of PCR Product. In order to remove primer dimer and other bands that could interfere with cloning, the expected SBIP24 band was cut from agarose gel and was purified using Qiagen Gel Extraction kit according to the manufacturer's instruction. The purified DNA was quantified using Nanodrop spectrophotometer and analyzed on a 1.2 % agarose gel.

Construction of pDONR221-SBIP24 Entry Clone. Entry clone was constructed by performing the BP reaction according to the manufacturer's instruction. The required amount (100 ng) of gel purified PCR product was mixed with 6 µl of TE buffer and 1 µl (150 ng) of pDONR221. Two microliter of BP clonase was added to the mixture and incubated at room temperature for 3 hours. Following incubation, proteinase K was added to the mixture to terminate the reaction.

Preparation of Competent DH5α Cell. A single colony of *E. coli* DH5α strain was inoculated in 3 ml of LB-broth and grown at 37 °C in a shaker (250 rpm) overnight. The next day 1 ml of overnight culture was diluted with 100 ml of fresh LB media in a 2 L flask and incubated at 37 °C in a shaker at 250 rpm until the OD600= 0.48 (~3 h). Then the bacterial culture was transferred to 2 chilled 50 ml falcon tubes. Culture was chilled on ice for 15 min and centrifuged at 3000 rpm for 10 min at 4 °C. All subsequent steps were conducted in sterile conditions while keeping samples on ice. The bacterial pellets in each tube were resuspended in 10 ml of ice cold sterile 0.1 M $CaCl_2$ and incubated on ice for 30 minutes. After incubation on ice, the bacterial suspensions were again centrifuged at 3000 rpm for 10 min at 4 °C. The supernatants were discarded and obtained bacterial pellets were resuspended in 2 ml of ice cold 0.1 M $CaCl_2$. Aliquots of 200 µl competent cells were added to precooled eppendorf tubes with 200 µl of 50% glycerol, mixed gently, and directly frozen in liquid nitrogen. Competent cells were stored at -80°C for future use.

Transformation of E. coli DH5α Competent Cell by BP Reaction. For transformation in *E.coli*, competent cells were thawed on ice for 15 minutes and 100 µl of DH5α competent cells were mixed gently with 1 µl of recombinant DNA (pDONR221+SBIP24) in a precooled eppendorf tube. The mixture was incubated on ice for 30 min. After incubation heat-shock was performed by incubating the sample in a 42 °C water bath for 45 sec and then immediately transferring back on ice and keeping there for 2 minutes. After transformation 250 µl of SOC media was added to the transformed bacterial cells and incubated at 37 °C for 1h in a shaker at 250 rpm. Transformed cells were diluted in 1:20 ratio with LB broth and the diluted *E. coli* cells were plated (20 µl and 100 µl) on LB agar plates with 50 µg/ml kanamycin using the autoclaved glass beads. The plates were incubated at 37 °C overnight.

Verification of SBIP24 Cloning into pDONR221 by Colony PCR. The following day colony PCR was performed as a fast method to screen for positive clones containing the target insert of SBIP24. Ten 0.2 ml PCR tubes with 40 µl of water each were labeled by numbers (1-10). On a fresh LB plate containing 50 µg/ml kanamycin (a master plate) a single bacterial colony from original plate was picked and streaked on the master plate, the same tip was put in a PCR tube containing 40 µl of sterile water and subsequently was used as a template for amplification of target insert using M13 forward and reverse primers (binding site present on pDONR221 plasmid). The same procedure was followed for a total of 10 individual colonies. For PER amplification, 10 µl of template (colony + water) was mixed with 2 µl 10x PCR Buffer, 2 µl dNTP (0.1M) mix, 1 µl M13 forward primer, 1 µl M13 reverse primer, 0.5 µl Taq polymerase, and 3.9 µl of water in an eppendorf tube. The PCR conditions were set with an initial denaturing step for 1 min at 94 °C followed by 30 cycles (94 °C for 30 seconds, 55 °C for 3 minutes, 72 °C for 1.5 minutes) and a final extension step at 72 °C for 5 minutes. PCR products were analyzed by agarose gel electrophoreses.

Recombinant Plasmid (pDONR221-SBIP24) Purification. Ten positive clones (checked by colony PCR) from the master plate were selected for plasmid purification using Qiagen miniprep kit according to the provider instruction. The isolated plasmids were quantitated using nanodrop spectrophotometer and the quality of plasmid DNA was checked in 0.8% agarose gel.

Sequencing of Recombinant Plasmid and BP Clone Selection. In order to verify the correct clone, the purified plasmid DNA's were sent for sequencing to the Yale University Sequencing facility. The plasmid was sequenced following 'Sanger' sequencing using M13 forward and reverse primers.

Construction of pDEST17-SBIP24. The clone that had the highest similarities with the predicted SBIP24 sequence (according to the sequencing result) was chosen for the LR reaction. SBIP24 was subcloned into the pDEST17 destination vector from pDONR221 by the LR reaction that is mediated by LR clonase. In an eppendorf tube 1µl of pDONR221-SBIP24 (94 ng/µl) was mixed with 6 µl of TE buffer and 1µl of pDEST17 (150 ng) vector, 2 µl of LR clonase was added to the reaction, mixed, and incubated at 25 ºC overnight. Transformed bacteria were plated on a LB agar plate containing 100µg/ml ampicillin and incubated overnight at 37 ºC

Recombinant Plasmid Isolation and Transformation of *E. coli* cells . Recombinant plasmid pDEST17-SBIP24 was isolated using the Qiagen Miniprep kit according to manufacturer's directions. DNA quality was analyzed by running 1 µl of plasmid DNA in a 0.8% agarose gel. The concentration of plasmid DNA was determined by Nanodrop spectrophotometer. Thirty nanogram of recombinant plasmid DNA was used to transform 100 µl of Magic competent cells (prepared as DH5α cells) by the heat shock method. Magic cells are BL21 (DE3) derived cells with extra codon for expression of eukaryotic proteins. Transformed bacteria were plated on to LB agar plate containing ampicillin (100 µg/ml) and kanamycin (10µg/ml) and were incubated at 37 ºC overnight.

Test for Small Scale Recombinant pDEST17-SBIP24 Protein Expression

Five colonies of recombinant bacteria from the LB-agar plate were inoculated into a 14-ml tube containing 3 ml of liquid LB with ampicillin (100 µg/ml) and kanamycin (10 µg/ml). Cells were grown for 3 hours at 37 ºC in a shaker at 250-300 rpm until the OD at 600 nm reached 0.6. One milliliter bacterial culture was removed in a 1.5-ml eppendorf tube and centrifuged at maximum speed for 3 minutes. The supernatant was discarded and the pellet was stored at -20 ºC. This served as the uninduced bacterial sample. The remaining culture

was induced with 1mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and incubated at 25 ºC in 250 rpm overnight shaking. The following day pellets were collected from the culture by centrifugation. Both induced and uninduced pellets were resuspended in 100 µl of 1x SDS-PAGE sample buffer (Appendix B), incubated in boiling water bath for 5 minutes, and centrifuged for 10 min at 13,000 rpm, the supernatants (10 µl) of the samples were loaded in a 12 % SDS-polyacrylamide gel along with 8µl of protein markers for size reference. In order to visualize the proteins gel was stained with coomassie blue and destained with destaining solution (Appendix B).

Recombinant pDEST17-SBIP24 Protein Solubility Test

Once the expression of SBIP24 was confirmed, the recombinant protein expressed in *E.coli* was subjected to solubility test. Five isolated colonies were and inoculated in 5 ml LB broth containing kanamycin (10µg/ml) as well as ampicillin (100 µg/ml). The culture was shaken at 250 rpm and 37 ºC until OD_{600}= 0.6. The pellet (uninduced) was collected from the 1 ml of bacterial culture by centrifugation. Then, 4 ml culture was induced with 1mM, 0.8mM, 0.5mM, 0.1mM (final) IPTG concentration, respectively. The induced bacterial culture was incubated at 17 ºC overnight at 250 rpm (~12 hour). The next morning pellets were collected and 100 µl of Ni-NTA-binding buffer (Appendix B) were added to the pellets. Bacterial cells were lysed by 5 times sonication for 10 sec each with 15 sec interval, at 20% amplitude. Then the samples were centrifuged at 13,000 rpm for 5 minutes at 4 ºC, the supernatants were mixed with 100 µl of 1x SDS-PAGE buffer. Before loading in 12% SDS polyacrylamide gel, samples were boiled for 5 minutes in water bath and centrifuged at 13000 rpm for 1 minute. In order to visualize the protein bands after SDS-PAGE, gel was stained with coomassie blue.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed by following Laemmli's protocol (Laemmli 1970). Samples for SDS-PAGE were mixed with 2x SDS sample buffer (Appendix B) containing β-ME and boiled for 5 minutes followed by a centrifugation at 12,000 rpm for 10 minutes at room temperature. The samples were subjected to electrophoresis for 45 minutes at 200 volts. Buffers and gels were prepared as described in Appendix B.

Western Blot

Western blot is used to detect proteins separated in SDS-PAGE with a specific antibody. To perform a Western blot after SDS-PAGE, gel was incubated in transfer buffer (Appendix B) for 15 minutes. Prior to Western blotting the PVDV (polyvinylidene difluoride) membrane was treated with 100% methanol for 15 seconds and rinsed with water. Then the gel and prepared PVDV membranes were sandwiched between pre-soaked (in 1X transfer buffer) sponges and Whatman 3 mm Chr papers. Care was taken to avoid any air bubbles trapped between the gel and membrane. The sandwich was placed in the buffer tank filled with 1X transfer buffer. A constant 100V for 1 hour at 4 ℃ was applied for transfer of proteins to the PVDV membrane. After protein transfer membrane was incubated in 100% methanol for 10 seconds and dried on a filter paper for 10 minutes. The membrane was incubated in 100% methanol for 10 seconds and rinsed with deionized water. To verify protein transfer, the membrane was stained with ponceau S stain (Appendix B). After the visualization of protein bands on membranes the ponceau S stain was washed off with 1x phosphate buffer saline (Appendix B). The primary antibody, anti-polyHistidine (Sigma) mouse monoclonal antibody, was prepared in blocking buffer in a 1:1000 dilution. The membrane, were then incubated in primary antibody overnight at 4 ℃ on a shaker. The following day, membranes were washed (with 1x PBS, 1x PBS containing 3% tween 20, and 1x PBS sequentially 3 times for 5 minutes each) to remove the unbound antibody. After the

washing the blot was probed again with secondary antibody for 1 hour at room temperature in a shaker. The secondary antibody, anti-mouse IgG in goat with HRP conjugate was diluted to 1:5000 in blocking buffer. After incubation the membrane was washed as described earlier. Finally, the signals on membranes were detected with ECL reagent, and captured on an x-ray film.

Cloning and Expression of SBIP24$\Delta^{31\text{-}393}$ (without Signal Peptide)

In many cases recombinant proteins with signal peptide expressed in *E.coli* accumulate in inclusion bodies. Therefore in order to enhance the solubility of recombinant SBIP24, the signal peptide has been removed from its full length. A forward primer, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGCTTCAACACTTCGTCCC-3′ (Primer no. 3 in Table. 1) had been designed for amplification of SBIP24$\Delta^{31\text{-}393}$. Only the nucleotides in N-terminal region were excluded to remove the putative signal peptide, the reverse primer (Primer no. 2 in Table. 1) that had been designed for cloning of full length SBIP24 was used.

PCR for Amplification of SBIP24Δ31-393. PCR was performed using the Advantage HF 2 PCR Kit (Clontech). The PCR reaction was (50 µl reaction) setup using 3 µl of tobacco leaf cDNA, 5µl of advantage 2 PCR buffer, 1 µl of 50X dNTP mix, 1 µl of 10 µM forward primer, 1µl of 10 µM reverse primer, 1µl of 50X advantage 2 polymerase, and 38 µl of autoclaved water. The PCR reaction was performed with an initial denaturing step for 1 min at 94 °C followed by 35 cycles (94 °C for 30 seconds, 68 °C for 6 minutes), and a final extension step at 68 °C for 6 minutes.

Construction of Entry clone. After PCR, DNA samples were run in a 0.8% gel, DNA bands were purified by the Qiagen Gel Purification kit as described earlier. To clone the fragment of $^{31\text{-}393}$ into pDONR221 same procedure as described earlier for BP reaction was followed. After the BP reaction DH5α competent cells were transformed and plated on LB-agar plates containing kanamycin (50 µg/ml) followed by overnight incubation at 37 °C. The

following day colony PCR was performed to screen the positive clones. Plasmid DNA were isolated from the positive colonies and sent for the DNA sequencing**.**

Construction of Expression Clone. After identification of the entry clone that had the highest similarity with SBIP24 in sequencing result, LR reaction was performed using the plasmid DNA of pDONR221- SBIP24$\Delta^{31\text{-}393}$and pDEST17 to create expression clone carrying SBIP24$\Delta^{31\text{-}393}$. The same protocol (described for LR reaction of SBIP24 with signal peptide) of LR reaction was followed for generating pDEST17- SBIP24$\Delta^{31\text{-}393}$expression clone**,** the recombinant plasmid DNA was purified by Qiagen Miniprep kit and used for transforming the protein expression host, BL21 (DE3) *E. coli* cell strains.

Expression of Recombinant pDEST17-SBIP24$\Delta^{31\text{-}39}$ Protein

Five milliliter cultures of recombinant bacteria were grown until OD_{600}=0.6 in liquid LB containing ampicillin. An aliquot of 1 ml bacterial culture was placed in an eppendorf tube and the pellet was collected for future reference. In the remaining 4ml culture the expression of recombinant protein was induced with the addition of IPTG to a concentration of 1mM for 3 hours at 37 ℃ at 250 rpm. After induction bacterial pellets were collected in 2 separate tubes, one was used for total protein expression analysis and the other one was used for protein solubility test. For expression detection the pellet was resuspended in 1x SDS sample buffer and analyzed by SDS-PAGE followed by Coomassie staining**.**

Solubility Test for pDEST17-SBIP24$\Delta^{31\text{-}39}$ Protein

For solubility test bacterial pellet was resuspended in 100 µL of Ni-NTA-binding buffer. Then the bacterial cells were broken down by an ultrasonicator; supernatant (containing soluble proteins) from bacterial pellets were collected after centrifugation at 13000 rpm for 5 minutes. Supernatant was mixed SDS sample buffer before loading in 1.2%

polyacrylamide SDS gel. Soluble and insoluble fractions of recombinant pDEST17-SBIP24$\Delta^{31\text{-}393}$ were analyzed by Western blot as previously described.

Optimization of Protein Solubility. Once the expression of soluble recombinant protein was confirmed, the expression conditions were optimized by changing fine-tuning the IPTG concentration, induction temperature, induction time, etc. In order to determine the optimal condition for solubility, after reaching the OD_{600} = 0.6, bacterial cultures were induced with IPTG (1mM or 0.1mM)) and were incubated at temperature (37 °C or 20 °C) for 3- 7 hours. Expression of protein was analyzed by Western blot previously described.

