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Localization of SIP470, a Plant Lipid Transfer Protein in Nicotiana tabacum

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Localization of SIP470, a Plant Lipid Transfer Protein in *Nicotiana tabacum*

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by

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ABSTRACT

Localization of SIP470, a Plant Lipid Transfer Protein in *Nicotiana tabacum*

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SABP2-interacting protein 470 (SIP470), a non-specific lipid transfer protein (nsLTP), was discovered in a yeast two-hybrid screening using SABP2 as bait and tobacco leaf proteins as prey. SABP2 is an important enzyme in systemic acquired resistance that converts salicylic acid to methyl salicylate. Localization studies are an important aspect to understanding the biological function of proteins. nsLTPs are generally considered apoplastic proteins and has been localized intracellularly and extracellularly. Transient expression shows highest expression of SIP470-eGFP at 2 days post infiltration into *Nicotiana benthamiana*. Confocal microscopy showed localization near the periphery of the cell. Subcellular localization using differential centrifugation showed that SIP470 is localized in the mitochondria. Mitochondria membranes are rich in lipids and have shown lipid exchange with the endoplasmic reticulum in mammalian systems. Co-localization of SIP470-eGFP+mCherry did not express complete co-localization in the targeted organelles. Co-localization pattern suggests possible localization in the endoplasmic reticulum.

DEDICATION

To my Lord and Savior, who directs my path and orders my steps.

To my parents Sonia Leader, Malcolm Leader and Jeffrey Andrews for their love and support. To my sister, Shatarah Cooper, I am forever grateful for your love, encouragement and support.

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INTRODUCTION

Plant defense systems are highly sophisticated which allows plants to survive in even the most unfavorable conditions. As autotrophs, they are fundamental in the food chain and most organisms are dependent on them for sustenance. Preservation of plants, particularly crops, is important due to biotic challenge from pathogens and viruses, animal pests, weeds and many others. In crops such as rice, potato and wheat loss potentials are as high as 6 – 28% (Oerke and Dehne 2004). Many strategies are used to improve plant resistance and sustainment. Conventional methods such as breeding, pedigree, and backcrossing have been used for decades (Miah et al. 2013). The use of chemicals such as pesticides, insecticides, and fungicides are widely utilized to control plant disease outbreaks. While these chemicals have proven effective, they can lead to adverse health issues to consumers and farmers over time (Kesavachandran et al. 2009). Bacteriophages are used as a biocontrol method against pathogens. This system has been advantageous because of its host specificity and its non-pathogenicity for humans (Czajkowski et al. 2014). To develop more resistant strains of crops or to enhance farming practices, a better understanding of plant immune systems is necessary.

Plant Immune System

Plants have developed a sophisticated and intricate defense system which has contributed to their overall success; despite being sessile organisms. Plant-microbe interactions can be mutualistic and hence beneficial for both organisms as well as it can be parasitic and deleterious for one of the organisms involved. In non-mutualistic

relationships, plants have various protection mechanisms. These mechanisms may include physical structures such as bark, trichomes, cuticle and leaf hair among others which protect the plant from invading pathogens. As a defense mechanism, plants can undergo physiological changes including suberization, callose deposition, lignification of the cell wall (Nurnberger et al. 2004).

Plants possess pattern recognition receptors (PRRs) that can detect pathogen-associated molecular patterns (PAMPs) present on microbes (Zipfel and Felix 2005; Jones and Dangl 2006). This detection activates PAMP-triggered immunity (PTI) which minimizes or stops colonization. Pathogens that have circumvented or suppressed PTI deploy effectors (called avirulence (avr) factor) which specifically interferes with PTI and leads to effector-triggered susceptibility (ETS) in plants. These effectors are recognized by nucleotide binding and leucine-rich repeats (NB-LRR) proteins which initiates effector-triggered immunity (ETI) (Jones and Dangl 2006). ETI is a more robust and accelerated PTI response at the site of infection that activates hypersensitive response through reactive oxygen species (ROS) production whereby initiating hormone defense signaling (Jones and Dangl 2006) and an increased expression of pathogenesis-related (PR) genes (Vlot et al. 2009). This form of plant response is known as ‘local’ defense. Plants also can acquire long-distance immunity from initial pathogen interactions, which is known as systemic acquired resistance (SAR).

