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Identification, Cloning, and Expression of Tobacco Responsive to Dehydration like Protein (RD22), SBIP-355 and Its Role in SABP2 Mediated SA Pathway in Plant Defense

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by

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ABSTRACT

Identification, Cloning, and Expression of Tobacco Responsive to Dehydration like Protein (RD22), SBIP-355 and Its Role in SABP2 Mediated SA Pathway in Plant Defense

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Abscisic acid (ABA) induces *RD22*, responsive to dehydration stress gene. Salicylic acid (SA) has been the focus of research due to its role against pathogens and abiotic stress. Interaction between ABA and SA signaling pathways is still poorly understood. SA-Binding Protein 2 (SABP2) converts methyl salicylate to SA. An attempt was made to identify proteins that interact with SABP2 using a yeast 2-hybrid screening. Several interactors were identified. One of them, SA-Binding Protein 2 Interacting Protein-355 (SBIP-355), showed high homology to *RD22*. Bioinformatic approaches showed that SBIP-355 contains a BURP domain. Phylogenetic analysis reveals that SBIP-355 clustered into the clade of RD22-like proteins. Thus, SBIP-355 gene might be a stress-inducible gene and encodes a dehydration-responsive protein, which is important for the stress tolerance of tobacco. The complementary DNA (cDNA) of tobacco SBIP-355 was cloned into pDEST-17 vector and then expressed in *E. coli* to detect the expression of SBIP-355 protein.

DEDICATION

I dedicate this thesis to my exceptional parents, who never stop giving of themselves in countless ways. Your constant love, prayers, trust, and guidance have given me the strength and patience to surpass all the obstacles.

I dedicate this thesis to my homeland, Saudi Arabia, the warmest womb.

I dedicate this thesis to my aunt and the closest friend during this journey, Ghada. You led me through the valley of darkness with light of hope and support.

I dedicate this thesis to the memory of Dr. Ghazi Algosaibi, who always inspires me to believe that all dreams are possible with determination.

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I'm always in debt to my parents for their constant believing and trusting my inner determination and for their honest prayers and blessings. I can't thank my lab members enough for their friendship and the moments we shared that created a nice working environment. I'm glad for this unique chance that let me meet friends from different backgrounds. I wish them all the best in their future personally and professionally.

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CHAPTER 1

INTRODUCTION

Crop plants regularly encounter harsh environmental abiotic stresses such as higher concentration of salt (salinity), water shortage (drought or dehydration), high wind, and extremes of temperatures such as cold or heat and humidity. These conditions have significant impact on growth, productivity, distribution, and development of plants. In fact, these stresses threaten the agricultural industry globally and cause losses worth millions of dollars annually due to damage of crops. Among various abiotic stresses, salinity and drought are the most intense environmental stresses. The latest report of the food and agriculture organization of the United Nations (FAO) demonstrates that crop production failure in 2013 ranges between 7 percent to 28 percent of the total production as a result of water deficit stresses, and it indicates that 36 countries around the world are in need of external assistance for food because of crop failures (FAO 2013). These costly impacts come in different forms such as economic, environmental, and social. Economically, it causes a decline in crop yield and consequent decline in agriculture-dependent industries such as textile, drugs, dairy and meat production. Subsequently, it leads to reduction in income for farmers and for a prolonged period, increase rate of unemployment in agriculture field and agriculture-dependent industries, and thus an increase in the market price of products. Environmentally, this reduction in plant growth results in a negative impact on animals in many different ways including shrinking their food supply, destruction of their habitat, and migration or decline in wildlife populations. Finally, the decline of agriculture can bring serious social consequences in people's health and safety such as anxiety or depression about economic losses, reduced quality of life, and increased poverty and social instability.

The main consequence of drought and salt stress is decreased growth and development of plants caused by a significant inhibition of photosynthesis, the process in which plants combine water, carbon dioxide, and light to make carbohydrates for energy. Reductions in photosynthetic components such as water impact plant growth negatively. Therefore, plants react by rapid closure of stomata to avoid further water loss via transpiration (Cornic 1994). Transpiration rate is regulated by Abscisic acid (ABA), a plant hormone known to reduce transpiration by inducing stomatal closure and greatly increase stomatal sensitivity to elevated CO_2 concentrations (Zhang et al. 1987). This enhances the protection of plants from damages induced by drought, salinity, and pathogenic attack.

Abscisic Acid

ABA is a plant stress phytohormone responsible for regulating many physiological events and supporting plants survival under many abiotic stress responses. Significant levels of ABA accumulate in response to drought and salt stresses and degrade after the release of stresses (Zhang et al. 2006). Studies have shown that plant adaption to abiotic stress increases by exogenous application of ABA (Bartels et al. 1990). Investigating the role of ABA in stress tolerance was carried out using mutants (Ingram and Bartels 1996). These mutants are *aba* (ABA deficient) mutations that affect synthesis of ABA, resulting in lower ABA levels; and *abi* (ABA-insensitive) mutations which impair ABA sensitivity (Rock and Zeevaart 1991). Studies with *aba* and *abi* helped to demonstrate that ABA plays a critical role in plant adaptation to stresses. For example, it was found that ABA induces closure of stomata by using an ABA-deficient tomato mutant, *flacca*. It was found to be unable to close stomata during drought stress and had

shown much less ABA content than wild-type tomatoes (Neill et al. 1986). On the other hand, The ABA's role in regulating plant adaption against stress is not well understood. In general, ABA is stimulated by stress to act via differential signal pathways, and then it expresses a number of genes that may regulate stress tolerance (Swamy and Smith 1999).

ABA Signaling Pathways

Genetic analysis of the ABA-deficient *Arabidopsis* mutants *los5/aba3* and *los6/aba1* showed that ABA plays critical role in the expression of stress-inducible genes. The expression of stress-responsive genes such as *RD29A*, *RD22*, *COR15A*, and *COR47* was severely reduced or completely blocked in the *los5* mutant (Xiong et al. 2001), while in *los6* the expression of *RD29A*, *RD19*, *COR15A*, *COR47*, *KIN1*, and *ADH* was lower than in wild-type plants (Xiong et al. 2002). Therefore, although ABA-independent signaling pathways may exist, an ABA-dependent signaling pathway plays an essential role in stress-responsive gene expression during osmotic stress (Fig.1) (Xiong et al. 2001).

Microarray analysis using *Arabidopsis thaliana* as a model plant showed that many drought-inducible genes were expressed and their mRNA levels are subsequently reduced by releasing the water deficit stress conditions. These genes include *RD* (responsive to dehydration), *ERD* (early responsive to dehydration) and *COR* (cold-regulated), *LTI* (low-temperature induced) and *KIN* (cold-inducible) (Shinozaki and Yamaguchi-Shinozaki 2000). It was suggested that this variety of stress-inducible genes gives a vision of how the responses of plants to dehydration and cold are complex and complicated. In addition, it was found that some of the stress-inducible genes are overexpressed in transgenic plants that exhibit enhanced stress tolerance, suggesting that their gene products may function in protecting cells from stress (Xiong et al. 2001).

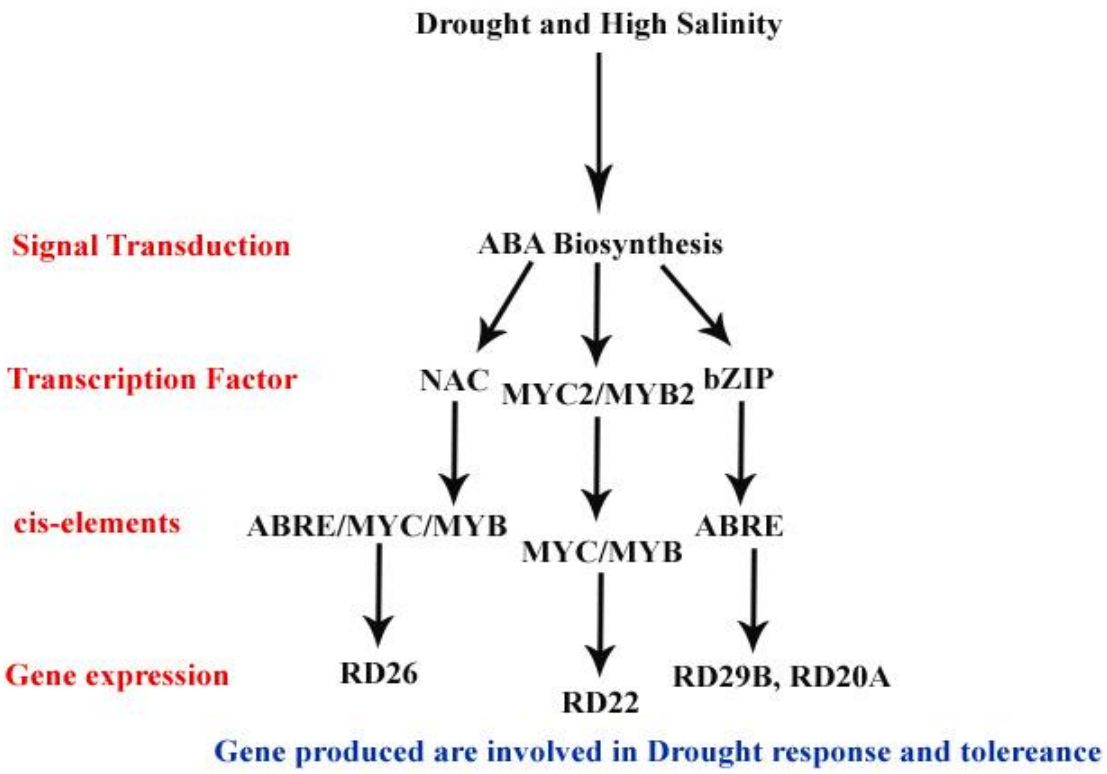

Figure 1: A Simplified Sketch of the Main Steps of ABA-Dependent Signaling Pathway of Dehydration Responsive Gene Expression. (NAC: (NAM-ATAF1,2-CUC2) transcription factor, MYC/MYB: MYC and MYB transcriptions factor families, *bZIP*: basic -leucine zipper transcription factors, *ABRE*; ABA-responsive elements ; and *RD26,RD22,RD29B,RD20A*: responsive to dehydration genes. (Figure adapted from Yamaguchi-Shinozaki and Shinozaki 2006).

Interaction Between Plant Hormones Signaling Pathways

The perception of drought stress triggers synthesis of ABA, which is considered the major regulatory hormone that activates transduction cascades during water deficit stress and leads to the activation of a large suite of stress-responsive genes and results in stomatal closure (Wilkinson and Davies 2010). It was found that these responses directly affect the biosynthesis or action of other hormones including salicylic acid (SA), ethylene (ET), jasmonic acid (JA), and cytokinins. These hormones can have either synergistic or antagonistic interactions with ABA,

leading to positive and negative functional results (Peleg and Blumwald 2011). Recent studies suggest that ABA has an important role in plant–pathogen defense signaling. ABA deficiency results in weaken plant defenses and causes enhanced pathogen susceptibility (Asselbergh et al. 2008; Cao et al. 2011). Also, ABA promotes stomata closure to inhibit the entry of pathogen. Along the same line, abiotic stress generally makes plants more sensitive to subsequent pathogen attack (Moeder and Yoshioka 2009). However, the mechanisms of involvement of ABA in plant–pathogen interactions are still ambiguous and need to be more investigated (Yasuda et al. 2008). Resistance response to many pathogens involves SA signaling pathway. The role of SA in the plant–pathogen resistance has been extensively investigated and widely documented. Recent valuable reports have found that SA also plays an important role in the response to abiotic stresses including drought and salt stress, and these reports suggested that SA has great agronomic potential to improve the stress tolerance of agriculturally important crops (Rivas-San Vicente and Plasencia 2011; Miura and Tada. 2014).

Salicylic Acid and Defense Signaling

SA had taken its name after its discovery as a major component in the extracts from willow (*Salix*) tree bark that had been used as a natural anti-inflammatory drug (*Aspirin*). SA has become the focus of plant research after exploring how it serves as a critical signal for activating disease resistance after pathogen attack in various plant species. This was first discovered in 1979, when tobacco plants were sprayed with aspirin that produced enhanced resistance to subsequent infection by tobacco mosaic virus (TMV) (White 1979). In addition, SA also plays an important role in other important functions in plants such as growth and development, stomatal

closure, seed germination, cell growth, respiration, and thermogenesis (Vlot et al. 2009; Rivas-San Vicente and Plasencia 2011).

Biosynthesis of SA

Biosynthesis of SA in plants has been under investigation for almost half a century (Chen et al. 2009). SA derivation occurs through the shikimate pathway through 2 different routes: the isochorismate synthase (ICS) catalyzed pathway and the phenylalanine ammonia-lyase (PAL) catalyzed pathway (Fig.2). Both of these pathways originate from chorismate, the end product of the shikimate pathway (Chen et al. 2009). In the ICS1 catalyzed pathway, chorismate is converted to isochorismate via isochorismate synthase (ICS), and then isochorismate is likely converted to SA via isochorismate pyruvate lyase (IPL). However, in the PAL catalyzed pathway, phenylalanine is converted to cinnamic acid via phenylalanine ammonia lyase (PAL), cinnamic acid is then converted to either coumaric acid to SA or benzoic acid (benzoate intermediates) to SA via benzoic acid-2- hydroxylase (BA2H) (Chen et al. 2009). To date the enzymes which required for the conversion of SA from cinnamate in the PAL pathway in plants have not been known (Chen et al. 2009).

Local and systemic acquired resistance (LAR and SAR) in plants require SA biosynthesis (Wildermuth et al. 2001). Mobility of SA is required for SAR to reach other parts of the plant for further signal transduction and to establish resistance. To achieve that, SA is first converted into methyl salicylate (MeSA) by SA-methyl transferase (SAMT) (Chen et al. 2009). MeSA is a volatile ester and critical phloem-mobile SAR signal that is dramatically increased upon pathogen infection (Kumar and Klessig 2003; Forouhar et al. 2005). Once MeSA has reached its

target tissue, it is converted back to SA by the esterase activity of SA binding protein 2 (SABP2) (Forouhar et al. 2005).

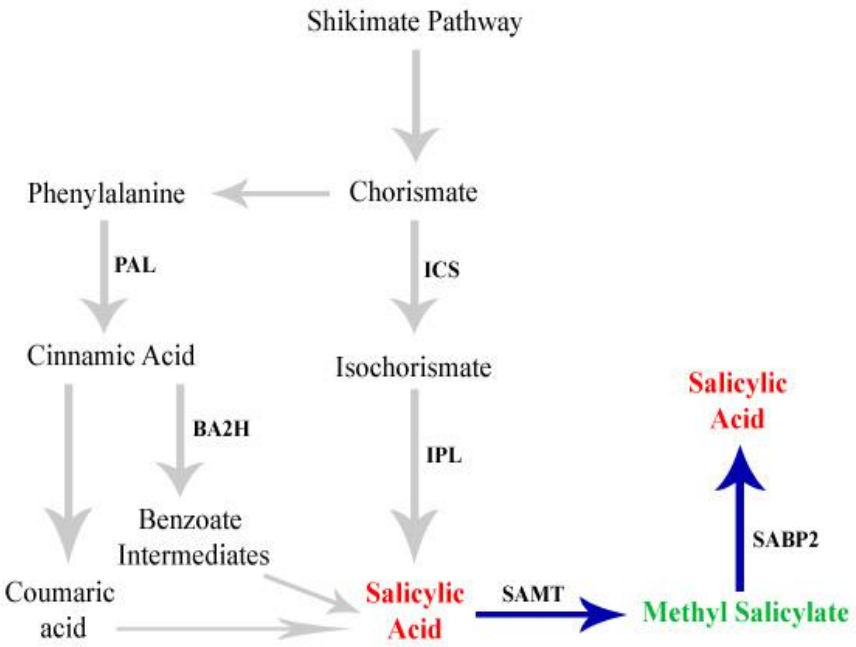

Figure 2: A Simplified Sketch of Pathways of SA biosynthesis in Plants. SA is synthesized and converted to mobile methyl salicylate (MeSA) by salicylic acid methyltransferase (SAMT) and then, MeSA is converted back to SA by SABP2. (Figure adapted from Chen et al. 2009).

Role of SA in LAR and SAR

Plants infected by a pathogen (this refers not only to microbial pathogens but any type of invasion such as nematodes, insects, or herbivores, as well as treatment with certain chemicals, or other types of stress) respond by expressing a set of defense-related genes, such as the genes encoding pathogenesis-related proteins (PR proteins) (Uknes et al. 1992; Sticher et al. 1997). It was found that these genes are expressed in both local infected and systemic noninfected leaves (Uknes et al. 1992). Plants containing a specific R gene are able to recognize a pathogen that

carries a corresponding avirulence (avr) gene that leads to localized cell death called the hyper-sensitive (HR) response. HR limits the spread of the pathogen (Glazebrook 1999; Rairdan and Delaney 2002). In the SAR pathway infection by a pathogen causes the host cell to trigger a signal to be spread throughout the plant; this signal triggers the activation of defense genes in uninfected tissues and results in showing enhanced resistance to future pathogen attack (Tiryaki and Tunaz 2004).

SA plays an indispensable role in activating SAR (Gaffney et al. 1993; Delaney et al. 1994). These findings come from experiments using plants transformed with bacterial *nahG* gene, which encodes the enzyme salicylate hydroxylase that converts SA to an inactive catechol (Gaffney et al. 1993; Tiryaki and Tunaz 2004). These experiments observed that plants expressing salicylate hydroxylase were unable to accumulate SA after pathogen attack and lacked the ability to activate *PR* genes, which are associated with the onset of SAR (Gaffney et al. 1993). Other findings support the notion that SA plays a role in the SAR pathway. It was demonstrated that the treatment of plants with SA leads to induction of SAR (White 1979; Metraux et al. 1991; Gaffney et al. 1993; Gorlach et al. 1996); whereas, the absence of SA in the plants made them unable to establish SAR (Gaffney et al. 1993; Delaney et al. 1994).