Purification of Recombinant SBIP24$\Delta^{31\text{-}393}$ by Nickel Affinity Purification

The recombinant his-tagged SBIP24$\Delta^{31\text{-}393}$protein was subjected to purification using Ni-NTA chromatography. A single colony of recombinant bacteria was inoculated in 3 ml LB medium containing 100 µg/ml ampicillin and incubated at 37 ºC and 250 rpm overnight. Next day the culture was diluted 100 times in fresh 500 ml LB medium containing ampicillin (100µg/ml) and was incubated at 37 ºC in 250 rpm until OD_{600} = 0.6. Protein expression was induced by 0.1mM IPTG (99.4 µl of 0.5 M IPTG in 497 ml culture) culture and incubating for 3 hours at 37 ºC and 250 rpm. The pellets were collected by centrifugation at 10,000 rpm for 10 min at 4 ºC and resuspended in 8 ml of Ni-NTA binding buffer (Appendix B) plus 80 µl of protease inhibitor (PMSF). Bacterial cells were broken 5 times in prechilled (4 ºC) French press under 15000 psi. The cell lysate was then centrifuged at 13,000 rpm at 4 ºC for 10 min. Fifty microliters of supernatant was stored at -20 ºC for future reference (Input). The rest of the supernatant was mixed with 1ml nickel resin overnight on a shaker at 4 ºC temperature. The resin with proteins was then transferred into a column for chromatography. After collecting the flow-through, the column was washed with Ni-NTA binding buffer (7 ml each time) (Appendix B) for twice and finally the bound SBIP24$\Delta^{31\text{-}393}$ was eluted with

elution buffer containing 250mM imidazole (pH 8). All steps of chromatography were performed at room temperature unless described otherwise.

Construction of pMDC123-SBIP24 Clone for Complementation of *Arabidopsis ssi2* Mutant Plant

In order to examine the ectopic expression of SBIP24 in *Arabidopsis ssi2* mutant plant, the fragment of SBIP24 from entry clone pDONR221-SBIP24 was cloned downstream of a cauliflower mosaic (CaMV) 35S promoter in gateway binary vector pMDC123 by LR reaction. The LR reaction was performed by adding 1 µl (150 ng) of pMDC123 plasmid DNA into a mixture of 2 µl (200 ng) of pDONR221-SBIP24 plasmid DNA, 5µl TE buffer, and 1 µl LR clonase. LR reaction was incubated at 25 °C for 5 hours followed by 10 minutes incubation with 1 µl of proteinase K at 37 °C. Subsequently DH5α competent cells were transformed by the LR reaction using heat shock method as previously described. 2 hundred microliter of SOC media was added to the transformed bacteria and kept in a 37 °C shaker for 1 hour at 250 rpm. Finally, the transformed bacteria were plated onto an LB-agar plate containing 50µg/ml kanamycin.

Semiquantitative RT PCR (Hypothesis II)

Infecting Tobacco Plant with TMV

Five weeks old one C3 (control) and 1-2 tobacco (SABP2 silenced) plant were transferred from the growth chamber to the pathogen treatment chamber one day prior to the TMV inoculation. TMV (1.4 µg/ml) was diluted in 50mM phosphate buffer (pH 7) before inoculation. Four layers of cheese cloth were cut into a square pieces and washed with distilled water and then soaked into diluted TMV solution. Three fully expanded upper leaves

of each plant were selected for infection. The selected leaves were dusted with an abrasive (carborundum) and were gently rubbed with cheese cloth soaked in diluted TMV solution.

Sample Collection

Leaf samples (leaf discs) were collected at 0, 24, 48, and 72 hpi (hours post infection) from the C3 and 1-2 tobacco plants, treated with TMV. Collected samples were snap frozen in liquid nitrogen and stored at -80 ºC until RNA isolation.

RNA Isolation and RT PCR

Total RNA were isolated by the previously described method using Trizol. The concentration of RNA was determined by the Nanodrop spectrophotometer. Total RNA (1 µg) was mixed with 1 µl of dT-14 (1 µg/µl) primer, and diluted with DEPC-treated water in a sterile RNase-free micro centrifuge tube to a total volume of 10 µl. The mixture was heated at 70 ºC for 10mins then immediately cooled on ice. To each sample, M-MLV 5X reaction buffer (4 µl), 10 mM dNTP (1 µl), 40 U/µl RNAsin (1 µl), M-MLV RT (1 µl), and DTT (2µl) nuclease-free water (1µl) were added respectively and mixed gently. The reaction mixture was incubated for 60 minutes at 42 ºC followed by a 70 ºC incubation for 10 minutes. Samples were stored at -20 ºC for future use. To determine the mRNA expression level of SBIP24, forward 5′-ATGCAGACATTCTTGAAT- 3′ primer (Primer no. 6 in Table1) and reverse 5′-GAGCTTAATCTCTCTACC- 3′ primer (Primer no.7 in Table 1) were designed to amplify 200 bp of its C terminal region and were amplified by PCR. To perform PCR, 1µl of cDNA from each sample was mixed with 1 µl of 10X Taq polymerase buffer, 1 µl of 2.5 mM dNTP, 0.2 µl of 10 U/µl Taq polymerase, 0.4 µl of 10 µM Fwd and Rev gene expression primer, and 6.4 µl of autoclaved water. The PCR condition was set with an initial denaturing step for 1 min at 94 °C followed by 35 cycles (94 °C for 30 seconds, 53 °C for 6 minutes), and a final extension step at 72 °C for 1 minutes. Same PCR settings and conditions were used for amplifying *PR1*, *SABP2, EF1-α* with their corresponding gene specific primers. For *EF1-α*

PCR was carried out for 30 cycles. Eight microliter of each PCR product was mixed with 2 µl of 6X loading buffer containing dye and loaded on 1.2% agarose gel, 8 µl of 100 base pair ladder (25ng/ µl) was also loaded on the gel as a marker. Finally, the gel was run at 100 volts for 45 minutes and visualized under UV light and photographed.

CHAPTER 3

RESULTS

Section I: Cloning and Expression of SBIP-24

Bioinformatic Analysis of SBIP-24

The full length sequence (SGN-U444515) of SBIP24 from the SGN database was identified by BLAST analysis using the partial sequence of SBIP24 (Fig. 3) obtained from yeast two-hybrid screening. According to the SGN-U444515, SBIP24 ORF would have 864 bp (Fig. 4) encoding for 288 amino acids. But, alignment of this sequence with other well-documented stearoyl CoA desaturase proteins (Fig. 5) in plants suggested that SGN-U444515 could be missing the C-terminal end. This leads to an investigation to isolate the full-length sequence containing the missing c-terminus. Finally, multiple nucleotide alignment of SGN-U444515 with other known delta 9 fatty acid desaturases indicated a single missing nucleotide (G) in the SGN-U444515 obtained from SGN (Fig. 6). This resulted in a shift in the open reading frame and changed the position of stop codon. Adding a 'G' in the SGN-U444515sequence resulted in SBIP24 to have 1182 nucleotides (Fig. 6) encoding for 393 amino acids (Fig. 7) with complete ORF and apparent full length (Fig. 8, 9). This analysis helped in designing the correct reverse primer.

```
AAGAGAGAGCTACCTTCATTTCTCATGGAAATACAGCTAGGCATGCTAAGGACCACGGGGA
CTTGAAACTAGCACAAGTATGTGGTATAATTGCCGCAGATGAGAAGCGTCATGAAACTGCA
TATACCAAGATTGTGGAGAAGCTATTTGAAGTCGACCCAGATGGCACCGTACTGGCTGTTG
CTGACATGATGAGGAAAAAAATCTCGATGCCGGCTCATCTGATGTATGATGGCAGGGATGA
CAACCTCTTCGAACACTTCTCTGCCGTAGCTCAACGCCTCGGCGTATACACTGCGAAGGATT
ATGCAGATATTCTTGAATTTCTGGTGGGAAGATGGGAAATGGAGAAATTGACTGGTCTTTC
TGGCGAAGGACACAAAGCGCAAGATTATGTATGTGGACTGGCTCCACGTATTAGAAAAATT
GGAGGAGAGGGGACAAGCCGGGGCCATGGATAAGGCTCCCCGTTCCTTTCAACTGGGTATT
TGGTAAAAAAATAACCTCTGAATGCTAAATGGTTGTAGG
```

Figure 3: Partial Sequence of SBIP24 Obtained from Yeast Two-Hybrid Screening**.**

>SGN-U444515 *Nicotiana tabacum*

TTTGGTGCATCTGTCTCTCTCCACCTTTCATTTCTCTTTCTGCTTTCTTTAACTTTTACCTTAGGCGGAC
CGAACGAGGTACTCTGAAAAAGCCATCGGAAAA**ATGGCTCTGAAACTCAATCCGACGACGTTTCAATCCA**
TTAAAACGACAACGTTTCCTTGTTCTCCACTCAGATCTCATAGAGTTTTCATGGCTTCAACACTTCGTCC
CCCGTCTGTGGAAGGTGGAAGTGTGAAGAAGCCATTCAGTCCTCCACGTGAGGTGCATGTTCAAGTTACC
CATTCCATGCCGCCAGAGAAGCGTGAAATCTTTGATTCCTTGCAAGATTGGGCTGAGAATAATATCTTGG
TGCACCTAAAGCCTGTCGAGAAGTGTTGGCAGGCCAGTGACTTTCTTCCGGATCCTGCATCGGAAGGATT
TGAGGAGCAGGTCAAGGAACTGAGGGAGAGATGCAAGGAAATTCCTGATGACTACTTTGTTGTATTAGTT
GGAGATATGATCACAGAGGAGGCTCTTCCAACTTATCAGACGATGCTTAACACCTTAGATGGCGTTCGTG
ATGAAACTGGTGCCAGCCTTACTCCTTGGGCTATTTGGACTAGGGCATGGACTGCCGAGGAAAATAGGCA
CGGTGACCTTCTCAACAAGTATCTGTATCTTTCTGGAAGAGTTGATATGAGGCAAATTGAAAAGACAATT
CAGTACCTCATTGGCTCAGGGATGGATCCTCGCACGGAAAACAACCCGTATTTGGGTTTTATCTACACTT
CCTTCCAAGAGAGAGCTACCTTCATTTCTCACGGAAATACAGCTAGGCATGCTAAGGAGCATGGGGACTT
GAAACTAGCACAGGTATGTGGTATAATTGCTGCAGATGAAAGCGCCATGAAACTGCATATACCAAGATTG
TGGAGAAGCTGTTTGAAGTCGATCCAGATGGCACTGTACTGGCTGTTGCTGACATGATGAGGAAAAAAAT
CTCGATGCCAGCTCATCTGATGTATGATGGCAGGGATGACAACCTCTTCGAACACTTCTCTGCTGTAGCT
CAACGCCTCGGCGTATACACTGCAAAGGATTATGCAGACATTCTTGAATTTCTGGTGGGAAGATGGGAAA
TGGAGAAATTGACTGGTCTTTCTGGCGAAGGACGCAAAGCGCAAGATTATGTATGTGGACTGGCTCCACG
TATTAGAAAATTGGAGGAGAGGGCACAAGCCAGGGCCAAGGAGAAGGCTCCCGTTCCTTTCAGCTGGGTA
TTTGGTAGAGAGATTAAGCTCTGAATGCTATATGGTTGTAGGGTTATGTTTACTTTTTGAACACCAATCG
CCATAGGAATGCTAGAAAAAAAAATTCAAGGTATGGTATCTATCAAGTAAGTATTTGTCATCCCTTTGTA
GGGAAGATGCTATGGAGTTAAAATTGCTCCACTAGAGTCCTGTAGATACCTTAAATTTTGTTTTTGTCTT
GCAATTGTTTTGGTAATATCAAGAAAAAGGACAGCCTTTAGTTTTACCTCCCCTTTAAGCGCATGTAAGT
TCTCTATGAGTTTGATATATGAAATGTTGAGTGGCTATGTATAAGAAGATTTAATAGCGGCCGCTGCAAT
GTTGAATTGGCCCTCTCGATGTGAACACCAGGTTCAAGGCAAATACAGAATTGTTACATCTTTAACAATC
ATACTTGAGCTGTCTGATGAGCATAATTCAGACTAGGCCGGCA

Figure 4: The Open Reading Frame of SBIP24 in SGN Database. Bold and underlined sequence are indicating the nucleotides of SBIP24 with start and stop codon (SGN-U444515). The nucleotide sequence in red represents the sequence obtained from yeast two-hybrid clone.

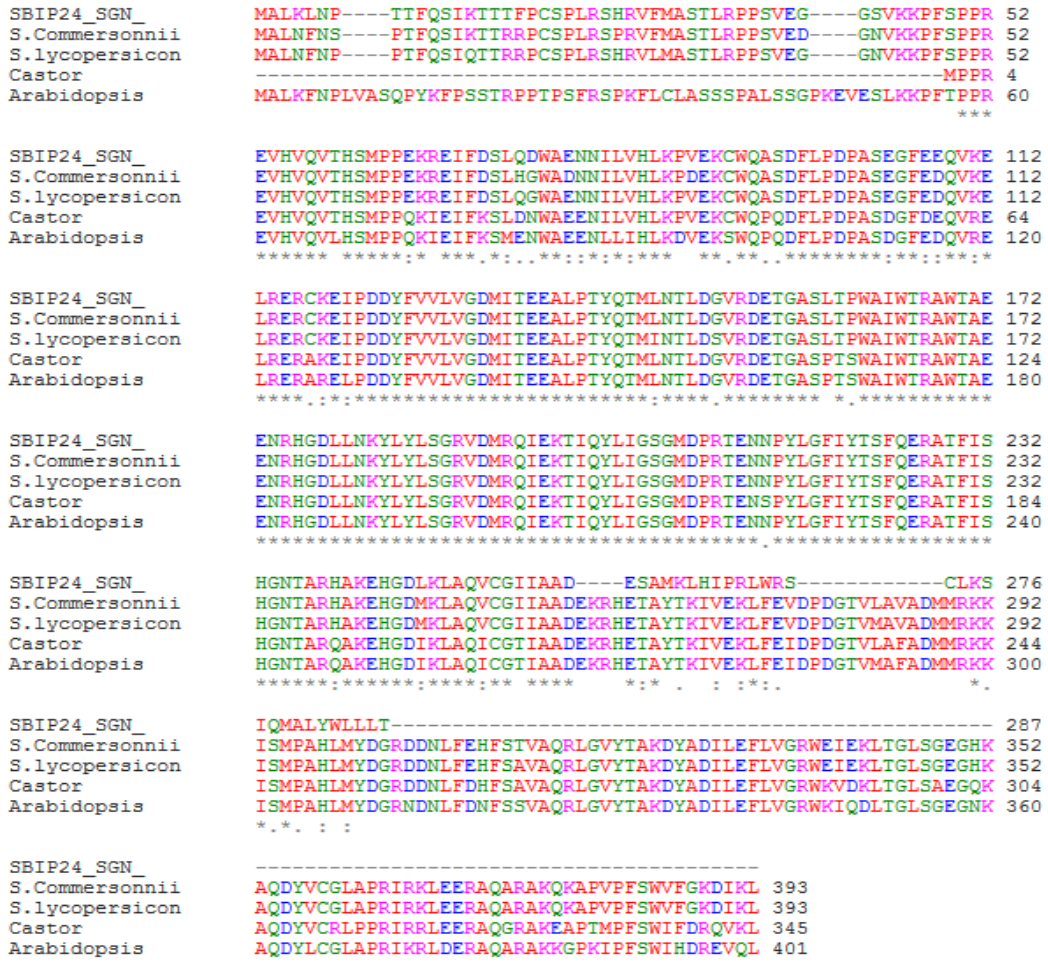

Figure 5: Multiple Amino Acid Sequence Alignments of SGN-U444515 with Other Known Stearoyl ACP Desaturases. Alignment shows that SGN-U444515 may have a missing part at the C terminus. (SBIP24_SGN = SGN-U444515, S. Commersonnii= *Solanum commersonnii,* S. lycopersicon= *Solanum lycopersicon*, Castor= *Ricinus communis*, Arabidopsis= *Arabidopsis thaliana*).