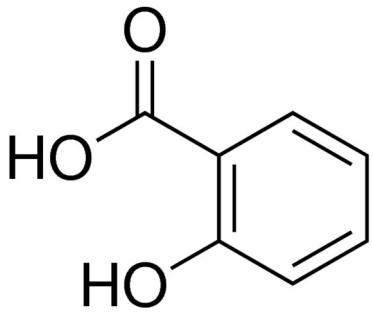


Figure 1: Chemical Structure of Salicylic Acid

Salicylic Acid and its Biosynthesis

Salicylic acid (SA) is a phenolic compound that is involved in many integral plant processes including allelopathy, lignin biosynthesis, enhanced pathogenic resistance, regulation of responses to environmental stresses (Dempsey et al. 2011) and thermogenesis (Vlot et al. 2009). SA is considered one of the most import signal hormones in plant defense regulation and has been studied extensively for several decades. It has numerous physiological roles in plants including seedling germination, flowering, thermotolerance, drought tolerance and photosynthesis (Kumar 2014).

Two distinct pathways have been discovered for the biosynthesis of SA which are the phenylalanine ammonia-lyase-mediated (PAL) pathway and isochorismate synthase-mediated (ICS) pathway. In the PAL-mediated pathway, SA is synthesized from ortho-couramic acid, a derivative of cinnamic acid (Dempsey et al. 2011). The ICS-mediated pathway is utilized by bacteria and SA is converted from chorismite via isochorismic acid intermediate (Chen et al. 2009; Dempsey et al. 2011). The IC pathway is species dependent as some bacteria such as *Yersinia enterocolitica* converts SA

from chorismate in a single step by isochorismic acid; meanwhile, in *Pseudomonas aeruginosa* and *P. fluorescens* this conversion requires isochorismate synthase (ICS) to convert chorismate to IC and then isochorismate pyruvate lyase (IPL) is used to convert IC to SA (Dempsey et al. 2011). Chorismate is produced in the plastids of plants, it is a product of the Shikimate pathway and it acts as a precursor of IC (Wildermuth et al. 2001). In *Arabidopsis,* two isochorismate synthase (ICS) homologous genes, ICS1 and ICS2, were identified and their expression levels were measured following pathogenically challenged leaves. ICS1 expression was correlated with *PR-1*, and SA-associated gene and well as with actual SA levels (Wildermuth et al. 2001; Dempsey et al. 2011). Single mutants *ics1* and *ics2* were subjected to UV light to induce SA production and had conflicting results as *ics1* exhibited a decrease in SA expression, and *ics2* showed an increase in the expression. Double mutant *ics1 ics2* plants expressed even lower levels of SA after the same treatment was administered.

Plant Lipid Transfer Proteins

Lipid transfer proteins were first identified for their ability to bind and transfer phospholipids across the membrane. The more recognized name, non-specific lipid transfer proteins (nsLTPs) was given upon determination that these protein's activities were not exclusive to phospholipids but a variety of lipids (Kader 1996). Lipid transfer proteins activity include binding to glycolipid-transfer proteins, sterol carrier proteins, fatty acid binding proteins, acyl-CoA-binding proteins and other lipid transfer proteins in vitro (Kader 1996; Carvalho and Gomes 2007). These proteins are abundant in higher plants and can constitute for up to 4% total soluble protein in plants (Liu et al. 2015). These proteins are small, ~10 kDa (Kader 1996), soluble and are cysteine-rich. Most

LTPs have highly conserved eight-cysteine motif that forms four disulfide bonds which shapes and stabilizes their structure. This compact structure allows LTPs to withstand the heat of 100°C without denaturation (Lindorff-Larsen and Winther 2001). In lipid transfer proteins, ideal fatty acid chains of 16-18C are preferred rather than larger carbons possibly due to the restrictive size of the hydrophobic cavity (Zachowski et al. 2001). Most nsLTPs are predicted to have an N-terminal signal peptide which can localize it in the apoplast (Edstam et al. 2011). There are two main classes of lipid transfer proteins in plants that are called Family 1 or LTP1 and Family 2 or LTP2 which are distinguishable by their molecular size and biochemical activity. The two families show low amino acid sequence identity (~30%) and similarity (Yeats and Rose 2008).

Family 1 LTP1 are 9-10 kDa that have 90-95 amino acids, and they have five conserved amino acid including Cys, Gly, Pro, Arg, and Tyr (Phe). LTP1s cavity has hydrophilic amino acids (Arg, Lys, Ser) which are in direct contact with the surface of a binding ligand. The hydrophobic cavity has an elongated tunnel-like shape which is determined by side chains consisting of Leu, Val, Ala, and Ile (Yeats and Rose 2008). These amino acids are responsible for the unique amphiphilic binding properties of many lipid transfer proteins. LTPs are found to be localized in cutin-coated organs and have a proposed function in cutin biosynthesis (Finkina et al. 2016). In LTP1s, intrinsic fluorescence is attributed to a Tyr79 amino acid residue.