Hara et al. (2011) reported drought induces expression of *PR1* and *PR2*, which are known SA-inducible genes. This findings demonstrates and supports that SA may have a role in drought tolerance and may lead to the induction of the protective mechanism.

SA-Binding Proteins (SABPs)

With a view to help elucidating SA role in disease resistance and clarify SA defense response mechanisms in plants, extensive studies have been carried out to investigate the

proteins that bind to SA. Several putative effector proteins have been identified in *Nicotiana tabacum*. The first, termed SA-binding protein (SABP), has a native molecular mass of 240 kDa. Further analysis of SABP revealed that it is catalase and its properties suggest that it may play a role in transmitting the SA signal during plant defense responses (Chen et al. 1993). SA levels in cells increase upon pathogen infection; therefore, they inhibit the H_2O_2 degrading activity of SABP because of binding to SA. Consequently, H_2O_2 accumulates in the plant cells and activates the hypersensitive response and, thus, leads to apoptotic cell death to limit pathogen growth (Conrath et al. 1995; Chen et al. 2003). The second salicylic acid-binding protein identified from tobacco was SABP2, which is a 29 kDa protein that displays high affinity for SA and catalyzes the conversion of inactive methyl salicylate (MeSA) into SA to induce SAR (Kumar and Klessig 2003; Forouhar et al. 2005). It also has been found that SABP2 plays an important role in LR and SAR following TMV infection (Kumar and Klessig 2003). Third SA-binding protein identified from tobacco was SABP3, which is chloroplast carbonic anhydrase (CA) (Wang et al. 2009). It was found that SABP3 has an antioxidant activity and is required for the induction of resistance in the plant against pathogen infection (Slaymaker et al. 2002).

Salicylic Acid Binding Protein2 (SABP2)

SABP2 is a 29-kDa tobacco soluble protein that belongs to α/β fold hydrolase super family. It is present in low abundance (10 fmol/mg) and binds SA with high affinity Kd = 90 nM). This binding is reversible and plays a crucial role in SA defense pathway via synthesis of SA from the biologically inactive form of MeSA (Kumar and Klessig 2003). Cloning of SABP2 encoding gene, purification, and characterization of its protein demonstrated that SABP2 is a

SA-stimulated lipase/esterase activity SABP2-silenced plants are defective in developing SAR (Kumar and Klessig 2008). It was recently reported that SABP2 is required for acibenzolar S-methyl (ASM), a synthetic analog of SA mediated activation of SAR in tobacco (Tripathi et al. 2010)

SABP2-Interacting Proteins (SBIPs)

The identification and characterization of SABP2 has opened the door to perform more biochemical and genetic analysis to identify and characterize more components and molecular mechanisms that might be involved in SABP2 mediated SA pathway signal transduction. In the light of this, an attempt was made to expand analysis of putative cellular proteins that interact with SABP2 and might play an important role directly or indirectly in SA pathway using yeast 2-hybrid screening (Y2H), which is an *in vivo* tool used for the identifying protein-protein interactions between two proteins called "bait" and "prey". Bait is the protein of interest and preys are their interaction partners (preys) (Brückner et al. 2009). In this screening SABP2 was used as bait (Fused to Gal4 binding domain) and tobacco leaf proteins as prey proteins (expressed as a fusion partner to Gal4 activation domain) (Fig.3). When a protein interacts with SABP2, the activating domain comes close to the binding domain and a transcription factor is activated upstream of the reporter gene allowing the yeast to grow on a selective medium, changes color to blue, and exhibits antibiotic resistance. Several interactors of SABP2 were identified and named as SABP2-Interacting Proteins (SBIPs), and each interactor was numbered by the number of the clone. One among these interacting proteins is SBIP-355. Bioinformatics

analysis shows that SBIP-355 has high homology to "Responsive to Dehydration 22" *(*RD22)-like protein.

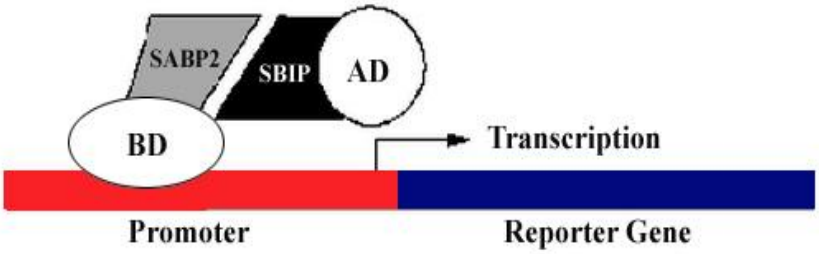

Figure 3: Yeast 2-Hybrid System. SABP2 and SBIP are 2 interacting proteins; BD and AD are Protein-binding domain and transcription activator domain, respectively.

Responsive to Dehydration-22 *(*RD22)-like Protein

RD22 gene is a responsive to dehydration gene of *Arabidopsis* and encodes a protein of unknown function (Yamaguchi-Shinozaki and Shinozaki 1993; Xiong et al. 2001). It is one of the drought-induced genes whose expression is mediated by ABA and requires de novo protein biosynthesis (Yamaguchi-Shinozaki and Shinozaki 1993). The promoter region of the *RD22* gene was analyzed in transgenic tobacco plants containing promoter-*p-glucuronidase* (GUS) fusion genes, and it was demonstrated that a 67-bp DNA fragment of the *RD22* promoter contains *cis*-acting elements involved in dehydration and ABA-responsive gene expression (Iwasaki et al. 1995). ABA activates *RD22* expression by activating the coding regions *RD22BP1* or *AtMYC2* of the transcription factor MYC and *AtMYB2* of MYB, which bind to *cis*-elements promoter region of *RD22*, and then they induces the expression of the *RD22* gene (Fig.4) (Abe et al. 1997). Because MYC and MYB proteins synthesized after accumulation of ABA and RD22 activation requires de novo protein biosynthesis, observation indicates that MYC and MYB transactivate the *rd22* gene under dehydration conditions (Abe et al. 1997).

RD22 has often been used as a marker gene for drought stress treatment in different plants. Reports have revealed that it is expressed only under water deficit conditions and ABA treatment, but not by cold or heat stress treatments (Yamaguchi-Shinozaki and Shinozaki 1993).

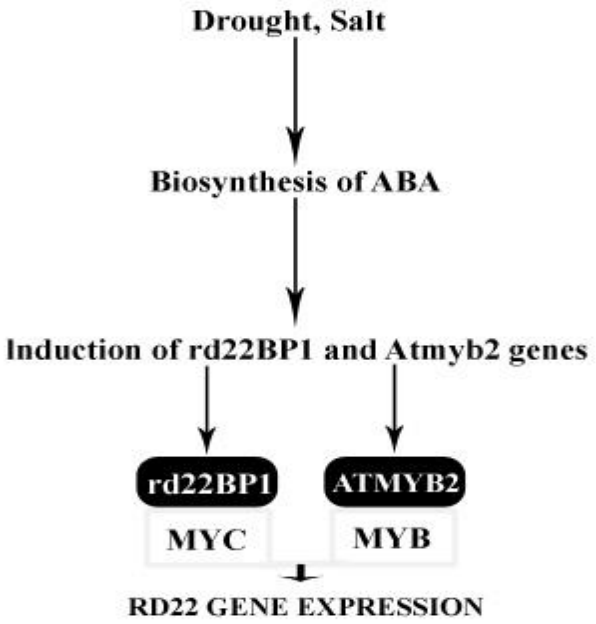

Figure 4: A Model for the lnduction of the *rd22* Gene under Water Deficit Stress Conditions. Dehydration or salt stress triggers the production of ABA, which induces the expression of *rd22BPl* and *Atmyb2*. *rd22BP1* and *ATMYB2* then bind to the MYC and MYB sites of the *rd22* promoter and activate the expression of the *rd22* gene. (Figure adapted from Abe et al. 1997).

MYB Transcription Factor

Seo et al. (2009) reported that R2R3-type MYB transcription factor MYB96 is required for proper expression of RD22. They used in their experiment a MYB96-overexpressing *Arabidopsis* mutant (*myb96-ox*) and a T-DNA insertional knockout mutant (*myb96-1d*). The RD22 expression was elevated by approximately 12-fold in the *myb96-ox* mutant but reduced by 3- to 4-fold in the *myb96-1d* mutant, these findings indicate that MYB96 is required for proper

expression of *RD22*. Although *RD22* was still induced by drought in the *myb96-1d* mutant, the transcript level was significantly lower than that in wild-type plants exposed to drought, which shows that the induction of RD22 is dependent at MYB96 under drought stress (Seo et al. 2009).

It was also reported that MYB96 plays diverse roles in plant responses to biotic and abiotic stresses and acts as a signaling link that integrates ABA and SA signals and interacts between them (Seo and Park 2010). MYB96 acts through the ABA-mediated abiotic stress signals signaling pathway to induce pathogen resistance by promoting SA biosynthesis and thus regulating stomata movement, drought tolerance, and disease resistance (Seo and Park 2010). In their work they tested pathogen resistance responses, expression levels of the SA biosynthetic, and signaling genes of the activation-tagging *myb96-1d* line and the *myb96-1* mutant. It was found that the activation-tagging *myb96-1d* line was resistant to drought and pathogen infection. However, the T-DNA insertional myb96-1 mutant was susceptible to drought and pathogen infection. However, SA biosynthetic gene SA Induction Deficient2 (*SID2*) was up-regulated, and the levels of SA were elevated in the activation-tagging myb96-1d line, which demonstrates that the increased SA biosynthesis levels, enhances pathogen resistance of the mutant. In addition, ABA and water deficit conditions induced the *SID2* gene. In contrast, the inductive effects of ABA were reduced in the *myb96-1* mutant, which indicates that the MYB96 is required for the *SID2* induction by ABA mediated abiotic stress signals (Seo and Park 2010).

BURP Domain-Containing Protein Family

Bioinformatics analysis showed that RD22-like protein belongs to the BURP domain-containing protein family. BURP domain is found at the C-terminus of several different plant

proteins and contains around 230 amino acid residues long (Peng et al. 2011). The BURP domain has taken its name from the proteins which were first identified: Brassica protein BNM2, which is expressed during the induction of microspore embryogenesis, Soybean USP-like proteins, a protein that is up-regulated by aluminium, *Arabidopsis RD22* drought induced protein and Tomato PG1beta, the beta-subunit of polygalacturonase isozyme 1 (PG1), which is expressed in ripening fruits (Xu et al. 2010). The molecular structure of the domain included four cystein-histidine (CH) repeats and 1 tryptophan residue. The spacing between the 4 CH residues is highly conserved, being X_{5}-CH-X_{10}-CH-$X_{23\text{-}27}$-CH-$X_{23\text{-}26}$-CH-X_{8}-W, where X is any amino acid (Xu et al. 2010). However, the difference mostly occurred in the region immediately downstream of the hydrophobic signal peptide. This region contains a short conserved segment and an optional segment of repeated units (Xu et al. 2010). The BNM2-like subfamily proteins are directly linked to the C-terminal region by a short conserved segment following the signal peptide (Hattori et al. 1998; Ding et al. 2009). The USP-like subfamily and RD22-like subfamily proteins have a region containing ~30 amino acid residues followed by a variable region. However, the variable region for the RD22-like subfamily proteins consists of repeat sequences (Granger et al. 2002). The PG1β-like family proteins can be distinguished from other protein members by the presence of multiple copies of a 14 amino-acid repeat sequence. As mentioned previously, BURP domain proteins share primary structural features; however, conditions under which they are expressed differ. It is suggested that the BURP domain localization is within the cell wall matrix. The molecular function of this domain is still unknown, and it is suggested that their functions may be plant specific because it is so far only found in plants (Xu et al. 2010).

Hypotheses

Hypothesis 1: SBIP-355 is a RD22-Like Protein of tobacco plants.

Analysis of the SBIP-355 partial sequence, which was obtained from Y2H screening, suggests that it is a BURP-domain containing protein and shares a high homology with other RD22 like proteins subfamily. So, it is crucial to verify if SBIP-355 is indeed a tobacco RD22 like protein.

Hypothesis 2: SBIP-355 interacts with SABP2 to alter the activity of SA mediated pathway in plants during abiotic stress.

Result of Y2H screen shows that SBIP-355 interacts with SABP2, leads us to determine if SABP2 has an effect in the *SBIP-355* gene expression during abiotic stress. Investigations are required to reach a conclusion about the interaction between SABP2 and SBIP-355.

CHAPTER 2

MATERIALS AND METHODS

Materials

Plant Materials

Before sowing the tobacco, *Nicotiana tabacum* cv. *Xanthi nc* seeds, soil containing peat moss (Fafard F-15, Agawam, MA) was autoclaved for 20 minutes. Fourteen days after sowing the tobacco seeds, the seedlings were transferred to 4 x 4 inch flats and were grown for 4 weeks. Subsequently, individual plantlets were transferred to 8-inch pots. All stages of planting were performed in a controlled PGW36 growth chamber (Conviron, Canada) set at 16 h of day/8 h of night cycle maintained at 22 °C. Experiments were carried out using 8- to 10-week old plants.

Chemicals and Reagents

Sodium dodecyl sulfate (SDS), ß-mercaptoethanol (ß-ME), tetramethylethylenediamine (TEMED), ammonium persulfate (APS), bovine serum albumin (BSA), coomassie blue R-250, coomassie brilliant blue G-250, ponceau-S, ethylene diaminetetra acetic acid (EDTA), TRIS base, glycine, glycerol, methanol, imidazole, Tween-20, Triton X-100, 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).

Buffers

Buffers used in this study are described in Appendix B.

Labware

All reusable plastic and glass labware were autoclaved before use. Labware were ordered from Sigma-Aldrich, St. Louis, MO, Thermo Scientific, Rockford, IL and Eppendorf, Hamburg, Germany.

Vectors

pDONR221 vector, pDEST17 vector and their reagents for Gateway BP and LR recombination reactions were obtained from Invitrogen (Carlsbad, CA).

Kits

Platinum *Pfx* DNA Polymerase Kit and PureLink Quick Plasmid Miniprep Kit were purchased from Invitrogen (Carlsbad, CA). QIAquick PCR Purification Kit was obtained from Qiagen (Valencia, CA).

Bactrial Strains

Chemically competent *E. coli* DH5α, TOP10, and BL21 (DE3) Mgk cells were used for transformations as cloning host or for protein expression. DH5α and BL21 (DE3) Magic cells were prepared in the lab (described in the method section), while TOP10 cells were purchased from Invitrogen (Carlsbad, CA).

Apparatus

Vortex and Shakers were purchased from Scientific Industries. Microcentrifuge and Thermal Cycler for PCR were obtained from Eppendorf. NanoDrop ND-1000 Spectrophotometer was purchased from Thermo Scientific. Gel Electrophoresis Apparatus for DNA separation was obtained from Fisher Biotech. Protein separation and Western Blotting System were obtained from Bio-Rad. Gel Documentation/Analysis System (UVP), Autoclave Machine, pH meter, Laboratory Fume Hoods, Mechanical Grinder, Top Loading Balance for weighing needs, and Absorbance Reader were used.

Oligonucleotides

The primers used for cloning of SBIP-355 were designed by following Gateway Cloning technology manual provided from Invitrogen, while the primers made to study gene expression were designed manually and analyzed by oligo analyser software. Primers were ordered from Eurofins MWG Operon. Upon receiving the primers, they were dissolved in sterile nuclease-free water and stored at -20 °C for future use. Listed below (Table. 1) are the primers used in this study or for future gene expression study.

Table 1: List of Primers Used in This Study or for Future Study

Primer #	Primers	Sequence (5´to 3´)	Purpose
DK525	attB1 Forward Primer	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC ATGGAGTTGAAGCTCCTT	Cloning of SBIP-355
DK526	attB2 Reverse Primer	GGGGACCACTTTGTACAAGAAAGCTGGGTGC TAGTTCTTAGGAACCCA	Cloning of SBIP-355
DK553	SBIP-355 Forward Primer	TAGTTTGCCACAAGCAGAATT	Gene expression analysis
DK554	SBIP-355 Forward Primer	AGAAACAACGGAGTCATACAT	Gene expression analysis
DK555	SBIP-355 Reverse Primer	GTTCTTAGGAACCCAAACAAT	Gene expression analysis
DK629	SBIP-355 Forward Primer	CAATTACGCCGCCAAAGATG	Gene expression analysis
DK630	SBIP-355 Reverse Primer	TCAGAGTCGGGCTTAACTGA	Gene expression analysis

Methods

Identification, Cloning, and Expression of SBIP-355

Bioinformatics Analysis

Partial sequence of SBIP-355 was obtained from the yeast 2-hybrid screening (Y2H). The full-length nucleotide sequence of SBIP-355 for *Nicotiana tabacum* was identified from the SOL Genomics Network (SGN) using the partial SBIP-355 nucleotide sequence (http://solgenomics.net; Bombarely et al. 2011). The full-length of nucleotide sequence was converted to Open Reading Frames (ORF) using ORF Finder tool from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). To translate nucleotide sequence into protein sequence, the Expert Protein Analysis System tool (ExPASy) (Swiss Institute of Bioinformatics) was used (www.expasy.org; Artimo et al. 2012). To identify homologous sequences of SBIP-355 from other plant species, Basic Local Alignment Search Tool (BLAST) from NCBI was used (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al. 1990). Alignment of SBIP-355 with similar proteins was performed using multiple sequence alignment tool (ClustalW2) operated by The European Bioinformatics Institute (EBI) (www.ebi.ac.uk/Tools/msa/clustalw2; Larkin et al. 2007). Conserved amino acids were identified by using conserved domain database (CDD) tool from NCBI (Marchler et al. 2011). Protein molecular weight of SBIP-355 was determined using the Protein Molecular Weight tool from Bioinformatics Organization (http://bioinformatics.org). To predict the presence and location of signal peptide cleavage site in SBIP-355 sequence, PrediSi tool from Technical University of Braunschweig was used (http://www.predisi.de; Hiller et al. 2004).). Phylogenetic tree using the

Neighbor-Joining method was constructed with Phylodendron, a phylogenetic tree drawing tool provided by Indiana University (http://iubio.bio.indiana.edu/treeapp).