Figure 6: Multiple Nucleotide Sequence Alignment of SBIP24 (SGN-U444515) with other Stearoyl ACP Desaurases of Solanaceae family indicates SGN-U44515 may have a deletion in its database. (S.Acuale= *Solanum acuale*, S.Tuberosum= *Solanum tuberosum*, S.Commersonii= *Solanum commersonii)*.

TTTGGTGCATCTGTCTCTCTCCACCTTTCATTTCTCTTTCTGCTTTCTTTAACTTTTACCTTAGGCGGA
CCGAACGAGGTACTCTGAAAAAGCCATCGGAAAAATGGCTCTGAAACTCAATCCGACGACGTTTCAATC
CATTAAAACGACAACGTTTCCTTGTTCTCCACTCAGATCTCATAGAGTTTTCATGGCTTCAACACTTCG
TCCCCCGTCTGTGGAAGGTGGAAGTGTGAAGAAGCCATTCAGTCCTCCACGTGAGGTGCATGTTCAAGT
TACCCATTCCATGCCGCCAGAGAAGCGTGAAATCTTTGATTCCTTGCAAGATTGGGCTGAGAATAATAT
CTTGGTGCACCTAAAGCCTGTCGAGAAGTGTTGGCAGGCCAGTGACTTTCTTCCGGATCCTGCATCGGA
AGGATTTGAGGAGCAGGTCAAGGAACTGAGGGAGAGATGCAAGGAAATTCCTGATGACTACTTTGTTGT
ATTAGTTGGAGATATGATCACAGAGGAGGCTCTTCCAACTTATCAGACGATGCTTAACACCTTAGATGG
CGTTCGTGATGAAACTGGTGCCAGCCTTACTCCTTGGGCTATTTGGACTAGGGCATGGACTGCCGAGGA
AAATAGGCACGGTGACCTTCTCAACAAGTATCTGTATCTTTCTGGAAGAGTTGATATGAGGCAAATTGA
AAAGACAATTCAGTACCTCATTGGCTCAGGGATGGATCCTCGCACGGAAAACAACCCGTATTTGGGTTT
TATCTACACTTCCTTCCAAGAGAGAGCTACCTTCATTTCTCACGGAAATACAGCTAGGCATGCTAAGGA
GCATGGGGACTTGAAACTAGCACAGGTATGTGGTATAATTGCTGCAGATGAAAGCGCCATGAAACTGC
ATATACCAAGATTGTGGAGAAGCTGTTTGAAGTCGATCCAGATGGCACTGTACTGGCTGTTGCTGACAT
GATGAGGAAAAAAATCTCGATGCCAGCTCATCTGATGTATGATGGCAGGGATGACAACCTCTTCGAACA
CTTCTCTGCTGTAGCTCAACGCCTCGGCGTATACACTGCAAAGGATTATGCAGACATTCTTGAATTTCT
GGTGGGAAGATGGGAAATGGAGAAATTGACTGGTCTTTCTGGCGAAGGACGCAAAGCGCAAGATTATGT
ATGTGGACTGGCTCCACGTATTAGAAAATTGGAGGAGAGGGCACAAGCCAGGGCCAAGGAGAAGGCTCC
CGTTCCTTTCAGCTGGGTATTTGGTAGAGAGATTAAGCTCTGAATGCTATATGGTTGTAGGGTTATGTT
TACTTTTTGAACACCAATCGCCATAGGAATGCTAGAAAAAAAAATTCAAGGTATGGTATCTATCAAGTA
AGTATTTGTCATCCCTTTGTAGGGAAGATGCTATGGAGTTAAAATTGCTCCACTAGAGTCCTGTAGATA
CCTTAAATTTTGTTTTTGTCTTGCAATTGTTTTGGTAATATCAAGAAAAAGGACAGCCTTTAGTTTTAC
CTCCCCTTTAAGCGCATGTAAGTTCTCTATGAGTTTGATATATGAAATGTTGAGTGGCTATGTATAAGA
AGATTTAATAGCGGCCGCTGCAATGTTGAATTGGCCCTCTCGATGTGAACACCAGGTTCAAGGCAAATA
CAGAATTGTTACATCTTTAACAATCATACTTGAGCTGTCTGATGAGCATAATTCAGACTAGGCCGGCA

Figure 7: Predicted Full Length Sequence of SBIP24.

MALKLNPTTFQSIKTTTFPCSPLRSHRVFMASTLRPPSVEGGSVKKPFSPPREVHVQVTHSMPP
EKREIFDSLQDWAENNILVHLKPVEKCWQASDFLPDPASEGFEEQVKELRERCKEIPDDYFVVL
VGDMITEEALPTYQTMLNTLDGVRDETGASLTPWAIWTRAWTAEENRHGDLLNKYLYLSGRVDM
RQIEKTIQYLIGSGMDPRTENNPYLGFIYTSFQERATFISHGNTARHAKEHGDLKLAQVCGIIA
ADERRHETAYTKIVEKLFEVDPDGTVLAVADMMRKKISMPAHLMYDGRDDNLFEHFSAVAQRLG
VYTAKDYADILEFLVGRWEMEKLTGLSGEGRKAQDYVCGLAPRIRKLEERAQARAKEKAPVPFS
WVFGREIKL

Figure 8: Amino Acid Sequence of Predicted SBIP-24 Translated by ExPASy Bioinformatic Tool.

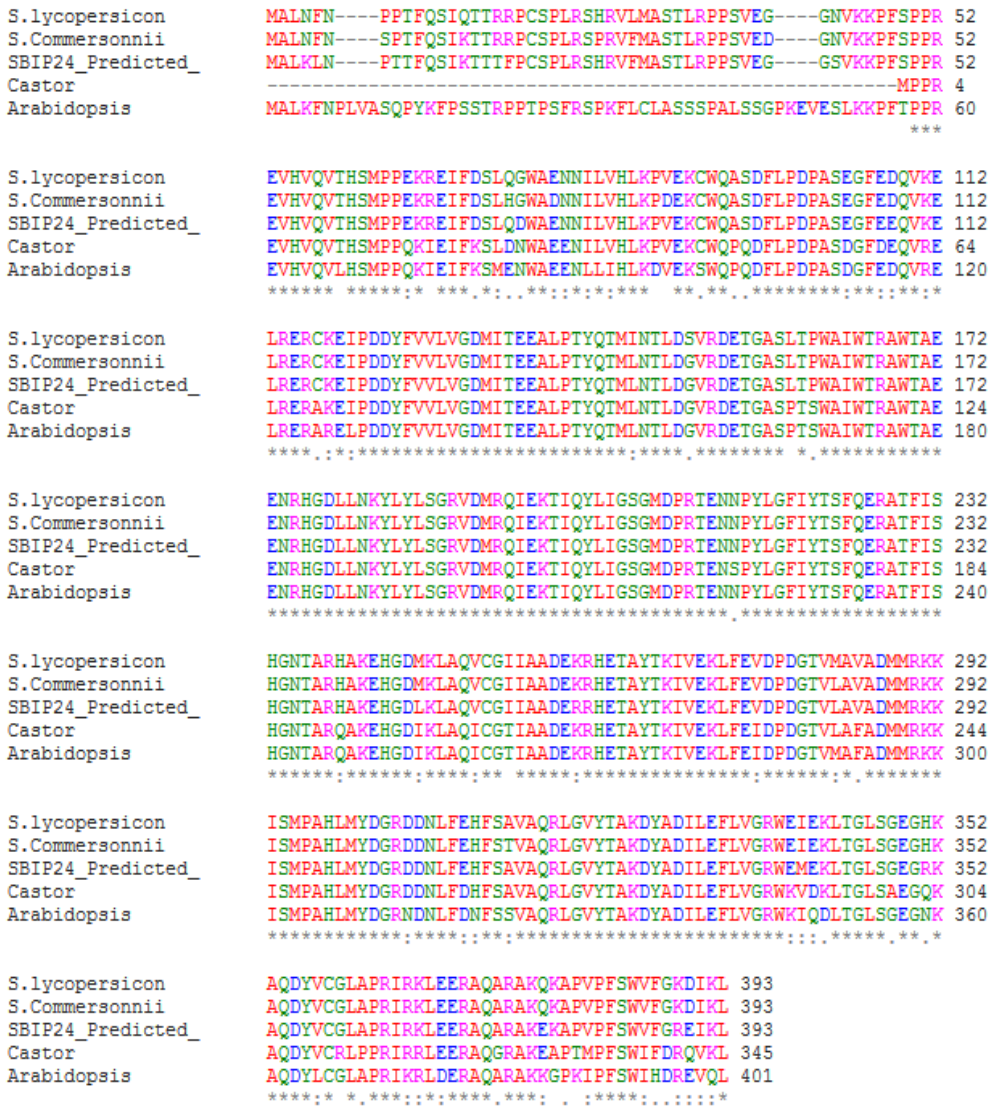

Figure 9: Multiple Amino Acid Sequence Alignment of Predicted SBIP24 with Other Stearoyl-CoA Desaturase, showing the missing part of SBIP24 (according to SGN) was recovered (by adding a "G" to the database sequence) in predicted SBIP24(S. lycopersicon= *Solanum lycopersicon*, S. Commersonnii= *Solanum commersonnii* , SBIP24_24= Predicted amino acid sequences of SBIP24, Castor= *Ricinus communis*, Arabidopsis= *Arabidopsis thaliana*)

Subcellular Localization of SBIP24. In *Arabidopsis*, *SSI2* and other isoforms of stearoyl-acyl carrier protein-desaturase genes have been found to have chloroplastic signal peptide (Kachroo et al. 2007). DNA sequence analysis of SBIP24 using ChloroP 1.1 and TatP 1.0 to determine the potential presence of a targeting signal peptide in SBIP24. Sequence

analysis revealed that SBIP24 has a 30 amino acids long chloroplast target peptide (Fig. 10, 11).

```
### chlorop v1.1 prediction results ##########################

Number of query sequences: 1

Name                    Length       Score  cTP       CS-     cTP-
                                                      score  length
-------------------------------------------------------------------
SBIP24                     393       0.569  Y      3.256     30
```

Figure 10: Result for Subcellular Localization of SBIP24 in ChloroP 1.1 Prediction Server. Result indicated SBIP24 has a 30 amino acid long chloroplast Target Peptide (cTP).

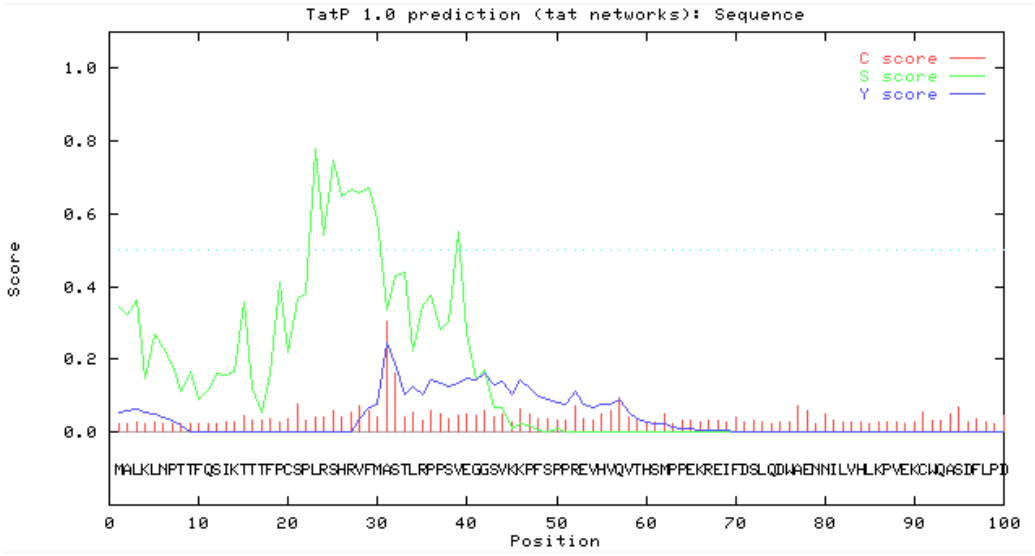

Figure 11: Cleavage Site of SBIP24 in TatP 1.0 Prediction Server. C score that indicates the raw cleavage site score is high in +1 (31st amino acid) position but low at all other positions. S-score, the signal peptide score, high at all positions before the cleavage site but low thereafter, and the Y score is indicating the combined cleavage site score. All the characteristics in the graph denote the cleavage site of SBIP24 is located between 30th and 31st amino acid of SBIP24.

Cloning and Heterologous Expression of SBIP24 in *E.coli* with Signal Peptide

The cloning of SBIP24 was accomplished by gateway cloning technology (Fig 12) that allowed rapid, directional cloning of gene of interest into an expression vector. This site-specific recombination-based cloning system was divided in 2 processes. The first process involved generation of the entry clone by the gateway BP reaction. Upon confirmation of a correct entry clone, the second process to generate a protein expression clone by LR reaction using the entry clone.