Family 2 LTP2 are 6-7 kDa proteins that have 65–70 amino acids and four conserved amino acids that include Cys, Gln, Pro, Tyr (Phe) (Yeats and Rose 2008). LTP2 has three alpha helices that are contained within the protein. The hydrophobic cavity forms a triangular box that has side chains Ala, Ile, Phe, Val, and Phe (Finkina et

al. 2016). A major distinguishing characteristic of Family 2 LTP2 is that their hydrophobic region can bind large molecules including sterols (Samuel et al. 2002). The general localization of Family 2 proteins are in suberin-coated organs, and it is believed that these proteins have a role in suberin biosynthesis (Finkina et al. 2016).

Lipid binding and Transfer

In plants, each nsLTP has a unique way of binding ligands in the hydrophobic cavity, which is dependent upon the amino acid residues lining the cavity, experimental settings, and spatial structure during in vitro analysis (Tassin-Moindrot et al. 2000). This specificity has resulted in single and multiple lipid molecules binding in the hydrophobic cavity of different plant species (Sodano et al.1997; Cheng et al. 2004). For other nsLTPs, there are bulky aromatic amino acids which obstruct the hydrophobic cavity and therefore, inhibit the definitive characteristic of lipid bind and transfer (Tassin et al. 1998). In vitro assay of lipid binding did not express the typical fluorescence relating to binding. However, there was interaction with the phospholipid membrane. It is suggested that the high plasticity in accommodating molecules the indicates nsLTPs likely role in cutin and wax formation (Tassin-Moindrot et al. 2000).

Localization of nsLTPs

The function of non-specific lipid transfer proteins was thought to transfer phospholipids in vivo, based on in-vitro models but early studies showed that extracellular localization was detected (Kader 1996). LTPs are predominantly extracellular proteins because as preproteins with a peptide signal they are secreted extracellularly. Extracellular localization has been observed in multiple plant species

including but not limited to *Arabidopsis* (Maldonado et al. 2002; Kielbowicz-Matuk et al. 2008), tobacco (Dani et al. 2005) and soybean (Djordjevic et al. 2007). Lipid transfer proteins have also been identified intracellularly (Kielbowicz-Matuk et al. 2008; Pagnussat et al. 2009). Because LTPs begin as preproteins before cleavage, the re-localization of HaAP10 in sunflower seeds before imbibition in the apoplast and then into intracellular organelles of oil bodies and glyoxysomes post imbibition might indicate the numerous roles of these proteins (Pagnussat et al. 2009). In *Arabidopsis*, LTP3 is localized in the cytoplasm (Guo et al. 2013) while LTP1 has been shown to be localized in the cell wall (Thoma 1994). Other cytoplasm localizations have been observed in durum wheat (Ahmed et al. 2017) and *N. benthamiana* (Pan et al. 2016).

Role of nsLTPs in Abiotic Stress

Plant lipid transfer proteins have long been associated with attributing to various environmental stresses. In many instances, the expression of LTPs in stress-induced environments have either been shown to be upregulated or downregulated whereby indicating a possible role. In *Arabidopsis*, LTP3 overexpression showed enhanced tolerance to freezing and drought while *ltp3* loss of function mutant was sensitive to drought (Guo et al. 2013). RNAi lines of LTP in fox millet was more susceptible to salt and drought tests compared to the overexpressing lines which had high recovery rates (Pan et al. 2016). In potato, STnsLTP1 exhibited high expression in all parts of the plant except the roots during heat, drought and salinity stress (Gangadhar et al. 2016). Recently in *Nicotiana tabacum*, NtLTP4 was overexpressed leading to higher tolerance to ROS as well as it was highly exp*ressed under salt* stress (Xu et al. 2018).

Arabidopsis LTP12

LTP12 is predicted to be a member of the pathogenesis-related protein, PR-14 family. LTP12 promoter is highly expressed in the tapetum of the anther during microspore and pollen development (Ariizumi et al. 2002) as well as it can also be expressed in the flower, petal, seeds and stamen. Overall, its expression in mature leaves and in seedlings is moderately low (Huang et al. 2013).