Total RNA Extraction from Tobacco Leaves Using TRIzol Reagent

To avoid contamination, leaf tissue samples were collected using sterilized cork borer, micro-centrifuge tubes, and disposable gloves. Three to 4 leaf discs were collected from 6- to 8-week old of tobacco XNN (wild-type plants). Samples were frozen immediately in liquid nitrogen in order to prevent degradation (Ian 2005). Using mechanical grinder, leaf samples were ground in presence of liquid nitrogen to obtain a fine powder. One milliliter of Tri Reagent (TRIzol) was added and mixed by inverting the tube to help cell lysis from tissue samples. After incubation for 5 minutes in room temperature, 200 µl of chloroform were added to the reaction and mixed by inverting the tube for 15 seconds, and incubated at room temperature for 2 minutes. This mixture was centrifuged at 12,000 g for 10 min at 4°C, and the aqueous phase was transfered into a new tube. RNA was precipitated by adding 500 µL of isopropyl alcohol to the solution and incubated at 28°C for 10 minutes. Then, it was centrifuged at 12,000 g for 10 min at 4°C. The supernatant was discarded and the pellet recovered. The RNA pellet was washed with 1 ml cold 75% ethanol and the tube was gently vortexed to wash the RNA. The tube was centrifuged at 7,500 *xg* for 5 min in 4°C. The supernatant was discarded and the pellet recovered was air-dried under a hood for about 10 to 15 min. RNA was dissolved into 43 µL diethyl dicarbonate (DEPC) treated water and placed in 55°C water bath for 5 min. Five microliter DNAse buffer and 2µl of DNAse unit were added remove the genomic DNA then it was incubated at 37°C for 20 minutes. All the steps were repeated with half volumes of TRIzol,

Chloroform, Isopropyl alcohol, and cold 75% Ethanol. After obtaining the pellet, it was resuspended in 20 µl of DEPC-treated water and placed at 55°C water bath for 10 minutes. The quality and quantity of the purified RNA was determined using NanoDrop ND-1000 UV-Vis Spectrophotometer at 260 nm. Finally, RNA was ready for cDNA synthesis.

cDNA Synthesis

After determining total RNA concentration, reverse transcription (RT) reaction was performed to make cDNA from the total RNA. To prime the first-strand of cDNA from the purified RNA sample, 2µl of oligo(dT18) was added to the sample and it was incubated at 75°C for 10 minutes in PCR machine. While sample was incubating, cDNA mix was prepared. cDNA mix contained of 4µl of RT 5X Buffer, 1µl of RT, 1µl of 10 mM deoxynucleoside triphosphates (dNTPs) mixture, 1µl of RNAse Inhibitor (RNasin) and 3µl of DEPC treated water. 10 µl of oligo dT +RNA after incubation in PCR machine and 10 µl of cDNA mixture were added together and mixed gently. The sample was incubated at 42°C for 60 minutes then 70°C for 10 minutes in PCR machine. The quality of the prepared cDNA was tested by amplifying tobacco EF1α housekeeping gene using PCR and then running on a 1.2% agarose gel. cDNA was stored at -20°C until the use time.

Gateway Recombination Cloning Technology

In order to express SBIP-355 protein, cloning of the gene into expression vector (pDEST17) was required. With a view to save time and effort that traditional restriction enzymes and ligase cloning methods usually consume, highly efficient and rapid Gateway Recombination

Cloning Technology from Invitrogen was used (Fig.5). This method is based on *in vitro* site specific recombination properties of bacteriophage lambda λ (*att* site). BP and LR recombination reactions are the 2 main basis of the Gateway Cloning System. BP recombination reaction exchanges the region between *attB* sites in the DNA attachment sites or PCR product (SBIP-355) with the region of vector containing *attP* sites (pDONR221) to create an entry vector by the catalysis of BP Clonase enzymes. Once integrated, the recombination site is termed *attL*. While, LR recombination reaction exchanges the region between *attL* sites in the entry clone and the *attR* site in the destination vector (pDEST17) to create an expression clone with *attB* sites by mediation of LR Clonase enzyme. Each *att* site has a unique specificity for its binding partner recombination *att* site (for example *αttBl* reacts with *αttPl*, or *αttLl* with *αttRl*) and does not cross-react with other recombination *att* sites (*αttBl* does not react with *attP2*). Additionally, the system has dual selection systems. By imposing antibiotic resistance selection for the desired construct and a selection (encoded by the *ccdB* gene) against starting molecules and intermediates, the desired clone is obtained (Curtis and Grossniklaus 2003).

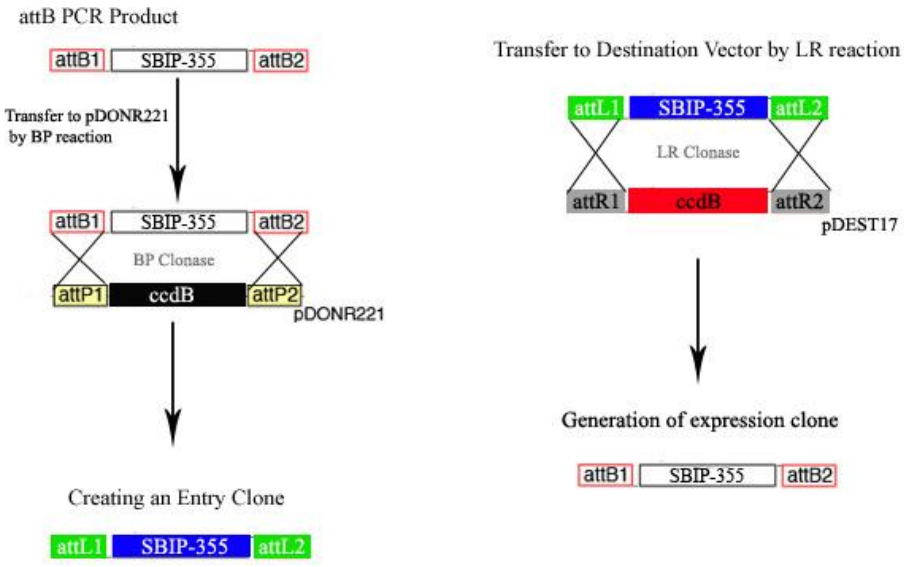

Figure 5: An Overview of Cloning of SBIP-355 into pDEST17 Using Gateway System.

Amplification of SBIP-355 Gene Using PCR

attB-containing primers were designed following Gateway Cloning technology manual provided by Invitrogen and used for cloning of SBIP-355. The full-length of SBIP-355 gene was amplified for cloning by PCR using the *att*B primers containing sites and tobacco XNN cDNA as template. The PCR was performed by using Platinum *Pfx* DNA Polymerase kit. The following reagents were added to a 0.2 ml PCR tubes: 1.5 µl 10 mM dNTP mixture (final concentration: 0.3 mM); 1 µl 50 mM MgSO4 (final concentration: 1 mM); 1.5 µl DK525 (final concentration: 0.3 µM); 1.5 µl DK526 (final concentration: 0.3 µM); 2 µl cDNA (final concentration: 10-200 ng); 5 µl 10× *Pfx* Amplification Buffer; 0.4 µl Platinum *Pfx* DNA Polymerase (1 Unit) and 37.1 µl water for 50 µl total reaction volume. PCR conditions were as follows: an initial denaturation step at 94 ℃ for 4 min was followed by amplification for 40 cycles (denaturation for 30 sec at 94 ℃, annealing for 30 sec at 55 ℃, extension for 2 min at 68 ℃) and final extension for 8 min at 68 ℃.

Analysis of SBIP-355 Amplification by Agarose Gel Electrophoresis

Agarose gel was run after PCR amplification of SBIP-355. Agarose gel (1.2%) was used to resolve ~1167bp of DNA. To prepare 1.2% agarose gel, the following recipe was used: 0.60 g of agarose was weighed and and placed into a 250ml flask filed with 50 ml of 1X TAE electrophoresis buffer; the agarose was melted by heating in microwave oven on high for 1 minute; the flask was cooled down in a 55℃ water bath for 10-15 minutes; after cooling down the mixture, 2.5 µl of Ethidium bromide (EtBr) dye (10mg/ml) was added to help visualize the DNA band under UV; gel mixture was poured into a gel casting tray containing a sample comb

and allowed to solidify for about 15-20 minutes; after the gel had solidified, the comb was carefully removed; the orientation of the tray was rotated so that the wells are toward the negative electrode to allow the DNA sample to migrate toward the positive electrode and then, the gel was covered with the of 1x Tris-Acetate EDTA (TAE) electrophoresis buffer (Appendix B); 6µl of 100 bp DNA Ladder (Concentration: 25 ng/µl) was added to the first well as a marker to help determine the size of the DNA; the DNA sample was prepared to be loaded by adding 2µl of 6X DNA dye with 5µl of the PCR product sample and pipetted into the second well; the lid and power leads were placed on the apparatus; the electric power was applied at 80 volts for 45-60 minutes. After separation, the DNA band was visualized under UV light at the correct size.

Purification of PCR Product Using PCR Purification Kit

After the successful PCR amplification of SBIP-355 was verified, PCR-amplified DNA was purified using QIAGEN QIAquick PCR purification kit following the manufacturer's protocol. The only modification being that final elution was performed using 30 µl of 10 mM Tris·Cl, pH 8.5. Purified DNA sample was quantified by spectrophotometer (NanoDrop ND-1000), and then it was examined by 1.2% agarose gel electrophoresis.

Cloning SBIP-355 into an Entry Vector (pDONR221) Using BP Reaction

The purified DNA was recombined with the pDONR221 entry vector using BP clonase enzyme mix from Invitrogen, following the manufacturer's protocol. The following reagents were added to perform BP recombination reaction: 1 µl of PCR-product, 1 µl of pDONR221

(150 ng/µl), 2 µl of BP Clonase II, and 6 µl of TE buffer. The mixture was incubated at room temperature for 1 hour. After incubation, One microliter of Proteinase K solution was added to the mixture to stop the reaction and was incubated for 10 min at 37°C.

Preparation of Chemically Competent *E. coli* DH5α Cells Using Calcium Chloride

In the first day a frozen glycerol stock of *E. coli* DH5α bacterial cells was streaked on an LB plate with no antibiotics, and then the plate was incubated overnight at 37°C. On the following day, a single colony of *E. coli* DH5α from the plate was selected and inoculated in 3 ml of fresh LB-broth and incubate overnight at 37ºC. The following day 1 ml of the overnight culture was transferred into a 1 L flask containing 500 ml LB culture and incubated at 37 °C in shaker at 250 rpm for 2 hrs, and then the optical density (OD) at 600 nm was measured every 15-20 minutes until it reaches 0.35-0.4 and was placed immediately on ice and chilled for 20-30 minutes. After cooling down the bacterial culture, it was transferred to sterile and ice-cold 50 ml polypropylene tubes. The cells were harvested by centrifugation at 4000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet was gently resuspend in 10 ml of ice cold 0.1 M $CaCl_2$ and stored on ice for 20-30 minutes. After that, the cells were recovered by centrifugation at 4000 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet was gently resuspend in 2 ml of ice cold 0.1 M $CaCl_2$ and stored on ice for 15-20 minutes, glycerol was added to 20% final concentration.. At this point cells were dispensed into aliquots of 200 µl into sterile chilled microcentrifuge tubes and were snapped frozed in liquid nitrogen. Finally, tubes of chemically competent *E. coli* DH5α cells were stored at -80°C.

Transformation of Chemically Competent *E. coli* DH5α Cells

One vial of chemically competent *E. coli* DH5α competent cell was thawed on ice for 10-15 minutes, and then 1µl of BP recombination reaction mix was added and swirled on ice gently and then incubated on ice for 30 min. After the incubation the cells ware heat-shocked for 30 sec at 42°C water bath without shaking and it was placed again on ice for 2 min. After cooling down, 250 µl of SOC media was added to the cells and the tube was capped tightly to incubate it at 37 °C shaker at 250 rpm for 1h. After that, 20 µl of the transformation mix was added to 180 µl of LB medium. On 2 prewarmed LB agar plates containing 50µg/ml kanamycin, 20 µl and 100 µl of the diluted transformation mix were spread using autoclaved glass beads. The plates were incubated overnight at 37 °C to allow for colony growth.

Confirmation of SBIP-355 Insertion into pDONR221 by Colony PCR

In order to verify presence of SBIP-355 in pDONR221 and determine if the cloning worked, colony PCR was performed. Colonies were chosen from the transformation plates and streaked with a pipette tip on a new LB agar plate containing 50µg/ml kanamycin as a master plate, then it was incubated at 37°C overnight. The same tip used for streaking was placed in PCR tubes containing 40 µl of autoclaved water. The primers specific for the pDONR221 vector were used (M13 forward and M13 reverse) for amplification. In a new 0.2 ml PCR tubes, 10 µl of resuspended colony was added with the following PCR reaction reagents: 2 µl of 10x PCR Buffer, 2 µl of dNTP (0.1M), 0.4 µl of M13 forward primer, 0.4 µl M13 reverse primer, 0.2 µl Taq polymerase, and 5 µl water for 10 µl total reaction. PCR conditions were as follows: an initial denaturation step at 94 °C for 4 min was followed by amplification for 30 cycles

(denaturation for 30 sec at 94 °C, annealing for 30 sec at 55 °C, extension for 1.15 min at 72 °C) and final extension for 8 min at 72 °C. The amplified products were tested by 0.8% agarose gel electrophoreses.

Purification of Plasmid DNA (pDONR221-SBIP-355) Using Plasmid Miniprep Kit

Invitrogen pure Link quick plasmid miniprep kit was used to purify the pDONR221 vector from *E. coli* DH5α that were identified as positive with the colony PCR. Purification was followed according to manufacturer's protocol. The purified plasmid DNA samples were quantified by spectrophotometer (NanoDrop ND-1000), and then they were examined by 0.8% agarose gel electrophoresis.

Sequencing of Purified Plasmid DNA (pDONR221-SBIP-355)

DNA sequencing was used to verify the exact sequence of SBIP-355 cloned into the pDONR221. The purified plasmid DNA samples were sent for sequencing to DNA Analysis Facility at Yale University. The samples were sequenced by Sanger method using M13 primers. Obtained sequences were compared with the predicted SBIP-355 sequence using clustalw2 tool to help align multiple sequences.

Construction of Expression Vector by Subcloning of pDONR221-SBIP-355 into pDEST17

After analysis of the sequencing results, the clone that showed the highest homology with the predicted SBIP-355 sequence was chosen for the LR recombination reaction that allows recombination between the entry clone (pDONR221) that contains the gene of interest (SBIP-355) flanked by attL sites and a destination vector (pDEST17) , which adds a 6X His tag fused on the N terminal of proteins; and contains attR sites to generate an expression clone by the aid of the enzyme LR Clonase I. LR recombination reaction was preformed following the manufacturer's protocol. The following reagents were added to perform the reaction: 1µl of pDONR221-SBIP-355 (120 ng/µl), 1 µl of pDEST17 (150 ng/µl), 2 µl of LR Clonase I and 6 µl of TE buffer. The mixture was incubated at room temperature for 1 hour. After incubation, 1 microliter of Proteinase K solution was added to the mixture to stop the reaction and was incubated for 10 min at 37°C. After that, 1µl of LR recombination reaction was used to transform 100 µl of Invitrogen TOP10 chemically competent cells by the heat shock method. Transformed cells were spread on a LB agar plate containing 100µg/ml ampicillin and incubated overnight at 37 ºC to allow for colony growth.

Purification of pDEST17-SBIP-355

Invitrogen pure Link quick plasmid miniprep kit was used to purify pDEST17-SBIP-355 from TOP10. Purification was followed according to manufacturer's protocol. The purified plasmid was quantified by spectrophotometer (NanoDrop ND-1000) and was analyzed by 0.8% agarose gel electrophoresis.

Transformation of pDEST17-SBIP-355 into Chemically Competent *E. coli* BL21 (DE3) Magic Cells

Chemically competent BL21 (DE3) Mgk cells have the T7 promoter expression system, which normally is capable of producing more protein than any other bacterial expression system (Guide to Gene Expression in BL21, 2014). Magic cells were prepared following the same protocol of preparation of DH5α cells. Transformations of pDEST17-SBIP-355 into competent cells were performed by adding 27 ng of plasmid DNA to 100 µl of Magic competent cells by the heat shock method. Transformed cells were spread on a LB agar plate containing 100µg/ml ampicillin and 10µg/ml kanamycin. Plate was incubated overnight at 37 ºC to allow for colony growth.

Test for Small Scale Recombinant Protein Expression pDEST17-SBIP-355 in *E. coli*

A colony of recombinant bacteria from the LB-agar plate was selected, inoculated into 3 ml of liquid LB containing ampicillin (100 µg/ml) and kanamycin (10 µg/ml), and incubated at 37 ºC for 3 hours with shaking at 250 rpm until the desired OD600 achieved 0.6. One milliliter of the culture was centrifuged at maximum speed for 3 minutes, and then the pellet was kept as an uniduced control at -20 ºC until the time of use. The rest of the bacterial culture was subjected to induction by adding 1mM isopropyl-β-D-thiogalactoside (IPTG) and incubated at 37 ºC for 3hrs with shaking at 250 rpm. Following incubation, centrifugation was performed to collect pellet from the bacterial culture. Induced and uninduced samples were resuspended in 100 µl of 1x SDS loading buffer (Appendix B). Then these samples incubated in boiling water bath for 5 minutes. After boiling they were centrifuged at 13,000 rpm for 10 minutes. Ten microliter of the

samples were loaded to detect protein expression onto a 12 % sodium dodecyl sulfate - polyacrylamide (SDS- PAGE) gel along with 8 µl of protein marker with a range of 14 kDa to 97 kDa for size reference, and then followed by western blot analysis for confirmation.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a method used for separating proteins according to their size. It was carried out by following Laemmli-SDS-PAGE method (Laemmli 1970). Before preparing the gel, the gel cast was assembled with 1.5 mm spacers and checked for leaks with distilled water. After that, 12 % polyacrylamide gels were prepared for separation according to the Laemmli's protocol (Appendix B). Then the supernatant of the prepared induced and uninduced samples were loaded into the gel wells. The gel was electrophoresed at constant 200V and proteins were allowed to separate for 1 hr and followed by Coomassie brilliant blue staining to visualize the proteins bands, and then the gel was destained with destaining solution (Appendix B).