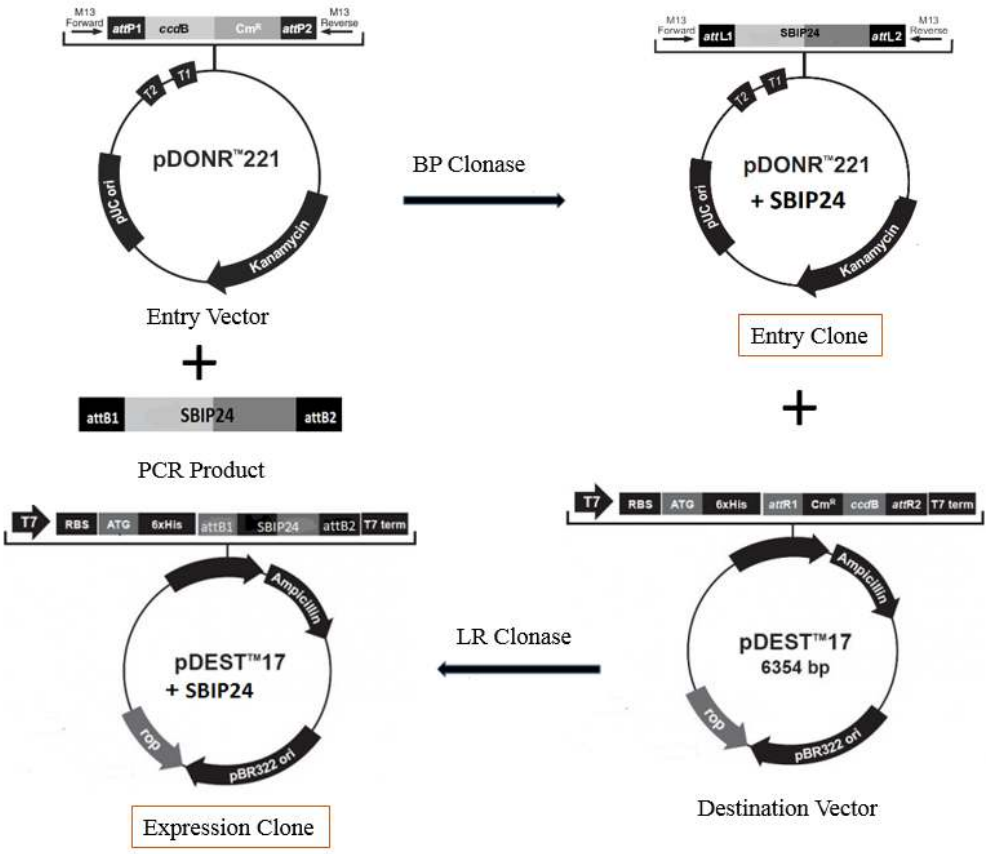

Figure 12: An Overview of Cloning of SBIP24 into pDEST17 Using Gateway System. SBIP24 with attB1 and attB2 sites was amplified from tobacco cDNA and was inserted into

the Gateway system via entry clone pDONR221 by BP reaction. Once the entry clone pDONR221-SBIP24 was formed, LR reaction of entry clone and destination vector transferred theSBIP24 fragment into expression clone pDEST17-SBIP24 (Calmels et al. 1991; Kulkarni and Deobagkar 2002)

Amplification of SBIP24. To amplify the 1182 bp SBIP24 fragment containing start and stop codons, cDNA from tobacco leaf was used as template and PCR was performed using Clontech Advantage Taq DNA polymerase (high fidelity). Fig. 13 shows the successful amplification of SBIP24 on a 1.2% agarose gel.

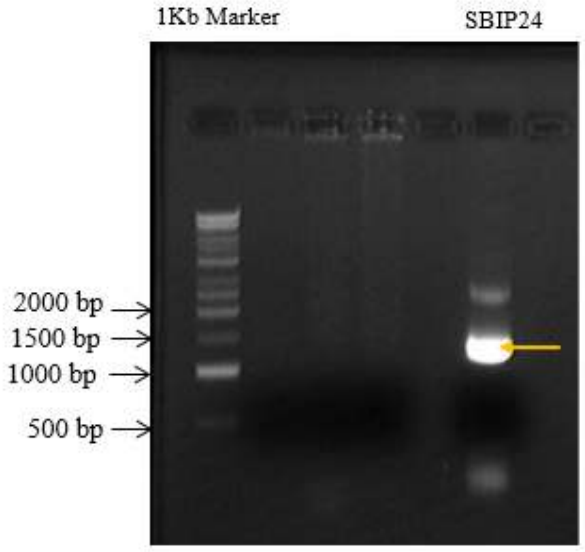

Figure 13: 1.2 % Agarose Gel Showing the Amplification of SBIP24. The gel was stained with EtBr.

Gel Purification of PCR Amplified SBIP24. PCR amplified SBIP24 were excised from the agarose gel and purified using Qiagen Gel Extraction Kit. The quality of DNA was confirmed by agarose gel electrophoresis (Fig 14).

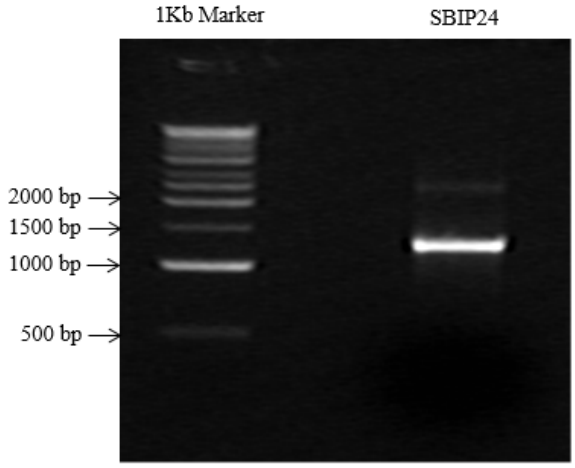

Figure 14: Image of 1.2% Agarose Gel Confirming the Quality of DNA Fragment by Gel Purification. The gel was stained with EtBr.

Confirmation of pDONR221-SBIP24 Entry Clone. The Gateway cloning system allows direct positive selection of colonies containing the gene insert by disruption of an active cytotoxic *ccdB* gene. As only cells containing recombinant DNA (where *ccdB* gene is replaced by gene of interest) can survive, the viability of a pDONR221-SBIP24 entry clone on a kanamycin plate validates the successful cloning of SBIP24 into pDONR221. Additionally, the insertion of SBIP24 into the pDONR221 was confirmed by colony PCR (Fig. 15) using M13 forward and reverse primers as well as M13 forward and SBIP24 reverse primers. Lanes 1-3 are showing the amplification of SBIP24 from pDONR221 using M13 forward and reverse primers. Lanes 4 and 5 are the amplification of SBIP24 from pDONR221 using M13 forward and SBIP24 reverse primers.

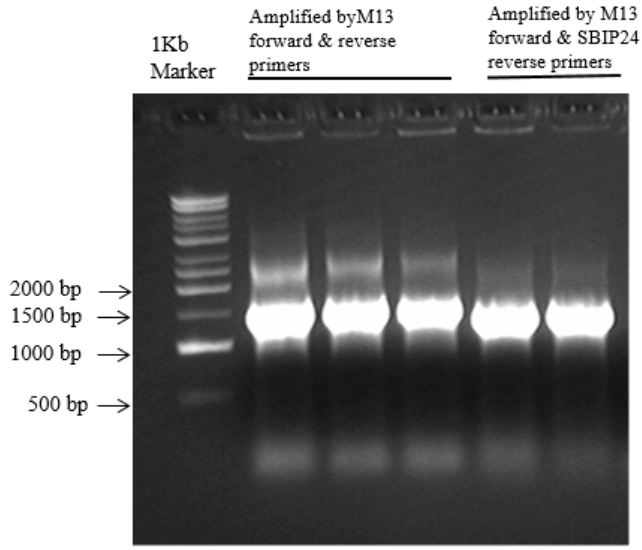

Figure 15: Screening of Insert of SBIP24 into pDONR221 by Colony PCR. Agarose gel showing PCR amplified fragment of SBIP24 from recombinant pDONR221-SBIP24 plasmids. Lanes 1-3 are showing the amplification of SBIP24 from pDONR221-SBIP24 clone using M13 forward and reverse primers. Lanes 4 and 5 are the amplification of SBIP24 from pDONR221-SBIP24 clone using M13 forward and SBIP24 reverse primers

Sequencing Result

Among the 10 positive clones analyzed by DNA sequencing, clone 8 had the insert that showed highest similarities with predicted SBIP24 sequence. Sequencing results revealed that there was a missing nucleotide "G" in SGN-U444515 (Fig. 16). Also, sequencing result of full length clone indicated changes resulting in 3 different amino acids in the pDONR221-SBIP24 compared to the predicted SBIP24 sequence based on SGN-U444515 (Fig. 17). Additionally, conserved domain analysis of SBIP24 suggested that the 3 amino acids that are different in predicted SBIP24 sequence was not located in the conserved region of stearoyl ACP desaturase (Fig. 18).

```
PredictedSBIP24      ATGGCTCTGAAACTCAATCCGACGACGTTTCAATCCATTAAAACGACAACGTTTCCTTGT 60
SGN-U444515          ATGGCTCTGAAACTCAATCCGACGACGTTTCAATCCATTAAAACGACAACGTTTCCTTGT 60
pDONR221-SBIP24      ATGGCTCTGAAACTCAATCCGACGACGTTTCAATCCATTAAAACGACAACGTTTCCATGT 60
                     ************************************************************ ***

PredictedSBIP24      TCTCCACTCAGATCTCATAGAGTTTTCATGGCTTCAACACTTCGTCCCCCGTCTGTGGAA 120
SGN-U444515          TCTCCACTCAGATCTCATAGAGTTTTCATGGCTTCAACACTTCGTCCCCCGTCTGTGGAA 120
pDONR221-SBIP24      TCTCCACTCAGATCTCATAGAGTTTTCATGGCTTCAACACTTCGTCCCCCGTCTGTGGAA 120
                     ************************************************************

PredictedSBIP24      GGTGGAAGTGTGAAGAAGCCATTCAGTCCTCCACGTGAGGTGCATGTTCAAGTTACCCAT 180
SGN-U444515          GGTGGAAGTGTGAAGAAGCCATTCAGTCCTCCACGTGAGGTGCATGTTCAAGTTACCCAT 180
pDONR221-SBIP24      GGTGGAAGTGTGAAGAAGCCATTCAGTCCTCCACGTGAGGTGCATGTTCAAGTTACCCAT 180
                     ************************************************************

PredictedSBIP24      TCCATGCCGCCAGAGAAGCGTGAAATCTTTGATTCCTTGCAAGATTGGGCTGAGAATAAT 240
SGN-U444515          TCCATGCCGCCAGAGAAGCGTGAAATCTTTGATTCCTTGCAAGATTGGGCTGAGAATAAT 240
pDONR221-SBIP24      TCCATGCCGCCAGAGAAGCGTGAAATCTTTGATTCCTTGCAAGATTGGGCTGAGAATAAT 240
                     ************************************************************

PredictedSBIP24      ATCTTGGTGCACCTAAAGCCTGTCGAGAAGTGTTGGCAGGCCAGTGACTTTCTTCCGGAT 300
SGN-U444515          ATCTTGGTGCACCTAAAGCCTGTCGAGAAGTGTTGGCAGGCCAGTGACTTTCTTCCGGAT 300
pDONR221-SBIP24      ATCTTGGTGCACCTAAAGCCTGTCGAGAAGTGTTGGCAGGCCAGTGACTTTCTTCCGGAT 300
                     ************************************************************

PredictedSBIP24      CCTGCATCGGAAGGATTTGAGGAGCAGGTCAAGGAACTGAGGGAGAGATGCAAGGAAATT 360
SGN-U444515          CCTGCATCGGAAGGATTTGAGGAGCAGGTCAAGGAACTGAGGGAGAGATGCAAGGAAATT 360
pDONR221-SBIP24      CCTGCATCGGAAGGATTTGAGGAGCAGGTCAAGGAACTGAGGGAGAGATGCAAGGAAATT 360
                     ************************************************************

PredictedSBIP24      CCTGATGACTACTTTGTTGTATTAGTTGGAGATATGATCACAGAGGAGGCTCTTCCAACT 420
SGN-U444515          CCTGATGACTACTTTGTTGTATTAGTTGGAGATATGATCACAGAGGAGGCTCTTCCAACT 420
pDONR221-SBIP24      CCTGATGACTACTTTGTTGTATTAGTTGGAGATATGATCACAGAGGAGGCTCTTCCAACT 420
                     ************************************************************

PredictedSBIP24      TATCAGACGATGCTTAACACCTTAGATGGCGTTCGTGATGAAACTGGTGCCAGCCTTACT 480
SGN-U444515          TATCAGACGATGCTTAACACCTTAGATGGCGTTCGTGATGAAACTGGTGCCAGCCTTACT 480
pDONR221-SBIP24      TATCAGACGATGCTTAACACCTTAGATGGCGTTCGTGATGGAACTGGTGCCAGCCTTACT 480
                     ************************************* ******************
```

Figure 16: Nucleotide Sequence Alignment of Predicted SBIP24 (with G), SGN-U444515 (Without 'G') and Recombinant pDONR221-SBIP24 Clone. Blue arrow is indicating the deletion of the 'G' in SGN data base.

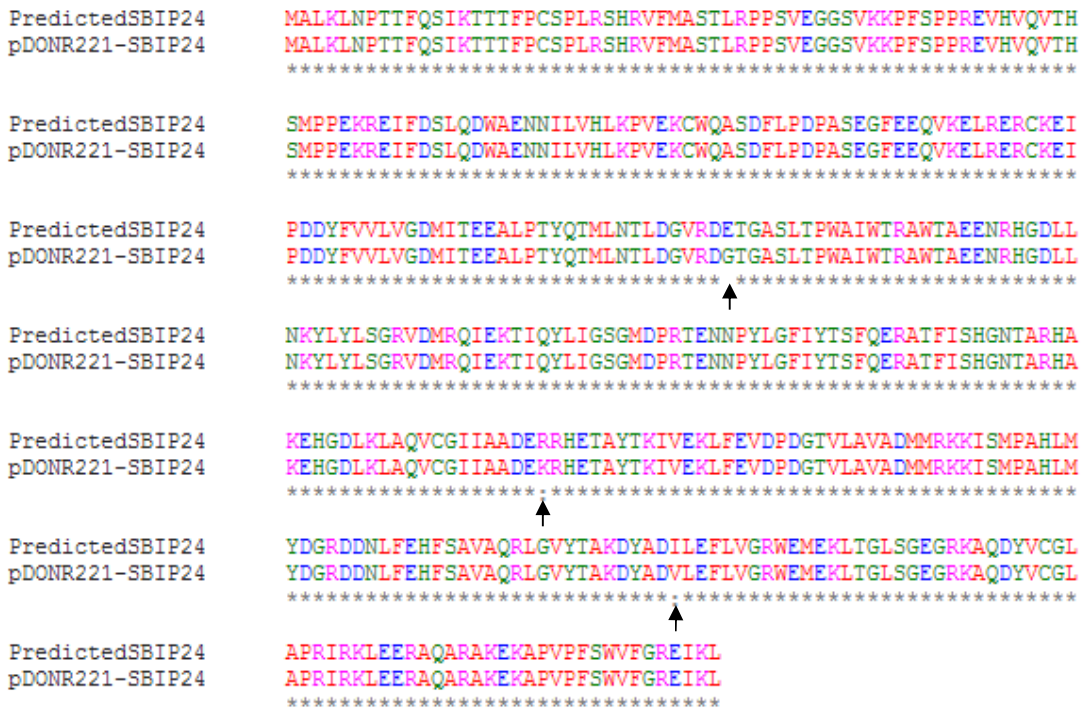

Figure 17: Amino Acid Sequence Alignment of Predicted SBIP24 and pDONR221-SBIP24 entry clone. Arrow is showing the amino acid change found in pDONR221-SBIP24 clone.