Arabidopsis ltp12 Mutant

Arabidopsis ltp12 is a knockout mutant with a T-DNA insertion in the LTP12 gene in *Arabidopsis thaliana* ecotype *Columbia-0* (Col-0). Expression of *ltp12* has been studied under various conditions including hormones, biotic and abiotic stress. In abscisic acid (ABA) and methyl jasmonate (MeJA) *ltp12* had a moderate expression at 3h post-treatment. In biotic stress, *ltp12* maintained a high expression in treatment with both avirulent, *Pseudomonas syringae. pv tomato DC3000 AvrRpm1* (*P.S. tomato DC3000 AvrRpm1*) stand virulent, *Pseudomonas syringae pv tomato DC3000* (*P.S. tomato DC3000*). Salinity stress of ltp12 resulted in a moderately sustained expression in the leaves and roots while osmotic stress induced with 300mM mannitol had a continuous moderate expression in the roots only up to 24h (Kilian et al. 2007). (http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp)

Salicylic Acid Binding Protein 2 (SABP2)

Salicylic acid binding protein 2 (SABP2) is one of several salicylic acid binding proteins identified in tobacco due to its interaction with SA (Du and Klessig 1997).

SABP2 is a soluble, 29 kDa protein that is a member of the α/β hydrolase superfamily which is distinguished by a catalytic triad consisting of Ser-81, His-238, and Asp-210 (Kumar and Klessig 2003; Forouhar et al. 2005; Park et al. 2007). SABP2 has a high affinity for SA (K_d = 90 nM) (Kumar and Klessig 2003). It exhibits esterase activity as it converts methyl salicylate into active SA through cleavage of the methyl group (Forouhar et al. 2005). Tobacco plants silenced in SABP2 resulted in suppression of both local immunity to the tobacco mosaic virus (TMV) and the development of SAR in plants (Kumar and Klessig 2003).

To further explore the role of SABP2 in plant defense against pathogen via SAR, a yeast two-hybrid screening was performed SABP2-interacting proteins. During this screening, SABP2 was used as the bait and tobacco leaf proteins were the prey. Several SABP2-interacting protein were identified including SIP470 (SABP2-Interacting Protein 470) (Kumar et al. unpublished).

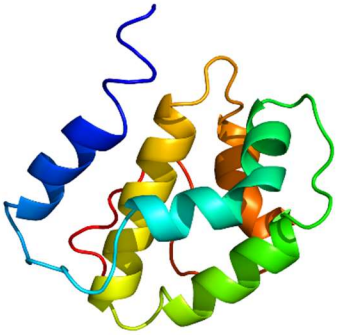


Figure 2: Predicted Structure of SIP470 (Chapagai 2014)

SABP2-Interacting Protein 470 (SIP470)

SIP470 is an 11 kDa protein that is predicted to be a member of the subfamily nsLTP1 and is likely localized in the extracellular region of the plant (Chapagai 2014). The predicted structure of SIP470 has five alpha-helices (Figure 2). One characteristic of LTPs is the ability to bind lipids. To verify the in silico predictions, a lipid binding assay using purified recombinant SIP470 and an artificial substrate, 2-*p*-toluidinonaphtalene-6-sulfonate (TNS) was incubated to measure the fluorescence of this interaction. SIP470 did bind to TNS, and the fluorescence increased as the concentration of TNS increased (Audam 2016). To understand possible functions of SIP470 in immunity in tobacco, RNAi-silenced transgenic tobacco lines were generated using pHELLSGATE8 gene-silencing vector (Audam 2016). To complement this, overexpression lines were generated using an estradiol-inducible expression system (Audam 2016).

To explore the possible roles of SIP470, *Arabidopsis* homologs with T-DNA insertion knockout mutants, *ltp2* and *ltp12* were obtained. The most similar *Arabidopsis* homologs to this tobacco protein were LTP2 and LTP12 with 53% and 46% identity, respectively (Chapagai 2014). Phenotypic growth characteristics were studied along with the wild-type control plant Columbia 0 (Col-0). It was observed that *ltp12* had significantly reduced height and inflorescence emergence compared to Col-0. Both mutants had significantly longer bolting time (Chapagai 2014). In addition, abiotic stress tests were performed and the *ltp12* mutant was more affected by H_2O_2, it was hypersensitive in abscisic acid and it did not recover after drought test (Audam 2016). Moreover, *ltp2* and *ltp12* mutant's response to biotic stress was tested. In basal

immunity, *ltp12* plants were compromised and showed high bacterial growth compared to wild-type, Col-0. Both, *ltp2* and *ltp12* mutants were still able to induce systemic acquired resistance upon pathogenic challenge (Audam 2016).