Western Blot Analysis

Western blot is a test used to detect desired proteins separated by SDS-PAGE using high-quality antibody directed against the desired proteins. The test is performed by transfer of protein from SDS-PAGE gel onto polyvinylidene difluoride membrane (PVDF). Prior to transferring of SDS-PAGE, the PVDV membrane was cut to the correct size for the SDS-PAGE gel and prepared for transfer by soaking in 100% methanol for 15 seconds and then rinsed with water. The western blot cassettes with sponge were soaked in a glass pan containing cold 1X transfer

buffer (Appendix B). SDS-PAGE gel was carefully removed from the glass plates and soaked in cold 1X transfer buffer for 10 min. The SDS-PAGE gel and PVDF membrane were sandwiched in the western blot cast between two Whatman papers. The cast was loaded into the tank and an ice pack was added before adding the cold 1X transfer buffer addition. Transfer was conducted at a constant 100V for 1 hour in the cold room (4 ºC). After transferring of proteins to the PVDE membrane, the membrane was rinsed in 100% methanol for 10 seconds, placed on a Whatman paper for 10 minutes to dry, and rinsed again in methanol for 10 seconds. The membrane was stained with ponceau stain for 2 minutes (Appendix B) to visualize the protein bands. The membrane was washed off with 1x phosphate buffer saline to remove off stain (Appendix B). Western blot is probed with adding the primary antibody against the target protein; therefore, the membrane was incubated in the primary antibody (anti-polyHistidine mouse monoclonal antibody prepared in blocking buffer in a 1:1000 dilution) and kept overnight at 4 °C on a horizontal shaker. Following incubation, the blocking buffer with the primary antibody was removed from the membrane. To remove unbound antibodies from the membrane, it was washed gently with (1x PBS buffer, 1x PBS + 3% tween 20 buffer, and then 1x PBS) consecutively 3 times for 5 minutes each in a horizontal shaker (Appendix B). After the washing, the blot was probed again by adding a secondary antibody (antimouse IgG in goat with HRP conjugate diluted to 1:5000 in blocking buffer). The incubation was for 1 hour room temperature on a horizontal shaker. After that, the same previous washing process was performed again. Finally, detection of signal took place in a dark room using enzyme linked chemiluminescence (ECL) detection reagents (A and B). ECL reagent A was mixed with reagent B in a 1:1 ratio and poured on the membrane with gentle shaking to cover it up for 1 minute. The membrane was placed between plastic wrap after discarding the fluid and then was put on autoradiography cassette

containing an X-ray film. It was exposed for 1 to 5 minutes. The membrane was developed (in developer, water, and fixer) consecutively once for 2 minute. The film was air dried and signal was recorded by photography.

CHAPTER 3

RESULTS

Bioinformatics Analysis of SBIP-355

The full length genomic sequence (SGN-U446947) of SBIP-355 (Fig. 6) was obtained from the SGN database of *Nicotiana tabacum* using the partial sequence of SBIP-355 (Fig. 7) that was obtained by sequencing yeast 2-hybrid clone. The sequence analysis shows that the full length sequence of SBIP-355 (SGN-U446947) is 1399 bp in length and 1167 bp of ORF. According to the SGN-U446947, SBIP-355 ORF encodes a protein of 388 amino acid residues (Fig. 8), containing a BURP domain (Fig. 9). Alignment of SBIP-355 with similar proteins showed that it contained a 230 amino acid long conserved BURP domain at its C-terminus (Fig.10). To investigate the evolutionary relationships of SBIP-355, a phylogenetic tree was generated from the amino acid sequences of multiple BURP domain-containing proteins from various plant species. The phylogenetic tree was analyzed by Phylodendron tool using the Neighbor-Joining method. The phylogenetic analysis showed that SBIP-355 was clustered into the subfamily that contains all the RD22-like proteins (Fig.11). PrediSi tool was employed to detect the presence and location of signal peptide cleavage sites in amino acid sequences of SBIP-355. Results indicated that SBIP-355 has a signal peptide, at the first 21 amino acids region (Fig. 12).

>SGN-U446947
CTTGTTAGTAGTCGAACTGTCCTTTTAAATTCATCAGAATTCTCTATCTTCTTTCTTTAATTTGCGTA
AGTTCAACC**ATGGAGTTGAAGCTCCTTCACGTCCTCACTTACCTTTCGTTGGCACTAGTGGCAAGTTA**
TGCAGCTCTTCCTCCTGCATCGAGCGGTCAAACTTATTGGAATACTAAGCTGCCTAATACGCCCATCC
CAAAGGCAATCAAAGATTCTCTGCAGCCAACTGGATTGACGGAGGACAAAAGCACTTCAGTGGAAGTA
GGCAAAGGCGGAGTAAACGTCAACACCGGCAAGGGGCACTCCGGCAGCGGCACTAACGTCAACGTTGG
CCACAAAGGTGTCGGCGTAAGCACTGGCAAGGGTCACTCCGGCAGGGGAGCCAACGTCAACGTCGGCC
ACAAAGGCGTGGGCGTAAGCACCGGGGGCGGAACCCACGTTGGCGTCGGCAAAGGAGGAGTCGGCGTG
ACCACTCCCGGCCACCACGGAAGGCCGCCGGTGTACGTCGGTGTACGTCCCGGACCGTCACCTTTTGT
TTACAATTACGCCGCCAAAGATGATCAACTTCACGACAACCCAAACGTCGCTCTTTTTTTCCTTGAGA
AAGACTTGCACCAAGGTAGTAACATGAACTTGCAGTTTGTGAAAACTACAAAAAATGCCGCCACTTTC
TTGCCTCGCCAAGTAGCAAATTCTATTCCCTTTTCGTCAAACAAGATGCCAGAAATTCTTAGCCAGTT
TTCAGTTAAGCCCGACTCTGAAGAAGCTCAGATCTTGAAGCAAACTGTCCAAGAATGTGAAGAGCCAG
GTATTAAAGGAGAAGAGAAGTACTGTGCTACTTCATTAGAATCCATGGTTGATTTCAGCACATCAAAG
TTAGGAAACAAAGTACAACCAGTATCAACAGAGACAGAGAAGGAAACACAAATGCAGAAATACACAAT
TCTAGGAGCCAAGAAAATGGGAAATGGGAAATCTGATGCTGTAGTTTGCCACAAGCAGAATTATGCAT
ATGCAGTTTTCTACTGTCATAAAACAGAAACAACGGAGTCATACATGGTTTCTTTGGTTGGTGCTGAT
GGAACAAAGGCTAAAGCAGTAGCTGTTTGCCATAAGGATACTTCAGCTTGGAATCCAAAACATTTGGC
TTTTAAAGTTCTTAAGGTTACACCTGGATCTGTTCCTGTTTGCCATTTCCTTCCTGAGGATCACATTG
TTTGGGTTCCTAAGAACTAGCTTTATACATAGATGATGTTAGTTTTATTATCTTTTTATTTGTGTTGT
TTTTATGTAATAATTCAGAGTATGTTGTGCTTATTATTACAGTTTTATCTTGTAATACTTCAGTATAC
GAATAAATAATTTCAGCATATATTTTAGCGGCCGCATGA

Figure 6: ORF of SBIP-355 in SGN Database. Sequence in red indicates the nucleotides of SBIP-355 with start and stop codon (SGN-U446947).

GAGCCAACGTCAACGTCGGCCACAAAGGCGTGGGCGTAAGCACCGGGGGCGGAACCCACGTTGGCGTCG
GCAAAGGAGGAGTCGGCGTGACCACTCCCGGCCACCACGGAAGGCCGCCGGTGTACGTCGGTGTACGTC
CCGGACCGTCACCTTTTGTTTACAATTACGCCGCCAAAGATGATCAACTTCACGACAACCCAAACGTCG
CTCTTTTTTTCCTTGAGGAAGACTTGCACCAAGGTAGTAACATGAACTTGCAGTTTGTGAAAACTACAA
AAAATGCCGCCACTTTCCTGCCTCGCCAAGTAGCAAATTCTATTCCCTTTTCGTCAAACAAGATGCCAG
AAATTCTTAGCCAGTTTTCAGTTAAGCCCGACTCTGAAGAAGCTCAGATCTTGAAGCAAACTGTCCAAG
AATGTGAAGAGCCAGGTATTAAAGGAGAAGAGAAGTACTGTGCTACTTCATTAGAATCCATGGTTGATT
TCAGCACATCAAAGTTAGGAAACAAAGTACAACCAGTATCAACAGAGACAGAGAAGGAAACACAAATGC
AGAAATACACAATTCTAGGAGCCAAGAAAATGGGAAATGGGAAATCTGATGCTGTAGTTTGCCACAAGC
AGAATTATGCATATGCAGTTTTCTACTGTCATAAAACAGAAACAACGGAGTCATACATGGTTTCTTTGG
TTGGTGCTGATGGAACAAAGGCTAAAGCAGTAGCTGTTTGCCATAAGGATACTTCAGCTTGGAATCCAA
AACATTTGGCTTTTAAAGTTCTTAAGGTTACACCTGGATCTGTTCCTGTTTGCCATTGCCTTCCTGAGG
ATCACATTGTTTGGGTTCCTAAGAACTAGCTTTATACATAGATGATGTTAGTTTTATTATCTTTTTATT
TGTGGTGGTTTTTATGTAATAATTCAGAGTATGTTGTGCTTATTATTACAGTTTTATCTTGTAATACTT
CAGTATACGAATAAATAATTTCAGCATATATTTTCTAAAAAAAAAAAAAAAAAAAAAAAAAAAACA

Figure 7: Partial Nucleotide Sequence of SBIP-355 obtained from Yeast Two-Hybrid Screening.

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MELKLLHVLTYLSLALVASYAALPPASSGQTYWNTKLPNTPIPKAIKDSLQPTGLTEDKSTSVEVGKGG
VNVNTGKGHSGSGTNVNVGHKGVGVSTGKGHSGRGANVNVGHKGVGVSTGGGTHVGVGKGGVGVTTPGH
HGRPPVYVGVRPGPSPFVYNYAAKDDQLHDNPNVALFFLEKDLHQGSNMNLQFVKTTKNAATFLPRQVA
NSIPFSSNKMPEILSQFSVKPDSEEAQILKQTVQECEEPGIKGEEKYCATSLESMVDFSTSKLGNKVQP
VSTETEKETQMQKYTILGAKKMGNGKSDAVVCHKQNYAYAVFYCHKTETTESYMVSLVGADGTKAKAVA
VCHKDTSAWNPKHLAFKVLKVTPGSVPVCHFLPEDHIVWVPKN
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Figure 8: Amino Acid Sequence of SBIP-355 Translated by ExPASy Tool.

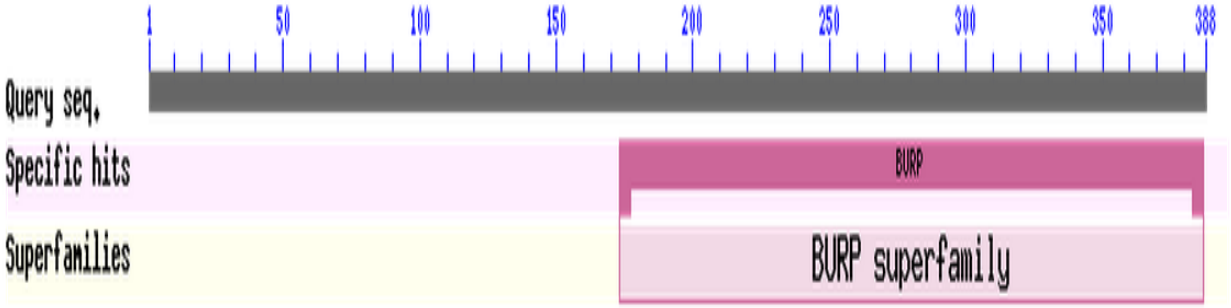


Figure 9: BLAST Search Result of SBIP-355 Protein

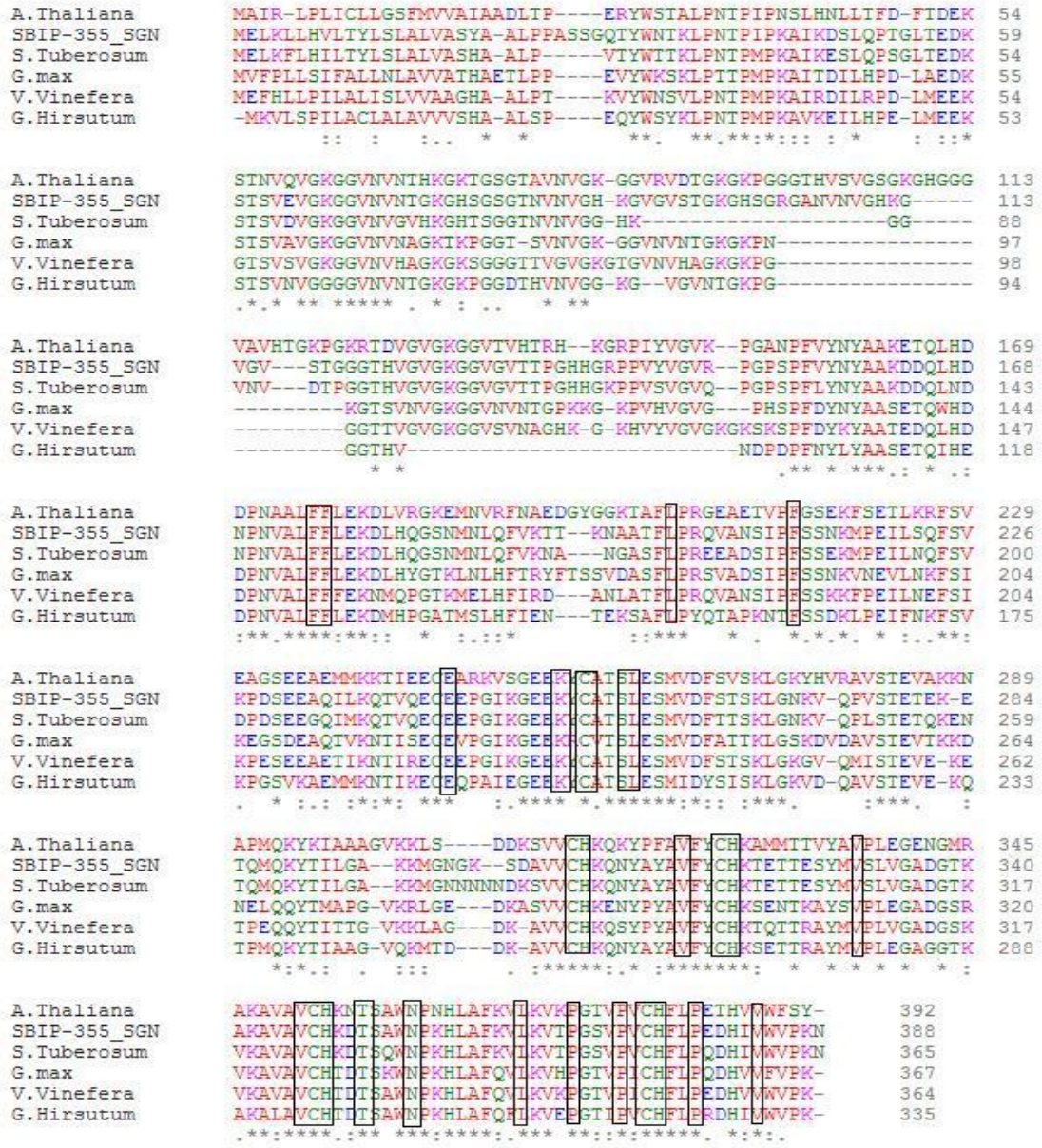


Figure 10: Multiple Amino Acid Sequence Alignments of SGN-U446947 with Similar Proteins Containing BURP domains. (SBIP-355 _SGN= SGN-U446947 ORF, S. Tuberosum= *Solanum tuberosum*, V. Vinefera= *Vitis vinefera*, G. Hirsutum= *Gossypium hirsutum* and A. Thaliana= *Arabidopsis thaliana. Solanum tuberosum* (81% identity), *Vitis vinefera* (61% identity), *Gossypium hirsutum* (60% identity), *Glycine max* (58% identity), and *Arabidopsis thaliana* (55% identity). Black boxes show conserved amino acid residues of BURP domain.