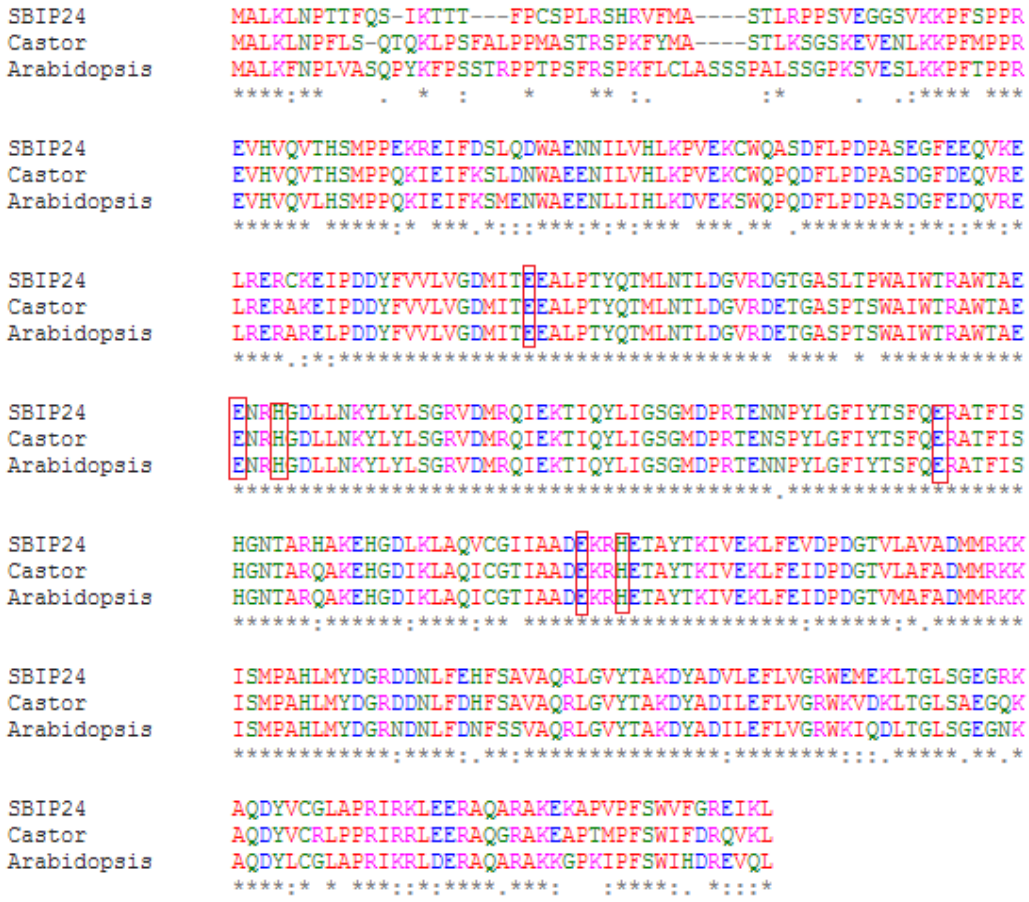

Figure 18: Amino Acid Sequence Alignment of Stearoyl ACP Desaturase of Tobacco (SBIP24), Arabidopsis and Castor stearoyl ACP desaturase. Red boxes indicate the amino acids that are conserved in stearoyl ACP desaturase protein**.**

Confirmation of pDEST17-SBIP24 Expression Clone. SBIP24 was successfully subcloned into pDEST17 by site-specific recombination between pDONR221-SBIP24 and pDEST-17 catalyzed by the LR clonase enzyme. Growth of recombinant pDEST17-SBIP24 on LB plates containing ampicillin as well as colony PCR (Fig. 19) confirms successful insertion of SBIP24.

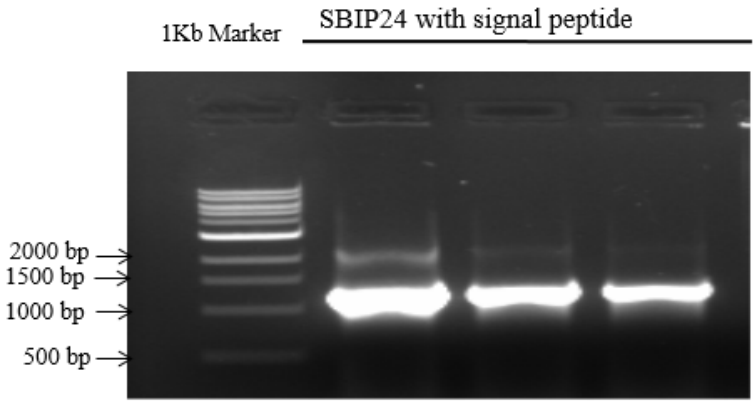

Figure 19: Image of a 1.2% Agarose Gel after Colony PCR for the Confirmation of SBIP24 Insert into pDEST17. The gel was stained with EtBr.

Small Scale Heterologous Expression of Recombinant SBIP24

To test the expression of SBIP24, 3 ml of pDEST17-SBIP24 in Mgk cells were induced with 1mM IPTG and incubated at 25°C overnight. Pellets were collected and processed (as described earlier in material and methods part) before analyzing on a 12 % SDS PAGE gel. After the SDS page gel electrophoreses, the gel was stained with coomassie blue. Fig. 20 confirms the expression of SBIP24 in *E. coli*.

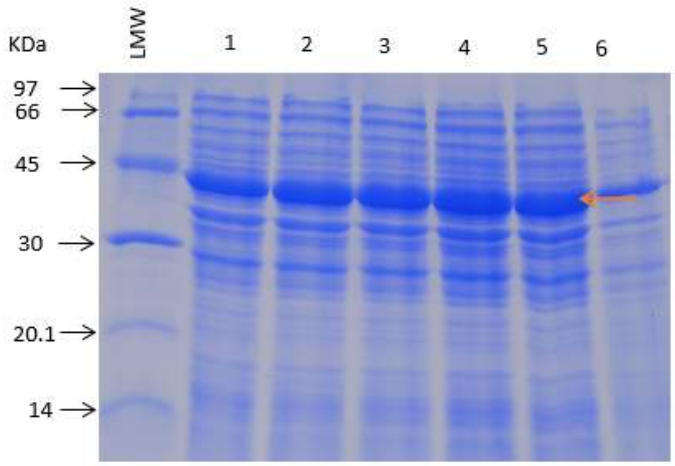

Figure 20: SDS-PAGE Analysis of pDEST17-SBIP24 (with signal peptide) Recombinant Protein Expression in E. coli. Protein molecular weight marker; Lane 1-5 showing protein expression with 1mM IPTG induction at 25°C overnight; Lane 6 showing protein expression from un-induced bacterial culture. Arrow is indicating the expected size of SBIP24 (45 KDa) fusion protein.

Solubility Test of Recombinant SBIP24

Several small scale solubility tests using various temperature (17 °C, 25 °C, 37 °C) conditions and IPTG concentrations have been performed but Western blot analysis did not detect any soluble SBIP24 recombinant protein. Low IPTG (0.5mM, 1mM, 0.1mM, 0.8mM) concentrations and reduced temperature (17°C) were applied to attempt to enhance the solubility of SBIP24. 2 gels with same samples were run parallel, one was used for coomassie blue staining and the other one was used for Western blot. It is clear from the Fig. 21 that most of the SBIP24 is expressing in insoluble form as very thick bands were visible in the lane containing insoluble recombinant proteins (both in coomassie staining and Western blot). No bands for soluble protein in the western blot indicate insufficient or no soluble protein expression of SBIP24.

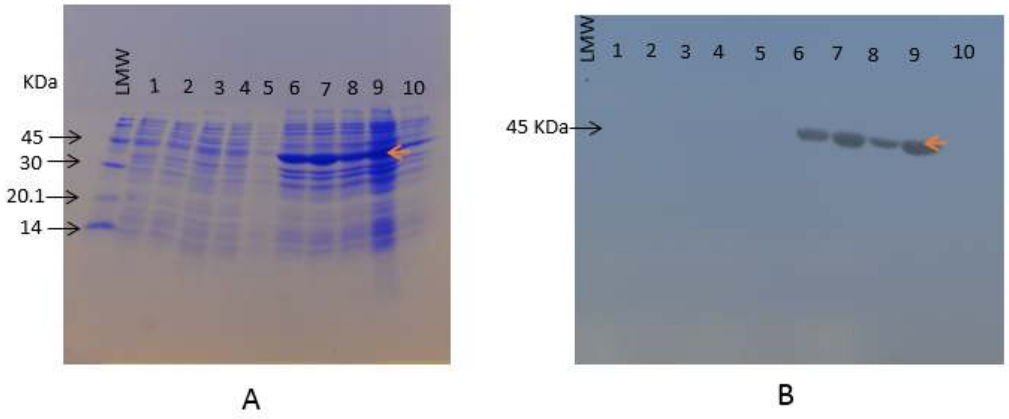

Figure 21: 12% SDS-Polyacrylamide Gel Electrophoresis (A) and Corresponding Western Blot (B) Analysis of Recombinant SBIP24; Low molecular marker; lane 1-4 induced insoluble protein by 0.5mM; 1mM, 0.1mM, 0.8 mM IPTG induction overnight at 17°C respectively; lane 5 un-induced soluble protein; lane 6-9 induced soluble protein by 0.5mM, 1mM, 0.1mM, 0.8 mM IPTG induction overnight at 17°C respectively; Lane 10 uninduced insoluble protein. Arrow indicates SBIP24**.**

Cloning of SBIP24$\Delta^{31\text{-}39}$

Because the majority of recombinant SBIP24 protein in *E.coli* cell was expressed as insoluble protein aggregate, an attempt to clone SBIP24 without signal peptide was made to increase the solubility of recombinant SBIP24.

Amplification of SBIP24Δ31-393 by PCR. In order to truncate the signal peptide from SBIP24, 90 nucleotides (Fig. 22) encoding first 30 amino acids (from N-terminal) (Fig. 23) were removed from full length SBIP24. The rest of the nucleotide sequence SBIP24$\Delta^{31\text{-}393}$ was successfully amplified from tobacco cDNA using the newly designed primers. The SBIP24$\Delta^{31\text{-}393}$ was gel purified using the Qiaquick gel purification kit. The concentration of the DNA was measured by using the Nanodrop spectrophotometer and the quality of DNA was verified by running it on a 1.2% agarose gel (Fig. 24).

GCTTCAACACTTCGTCCCCCGTCTGTGGAAGGTGGAAGTGTGAAGAAGCCATTCAGTCCTCCACGTGAGG
TGCATGTTCAAGTTACCCATTCCATGCCGCCAGAGAAGCGTGAAATCTTTGATTCCTTGCAAGATTGGGC
TGAGAATAATATCTTGGTGCACCTAAAGCCTGTCGAGAAGTGTTGGCAGGCCAGTGACTTTCTTCCGGAT
CCTGCATCGGAAGGATTTGAGGAGCAGGTCAAGGAACTGAGGGAGAGATGCAAGGAAATTCCTGATGACT
ACTTTGTTGTATTAGTTGGAGATATGATCACAGAGGAGGCTCTTCCAACTTATCAGACGATGCTTAACAC
CTTAGATGGCGTTCGTGATGGAACTGGTGCCAGCCTTACTCCTTGGGCGATTTGGACTAGGGCATGGACT
GCCGAGGAAAATAGGCACGGTGACCTTCTCAACAAGTATCTGTATCTTTCTGGAAGAGTTGATATGAGGC
AAATTGAAAAGACAATTCAGTACCTCATTGGCTCAGGGATGGATCCTCGCACGGAAAACAACCCGTATTT
GGGTTTTATCTACACTTCCTTCCAAGAGAGAGCTACCTTCATTTCTCACGGAAATACAGCTAGGCATGCT
AAGGAGCATGGGGACTTGAAACTAGCACAGGTATGTGGTATAATTGCTGCAGATGAGAAGCGCCATGAAA
CTGCATATACCAAGATTGTGGAGAAGCTGTTTGAAGTCGATCCAGATGGCACTGTACTGGCTGTTGCTGA
CATGATGAGGAAAAAAATCTCGATGCCAGCTCATCTGATGTATGACGGCAGGGATGACAACCTCTTCGAA
CACTTCTCTGCTGTAGCTCAACGCCTCGGCGTATACACTGCAAAGGATTATGCAGACGTTCTTGAATTTC
TGGTGGGAAGATGGGAAATGGAGAAATTGACTGGTCTTTCTGGCGAAGGACGCAAAGCGCAAGATTATGT
ATGTGGACTGGCTCCACGTATTAGAAAATTGGAGGAGAGGGCACAAGCCAGGGCCAAGGAGAAGGCTCCC
GTTCCCTTCAGCTGGGTATTTGGTAGAGAGATTAAGCTCTGA

Figure 22: Nucleotide Sequence of SBIP24$\Delta^{31\text{-}393}$ (1092bp).

ASTLRPPSVEGGSVKKPFSPPREVHVQVTHSMPPEKREIFDSLQDWAENNILVHLKPVEKCWQASDFLPD
PASEGFEEQVKELRERCKEIPDDYFVVLVGDMITEEALPTYQTMLNTLDGVRDGTGASLTPWAIWTRAWT
AEENRHGDLLNKYLYLSGRVDMRQIEKTIQYLIGSGMDPRTENNPYLGFIYTSFQERATFISHGNTARHA
KEHGDLKLAQVCGIIAADEKRHETAYTKIVEKLFEVDPDGTVLAVADMMRKKISMPAHLMYDGRDDNLFE
HFSAVAQRLGVYTAKDYADVLEFLVGRWEMEKLTGLSGEGRKAQDYVCGLAPRIRKLEERAQARAKEKAP
VPFSWVFGREIKL

Figure 23: Amino Acid Sequences of SBIP24$\Delta^{31\text{-}393}$ (363 amino acids).