Hypotheses

Hypothesis 1: Tobacco SIP470 has a role in biotic and abiotic stress signaling

Based on the preliminary studies using *Arabidopsis homologs (ltp2* and *ltp12*) of SIP470 for their role in innate immunity and abiotic stress, it is hypothesized that tobacco SIP470 has a in biotic and abiotic stress signaling in plants. Lipid transfer proteins role in environmental stress adaptations (Guo et al. 2013; Pan et al. 2016) and biotic challenges (Carvalho et al. 2006) have been well studied in higher plants.

Upon verification of SIP470-silenced and overexpressor lines, immunity studies including basal resistance, systemic acquired resistance and various abiotic tests will be performed. Additionally, to complete the biochemical characterization of *Arabidopsis* LTP12 cloning of this gene using the Gateway system will be performed. After cloning, protein would be produced, purified as used for lipid binding analysis using TNS substrate.

Hypothesis 2: SIP470 is localized in the apoplast

Plant lipid transfer proteins are generally recognized as apoplastic proteins (Liu et al. 2015). This is largely because LTPs are produced as preproteins with an N-terminal signal peptide which when matured are exported to the secretory pathways into

the apoplastic space (Carvalho and Gomes 2007; Salminen et al. 2016). A hallmark of LTPs is their ability to bind lipids and transfer them between donor and acceptor membranes. SIP470 is a lipid transfer protein (Audam 2016) and it is likely to be localized in the apoplast as a mature protein.

CHAPTER 2

MATERIALS AND METHODS

Plant Material

In this study, *Arabidopsis thaliana* mutant plant, *ltp12* (CS736658) and Columbia (Col-0), a wild-type control plant was used along with two species of tobacco, *Nicotiana tabacum* cv. Xanthi-nc NN (XNN) and *Nicotiana benthamiana* were all grown from seeds. All seeds were grown in a growth chamber (PGW 36, Conviron, Canada) with a 16-hour light cycle and a fixed temperature of 22°C. The soil (Fafard F-15, Agawam, MA) was steam sterilized by an autoclave for 20 minutes and then cooled to room temperature. For *Arabidopsis*, the soil was filled into 4 x 4-inch plastic flats, and about 20 seeds were sown. The flats with seeds were covered and placed at 4°C for two days to break dormancy. On the third day, the flats were transferred to the growth chamber with 16-hour light cycle and a fixed temperature of 22°C. After seven days, the seedlings were transferred into individual 4 x 4-inch plastic flats (2 seedlings per flat) and were grown for 3 to 4 weeks. For tobacco, about 20 seeds were sown on autoclaved soil and transferred to the growth chamber. After 10-14 days, seedlings were transplanted into 4 x 4 plastic flats (2 seedlings per flat) filled with autoclaved soil. After 3-4 weeks, individual plants were transferred into 8-inch pots and grown until used for experiments.

Chemicals and Reagents

Sodium dodecyl sulfate (SDS), ß-mercaptoethanol (ß-ME), ammonium persulfate (APS) tetramethylethylenediamine (TEMED), Ponceau-S, Coomassie brilliant blue R-250,

phenylmethylsulfonyl fluoride (PMSF), agar (Acros Organics), magnesium chloride ($MgCl_2$), sodium chloride (NaCl), sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), sucrose (Bioworld), EDTA, acrylamide, bis-acrylamide imidazole, methanol, ethanol, ß-estradiol, guanidine thiocyanate (Sigma-Aldrich), chloroform, phenol, isopropanol, isoamyl alcohol, Tween 20, bleach, ECL western blotting substrate (Thermo Scientific), low molecular weight marker (GE healthcare), mouse monoclonal anti-poly-Histidine antibody, rabbit monoclonal anti-eGFP antibody, rabbit monoclonal anti-GST antibody, HRP conjugated goat anti-mouse IgG, HRP conjugated goat anti-rabbit IgG.