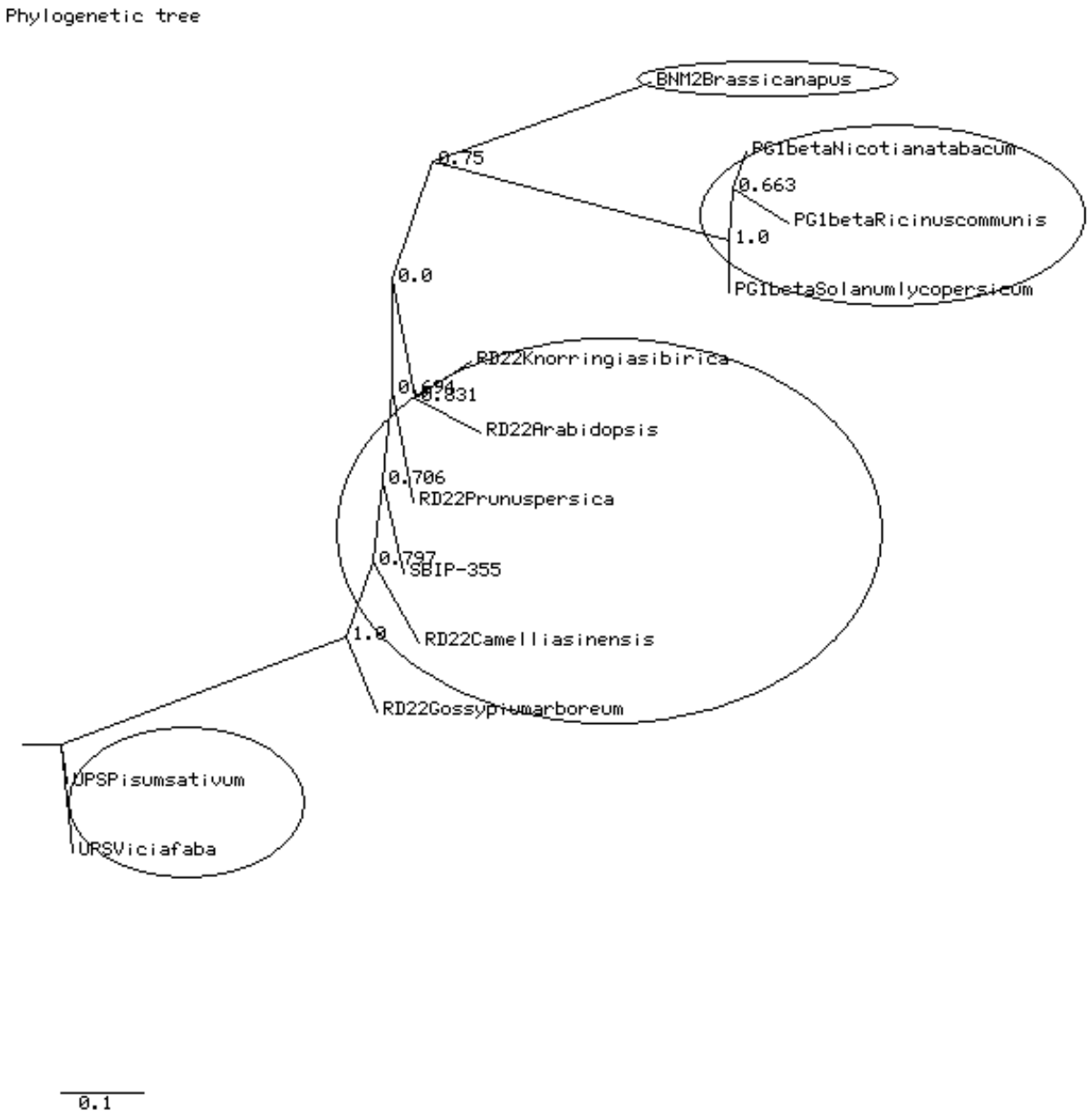
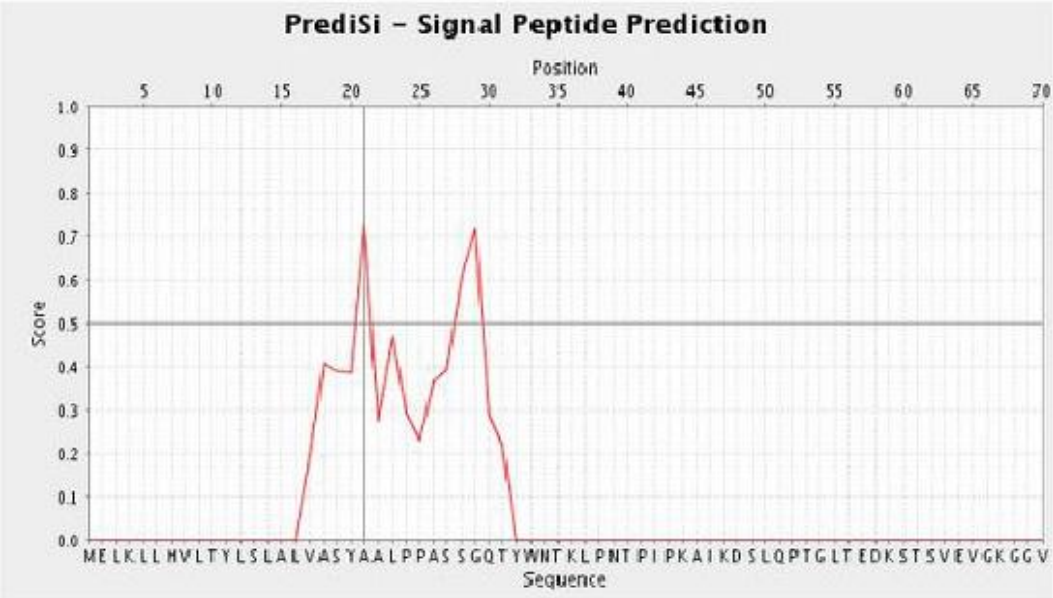


Figure 11: Phylogenetic Tree of Multiple Proteins of BURP Domain-Containing Protein Family. It was generated using the Neighbor-Joining method. The proteins are BNM2, *Brassica napus*; PG1 beta, *Nicotiana tabacum*; PG1 beta, *Ricinus communis*; PG1beta, *Solanum lycopersicum*; RD22,*Knorringia sibirica*; RD22, *Arabidopsis*; RD22, *Prunus persica*; RD22, *Camellia sinensis*; RD22, *Gossypium arboreum*; UPS, *Pisum sativum* and UPS, *Vicia faba*. SBIP-355 is clustered into the subfamily that contains all the RD22-like proteins

Details:

Matrix:	Eukarya
Truncation:	70 residues
Cleavage position:	21
Score:	0.7285
Secreted protein:	**predicted for secretion**



SBIP-355:

```
MELKLLHVLTYLSLALVASYAALPPASSGQTYWNTKLPNTPIPKAIKDSLQPTGLTEDKSTSVEVGKGGV
|   |   |   |   |   |   |   |   |   |   |   |   |   |   |
1       10      20      30      40      50      60      70
```

Figure 12: Result of Signal Peptide Detection of SBIP-355. The method is based on a position weight matrix approach. Analysis shows the presence of signal peptide in the (first 21 amino acids). In addition, It is predicted that SBIP-355 is a secretory protein

Total RNA Isolation from XNN Tobacco Using TRIzol Method and cDNA Synthesis

The isolated RNA was quantified using NanoDrop ND-1000 UV-Vis Spectrophotometer at 260 nm (Fig.13). The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of RNA from contamination with protein in the sample and the 260/230 ratio is used as a secondary measure of RNA purity from contamination with salts and phenols. For RNA, the ideal 260/280 ratio is between 1.8-2.0 and 260/230 ratio and it should be very close to 2.0 (Imbeaud et al. 2005). The synthesis of cDNA was performed using the isolated RNA by reverse transcription, tested by amplification of the tobacco housekeeping gene *EF1α* using PCR and measured for integrity using 1.2% Agarose gel (Fig.14).

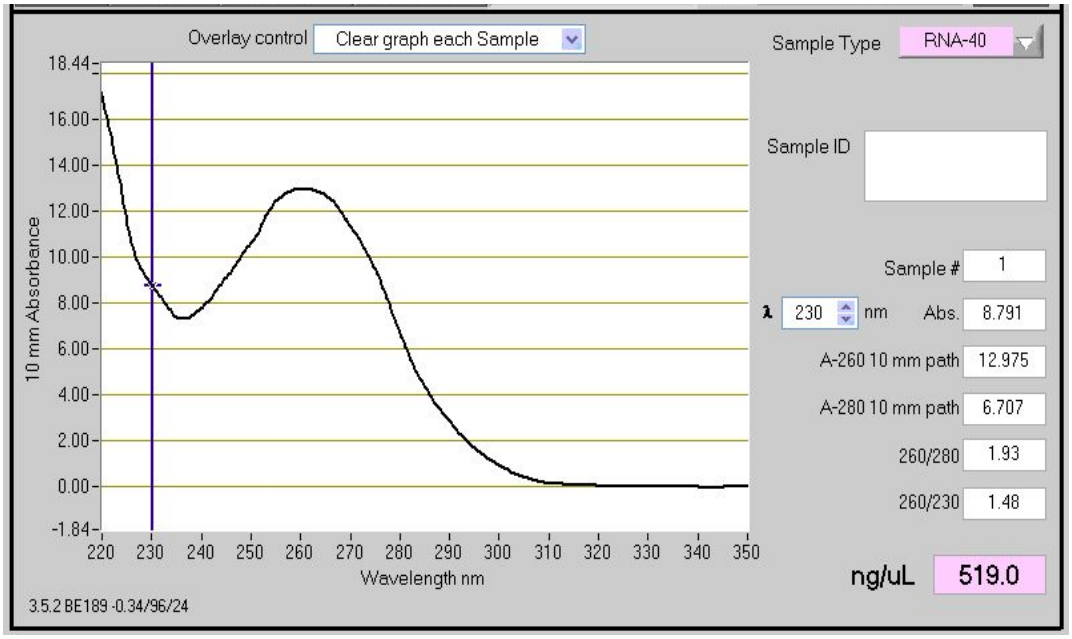


Figure 13: Quantitation of RNA Isolated from Tobacco XNN Leaves using NanoDrop Spectrophotometer. RNA Concentration= 519.0 ng/µL, 260/280 Ratio= 1.93 and 260/230= 1.84

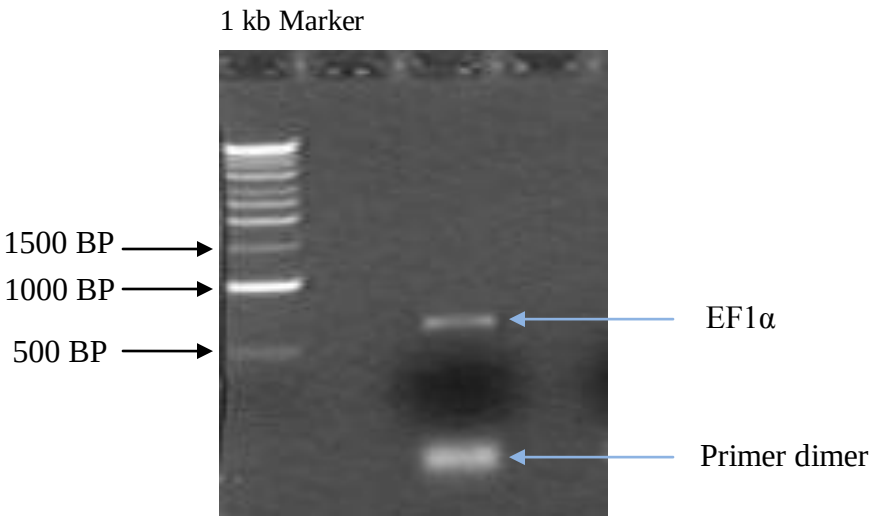


Figure 14: 1.2 % Agarose Gel Stained with EtBr for the Amplification of EF1α

Cloning of SBIP-355 by Gateway Cloning Technology and Expression in *E.coli*

Amplification of SBIP-355 Using PCR

PCR was performed to amplify SBIP-355 (1167bp) using cDNA from tobacco XNN leaf as template and Platinum *Pfx* DNA polymerase. Then, the amplification of SBIP-355 was tested for integrity on a 1.2% agarose gel (Fig.15).

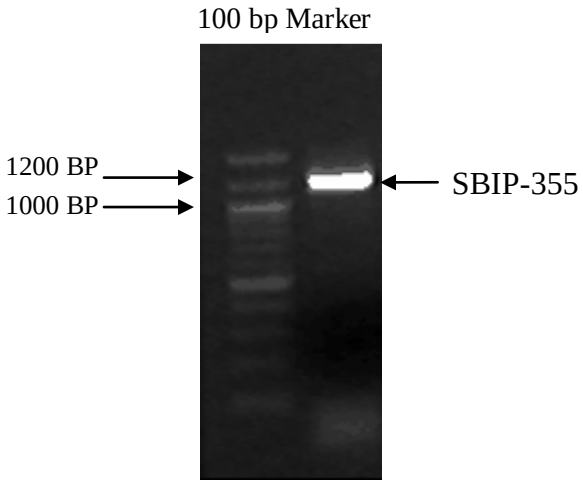


Figure 15: 1.2 % Agarose Gel Stained with EtBr for the Amplification of SBIP-355

Purification of PCR Amplified SBIP-355 Using PCR Purification Kit

PCR amplified SBIP-355 was purified using QIAGEN QIAquick PCR purification kit. The concentration of the purified DNA was measured using the NanoDrop ND-100 Spectrophotometer, and then the quality of purified DNA was tested on 1.2% agarose gel (Fig.16).

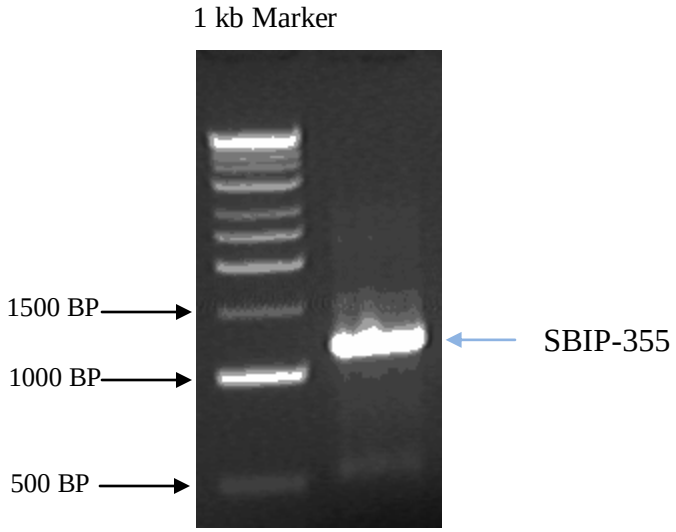


Figure 16: 1.2 % Agarose Gel Stained with EtBr for the Quality of Purified SBIP-355

Cloning of SBIP-355 into pDONR221 Using BP Recombination Reaction

BP recombination reaction was performed with the purified SBIP-355 and pDONR221 was used to generate an entry clone. After successful transformation, colonies were tested for the presence of insert via colony PCR using M13 forward and reverse primers that flanked the insertion site and also by using M13 forward and SBIP-355 reverse primers. PCR amplified fragments of SBIP-355 from recombinant pDONR221-SBIP-355 plasmids were tested for integrity on a 0.8% agarose gel (Fig.17).

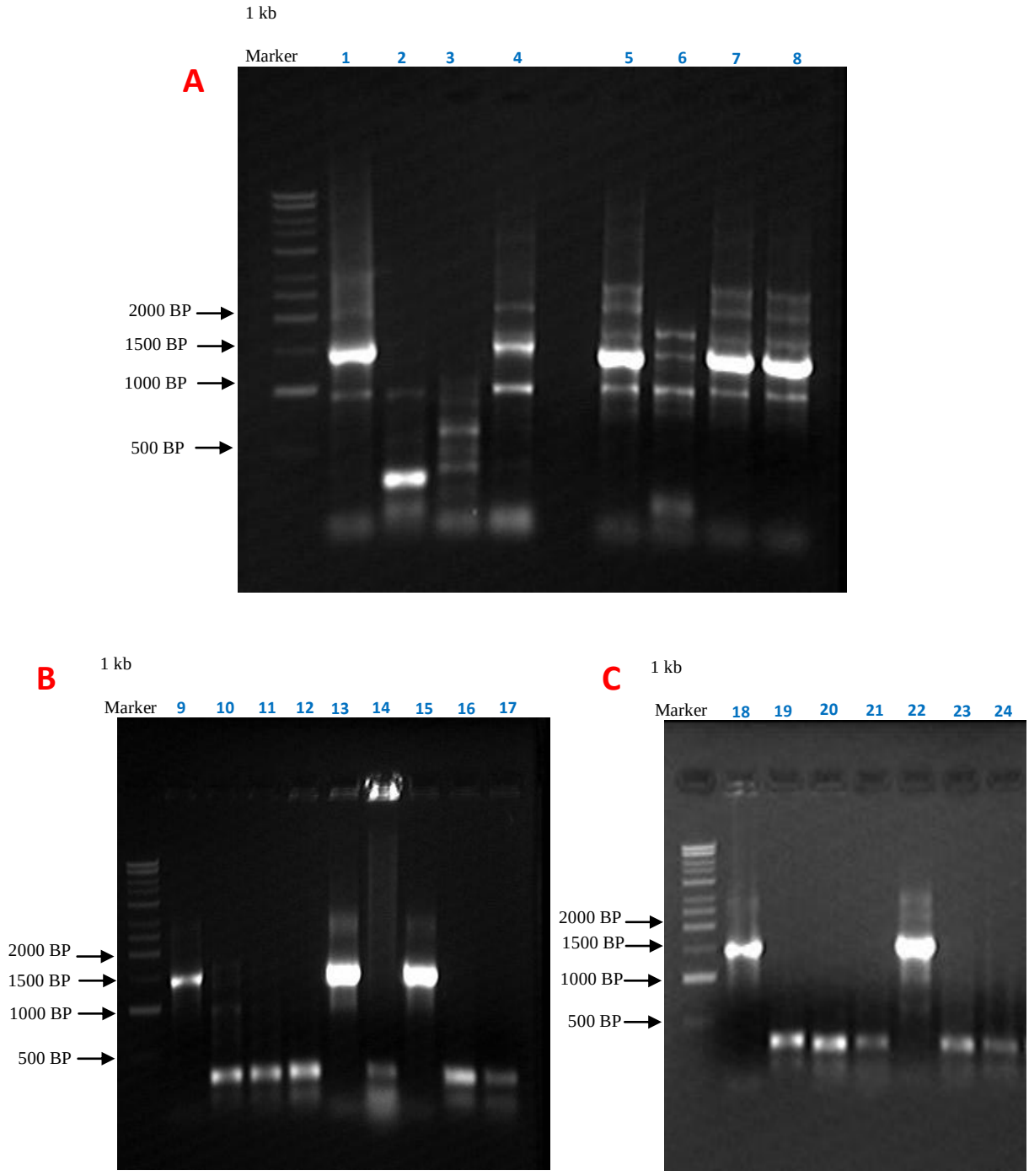


Figure 17: 0.8% Agarose Gel Stained with EtBr for the Confirmation of the Insertion of SBIP-355 into pDONR221 by Colony PCR. Fig A is showing the amplification of SBIP-355 from pDONR221-SBIP-355 clone using M13 F and R primers. Fig B and C are the amplification of SBIP-355 from pDONR221-SBIP-355 clone using M13 F and SBIP-355 R primer. Seven positive clones were sent for sequencing.