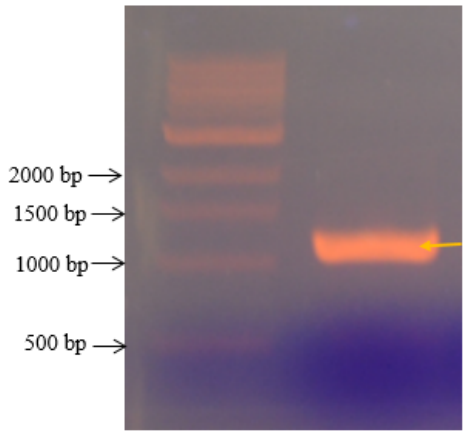

Figure 24: An Image of 1.2% Agarose Gel Showing the Band of Gel Purified SBIP24$\Delta^{31\text{-}393}$.

Detection of Entry clone pDONR221- SBIP24Δ31-393 by Colony PCR. To identify the positive clones with insert, colony PCR was performed following transformation of the recombination reaction (BP reaction) between entry vector (pDONR221) and target insert SBIP24$\Delta^{31\text{-}39}$. Fig. 25 shows the presence of SBIP24$\Delta^{31\text{-}39}$ in the pDONR221.

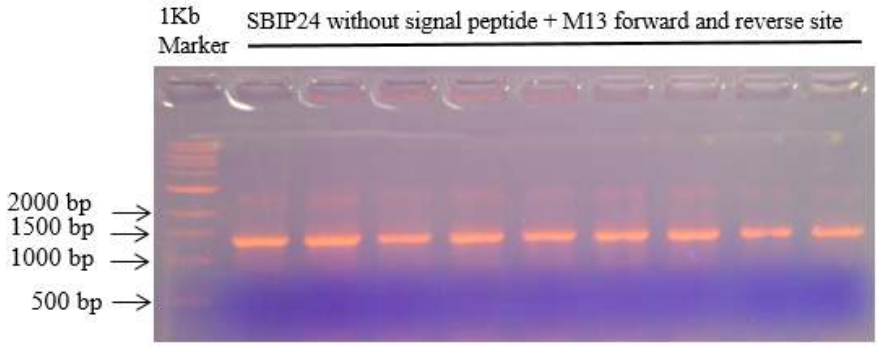

Figure 25: Screening of Positive Clone Containing SBIP24$\Delta^{31\text{-}393}$ Fragment by Colony PCR.1.2 % agarose gel showing PCR products after colony PCR of recombinant bacterial DNA (from different colony) using M13 forward and reverse primer**.**

Sequencing result. Plasmid DNAs extracted from SBIP24$\Delta^{31\text{-}39}$ were sequenced. To verify the correct nucleotide sequence chromatograms of each clone were examined carefully. Sequencing results shows colony#4 had the best match with SBIP24 (Fig. 26) but that has one nucleotide change (Fig. 27).

```
SBIP24            ATGGCTCTGAAACTCAATCCGACGACGTTTCAATCCATTAAAACGACAACGTTTCCATGT
SBIP24Δ31-393     ------------------------------------------------------------


SBIP24            TCTCCACTCAGATCTCATAGAGTTTTCATGGCTTCAACACTTCGTCCCCCGTCTGTGGAA
SBIP24Δ31-393     ------------------------------GCTTCAACACTTCGTCCCCCGTCTGTGGAA
                                                 ****************************

SBIP24            GGTGGAAGTGTGAAGAAGCCATTCAGTCCTCCACGTGAGGTGCATGTTCAAGTTACCCAT
SBIP24Δ31-393     GGTGGAAGTGTGAAGAAGCCATTCAGTCCTCCACGTGAGGTGCATGTTCAAGTTACCCAT
                  ************************************************************

SBIP24            TCCATGCCGCCAGAGAAGCGTGAAATCTTTGATTCCTTGCAAGATTGGGCTGAGAATAAT
SBIP24Δ31-393     TCCATGCCGCCAGAGAAGCGTGAAATCTTTGATTCCTTGCAAGATTGGGCTGAGAATAAT
                  ************************************************************

SBIP24            ATCTTGGTGCACCTAAAGCCTGTCGAGAAGTGTTGGCAGGCCAGTGACTTTCTTCCGGAT
SBIP24Δ31-393     ATCTTGGTGCACCTAAAGCCTGTCGAGAAGTGTTGGCAGGCCAGTGACTTTCTTCCGGAT
                  ************************************************************

SBIP24            CCTGCATCGGAAGGATTTGAGGAGCAGGTCAAGGAACTGAGGGAGAGATGCAAGGAAATT
SBIP24Δ31-393     CCTGCATCGGAAGGATTTGAGGAGCAGGTCAAGGAACTGAGGGAGAGATGCAAGGAAATT
                  ************************************************************

SBIP24            CCTGATGACTACTTTGTTGTATTAGTTGGAGATATGATCACAGAGGAGGCTCTTCCAACT
SBIP24Δ31-393     CCTGATGACTACTTTGTTGTATTAGTTGGAGATATGATCACAGAGGAGGCTCTTCCAACT
                  ************************************************************

SBIP24            TATCAGACGATGCTTAACACCTTAGATGGCGTTCGTGATGGAACTGGTGCCAGCCTTACT
SBIP24Δ31-393     TATCAGACGATGCTTAACACCTTAGATGGCGTTCGTGATGGAACTGGTGCCAGCCTTACT
                  ************************************************************

SBIP24            CCTTGGGCGATTTGGACTAGGGCATGGACTGCCGAGGAAAATAGGCACGGTGACCTTCTC
SBIP24Δ31-393     CCTTGGGCGATTTGGACTAGGGCATGGACTGCCGAGGAAAATAGGCACGGTGACCTTCTC
                  ************************************************************

SBIP24            AACAAGTATCTGTATCTTTCTGGAAGAGTTGATATGAGGCAAATTGAAAAGACAATTCAG
SBIP24Δ31-393     AACAAGTATCTGTATCTTTCTGGAAGAGTTGATATGAGGCAAATTGAAAAGACAATTCAG
                  ************************************************************

SBIP24            TACCTCATTGGCTCAGGGATGGATCCTCGCACGGAAAACAACCCGTATTTGGGTTTTATC
SBIP24Δ31-393     TACCTCATTGGCTCAGGGATGGATCCTCGCACGGAAAACAACCCGTATTTGGGTTTTATC
                  ************************************************************

SBIP24            TACACTTCCTTCCAAGAGAGAGCTACCTTCATTTCTCACGGAAATACAGCTAGGCATGCT
SBIP24Δ31-393     TACACTTCCTTCCAAGAGAGAGCTACCTTCATTTCTCACGGAAATACAGCTAGGCATGCT
                  ************************************************************
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SBIP24            AAGGAGCATGGGGACTTGAAACTAGCACAGGTATGTGGTATAATTGCTGCAGATGAGAAG
SBIP24Δ31-393     AAGGAGCATGGGGACTTGAAACTAGCACAGGTATGTGGTATAATTGCTGCAGATGAGAAG
                  ************************************************************

SBIP24            CGCCATGAAACTGCATATACCAAGATTGTGGAGAAGCTGTTTGAAGTCGATCCAGATGGC
SBIP24Δ31-393     CGCCATGAAACTGCATATACCAAGATTGTGGAGAAGCTGTTTGAAGTCGATCCAGATGGC
                  ************************************************************

SBIP24            ACTGTACTGGCTGTTGCTGACATGATGAGGAAAAAAATCTCGATGCCAGCTCATCTGATG
SBIP24Δ31-393     ACTGTACTGGCTGTTGCTGACATGATGAGGAAAAAAATCTCGATGCCAGCTCATCTGATG
                  ************************************************************

SBIP24            TATGACGGCAGGGATGACAACCTCTTCGAACACTTCTCTGCTGTAGCTCAACGCCTCGGC
SBIP24Δ31-393     TATGACGGCAGGGATGACAACCTCTTCGAACACTTCTCTGCTGTAGCTCAACGCCTCGGC
                  ************************************************************

SBIP24            GTATACACTGCAAAGGATTATGCAGACGTTCTTGAATTTCTGGTGGGAAGATGGGAAATG
SBIP24Δ31-393     GTATACACTGCAAAGGATTATGCAGACGTTCTTGAATTTCTGATGGGAAGATGGGAAATG
                  ****************************************** ******************

SBIP24            GAGAAATTGACTGGTCTTTCTGGCGAAGGACGCAAAGCGCAAGATTATGTATGTGGACTG
SBIP24Δ31-393     GAGAAATTGACTGGTCTTTCTGGCGAAGGACGCAAAGCGCAAGATTATGTATGTGGACTG
                  ************************************************************

SBIP24            GCTCCACGTATTAGAAAATTGGAGGAGAGGGCACAAGCCAGGGCCAAGGAGAAGGCTCCC
SBIP24Δ31-393     GCTCCACGTATTAGAAAATTGGAGGAGAGGGCACAAGCCAGGGCCAAGGAGAAGGCTCCC
                  ************************************************************

SBIP24            GTTCCCTTCAGCTGGGTATTTGGTAGAGAGATTAAGCTCTGA
SBIP24Δ31-393     GTTCCCTTCAGCTGGGTATTTGGTAGAGAGATTAAGCTCTGA
                  *******************************************
```

Figure 26: Nucleotide Sequence Alignment of SBIP24$\Delta^{31\text{-}39}$ (from entry clone) and SBIP24.

```
SBIP24Δ31-393     ----------------------------ASTLRPPSVEGGSVKKPFSPPREVHVQVTH
SBIP24            MALKLNPTTFQSIKTTTFPCSPLRSHRVFMASTLRPPSVEGGSVKKPFSPPREVHVQVTH
                                               ********************************

SBIP24Δ31-393     SMPPEKREIFDSLQDWAENNILVHLKPVEKCWQASDFLPDPASEGFEEQVKELRERCKEI
SBIP24            SMPPEKREIFDSLQDWAENNILVHLKPVEKCWQASDFLPDPASEGFEEQVKELRERCKEI
                  ************************************************************

SBIP24Δ31-393     PDDYFVVLVGDMITEEALPTYQTMLNTLDGVRDGTGASLTPWAIWTRAWTAEENRHGDLL
SBIP24            PDDYFVVLVGDMITEEALPTYQTMLNTLDGVRDGTGASLTPWAIWTRAWTAEENRHGDLL
                  ************************************************************

SBIP24Δ31-393     NKYLYLSGRVDMRQIEKTIQYLIGSGMDPRTENNPYLGFIYTSFQERATFISHGNTARHA
SBIP24            NKYLYLSGRVDMRQIEKTIQYLIGSGMDPRTENNPYLGFIYTSFQERATFISHGNTARHA
                  ************************************************************

SBIP24Δ31-393     KEHGDLKLAQVCGIIAADEKRHETAYTKIVEKLFEVDPDGTVLAVADMMRKKISMPAHLM
SBIP24            KEHGDLKLAQVCGIIAADEKRHETAYTKIVEKLFEVDPDGTVLAVADMMRKKISMPAHLM
                  ************************************************************

SBIP24Δ31-393     YDGRDDNLFEHFSAVAQRLGVYTAKDYADVLEFLMGRWEMEKLTGLSGEGRKAQDYVCGL
SBIP24            YDGRDDNLFEHFSAVAQRLGVYTAKDYADVLEFLVGRWEMEKLTGLSGEGRKAQDYVCGL
                  **********************************:*************************
```

Figure 27: Amino Acid Sequence Alignment of SBIP24$\Delta^{31\text{-}393}$ (from entry clone) and SBIP24.

Detection of pDEST17- SBIP24Δ31-39 Expression Clone. Colony PCR was also performed to determine cloning of SBIP24$\Delta^{31\text{-}393}$ into pDEST17. Fig. 28 confirms the target insert by showing the amplification of SBIP24$\Delta^{31\text{-}393}$ from recombinant pDEST17-SBIP24$\Delta^{31\text{-}393}$ plasmid construct.

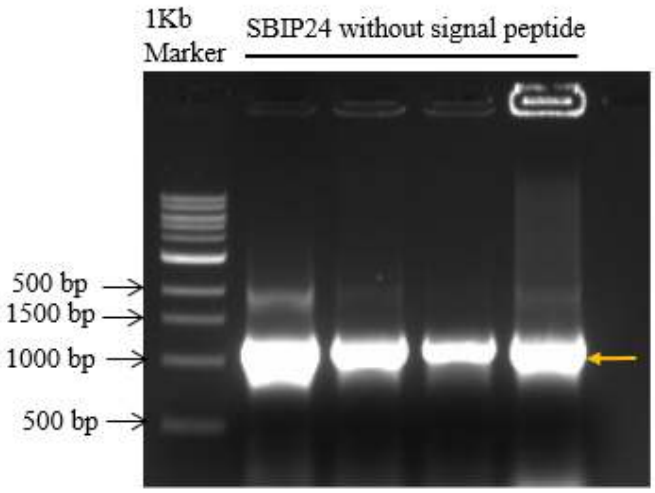

Figure 28: Image of a 0.8% Agarose Gel Electrophoresis Showing the Amplification of SBIP24$\Delta^{31\text{-}39}$ in Colony PCR Using the Recombinant pDEST17- SBIP24$\Delta^{31\text{-}39}$ Colony.

Expression Analysis of Recombinant SBIP24 $\Delta^{31\text{-}393}$ Protein

In order to confirm the expression of recombinant SBIP24$\Delta^{31\text{-}393}$ protein, a small scale expression screening test was performed by using BL21 (DE3) *E.coli* carrying pDEST17-SBIP24$\Delta^{31\text{-}393}$ plasmid. The bacterial culture was induced with 1mM IPTG. Expression of recombinant protein was analyzed from the preinduction and the postinduction pellets containing pDEST17-SBIP24$\Delta^{31\text{-}393}$ construct. Fig. 29 confirms the expression of recombinant SBIP24$\Delta^{31\text{-}393}$ by showing a clear ~41KDa protein band in IPTG induced sample compared to the uninduced sample.

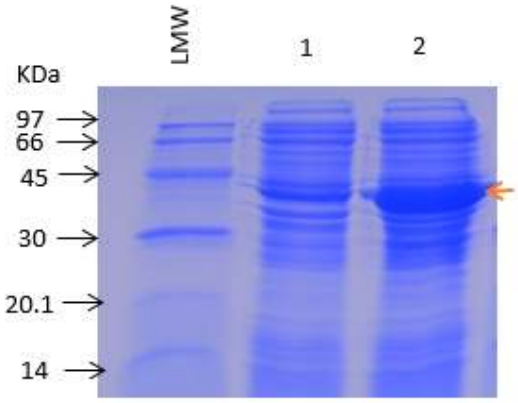

Figure 29: Coomassie-Stained 12% SDS PAGE of Recombinant SBIP24$\Delta^{31\text{-}393}$ Protein Expression. LMW= low molecular weight standard proteins; Lane 1, Un-induced protein; Lane 2, Induced protein. Arrow is indicates the expected SBIP24$\Delta^{31\text{-}393}$ protein.