Other Materials

Centrifuge (Sorvall RC5B), ultracentrifuge (Sorvall ultra speed T-865), Master Cycler (Eppendorf, NY), spectrophotometer, French press (Thermos Scientific), FastPrep-24 (MP Biomedicals), ND-1000 Nanodrop spectrophotometer (Thermo scientific), UV transilluminator (UVP Bioimaging Systems), pH meter (Beckman), Pierce® GST Spin column (Thermo Fischer), LI-COR C-DIGIT Western blot imager (LI-COR), 1 ml syringes (BD Syringes), Ultrasonicator (Fisher Scientific), cheesecloth, and miracloth (Calbiochem), water bath (Fisher Scientific), mechanical grinder (IKA® RW 20 digital).

Antibiotics

Rifampicin, gentamycin, spectinomycin, hygromycin (Sigma-Aldrich); kanamycin, carbenicillin (BioWorld).

Oligonucleotides (Primers)

Table 1. List of primers used in this study

Primer	Sequence	Purpose
DK672 (Forward)	GGCCCTCTAGGGAGGTGT	Screening/detection
DK673 (Reverse)	TCACTGGACCCTGGAGCA	Screening/detection
DK675 (Reverse)	ACTAGTTCACTGGACCCTGGAGCA	Amplification of pSITE-2CA-SIP470
DK677 (Forward)	AAGGAAGTTCATTTCATTTGGAGAG	Amplification of pSITE-2CA-SIP470
DK461 (Forward)	TTAGTTTCTTTTCAATCGCCG	cDNA synthesis of ltp12
DK462 (Reverse)	GCTGGCAAGTTTGATTAGTCG	cDNA synthesis of ltp12
DK753 (Forward)	ACCATCCAGTGTGGGACAGT	Amplification of LTP12
DK754 (Reverse)	TCACACGGCAGTCGATATACTG	Amplification of LTP12
DK736 (Forward)	CATATGATGGCGTTTACTCCGAAGATC	Full-length LTP12 for cloning
DK737 (Reverse)	CTCGAGTCACACGGCAGTCGATATACT	Full-length LTP12 for cloning

Screening of SIP470 Silenced Transgenic lines

Screening of the RNAi silenced lines were performed via RT-PCR analysis. Two growth methods were used for the screening of the silenced plants. In the first method, seeds were grown on half-strength MS media containing kanamycin (100 µg/mL). After three weeks, the seedlings were transferred to the soil kept in a growth chamber maintained at 22°C. In the second method, about twenty seeds were sowed directly in the soil and were allowed to germinate and grown as previously stated.

Total RNA Isolation from SIP470 Silenced Transgenic lines

For total RNA isolation, three leaf disks (cork borer #6) were collected from 4-week-old plants, placed into Eppendorf tubes and flash frozen in liquid nitrogen. The samples were ground using a mechanical grinder (IKA® RW20 digital) and 500 µL of Guanine HCl buffer (Appendix B) + 3.45 µL β-mercaptoethanol was added and incubated at room temperature for 10 minutes then centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected into a new tube and 500 µL water-saturated phenol and 500 µL chloroform: isoamyl alcohol (Appendix B) was added and mixed well by inverting 7-10 times. The mixture was incubated on a shaker at room temperature for 10 minutes and then centrifuged at 10,000 rpm for 10 minutes. To the aqueous phase, equal volume of chloroform: isoamyl alcohol was added, mixed well by inverting 7-10 times and incubated at room temperature on a shaker for 10 minutes. The mixture was centrifuged at 10,000 rpm for 10 minutes at 4°C. Again, the aqueous phase was collected, 1/10 volume of 3M sodium acetate and an equal volume of cold isopropanol was added. The content of the tubes were mixed by inverting and incubated at -20°C for 15 minutes. Following incubation, samples were centrifuged at 10,000 rpm for 10 minutes at 4°C. To the total RNA pellet, 500 µL of 75% cold ethanol was added, the tube was vortexed and incubated on ice for 5 minutes. The sample was centrifuged at 7,500 x g for 5 minutes at 4°C followed by air drying under a heated lamp for 10 minutes. The pellet was resuspended in 43 µL DEPC treated sterile water.

To the resuspended total RNA, 2 µL of RNase free-DNase (Promega) and 5 µL of 10x DNAse was added, mixed and incubated at 37°C for 20 minutes. Two hundred microliters of TRIzol was added to the DNAse treated total RNA and incubated at room

temperature for 5 minutes. After incubation, 100 µL chloroform was added and mixed by inverting tube, and incubated for three minutes. This mixture was centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous phase was transferred to a new tube and 200 µL isopropanol was added, mixed, and the mixture was incubated at room temperature for 20 minutes. This mixture was centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the resulting pellet was washed in 500 µL ice-cold ethanol, gently mixed and centrifuged at 7,500 rpm for 5 minutes at 4°C as described earlier. The resulting pellet was air dried for 10 minutes. The pellet was resuspended in 20 µL DEPC treated water. The total RNA concentration was measured using nanodrop spectrophotometer.