Purification of Plasmid DNA (pDONR221-SBIP-355) Using Plasmid Miniprep Kit

Plasmids were isolated from bacterial culture using Invitrogen Pure Link Quick Plasmid Miniprep kit. The concentration of the plasmid DNA was measured by the NanoDrop ND-100 Spectrophotometer and the quality was tested on 0.8% agarose gel (Fig.18).

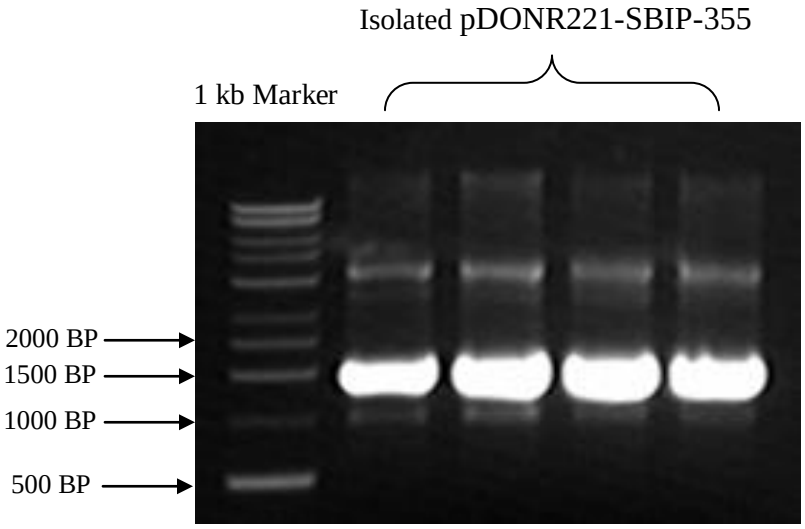


Figure 18: 0.8% Agarose Gel Stained with EtBr for the Confirmation of the Quality of the Isolated pDONR221-SBIP-355 using Plasmid Miniprep Kit

DNA Sequencing Results

Positive clones were sent for sequencing and aligned with SBIP-355 nucleotide sequence based on SGN-U446947 and with the partial nucleotide sequence of SBIP-355 obtained from yeast 2-hybrid screening (Fig. 19). Furthermore, they were aligned with amino acid sequence by translation of nucleotides sequences into amino acid sequence using ExPASy translation tool (Fig. 20). Based on the analysis, clone no.1 is the most highly homologous clone with SBIP-355

nucleotide sequences (Fig. 21) and amino acid sequence (Fig. 22) among the rest of the sequenced clones and was selected for the protein expression step. Sequencing alignment of clone no.1 sequence with SBIP-355 sequence based on SGN-U446947 shows mismatch in 3 nucleotides; however, clone no.1 alignment with amino acid sequence of SBIP-355 shows a perfect match with SBIP-355 sequence based on SGN-U446947 and 1 amino acid mismatch with amino acids sequence of SBIP-355 obtained from yeast 2-hybrid screening. Further reviews give a conclusion that the nucleotides mutations in the DNA sequence are silent mutation and they code for the same amino acid because DNA alignment doesn't take into account the redundancy of amino acid codons. Other sequenced clones showed high similarities with SBIP-355 and they are proposed to be different isoforms of SBIP-355 because it is evident that *Nicotiana tabacum* is a tetraploid genome structure that consists of 4 sets of chromosomes (Cameron 1952).

```
1               ATGGAGTTGAAGCTCCTTCACGTCCTCACTTACCTTTCGTTGGCACTAGTGGCAAGTTAT 60
4               ATGGAGTTGAAGCTCCTTCACATCCTTACCTACCTTTCTTTGGCACTAGTGGCAAGTTAT 60
9               ATGGAGTTGAAGCTCCTTCACATCCTTACCTACCTTTCTTTGGCACTAGTGGCAAGTTAT 60
16              ATGGAGTTGAAGCTCCTTCACATCCTTACCTACCTTTCTTTGGCACTAGTGGCAAGTTAT 60
29              ------------------------------------------------------------
SGN             ATGGAGTTGAAGCTCCTTCACGTCCTCACTTACCTTTCGTTGGCACTAGTGGCAAGTTAT 60
SBIP            ------------------------------------------------------------

1               GCAGCTCTTCCTCCTGCATCGAGCGGTCAAACTTATTGGAATACTAAGCTGCCTAATACG 120
4               GCAGCTCTTTCTC---CATCGAGCGGTCAAACTTATTGGAATACTAAACTGCCTAATACT 117
9               GCAGCTCTTTCTC---CATCGAGCGGTCAAACTTATTGGAATACTAAACTGCCTAATACT 117
16              GCAGCTCTTTCTC---CATCGAGCGGTCAAACTTATTGGAATACTAAACTGCCTAATACT 117
29              ------------------------------------------------------------
SGN             GCAGCTCTTCCTCCTGCATCGAGCGGTCAAACTTATTGGAATACTAAGCTGCCTAATACG 120
SBIP            ------------------------------------------------------------

1               CCCATCCCAAAGGCAATCAAAGATTCTCTGCAGCCAACTG---------GATTGACGGAG 171
4               CCCATGCCAAAGGCAATCAAAGATTCTCTTCAGCCAACTGATTTCGCAGGATTGACGGAG 177
9               CCCATGCCAAAGGCAATCAAAGATTCTCTTCAGCCAACTG---------GATTGACGGAG 168
16              CCCATGCCAAAGGCAATCAAAGATTCTCTTCAGCCAACTG---------GATTGATGGAG 168
29              ------------------------------------------------------------
SGN             CCCATCCCAAAGGCAATCAAAGATTCTCTGCAGCCAACTG---------GATTGACGGAG 171
SBIP            ------------------------------------------------------------

1               GACAAAAGCACTTCAGTGGAAGTAGGCAAAGGCGGAGTAAACGTCAACACCGGCAAGGGG 231
4               GACAAAACCACTTCAGTGGTAGTAGGCAAAGGCGGAGTAAACGTCAACACCGGCAAAGGG 237
9               GACAAAACCACTTCAGTGGTAGTAGGCAAAGGCGGAGTAAACGTCAACACCGGCAAAGGG 228
16              GACAAAACCACTTCAGTGGTAGTAGGCAAAGGCGGAGTAAACGTCAACACCGGCAAAGGG 228
29              ------------------------------------------------------------
SGN             GACAAAAGCACTTCAGTGGAAGTAGGCAAAGGCGGAGTAAACGTCAACACCGGCAAGGGG 231
SBIP            ------------------------------------------------------------
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1             CACTCCGGCAGCGGCACTAACGTCAACGTTGGCCACAAAGGTGTCGGCGTAAGCACTGGC 291
4             CACTCCGGCAGCGGCACTAACGTCAACGTTGGCCGCAAAGGCGTCGGCGTAAGCACCGGC 297
9             CACTCCGGCAGCG---------------------------------------------- 241
16            CACTCCGGCAGCGGCACTAACGTCAACGTTGGCCACAAAGGCGTCGGCGTAAGCACCGGC 288
29            ------------------------------------------------------------
SGN           CACTCCGGCAGCGGCACTAACGTCAACGTTGGCCACAAAGGTGTCGGCGTAAGCACTGGC 291
SBIP          ------------------------------------------------------------

1             AAGGGTCACTCCGGCAGGGGAG---CCAACGTCAACGTCGGCCACAAAGGCGTGGGCGTA 348
4             AAGGGTCACTCCGGCAGGGGGGGCACCAACGTCAACGTCGGCCACAAAGGCGTCGGCGTA 357
9             ------------------GCA---CTAACGTCAACGTTGGCCACAAAGGCGTCGGCGTA 279
16            AAGGGTCACTCC-------GAG---CCAACGTCAACGTCGGCCACAAAGGCGTGGGCGTA 338
29            ------------------------------------------------------------
SGN           AAGGGTCACTCCGGCAGGGGAG---CCAACGTCAACGTCGGCCACAAAGGCGTGGGCGTA 348
SBIP          ------------------GAG---CCAACGTCAACGTCGGCCACAAAGGCGTGGGCGTA 38

1             AGCACCGG-----------------GGGCGGAACCCACGTTGGCGTCGGCAAAGGAGGA 390
4             AGCACCGG-----------------GGGCGGAACCCACGTCGGCGTCGGCAAAGGAGGA 399
9             AGCACCGGCAAGGGTCACTCCGGCAGGGGGGGCACCAACGTCAACGTCGGCCACAAAGGC 339
16            AGCACCGG-----------------GGGCGGAACCCACGTCGGCGTCGGCAAAGGAGGA 380
29            ------------------------------------------------------------
SGN           AGCACCGG-----------------GGGCGGAACCCACGTTGGCGTCGGCAAAGGAGGA 390
SBIP          AGCACCGG-----------------GGGCGGAACCCACGTTGGCGTCGGCAAAGGAGGA 80

1             GTCGGCGTGACCACTCCCGGCCACCACGGAAGGCCGCCGGTGTACGTCGGTGTACGTCCC 450
4             GTGGGCGTGACCACCCCCGGCCACCACGGAAGGCCGCCGGTCTACGTCGGTGTACGCCCC 459
9             GTCGGCGTAAGCAC---------CGGGGGAAGGCCGCCGGTCTACGTCGGTGTACGCCCC 390
16            GTGGGCGTGACCACCCCCGGCCACCACGGAAGGCCGCCGGTCTACGTCGGTGTACGCCCC 440
29            ------------------------------------------------------------
SGN           GTCGGCGTGACCACTCCCGGCCACCACGGAAGGCCGCCGGTGTACGTCGGTGTACGTCCC 450
SBIP          GTCGGCGTGACCACTCCCGGCCACCACGGAAGGCCGCCGGTGTACGTCGGTGTACGTCCC 140

1             GGACCGTCACCTTTTGTTTACAATTACGCCGCCAAAGATGATCAACTTCACGACAACCCA 510
4             GGACCATCACCGTTTGTTTACAATTACGCCGCCAAAGATGATCAACTTCACGACAACCCA 519
9             GGACCATCACCGTTTGTTTACAATTACGCCGCCAAAGATGATCAACTTCACGACAACCCA 450
16            GGACCATCACCGTTTGTTTACAATTACGCCGCCAAAGATGATCAACTTCACGACAACCCA 500
29            -----------------------------------ATGATCAACTTCACGACAACCCA 23
SGN           GGACCGTCACCTTTTGTTTACAATTACGCCGCCAAAGATGATCAACTTCACGACAACCCA 510
SBIP          GGACCGTCACCTTTTGTTTACAATTACGCCGCCAAAGATGATCAACTTCACGACAACCCA 200
                                                ***********************

1             AACGTCGCTCTTTTTTTCCTTGAGAAAGACTTGCACCAAGGTAGTAACATGAACTTGCAG 570
4             AACGTCGCTCTTTTTTTCCTCGAGAAGGACTTGCACCAAGGGAGTAACATGAACTTACAA 579
9             AACGTCGCTCTTTTTTTCCTCGAGAAGGACTTGCACCAAGGGAGTAACATGAACTTACAA 510
16            AACGTCGCTCTTTTTTTCCTCGAGAAGGACTTGCACCAAGGGAGTAACATGAACTTACAA 560
29            AACGTCGCTCTTTTTTTCCTCGAGAAGGACTTGCACCAAGGGAGTAACATGAACTTACAA 83
SGN           AACGTCGCTCTTTTTTTCCTTGAGAAAGACTTGCACCAAGGTAGTAACATGAACTTGCAG 570
SBIP          AACGTCGCTCTTTTTTTCCTTGAGGAAGACTTGCACCAAGGTAGTAACATGAACTTGCAG 260
              ******************** *** * ************** ************** **

1             TTTGTGAAAACTACAAAAAATGCCGCCACTTTCTTGCCTCGCCAAGTAGCAAATTCTATT 630
4             TTTGTGAAAACTACAAATACTGCCGCCACTTTCTTGCCTCGCCAAGTCGCAAATTCTATT 639
9             TTTGTGAAAACTACAAATACTGCCGCCACTTTCTTGCCTCGCCAAGTCGCAAATTCTATT 570
16            TTTGTGAAAACTACAAATACTGCCGCCACTTTCTTGCCTCGCCAAGTCGCAAATTCTATT 620
29            TTTGTGAAAACTACAAATACTGCCGCCACTTTCTTGCCTCGCCAAGTCGCAAATTCTATT 143
SGN           TTTGTGAAAACTACAAAAAATGCCGCCACTTTCTTGCCTCGCCAAGTAGCAAATTCTATT 630
SBIP          TTTGTGAAAACTACAAAAAATGCCGCCACTTTCCTGCCTCGCCAAGTAGCAAATTCTATT 320
              ***************** * ************* ************* ************
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```
1             CCCTTTTCGTCAAACAAGATGCCAGAAATTCTTAGCCAGTTTTCAGTTAAGCCCGACTCT 690
4             CCCTTTTCGTCAAACAAGATGCCAGAAATTCTTAATCAGTTTTCAGTTAAACCCGACTCT 699
9             CCCTTTTCGTCAAACAAGATGCCAGAAATTCTTAATCAGTTTTCAGTTAAACCCGACTCT 630
16            CCCTTTTCGTCAAACAAGATGCCAGAAATTC------------------------------ 651
29            CCCTTTTCGTCAAACAAGATGCCAGAAATTCTTAATCAGTTTTCAGTTAAACCCGACTCT 203
SGN           CCCTTTTCGTCAAACAAGATGCCAGAAATTCTTAGCCAGTTTTCAGTTAAGCCCGACTCT 690
SBIP          CCCTTTTCGTCAAACAAGATGCCAGAAATTCTTAGCCAGTTTTCAGTTAAGCCCGACTCT 380
              ******************************

1             GAAGAAGCTCAGATCTTGAAGCAAACTGTCCAAGAATGTGAAGAGCCAGGTATTAAAGGA 750
4             GAAGAAGCTCAGATCTTGAAGCAGACTGTACAAGAATGTGAACAGCCAGGTATTAAAGGA 759
9             GAAGAAGCTCAGATCTTGAAGCAGACTGTACAAGAATGTGAACAGCCAGGTATTAAAGGA 690
16            ------------------------------AAGAATGTGAACAGCCAGGTATTAAAGGA 680
29            GAAGAAGCTCAGATCTTGAAGCAGACTGTACAAGAATGTGAACAGCCAGGTATTAAAGGA 263
SGN           GAAGAAGCTCAGATCTTGAAGCAAACTGTCCAAGAATGTGAAGAGCCAGGTATTAAAGGA 750
SBIP          GAAGAAGCTCAGATCTTGAAGCAAACTGTCCAAGAATGTGAAGAGCCAGGTATTAAAGGA 440
                                          *********** ****************

1             GAAGAGAAGTACTGTGCTACTTCATTAGAATCCATGGTTGATTTCAGCACATCAAAGTTA 810
4             GAAGAGAAGTATTGTGCTACTTCATTAGAATCCATGGTTGATTTCAGCACATCAAAGTTA 819
9             GAAGAGAAGTATTGTGCTACTTCATTAGAATCCATGGTTGATTTCAGCACATCAAAGTTA 750
16            GAAGAGAAGTATTGTGCTACTTCATTAGAATCCATGGTTGATTTCAGCACATCAAAGTTA 740
29            GAAGAGAAGTATTGTGCTACTTCATTAGAATCCATGGTTGATTTCAGCACATCAAAGTTA 323
SGN           GAAGAGAAGTACTGTGCTACTTCATTAGAATCCATGGTTGATTTCAGCACATCAAAGTTA 810
SBIP          GAAGAGAAGTACTGTGCTACTTCATTAGAATCCATGGTTGATTTCAGCACATCAAAGTTA 500
              *********** **********************************************

1             GGAAACAAAGTACAACCAGTATCAACAGAGACAGAGAAGGAAACACAAATGCAGAAATAC 870
4             GGAAACAAAGTGCAACCAGTGTCAACAGAGACAGAGAAGGAAACTCAAATGCAGAAATAC 879
9             GGAAACAAAGTGCAACCAGTGTCAACAGAGACAGAGAAGGAAACTCAAATGCAGAAATAC 810
16            GGAAACAAAGTGCAACCAGTGTCAACAGAGACAGAGAAGGAAACTCAAATGCAGAAATAC 800
29            GGAAACAAAGTGCAACCAGTGTCAACAGAGACAGAGAAGGAAACTCAAATGCAGAAATAC 383
SGN           GGAAACAAAGTACAACCAGTATCAACAGAGACAGAGAAGGAAACACAAATGCAGAAATAC 870
SBIP          GGAAACAAAGTACAACCAGTATCAACAGAGACAGAGAAGGAAACACAAATGCAGAAATAC 560
              *********** ******** ********************** ***************

1             ACAATTCTAGGAGCCAAGAAAATGGGAAATGGGAAATCTGATGCTGTAGTTTGCCACAAG 930
4             ACAATTCTAGGAGCCAAGAAAATGGGAAATGGGAAATCTGATGCTGTAGTTTGCCACAAG 939
9             ACAATTCTAGGAGCCAAGAAAATAGGAAATGGGAAATCTGATGCTGTAGTTTGCCACAAG 870
16            ACAATTCTAGGAGCCAAGAAAATAGGAAA------------------------------- 829
29            ACAATTCTAGGAGCCAAGAAAATAGGAAATGGGAAATCTGATGCTGTAGTTTGCCACAAG 443
SGN           ACAATTCTAGGAGCCAAGAAAATGGGAAATGGGAAATCTGATGCTGTAGTTTGCCACAAG 930
SBIP          ACAATTCTAGGAGCCAAGAAAATGGGAAATGGGAAATCTGATGCTGTAGTTTGCCACAAG 620
              ********************** *****

1             CAGAATTATGCATATGCAGTTTTCTACTGTCATAAAACAGAAACAACGGAGTCATACATG 990
4             CAGAATTATGCATATGCAGTTTTCTACTGTCATAAAACAGAAACAACGGAGTCATACATG 999
9             CAGAATTATGCATATGCAGTTTTCTACTGTCATAAAACAGAAACAACGGAGTCATACATG 930
16            ------------------------------CATAAAACAGAAACCACAGAGTCATACATG 859
29            CAGAATTATGCATATGCAGTTTTCTACTGTCATAAAACAGAAACCACAGAGTCATACATG 503
SGN           CAGAATTATGCATATGCAGTTTTCTACTGTCATAAAACAGAAACAACGGAGTCATACATG 990
SBIP          CAGAATTATGCATATGCAGTTTTCTACTGTCATAAAACAGAAACAACGGAGTCATACATG 680
                                          ************** ** ************

1             GTTTCTTTGGTTGGTGCTGATGGAACAAAGGCTAAAGCAGTAGCTGTTTGCCATAAGGAT 1050
4             GTTTCTTTGGTTGGTGCTGATGGAACAAAGGCTAAAGCAGTAGCTGTTTGCCATAAGGAT 1059
9             GTTTCTTTGGTTGGTGCTGATGGAACAAAGGCTAAAGCAGTAGCTGTTTGCCATAAGGAT 990
16            GTTTCTTTGGTTGGTGCTGATGGAACAAAG------------------------------ 889
29            GTTTCTTTGGTTGGTGCTGATGGAACAAAGGCTAAAGCAGTAGCTGTTTGCCATAAGAAT 563
SGN           GTTTCTTTGGTTGGTGCTGATGGAACAAAGGCTAAAGCAGTAGCTGTTTGCCATAAGGAT 1050
SBIP          GTTTCTTTGGTTGGTGCTGATGGAACAAAGGCTAAAGCAGTAGCTGTTTGCCATAAGGAT 740
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1               ACTTCAGCTTGGAATCCAAAACATTTGGCTTTTAAAGTTCTTAAGGTTACACCTGGATCT 1110
4               ACTTCAGCTTGGAATCCAAAGCATTTGGCTTTTAAAGTTCTTAAGGTTACACCGGGATCT 1119
9               ACTTCAGCTTGGAATCCAAAACATTTGGCTTTTAAAGTTCTTAAGGTTACACCTGGATCT 1050
16              ----------------------------TTTAAAGTTCTTAAGGTTACACCGGGATCT 919
29              ACTTCAGCTTGGAATCCAAAGCATTTGGCTTTTAAAGTTCTTAAGGTTACACCGGGATCT 623
SGN             ACTTCAGCTTGGAATCCAAAACATTTGGCTTTTAAAGTTCTTAAGGTTACACCTGGATCT 1110
SBIP            ACTTCAGCTTGGAATCCAAAACATTTGGCTTTTAAAGTTCTTAAGGTTACACCTGGATCT 800
                                          ********************** ******

1               GTTCCTGTTTGCCATTTCCTTCCTGAGGATCACATTGTTTGGGTTCCTAAGAACTAG 1167
4               GTTCCTGTTTGCCATTTCCTTCCTGAGGATCACATTGTTTGGGTTCCTAAGAACTAG 1176
9               GTTCCTGTTTGCCATTTCCTTCCTGAGGATCACATTGTTTGGGTTCCTAAGAACTAG 1107
16              GTTCCTGTTTGCCATTTCCTTCCTGAGGATCACATTGTTTGGGTTCCTAAGAACTAG 976
29              GTTCCTGTTTGCCATTTCCTTCCTGAGGATCACATTGTTTGGGTTCCTAAGAACTAG 680
SGN             GTTCCTGTTTGCCATTTCCTTCCTGAGGATCACATTGTTTGGGTTCCTAAGAACTAG 1167
SBIP            GTTCCTGTTTGCCATTGCCTTCCTGAGGATCACATTGTTTGGGTTCCTAAGAACTAG 857
                 **************** ****************************************
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Figure 19: Nucleotide Sequence Alignment of the Sequenced Clones (1,4,9,16, and 29) (Recombinant pDONR221-SBIP-355) with SBIP-355 Nucleotide sequence. The alignment is based on SGN-U446947 (SGN) and the partial nucleotide sequence of SBIP-355 obtained from yeast 2-hybrid screening (SBIP).