Solubility test

After confirmation of protein expression, a solubility test was carried out. The collected protein was separated into insoluble and soluble fraction that were run in 12 % SDS PAGE gel and stained with coomassie blue. Fig. 30A shows that the majority of recombinant SBIP24 protein is expressing in insoluble form while trace amounts of protein may be in soluble form. Western blot analysis using anti-polyHistidine antibodies detected expression of recombinant protein in soluble form (Fig. 30B).

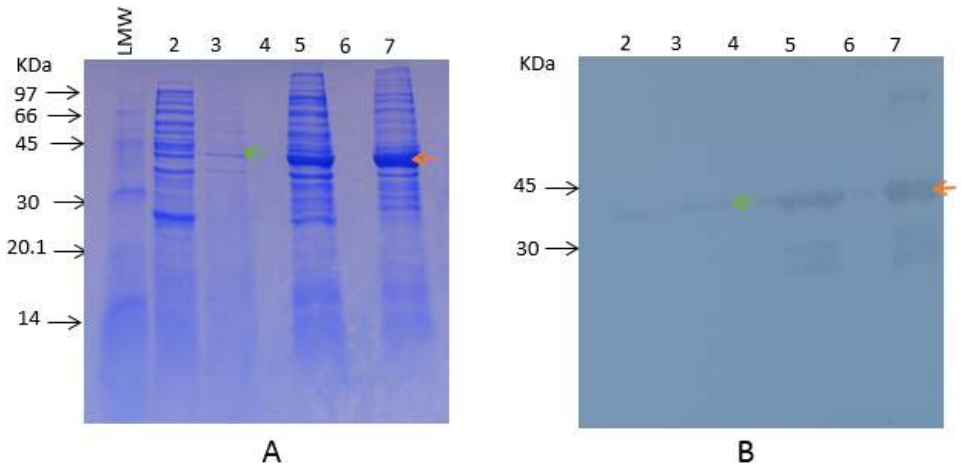

Figure 30: Coomassie Blue- Stained 12% SDS gel (A) and Corresponding Western Blot (B) Analysis Showing Soluble Expression of SBIP24Δ$^{31\text{-}393}$protein. Recombinant SBIP24Δ^{31} was detected using anti-polyHistidine antibody. Lane 1 low molecular weight, 2 and 3 shows the soluble recombinant SBIP24Δ$^{31\text{-}393}$ protein, lane 4 blank, lane 5 insoluble recombinant SBIP24Δ$^{31\text{-}393}$protein, lane 6 blank, Lane 7 insoluble recombinant SBIP24Δ$^{31\text{-}393}$protein. Lane #2, 3 in Fig 30B indicate presence of soluble recombinant SBIP24Δ$^{31\text{-}393}$.

Condition Optimization for Better Solubility. In order to obtain sufficient protein for enzyme analysis, various IPTG concentrations, induction temperatures, and duration of IPTG induction were used. Though soluble expression of recombinant is not prominent in the coommasie stained SDS page gel, Western blot analysis verifies soluble SBIP24Δ$^{31\text{-}393}$ recombinant protein (Fig. 31).

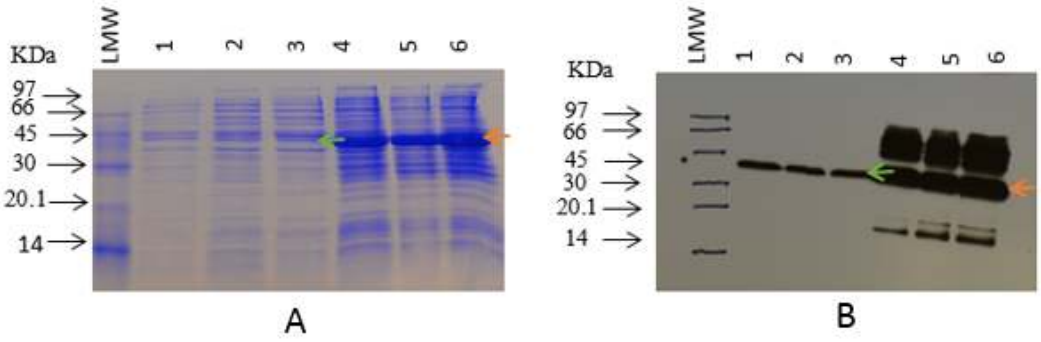

Figure 31: Coomassie Stained 12% SDS-PAGE Gel (A) and Corresponding Western blot (B) Analysis Showing the Soluble Protein Expression of Induced recombinant SBIP24Δ$^{31\text{-}393}$ Using Different IPTG Concentrations, Postinduction Time and Temperatures. Recombinant SBIP24Δ$^{31\text{-}393}$ was detected using anti-polyHistidine antibody. Low molecular weight marker; Lane 1, Soluble protein from 1mM IPTG induction for 5 hours at 20 ºC. Lane 2; Soluble protein by 0.1mM IPTG induction for 5 hours at 20º C.; Lane 3 Soluble protein by 0.1mM

IPTG induction for 3 hours at 37º C; Lane 4, Insoluble protein by 1mM IPTG induction for 5 hours at 20 ºC. Lane 5; Insoluble protein by 0.1mM IPTG induction for 5 hours at 20 ºC.; Lane6 Insoluble protein by 0.1mM IPTG induction for 3 hours at 37º C.

Purification of Recombinant SBIP24$\Delta^{31\text{-}393}$ by Nickel Affinity Chromatography

To purify the soluble recombinant SBIP24$\Delta^{31\text{-}393}$ protein Ni-NTA affinity chromatography was performed by adding Ni-NTA binding buffer (buffer containing 10 mM imidazole) prior to the elution of column with buffer containing 250 mM imidazole. SDS gel electrophoreses followed by coomassie blue staining were performed to analyze the elution profile of soluble recombinant SBIP24$\Delta^{31\text{-}39}$ protein. Fig. 31 shows majority of soluble recombinant SBIP24$\Delta^{31\text{-}393}$ was eluted in E2 through E6 fractions. Fig. 32 also shows a number of bands other than recombinant SBIP24$\Delta^{31\text{-}393}$ are present in purified protein samples**.** To verify the existence of soluble in purified protein samples, Western blot analysis was done using the crude and eluted proteins. Fig. 33 confirms the presence of recombinant SBIP24$\Delta^{31\text{-}393}$ protein in purified protein sample.

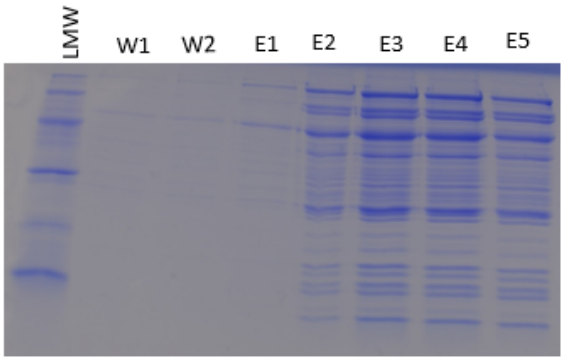

Figure 32: Ni-NTA Chromatography Results for Soluble Recombinant SBIP24$\Delta^{31\text{-}393}$. LMW = Low molecular weight, W1-W2= protein samples washed with Ni-NTA binding buffer, E1-E5= eluted protein samples from agarose beads using elusion buffer with 250mM Imidazole.

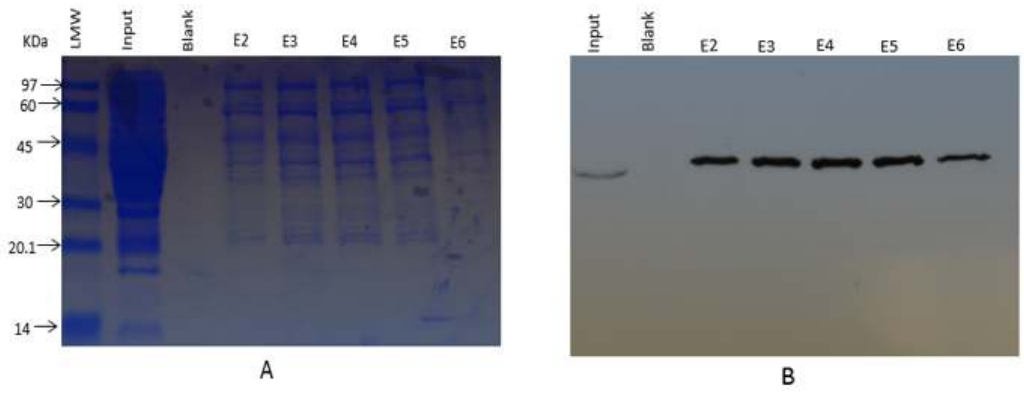

Figure 33: Coomassie Stained 12% SDS-PAGE Gel (A) and Corresponding Western blot (B) Analysis of Partially Purified Recombinant SBIP24$\Delta^{31\text{-}393}$. LMW= low molecular weight, Input= crude proteins, E2- E6= eluted protein samples.

Verification of pMDC123-SBIP24 Construct for Complementation of *Arabidopsis ssi2* Mutant Plant

To determine whether SBIP24 can complement for the loss of *Arabidopsis SSI2* gene in *ssi2* mutant plants, SBIP24 was cloned under the control of constitutive CaMV35S promoter in binary vector pMDC123. Amplification of SBIP24 (Fig. 34) from pMDC123-SBIP24 colony using SBIP24 forward and reverse primers confirms the insertion of SBIP24 into pMDC123 binary vector. However, further confirmation by restriction digestion and sequencing is required because both pDONR221 and pMDC123 have kanamycin resistance gene which makes it difficult to select recombinant clones based on antibiotic selection.

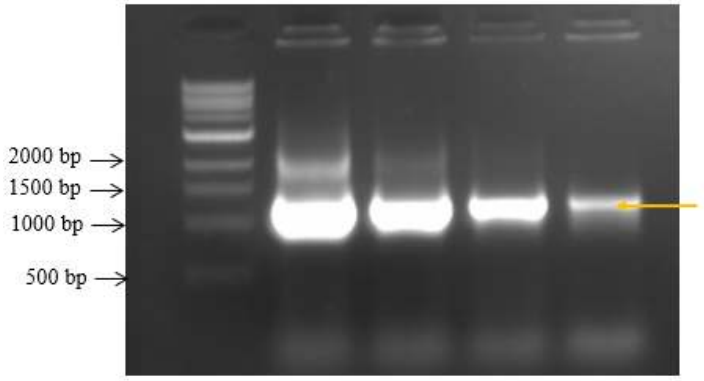

Figure 34: Image of a 0.8% Agarose Gel Electrophoresis Showing Amplification of SBIP24 Using the Recombinant Colony of pMDC123-SBIP24 as a Template.

Section II: Gene Expression of SABP2 and SBIP24 in Tobacco Transgenic Plant (hypothesis II)

To determine whether silencing of *SABP2* affects the mRNA expression of *SBIP24* in normal or infected (treated with TMV) tobacco plants, C3 and 1-2 tobacco plants have been infected with TMV (Fig. 35). RNAs were isolated from the leaf samples of infected plants at different time points after infection; RT-PCR was performed with the isolated RNAs using gene-specific primers. Tobacco *EF1-α* gene was used as a control; *PR1*, *SABP2* genes were amplified to validate the infection and silencing of *SABP2* respectively. Fig. 36 shows the expression of *SBIP24* was down-regulated at 72 hpi in C3 plants; this was not observed in 1-2 plant. These results need verification through another independent experiment.

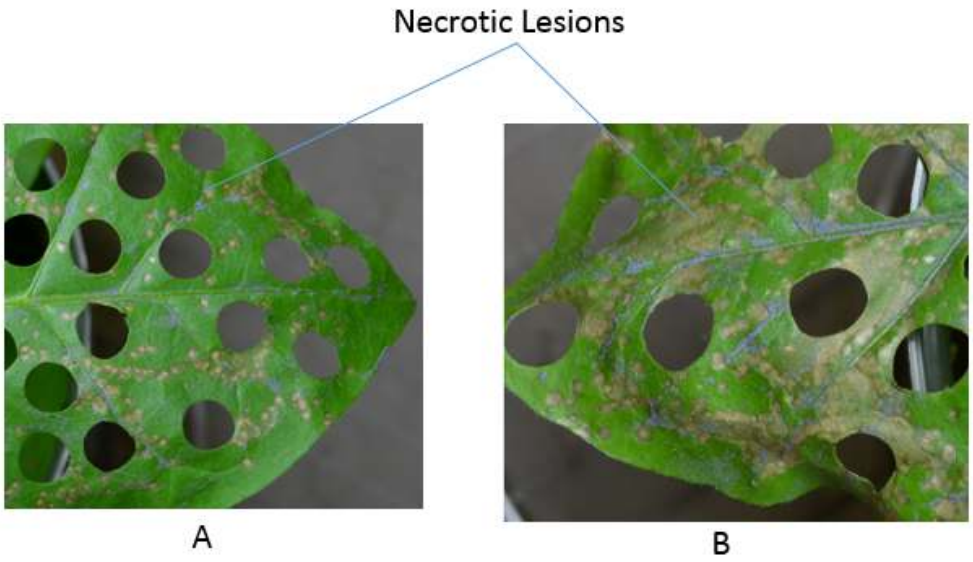

Figure 35: TMV Infection in C3 (A) and 1-2 (B) Tobacco Plants at 72 hpi. The size of necrotic lesion is bigger in 1-2 plant compared to C3 plant indicating C3 plant is more resistance than 1-2 *(SABP2* silenced*)* plant against TMV.

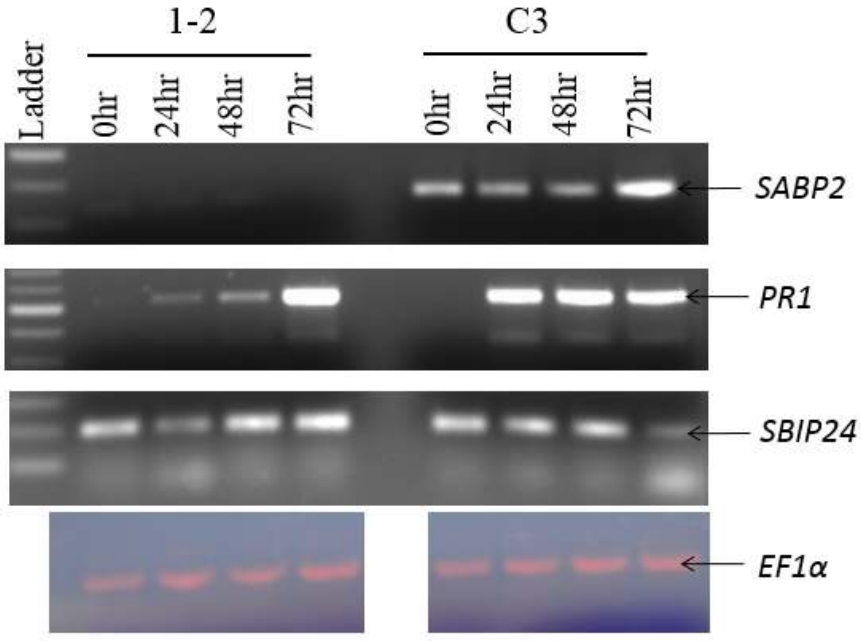

Figure 36: Expressions of *SBIP24* and *SABP2* in 1-2 and C3 Tobacco Plants upon TMV Infection. Semiquantitative RT PCR analysis of RNA extracted from TMV infected C3 and 1-2 tobacco leaves at 0, 24, 48, and 72hpi; *EF1-α* was used as control to normalize the amount of cDNA; *PR1* was used as confirmation of pathogen infection.