First-strand cDNA Synthesis

Following the nanodrop reading, total RNA concentration was calculated. One microgram total RNA was diluted to 8 µL with DEPC-treated water. To the RNA, 2 µL of oligo-dT (0.5 µg/mL) was added, mixed and incubated at 70°C in a thermocycler for 10 minutes and allowed to cool to 4°C. To complete cDNA synthesis, 10 µL of RT-mix (0.25 µL 5x RT maxima, 4 µL RT buffer, 0.5 µL RNase inhibitor)), 1 µL of 10 mM dNTP and 4.25 µL DEPC treated water) was added to RNA mixture to make up 20 µL. The sample was mixed and incubated in a thermocycler for 60 minutes at 42°C followed by 10 minutes at 70°C.

PCR analysis

Following cDNA synthesis, PCR amplification was used to analyze the SIP470 mRNA expression. The PCR mixture contained 6 µL sterile water, 1 µL 10X dNTP, 1 µL 10X Taq polymerase buffer, 0.2 µL Taq polymerase, 0.4 µL of 10 µM DK753 primer, 0.4

µL 10 µM DK754 primer, and 1 µL of cDNA sample. PCR reaction was performed using a thermocycler for 30 cycles with denaturation temperature of 94°C for 30 seconds, annealing temperature of 55°C for 30 seconds, extension temperature of 72°C for 30 seconds and a final extension temperature of 72°C for 7 minutes after the completed cycles. PCR product was visualized on 1.5% agarose gel containing 5% ethidium bromide. For loading control, actin and EF1α gene were amplified followed by SIP470 gene specific primers (DK677 and DK675) for PCR amplification.

Agarose Gel Electrophoresis

Agarose gel (1.5%) was prepared by mixing 0.75 g agarose into 50 mL 1X TAE buffer (Appendix A), heating until dissolved and cooling down in 55°C water bath for 15 minutes. To the solution, 2.5 µL (5 mg/mL) of ethidium bromide was added and the mixture was poured into an agarose gel tray. Eight microliters of PCR product and 2 µL of 6X DNA loading dye was loaded into the wells of an agarose gel. The gel was run at 90 volts for 25-30 minutes and then visualized using a UV trans-illuminator.

Screening of SIP470 Overexpressing Transgenic Tobacco lines

The SIP470 overexpressing transgenic tobacco lines were previously generated (Audam 2016). The overexpressing lines were analyzed for SIP470 expression by inducing the expression of myc-tagged SIP470 in leaf tissue by treating with β-estradiol. Two leaf disks were collected from 4-5-week old plants using cork borer #6. The leaf discs were treated by floating on 50 µM β-estradiol solution for 24 hours under continuous light. Leaf disks were collected and ground using a mechanical grinder and 100 µL of 1X SDS dye was added and processed for SDS-PAGE as previously

described. Western blot analysis was performed using an anti-Myc tag primary antibody (monoclonal).

Cloning of *Arabidopsis* LTP12

To clone LTP12, RT-PCR amplification was used. For total RNA isolation, three leaf disks samples were collected from *Arabidopsis* plants, flash frozen and homogenized using a mechanical grinder (IKA® RW 20 digital) at 1075 rpm. After grinding, total RNA was isolated followed by the synthesis of cDNA as described previously. Agarose gel electrophoresis was used to visualize the results on 1.5% agarose gel as described earlier. The primers used in this are DK753 and DK754, DK461 and DK462, DK736 and DK737.

Subcellular Localization of SIP470

The main approach to study the subcellular localization of SIP470 was to visualize it by confocal microscopy. For this, a recombinant SIP470-tagged with eGFP was generated as described below.

Cloning of SIP470 into pSITE-2CA

The full-length SIP470 (345 bp length) was previously cloned into the entry vector, pDONR221 using Gateway (Invitrogen) cloning system and verified through DNA sequencing (Chapagai 2014).

SIP470 sequence verified entry clone was subcloned into pSITE-2CA by Gateway LR reaction (Invitrogen) following manufacturer's instructions. The reaction mixture contained 1µL entry clone pDONR221-SIP470 (381.61 ng/µL), 1µL destination

vector (150 ng/µL), 1µL LR clonase enzyme and TE buffer (pH 8.0) to a final volume of 8.0 µL. This mixture was incubated overnight at room temperature and the next morning 1.0 µL proteinase K (Invitrogen) was added to the reaction mixture and mixed. The mixture was incubated at 37°C for 10 minutes. The reaction was kept on ice at 4°C and then transformed into TOP10 chemically competent cells.