```
1               MELKLLHVLTYLSLALVASYAALPPASSGQTYWNTKLPNTPIPKAIKDSLQPTGLT---E 57
4               MELKLLHILTYLSLALVASYAALS-PSSGQTYWNTKLPNTPMPKAIKDSLQPTDFAGLTE 59
9               MELKLLHILTYLSLALVASYAALS-PSSGQTYWNTKLPNTPMPKAIKDSLQPTGLT---E 56
16              ------------------------------------------------------------
SGN             MELKLLHVLTYLSLALVASYAALPPASSGQTYWNTKLPNTPIPKAIKDSLQPTGLT---E 57
29              ------------------------------------------------------------
SBIP            ------------------------------------------------------------

1               DKSTSVEVGKGGVNVNTGKGHSGSGTNVNVGHKGVGVSTGKGHSGRG-ANVNVGHKGVGV 116
4               DKTTSVVVGKGGVNVNTGKGHSGSGTNVNVGRKGVGVSTGKGHSGRGGTNVNVGHKGVGV 119
9               DKTTSVVVGKGGVNVNTGKGHSGSG----------------------TNVNVGHKGVGV 93
16              ------------------------------------------------------------
29              ------------------------------------------------------------
SGN             DKSTSVEVGKGGVNVNTGKGHSGSGTNVNVGHKGVGVSTGKGHSGRG-ANVNVGHKGVGV 116
SBIP            ---------------------------------------------ANVNVGHKGVGV 12

1               STG------GGTHVGVGKGGVGVTTPGHHGRPPVYVGVRPGPSPFVYNYAAKDDQLHDNP 170
4               STG------GGTHVGVGKGGVGVTTPGHHGRPPVYVGVRPGPSPFVYNYAAKDDQLHDNP 173
9               STGKGHSGRGGTNVNVGHKGVGVSTG---GRPPVYVGVRPGPSPFVYNYAAKDDQLHDNP 150
16              ------------------------------------------------------------
29              ------------------------------------------------------------
SGN             STG------GGTHVGVGKGGVGVTTPGHHGRPPVYVGVRPGPSPFVYNYAAKDDQLHDNP 170
SBIP            STG------GGTHVGVGKGGVGVTTPGHHGRPPVYVGVRPGPSPFVYNYAAKDDQLHDNP 66
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1               NVALFFLEKDLHQGSNMNLQFVKTTKNAATFLPRQVANSIPFSSNKMPEILSQFSVKPDS 230
4               NVALFFLEKDLHQGSNMNLQFVKTTNTAATFLPRQVANSIPFSSNKMPEILNQFSVKPDS 233
9               NVALFFLEKDLHQGSNMNLQFVKTTNTAATFLPRQVANSIPFSSNKMPEILNQFSVKPDS 210
16              ----------------MELKLLHILTYLSLAL---VASYAALSPSSGQTYWN--TKLPNT  39
29              ----------------MNLQFVKTTNTAATFLPRQVANSIPFSSNKMPEILNQFSVKPDS  44
SGN             NVALFFLEKDLHQGSNMNLQFVKTTKNAATFLPRQVANSIPFSSNKMPEILSQFSVKPDS 230
SBIP            NVALFFLEEDLHQGSNMNLQFVKTTKNAATFLPRQVANSIPFSSNKMPEILSQFSVKPDS 126
                               *:*:::: . : * **. .:*...    . : *::

1               EEAQILKQTVQECEEPGIKGEEKYCATSLESMVDFSTSKLGNKVQPVSTETEKETQMQKY 290
4               EEAQILKQTVQECEQPGIKGEEKYCATSLESMVDFSTSKLGNKVQPVSTETEKETQMQKY 293
9               EEAQILKQTVQECEQPGIKGEEKYCATSLESMVDFSTSKLGNKVQPVSTETEKETQMQKY 270
16              PMPKAIKDSLQPTG----------------LMEDKTTSVVVGKGG-VNVNTGKG------  76
29              EEAQILKQTVQECEQPGIKGEEKYCATSLESMVDFSTSKLGNKVQPVSTETEKETQMQKY 104
SGN             EEAQILKQTVQECEEPGIKGEEKYCATSLESMVDFSTSKLGNKVQPVSTETEKETQMQKY 290
SBIP            EEAQILKQTVQECEEPGIKGEEKYCATSLESMVDFSTSKLGNKVQPVSTETEKETQMQKY 186
                  .: :*:::*                    * * :** : .*   *..:* *

1               TILGAKKMGNGKSDAVVCHKQNYAYAVFYCHKTETTESYMVSLVGADGTKAKAVAVCHKD 350
4               TILGAKKMGNGKSDAVVCHKQNYAYAVFYCHKTETTESYMVSLVGADGTKAKAVAVCHKD 353
9               TILGAKKIGNGKSDAVVCHKQNYAYAVFYCHKTETTESYMVSLVGADGTKAKAVAVCHKD 330
16              ------HSGSG-TNVNVGHKG----------------------VGVSTGKGHSEPTSTSA 107
29              TILGAKKIGNGKSDAVVCHKQNYAYAVFYCHKTETTESYMVSLVGADGTKAKAVAVCHKN 164
SGN             TILGAKKMGNGKSDAVVCHKQNYAYAVFYCHKTETTESYMVSLVGADGTKAKAVAVCHKD 350
SBIP            TILGAKKMGNGKSDAVVCHKQNYAYAVFYCHKTETTESYMVSLVGADGTKAKAVAVCHKD 246
                      : *.* ::. * **                        **..  *.:: ... .

1               TSAWNPKHLAFKVLKVTPGSVPVCHFLPEDHIVWVPKN 388
4               TSAWNPKHLAFKVLKVTPGSVPVCHFLPEDHIVWVPKN 391
9               TSAWNPKHLAFKVLKVTPGSVPVCHFLPEDHIVWVPKN 368
16              TKAWA---------------------------------- 112
29              TSAWNPKHLAFKVLKVTPGSVPVCHFLPEDHIVWVPKN 202
SGN             TSAWNPKHLAFKVLKVTPGSVPVCHFLPEDHIVWVPKN 388
SBIP            TSAWNPKHLAFKVLKVTPGSVPVCHCLPEDHIVWVPKN 284
                *.**
```

Figure 20: Amino Acid Sequence Alignment of the Sequenced Clones (1,4,9,16, and 29) (Recombinant pDONR221-SBIP-355) with SBIP-355 Amino Acid Sequence. The alignment is based on SGN-U446947 (SGN) and the partial amino acid sequence of SBIP-355 obtained from yeast 2-hybrid screening (SBIP).

```
1               ATGGAGTTGAAGCTCCTTCACGTCCTCACTTACCTTTCGTTGGCACTAGTGGCAAGTTAT 60
SGN             ATGGAGTTGAAGCTCCTTCACGTCCTCACTTACCTTTCGTTGGCACTAGTGGCAAGTTAT 60
SBIP            ------------------------------------------------------------


1               GCAGCTCTTCCTCCTGCATCGAGCGGTCAAACTTATTGGAATACTAAGCTGCCTAATACG 120
SGN             GCAGCTCTTCCTCCTGCATCGAGCGGTCAAACTTATTGGAATACTAAGCTGCCTAATACG 120
SBIP            ------------------------------------------------------------


1               CCCATCCCAAAGGCAATCAAAGATTCTCTGCAGCCAACTGGATTGACGGAGGACAAAAGC 180
SGN             CCCATCCCAAAGGCAATCAAAGATTCTCTGCAGCCAACTGGATTGACGGAGGACAAAAGC 180
SBIP            ------------------------------------------------------------


1               ACTTCAGTGGAAGTAGGCAAAGGCGGAGTAAACGTCAACACCGGCAAGGGGCACTCCGGC 240
SGN             ACTTCAGTGGAAGTAGGCAAAGGCGGAGTAAACGTCAACACCGGCAAGGGGCACTCCGGC 240
SBIP            ------------------------------------------------------------


1               AGCGGCACTAACGTCAACGTTGGCCACAAAGGTGTCGGCGTAAGCACTGGCAAGGGTCAC 300
SGN             AGCGGCACTAACGTCAACGTTGGCCACAAAGGTGTCGGCGTAAGCACTGGCAAGGGTCAC 300
SBIP            ------------------------------------------------------------


1               TCCGGCAGGGGAGCCAACGTCAACGTCGGCCACAAAGGCGTGGGCGTAAGCACCGGGGGC 360
SGN             TCCGGCAGGGGAGCCAACGTCAACGTCGGCCACAAAGGCGTGGGCGTAAGCACCGGGGGC 360
SBIP            ----------GAGCCAACGTCAACGTCGGCCACAAAGGCGTGGGCGTAAGCACCGGGGGC 50
                          **********************************************


1               GGAACCCACGTTGGCGTCGGCAAAGGAGGAGTCGGCGTGACCACTCCCGGCCACCACGGA 420
SGN             GGAACCCACGTTGGCGTCGGCAAAGGAGGAGTCGGCGTGACCACTCCCGGCCACCACGGA 420
SBIP            GGAACCCACGTTGGCGTCGGCAAAGGAGGAGTCGGCGTGACCACTCCCGGCCACCACGGA 110
                ************************************************************


1               AGGCCGCCGGTGTACGTCGGTGTACGTCCCGGACCGTCACCTTTTGTTTACAATTACGCC 480
SGN             AGGCCGCCGGTGTACGTCGGTGTACGTCCCGGACCGTCACCTTTTGTTTACAATTACGCC 480
SBIP            AGGCCGCCGGTGTACGTCGGTGTACGTCCCGGACCGTCACCTTTTGTTTACAATTACGCC 170
                ************************************************************


1               GCCAAAGATGATCAACTTCACGACAACCCAAACGTCGCTCTTTTTTTCCTTGAGAAAGAC 540
SGN             GCCAAAGATGATCAACTTCACGACAACCCAAACGTCGCTCTTTTTTTCCTTGAGAAAGAC 540
SBIP            GCCAAAGATGATCAACTTCACGACAACCCAAACGTCGCTCTTTTTTTCCTTGAGGAAGAC 230
                ************************************************** *****


1               TTGCACCAAGGTAGTAACATGAACTTGCAGTTTGTGAAAACTACAAAAAATGCCGCCACT 600
SGN             TTGCACCAAGGTAGTAACATGAACTTGCAGTTTGTGAAAACTACAAAAAATGCCGCCACT 600
SBIP            TTGCACCAAGGTAGTAACATGAACTTGCAGTTTGTGAAAACTACAAAAAATGCCGCCACT 290
                ************************************************************


1               TTCTTGCCTCGCCAAGTAGCAAATTCTATTCCCTTTTCGTCAAACAAGATGCCAGAAATT 660
SGN             TTCTTGCCTCGCCAAGTAGCAAATTCTATTCCCTTTTCGTCAAACAAGATGCCAGAAATT 660
SBIP            TTCCTGCCTCGCCAAGTAGCAAATTCTATTCCCTTTTCGTCAAACAAGATGCCAGAAATT 350
                *** ********************************************************


1               CTTAGCCAGTTTTCAGTTAAGCCCGACTCTGAAGAAGCTCAGATCTTGAAGCAAACTGTC 720
SGN             CTTAGCCAGTTTTCAGTTAAGCCCGACTCTGAAGAAGCTCAGATCTTGAAGCAAACTGTC 720
SBIP            CTTAGCCAGTTTTCAGTTAAGCCCGACTCTGAAGAAGCTCAGATCTTGAAGCAAACTGTC 410
                ************************************************************


1               CAAGAATGTGAAGAGCCAGGTATTAAAGGAGAAGAGAAGTACTGTGCTACTTCATTAGAA 780
SGN             CAAGAATGTGAAGAGCCAGGTATTAAAGGAGAAGAGAAGTACTGTGCTACTTCATTAGAA 780
SBIP            CAAGAATGTGAAGAGCCAGGTATTAAAGGAGAAGAGAAGTACTGTGCTACTTCATTAGAA 470
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                ************************************************************

1               TCCATGGTTGATTTCAGCACATCAAAGTTAGGAAACAAAGTACAACCAGTATCAACAGAG 840
SGN             TCCATGGTTGATTTCAGCACATCAAAGTTAGGAAACAAAGTACAACCAGTATCAACAGAG 840
SBIP            TCCATGGTTGATTTCAGCACATCAAAGTTAGGAAACAAAGTACAACCAGTATCAACAGAG 530
                ************************************************************

1               ACAGAGAAGGAAACACAAATGCAGAAATACACAATTCTAGGAGCCAAGAAAATGGGAAAT 900
SGN             ACAGAGAAGGAAACACAAATGCAGAAATACACAATTCTAGGAGCCAAGAAAATGGGAAAT 900
SBIP            ACAGAGAAGGAAACACAAATGCAGAAATACACAATTCTAGGAGCCAAGAAAATGGGAAAT 590
                ************************************************************

1               GGGAAATCTGATGCTGTAGTTTGCCACAAGCAGAATTATGCATATGCAGTTTTCTACTGT 960
SGN             GGGAAATCTGATGCTGTAGTTTGCCACAAGCAGAATTATGCATATGCAGTTTTCTACTGT 960
SBIP            GGGAAATCTGATGCTGTAGTTTGCCACAAGCAGAATTATGCATATGCAGTTTTCTACTGT 650
                ************************************************************

1               CATAAAACAGAAACAACGGAGTCATACATGGTTTCTTTGGTTGGTGCTGATGGAACAAAG 1020
SGN             CATAAAACAGAAACAACGGAGTCATACATGGTTTCTTTGGTTGGTGCTGATGGAACAAAG 1020
SBIP            CATAAAACAGAAACAACGGAGTCATACATGGTTTCTTTGGTTGGTGCTGATGGAACAAAG 710
                ************************************************************

1               GCTAAAGCAGTAGCTGTTTGCCATAAGGATACTTCAGCTTGGAATCCAAAACATTTGGCT 1080
SGN             GCTAAAGCAGTAGCTGTTTGCCATAAGGATACTTCAGCTTGGAATCCAAAACATTTGGCT 1080
SBIP            GCTAAAGCAGTAGCTGTTTGCCATAAGGATACTTCAGCTTGGAATCCAAAACATTTGGCT 770
                ************************************************************

1               TTTAAAGTTCTTAAGGTTACACCTGGATCTGTTCCTGTTTGCCATTTCCTTCCTGAGGAT 1140
SGN             TTTAAAGTTCTTAAGGTTACACCTGGATCTGTTCCTGTTTGCCATTTCCTTCCTGAGGAT 1140
SBIP            TTTAAAGTTCTTAAGGTTACACCTGGATCTGTTCCTGTTTGCCATTGCCTTCCTGAGGAT 830
                ******************************************** *************

1               CACATTGTTTGGGTTCCTAAGAACTAG 1167
SGN             CACATTGTTTGGGTTCCTAAGAACTAG 1167
SBIP            CACATTGTTTGGGTTCCTAAGAACTAG 857
                ***************************
```

Figure 21: Nucleotide Sequence Alignment of Clone #1 the Highest Homologue with SBIP-355 Nucleotide Sequence. The alignment is based on SGN-U446947 (SGN) and the partial nucleotide sequence of SBIP-355 obtained from yeast 2-hybrid screening (SBIP).