CHAPTER 4

DISCUSSION

The SA signaling pathway plays an extensive role in plant disease resistance. Identification of SABP2 the high affinity tobacco enzyme catalyzing conversion of MeSA to SA has opened a new area of research that could untangle many mysteries of the SA pathway (Kumar 2014). Recognition of SABP2 interacting proteins (SBIPs) and their functions are required for providing a better understanding of the SA pathway. So the approach of cloning, expressing, and analyzing functions of SBIPs from tobacco plant should hold significant biological potential to unravel the unknown molecular mechanism of the SA pathway. To elucidate the mechanism of SABP2 mediated SA pathway in plant disease resistance, this project started off with the aim of cloning, expressing, purifying, and bio-characterizing one of the SABP2 interacting proteins, SBIP24.

The full-length sequence of SBIP24 posted in the SGN (tobacco unigene sequence database), indicates it is likely codes for a stearoyl CoA ACP desaturase. Due to a missing nucleotide in the SGN database sequence, the corresponding ORF is only 861 nucleotides long (Fig. 4). However, multiple amino acid sequences as well as nucleotide sequence alignments with other documented stearoyl ACP desaturases (Fig. 6 and 7) suggests SBIP24 should have approximately 1182 nucleotides (coding for 393 amino acids) (Fig. 7 and 8). Bioinformatics analysis also revealed that SBIP24 is a prospective stearoyl-acyl-carrier and it shares the highest (93%) identity with *Solanum acaule* stearoyl-acyl desaturase mRNA, complete DNA sequence (JX412962) (Zhang et al. 2000; Morgulis et al. 2008). Protein BLAST analysis of SBIP24 with nonredundant protein sequences indicates the encoded protein sequence of SBIP24 shows the highest 95% identities with stearoyl-acyl desaturase protein of *Solanum lycopersicum* (XP 004234817.1), *Solanum acaule* (AFS68797.1)

followed by *Solanum commersonii* (Q41319.2), *Solanum cardiophyllum* (AFS 68798.1) and *Solanum tuberosum* (XP 006352145.1) (Fig 8) (Altschul et al. 2005). According to posted sequences of *Arabidopsis* stearoyl ACP desaturases (Kachroo et al. 2007) and the ChloroP 1.1 prediction server, SBIP24 is potentially localized in chloroplast (Fig. 10 and 11). This strengthens our notion that SBIP24 and SABP2 could have possible interaction because recent study demonstrates that SABP2 is likely localized in the chloroplast (Fai and Kumar, unpublished).

Along with modulating saturated and mono-unsaturated fatty acid biosynthesis in plant cells, stearoyl-CoA- desaturase is known for its association with plant defense responses (Kachroo and Kachroo 2009; Savchenko et al. 2010). How it is regulating the defense signal in plants is still not clear. Although, *ssi2 Arabidopsis* mutant plants (lacking in stearoyl ACP desaturase gene) accumulates high level of SA and exhibit enhanced resistance to biotrophic pathogens, studies suggests that the *SSI2*-generated signal affects defense signaling in either a SA-dependent, NPR1-independent defense pathway, or an SA and NPR1-independent defense pathway (Shah et al. 2001). On the other hand, the *ssi2* mutant shows increased susceptibility to necrotrophic pathogens suggesting the role of SSI2 in crosstalk between SA and JA mediated pathways. More interestingly, recently azelaic acid that is derived from oleic acid has been shown to prime SA biosynthesis. The finding of the study that azelaic acid is unable to induce resistance in SAR defective SA pathway mutants (Jung et al. 2009) further substantiates that the stearoyl ACP desaturase plays a complex but a very critical role in SA mediated defense responses in plant. So, the characterization of tobacco SBIP24 and its relation to SABP2 are essential for revealing a molecular mechanism underlying the complex network of plant defense resistance.

The SBIP24 gene isolated from tobacco cDNA was successfully cloned (Fig. 19) into pDEST17 expression vector using gateway cloning technology. Even though the recombinant

SBIP24 in pDEST17 was expressed successfully in *E. coli* (Fig. 20), it was mostly in insoluble form (Fig. 21). As a prokaryotic expression system, *E.coli* possesses some disadvantages and limitations. Over-expression of recombinant protein in *E.coli* induces stress response that leads it to accumulate the recombinant proteins in insoluble form as inclusion bodies (Sorensen and Mortenson 2005; Terpe 2006; Sahdev et al. 2008). Also, recombinant proteins with their natural N-terminus signal peptide could be targeted to the periplasmic space while expressing in *E. coli* (Luo et al. 2009; Singh et al. 2013). Besides the disadvantages, *E. coli* expression systems also offer some advantages like rapid expression of target protein feasible purification strategies. These advantages have made it ideal system for expression of target proteins (Sorensen and Mortenson 2005; Terpe 2006; Sahdev et al. 2008). As most of the SBIP24 was expressing in insoluble forms, several efforts including the optimization of IPTG concentration, induction temperature, and induction time were made to enhance the solubility of SBIP24 in *E coli* cell. Failure of all endeavors encouraged us to clone SBIP24 into *E. coli* without a signal peptide. In pursuance of expressing SBIP24 in soluble form, 30 N-terminal amino acids predicted by chloroP software as a signal peptide from full length SBIP24, were removed by cloning using RT-PCR. Finally SBIP24 without a signal peptide **(**SBIP24$\Delta^{31\text{-}39}$**)** was cloned (Fig. 28) and recombinant protein expressed (Fig. 29) in soluble form in *E.coli* (Fig. 31). Also SBIP24$\Delta^{31\text{-}39}$ was partially purified using Ni-NTA affinity chromatography (Fig. 32 and 33).

The principal goal of this project was to characterize SBIP24 as a stearoyl-CoA-desaturase protein. Because of time limitation and unexpected negative results, this project requires more time to reach its goal. But the cloning as well as the expression of SBIP24 as a soluble protein in *E.coli* and the construction of a binary vector with SBIP24 (Fig. 34) (needs to be verified) for complementation assay will surely provide the foundation to take the project forward.

Along with the cloning and expression of SBIP24, an attempt was made to determine if *SABP2* affected the expression of *SBIP24*. Accordingly, semiquantitative RT-PCR was performed to determine the expression of *SBIP24* in 1-2 (*SABP2* silenced) and C3 (containing the empty silencing vector) transgenic tobacco plants infected with TMV. Results suggest that *SABP2* may affect *SBIP24* expression as seen by the down regulation of *SBIP24* expression at 72 hpi in a C3 (control) tobacco plant compared to 1-2 (*SABP2* silenced) tobacco plant (Fig. 36). Interestingly, the expression of *SABP2* is highest at 72 hpi in C3 plant. It is also possible that *SBIP24* is affecting the expression of *SABP2*. This result needs further verification through independent experiment. Also, inclusion of mock inoculated samples in the experimental design will present a clearer picture of expression levels of *SBIP24*. On the other hand, whether *SBIP24* regulates the expression of *SABP2* or not also needs to be analyzed. So, further investigations are required to reach a conclusion about the relation of *SABP2* and *SBIP24* mRNA expression.

Future Directions

To reach to the principal goal of the characterization of SBIP24, the first step of cloning and expression of SBIP24 into *E.coli* has been completed. Future experiments should be the further purification and testing the desaturase activity of SBIP24. The desaturase activity of SBIP24 could be tested invivo as well as invitro. Mature SBIP24 protein (SBIP24$\Delta^{31\text{-}393}$) would be used for examining the desaturase activity of SBIP24 invitro where the recombinant pDEST17- SBIP24 bacteria (with signal peptide) would be used for measuring desaturase enzymatic activity in vivo by providing the substrate, stearic acid (18:0), in the culture medium. Because *E. coli* does not have a stearoyl CoA desaturase gene, the difference between the 18:0-18:1 content of wild type bacteria and recombinant bacteria (expressing SBIP24) would indicate whether SBIP24 is a stearoyl CoA desaturase or not. Another possible approach to test the hypothesis that SBIP24 is a stearoy-CoA desaturase enzyme is through the complementation of *Arabidopsis ssi2* mutant plants (stunted in growth compare to wild type plant) with tobacco SBIP24. SBIP24 has been already cloned into a gateway binary vector pMDC 123 (needs verification). The recombinant pMDC123-SBIP24 could be used to transform *Arabidopsis ssi2* mutant plant by floral dip method (Clough and Bent 1998). In addition to all these experiments, the physical interaction between SBIP-24 and SABP2 should be independently validated by performing pull down assays using recombinant SABP2 and SBIP24 (Einarson et al. 2007).

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APPENDICES

Appendix A – Abbreviations

SABP2 - Salicylic acid binding protein 2

SBIP24 - SABP2 Interacting Protein-24

SBIP24 $\Delta^{31\text{-}393}$ – Mature SBIP24 (Without the signal peptide)

SACPD - Stearoyl-ACP Desaturase

C3 - Control plants (*Nicotiana tabacum* cv Xanthi nc, containing empty silencing vector)

NahG - Plants expressing salicylate hydroxylase which converts SA to catechol.

1-2 - SABP2 - silenced plants (transgenic *N.t*. cv Xanthi nc in which *SABP2* gene expression is silenced by RNA interference).

PRRs - Pattern recognition receptors

PAMPs - Pathogen-associated molecular patterns

R protein - Resistance protein

Avr - Avirulence

ICS 1 - Isochorismate synthase 1

BA2H - Benzoic-2-hydroxylase

HR - Hypersensitive response

SA - Salicylic acid

JA - Jasmonic acid

ET - Ethylene

ISR - Induced systemic resistance

SAR - Systemic acquired resistance

SAMT - Salicylic acid methyl transferase

MeSA - Methyl salicylate

MUFA- Mono unsaturated fatty acid

SCD- Stearoyl CoA desaturase

SSI2- Suppressor of salicylic acid insensitive 2

SDS PAGE - Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

TMV - Tobacco mosaic virus

PR - Pathogenesis-related

BTH - Benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid S-methyl ester

NPR1 - Non-expresser of pathogenesis-related protein 1

IPL - Isopyruvate lyase

βME - βeta mercaptoethanol

EFalpha1 - Elongation Factor alpha 1

PAD4 - Phytoalexin Deficient 4

R-genes - Resistance genes

TAE - Tris-Acetate EDTA

KDa - Kilo Dalton

OD - Optical Density

UV - Ultra violet

µg - micro gram

µl - micro litre

ml - milli litre

mM - milli Molar

Appendix B – Buffers and Reagents

10x Phosphate Buffer Saline (10x PBS)

Sodium Chloride (76g), M.W. = 58.44g/mol, final concentration = 1.3M

Sodium Phosphate dibasic (10g), M.W. = 141.96g/mol, final concentration = 70mM

Sodium Phosphate monobasic (4.1g), M.W. = 119.96g/mol, final concentration = 30mM

For 1x PBS (1 L), 100mL of 10x PBS was diluted in 900mL of water.

For 1x PBS (1 L) with 3% Tween 20, dilute 100mL of 10x PBS in 870mL, then add 30mL of tween 20.

Western Blotting Blocking Buffer (100mL)

1x PBS buffer, 100mL

Dry Milk (1g), final concentration = 1%

BSA (3g), final concentration = 3%

4x SDS-PAGE Separating gel buffer (500mL)

Tris base (90.85g), M.W. = 121.1g/mol, final concentration = 1.5M

Adjust pH to 8.8

Add SDS (0.2g), final concentration = 0.04%

4x SDS-PAGE Stacking gel buffer (500mL)

Tris base (30.28), M.W. = 121.1g/mol, final concentration = 0.5M

Adjust pH to 6.8

Add SDS (0.2g), final concentration = 0.04%

10x SDS-PAGE Running Buffer (1 L)

Tris base (30g), M.W. = 121.1g/mol

Glycine (144g), M.W. 75.07g/mol

SDS (10g)

10x Western Blotting Transfer Buffer (1L)

Tris base (30.3g), M.W. = 121.1g/mol, final concentration = 125mM

Glycine (72.06g), M.W. = 75.07g/mol, final concentration 960mM

For western, 1x transfer buffer is prepared by mixing 100mL of 10x transfer buffer, 100mL of 100% methanol, and 800mL of cold water.

2x SDS-PAGE Loading Dye (100mL)

1M Tris-Cl, pH 6.8 (10mL), final concentration = 100mM

SDS (0.4g), final concentration = 0.4%

Glycerol (20mL), final concentration, 20%

Bromophenol blue (0.2g), final concentration = 0.2%, 5mL of β-mercaptoethanol (βME) was added before use.

Ponceau S Stain (100mL)

Ponceau S (0.1g), final concentration = 0.1%

Acetic acid = 5 ml (Final conc. = 5%)

0.1% Diethylpyrocarbonate (DEPC) Treated Water

Diethyl pyrocarbonate = 0.1 ml

Distilled water = 100 ml

Incubated for ~12 hours at 37^0C, Autoclaved for 15 minutes

50X Tris Acetate EDTA Buffer

121.0 g Tris base

28.55mL glacial acetic acid

50.0mL 0.5 M EDTA (pH 8.0)

Distilled water was added to bring to 500mL volume

1.2% Agarose gel

0.60 g agarose

50ml distilled water

2.5µl ethidium bromide (10 mg/mL)

100mM Phosphate (sodium) buffer

39ml Stock solution A (27.6g/L monobasic Na-phosphate)

61ml Stock solution B (28.4g/L dibasic Na-phosphate)

Diluting to total volume 200ml (pH 7)

50mM was made by diluting 50ml of 100mM phosphate buffer in 100ml distilled water

1xNi-NTA binding Buffer

6.896 g NaH_2PO_4 (Monobasic)

17.53 g NaCl

0.6808 g Imidazole (pH 8.0)

Destaining solution for coomassie brilliant blue

500 ml distilled water

400 ml methanol

100 ml acetic acid

Coomassie Brilliant Blue staining solution

500ml Methanol

100 ml acetic acid

400 ml H_2O

1 g of Coomassie Brilliant Blue was dissolved in 1 liter of the solution

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