```
1               MELKLLHVLTYLSLALVASYAALPPASSGQTYWNTKLPNTPIPKAIKDSLQPTGLTEDKS 60
SGN             MELKLLHVLTYLSLALVASYAALPPASSGQTYWNTKLPNTPIPKAIKDSLQPTGLTEDKS 60
SBIP            ------------------------------------------------------------

1               TSVEVGKGGVNVNTGKGHSGSGTNVNVGHKGVGVSTGKGHSGRGANVNVGHKGVGVSTGG 120
SGN             TSVEVGKGGVNVNTGKGHSGSGTNVNVGHKGVGVSTGKGHSGRGANVNVGHKGVGVSTGG 120
SBIP            -------------------------------------------ANVNVGHKGVGVSTGG 16
                                                           ****************
```

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1               GTHVGVGKGGVGVTTPGHHGRPPVYVGVRPGPSPFVYNYAAKDDQLHDNPNVALFFLEKD 180
SGN             GTHVGVGKGGVGVTTPGHHGRPPVYVGVRPGPSPFVYNYAAKDDQLHDNPNVALFFLEKD 180
SBIP            GTHVGVGKGGVGVTTPGHHGRPPVYVGVRPGPSPFVYNYAAKDDQLHDNPNVALFFLEED 76
                ************************************************************:*

1               LHQGSNMNLQFVKTTKNAATFLPRQVANSIPFSSNKMPEILSQFSVKPDSEEAQILKQTV 240
SGN             LHQGSNMNLQFVKTTKNAATFLPRQVANSIPFSSNKMPEILSQFSVKPDSEEAQILKQTV 240
SBIP            LHQGSNMNLQFVKTTKNAATFLPRQVANSIPFSSNKMPEILSQFSVKPDSEEAQILKQTV 136
                ************************************************************

1               QECEEPGIKGEEKYCATSLESMVDFSTSKLGNKVQPVSTETEKETQMQKYTILGAKKMGN 300
SGN             QECEEPGIKGEEKYCATSLESMVDFSTSKLGNKVQPVSTETEKETQMQKYTILGAKKMGN 300
SBIP            QECEEPGIKGEEKYCATSLESMVDFSTSKLGNKVQPVSTETEKETQMQKYTILGAKKMGN 196
                ************************************************************

1               GKSDAVVCHKQNYAYAVFYCHKTETTESYMVSLVGADGTKAKAVAVCHKDTSAWNPKHLA 360
SGN             GKSDAVVCHKQNYAYAVFYCHKTETTESYMVSLVGADGTKAKAVAVCHKDTSAWNPKHLA 360
SBIP            GKSDAVVCHKQNYAYAVFYCHKTETTESYMVSLVGADGTKAKAVAVCHKDTSAWNPKHLA 256
                ************************************************************

1               FKVLKVTPGSVPVCHFLPEDHIVWVPKN 388
SGN             FKVLKVTPGSVPVCHFLPEDHIVWVPKN 388
SBIP            FKVLKVTPGSVPVCHCLPEDHIVWVPKN 284
                *************** ************
```

Figure 22: Amino Acid Sequence Alignment of Clone #1 the Highest Homologue with SBIP-355 Amino Acid Sequence. The alignments arebased on SGN-U446947 (SGN) and the partial amino acid sequence of SBIP-355 obtained from yeast 2-hybrid screening

Subconing of Recombinant pDONR221-SBIP-355 into pDEST17 Expression Vector Using LR Recombination Reaction

LR recombination reaction was performed with the Recombinant pDONR221-SBIP-355 and Gateway destination vector pDEST17 for protein expression in *E.coli*. Colony PCR was carried out by using SBIP-355 forward and reverse primers to confirm successful insertion of SBIP-355 into pDEST17 (Fig.23). Plasmid was isolated from bacterial culture using Invitrogen Pure Link Quick Plasmid Miniprep kit, measured by the NanoDrop Spectrophotometer to quantify the concentration and tested for the quality on 0.8% agarose gel (Fig.24).

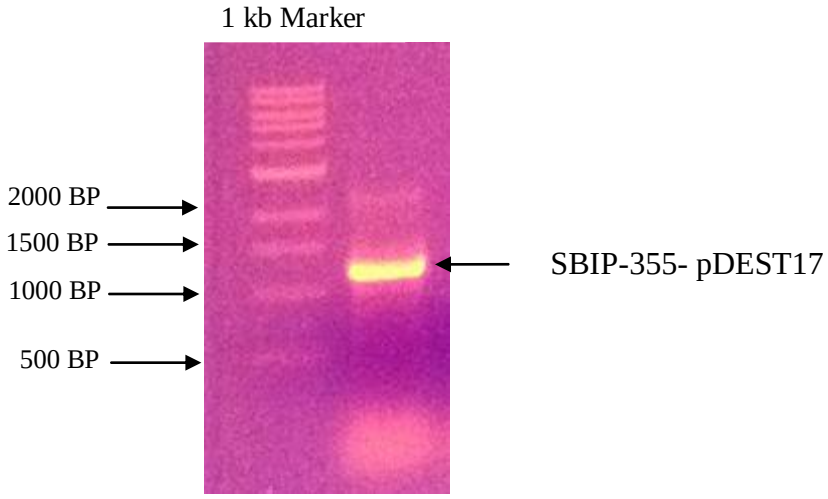

Figure 23: 0.8% Agarose Gel Stained with EtBr for the Confirmation of the Insertion of SBIP-355 into pDEST17.

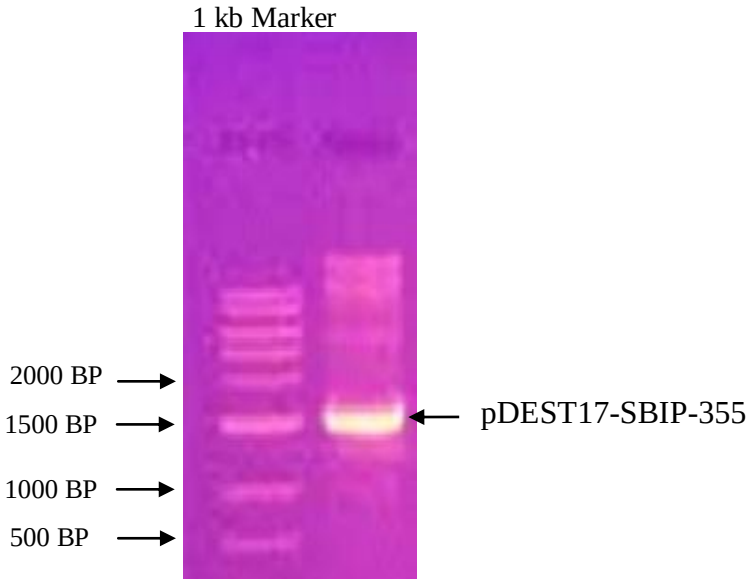

Figure 24: 0.8% Agarose gel Stained with EtBr for the Confirmation of the Quality of the Isolated pDEST17-SBIP-355 using Plasmid Miniprep Kit.

Small Scale Expression of Recombinant SBIP-355 Protein in *E.coli*

To confirm the expression of recombinant SBIP-355 protein, a small scale expression screening test was performed by using *E.coli* BL21 (DE3) Magic cells carrying pDEST17-SBIP-355. The expression was tested using a sample of bacterial culture induced with 1mM IPTG and uninduced sample as a control. It was analyzed on a 12 % SDS PAGE gel; stained with Coomassie blue to visualize the bands of proteins (Fig. 25.A). Following this, the gel was transferred to confirm the expression using western blot analysis to detect SBIP-355. Result from this analysis shows that a protein with MW ~41 kDa is present (Fig. 25.C).

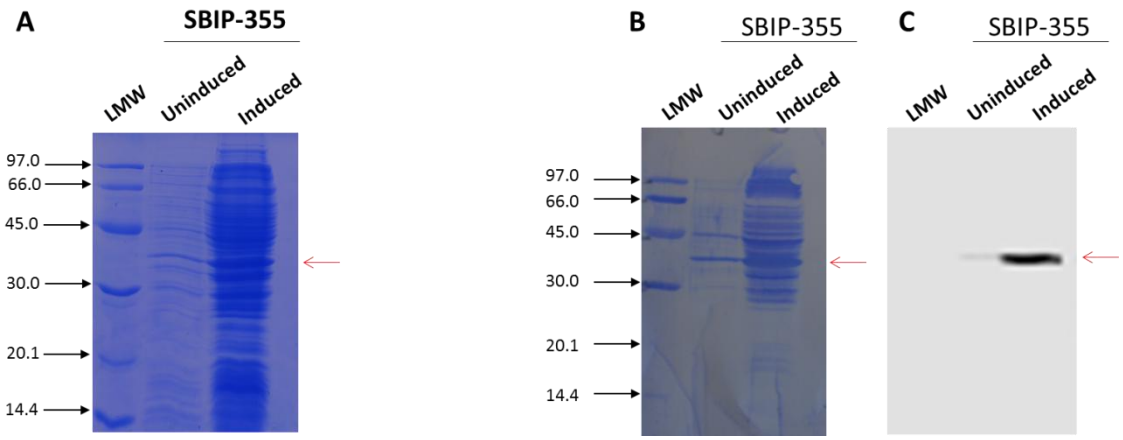

Figure 25: Expression of SBIP-355. (A) SDS-PAGE analysis of pDEST17-SBIP-355 Recombinant Protein Expression in *E. coli*. LMW: low molecular weight protein marker; Uninduced lane shows SBIP-355 protein expression without IPTG induction; Induced lane shows SBIP-355 protein expression with 1 mM IPTG induction. The red arrows are indicating the expected size of SBIP-355 (~41kDa). (B) Corresponding PVDE membrane restained with coomassie blue following immunoblotting. (C) Western blot analysis using anti-His antibodies. Blot was developed using Western Sure reagent from Licor and scanned using digital C-DiGit scanner.

CHAPTER 4

DISCUSSION

The members of plant specific BURP domain protein family are growing rapidly and over 270 deduced proteins from different species of plants have been submitted to the GenBank database to date (Peng et al. 2011). Their functions are still unknown; however, it is suggested that they are involved in cellular secretion pathway during embryogenesis, seed, fruit, and root development (Peng et al. 2011).

SBIP-355 from *Nicotiana tabacum* is a novel gene encoding a BURP domain-containing protein. In this study it was successfully isolated and cloned using Gateway cloning system, and then, SBIP-355 protein was expressed in *E.coli*. The sequence analysis shows that the SBIP-355 gene is 1399 bp in length and 1167 bp of ORF. BLAST search demonstrates that SBIP-355 shares high amino acid sequence identity to other BURP domain containing proteins. The coding sequence of SBIP-355 encodes a 388 amino acid polypeptide with a calculated molecular mass of ~41 kDa. Signal peptide prediction shows that SBIP-355 protein is a secreted protein. It also shows it has a signal peptide, the first 21 amino acids region. The BURP domain of SBIP-355 protein consisted of around 230 amino acids and has typical characteristics, such as 2 phenylalanines (FF) and four cysteine-histidine (CH) motifs (Figure.11) (Peng et al. 2011).

To investigate the evolutionary relationships of SBIP-355, a phylogenetic tree was generated from the amino acid sequences of BURP domain-containing proteins from various plant species. Phylogenetic analysis reveals that SBIP-355 clustered into the clade with several RD22-like proteins. Some of the RD22-like proteins from different plants have been isolated and studied. It was found that they are stress-responsive. *RD22* from *Arabidopsis*, 4 genes from

Bruguiera gymnorrhiza (*BgBDC1*, 2, 3, and 4), *PpRD22* from *Prunus persica* and *GhRDL* from *Gossypium hirsutum* were expressed under water deficit conditions and abscisic acid treatment (ABA) (Callahan et al. 1993; Li et al. 2002; Banzai et al. 2011). Thus, SBIP-355 might be a stress-inducible gene and encode a dehydration-responsive protein, which is important for the stress tolerance in tobacco.

As mentioned previously, plant growth is severely affected by harsh environmental conditions. Many stress-responsive genes from different protein families have been analyzed and used in genetic engineering to support plants against the stress tolerances. BURP domain-containing proteins have shown great possibility in the uses for stress-resistant transgenic crops. Thus, the isolation and analysis of SBIP-355 is relevant and would provide a base for further studies to investigate its role and functions and how to better use that in agriculture.

Future Directions

To achieve to the goal of this research and determine if SABP2 has an effect in the expression of SBIP-355, investigations are required to reach a conclusion about the interaction between SABP2 and SBIP-355. Future experiments should be performed using (1-2) plants in which SABP2 gene expression is silenced by RNA interference, and also by using (C3) plants from plant lines containing empty silencing vector (pHANNIBAL) and *Nicotiana tabacum* cv. *Xanthi nc* (Kumar and Klessig 2003); inoculated with various concentration of NaCl and then collection of leaves samples within different set of times to measure the expression levels of SBIP-355 and SABP2. In addition, the physical interaction between SBIP-355 and SABP2 should be confirmed by performing pull down assays using recombinant SABP2 and SBIP-355.

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APPENDICES

Appendix A – Abbreviations

1-2 - SABP2 Silenced Plants

Avr - Avirulence

ABA- Abscisic acid

BA2H - Benzoic-2-hydroxylase

βME - βeta mercaptoethanol

cDNA-Complementary DNA

DNA-Deoxyribonucleic acid

dNTP-deoxyribonucleoside triphosphate

E. coli-Escherichia coli

EFalpha1 - Elongation Factor alpha 1

ET – Ethylene

EtBr-Etidium bromide

HR - Hypersensitive response

ICS - Isochorismate synthase

IPTG-Isopropylthiogalactosidase

JA - Jasmonic acid

Kb-kilo base

kDa - Kilo Dalton

LB-Luria-Bertani broth

MeSA - Methyl salicylate

min-minutes(s)

ml - milli litre

mM - milli Molar

mRNA-messenger RNA

NahG Plants expressing salicylate hydroxylase which converts SA to catechol

NCED-9-cis epoxycarotenoid dioxygenase

nm-nano meter.

OD - Optical Density

PR - Pathogenesis Resistance genes

R-genes - Resistance genes

PCR-Polymerase Chain Reaction

RNA-Ribonucleic acid

SA - Salicylic acid

SABP2 - Salicylic acid binding protein 2

SAR - Systemic acquired resistance

SAMT - Salicylic acid methyl transferase

SBIP-355 - SABP2 Interacting Protein-355

SDS PAGE - Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

TAE - Tris-Acetate EDTA

TMV - Tobacco mosaic virus

UV - Ultra violet

µg - micro gram

µl - micro litre

XNN- *Nicotiana tabacum* cv. Xanthi nc (Wild-type tobacco plants)

ZEP-Zeaxanthin epoxidase

Appendix B – Buffers and Reagents

0.1% Diethyl Pyrocarbonate Treated Water (DEPC)

Diethyl pyrocarbonate = 0.1 ml

Distilled water = 100 ml

It was incubated for overnight at 37°C and the following day, it was autoclaved for 15 minutes.

1.2% Agarose gel

0.60 g agarose

50 ml distilled water

2.5µl ethidium bromide (10 mg/ml)

To prepare 0.8% Agarose gel: 0.40 g agarose was added.

50X Tris Acetate EDTA (TAE) Electrophoresis Buffer:

242.0 g Tris base

57.1 ml Glacial acetic acid (M.W.: 60.05).

100 ml 0.5 M EDTA (pH 8.0 at 25°C)

Distilled water was added to bring the volume to 1L

To prepare 1L 1X TAE Buffer: 980 ml distilled water was added to 20 ml 50X TAE Buffer.

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 by adding NaOH

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 by adding NaOH

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)

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 prior to use.

10x Phosphate Buffer Saline (10x PBS)

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

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

Sodium Phosphate monobasic (4.1g), M.W. = 119.96g/mol, final conc. = 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 conc. = 1.0%

BSA (3g), final conc. = 3.0%

10x Western Blotting Transfer Buffer (1L)

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

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

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

Coomassie Blue staining solution

1 g of Coomassie Blue

400 ml H_2O

500 ml methanol

100 ml acetic acid

Destaining solution for coomassie blue

500 ml distilled H_2O

400 ml methanol

100 ml acetic acid

Ponceau S Stain (100mL)

Ponceau S (0.1g), final conc. = 0.1%

Acetic acid = 5 ml, final conc. = 5.0%

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