Preparation of TOP10 Chemically Competent *E.* coli Cells

TOP10 *E. coli* was streaked (from glycerol stock stored at -80°C) onto an LB media plate and incubated overnight at 37°C. The next day, a single isolated colony was picked and inoculated in 2 mL LB broth and incubated on a shaker at 250 rpm overnight at 37°C. Next morning, 0.5 ml of overnight culture was added to 50 mL fresh LB broth and incubated on a shaker at 250 rpm for 1.5 - 2 hours at 37°C until the bacterial growth OD_{600} reached 0.5 - 0.6. Once the desired OD was obtained, the culture was centrifuged for 10 min at 3000 x g at 4°C. The supernatant was discarded, and the resulting bacterial pellet was resuspended in sterile ice-cold 0.1 M $CaCl_2$. The suspension was gently mixed by pipetting and swirling. The suspended cells were centrifuged at 3000 x g at 4°C for 10 minutes. The resulting supernatant was discarded and the cells were resuspended in 2 mL of 0.1M $CaCl_2$ at the same stated conditions. Glycerol stocks were made by gently mixing chemically competent cells and sterile glycerol in an Eppendorf tube to a final concentration of 20%. The stock was flash frozen in liquid nitrogen and stored at -80°C for future use.

Transformation of pSITE-2CA-SIP470 into TOP10 Chemically Competent *E.* coli

Two microliters of LR reaction mixture was added to 100 µL of competent cells, gently tapped/swirled to mix and incubated on ice. After 30 minutes, the mixture was incubated in a 42°C water bath for 45 seconds then incubated on ice for an additional 5 minutes. Seven hundred fifty microliters of SOC media (Appendix B) was added to the tube and incubated in a shaker at 250 rpm at 37°C for 1 hour. Following incubation, 50 µL, and 150 µL of bacterial cells were plated separately on LB agar plates containing spectinomycin (100 µg/mL) and incubated at 37°C overnight.

Screening of Transformed pSITE-2CA-SIP470 Colonies using Colony PCR

To test for the presence of pSITE-2CA-SIP470 in the transformed cells, colony PCR was used. A sterile pipette tip was used to touch a single colony which was then streaked on LB agar plates containing spectinomycin (100 µg/mL) and then the tip was gently rinsed by repeatedly pipetting in 20 µL of sterile, autoclaved water in a 0.2 mL (PCR) tube. Ten microliters of the bacterial mixture was used for PCR amplification. The PCR mix contained 10 µL of bacterial suspension, 2 µL 10X PCR buffer, 2 µL dNTP, 4.8 µL sterile water, 0.4 µL 10X Taq polymerase, 0.4 µL DK677 forward primer, 0.4 µL DK675 reverse primer for a total volume of 20 µL. The conditions of the PCR were set at 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds (annealing), 72°C for 1 minute (extension) and a final extension 72°C for 7 minutes. Ten microliters of the amplified PCR products were analyzed on a 0.8% agarose gel and photographed.

Isolation of pSITE-2CA-SIP470 Plasmid DNA

Positive colonies (containing pSITE-2CA-SIP470) were grown in 5 mL of LB broth containing spectinomycin (100 µg/mL) overnight at 37°C. The overnight culture was centrifuged at 18,800 x g for 60 seconds and the resulting supernatant was discarded. The bacterial pellet was resuspended by pipetting in 500 µL of ice-cold STE solution. The resuspended pellet was centrifuged at 13,600 x g for 60 seconds at 4°C. The resulting supernatant was discarded, and the pellet was completely resuspended in 500 µL STET solution by continuous raking of the Eppendorf tube across a test tube rack followed by vortexing. Twenty-five microliters of fresh lysozyme were added to the resuspended solution and mixed by continuous inversion of tube. The mixture was placed into a boiling water bath for 45 seconds to terminate the reaction. The solution was cooled to room temperature for 5 minutes. The solution was centrifuged at 13,600 x g for 15 minutes at 4°C. A sterile toothpick was used to remove the pellet and to the resulting supernatant 400 µL of Phenol:Chloroform:Isoamyl alcohol (25:24:1) was added. The solution was thoroughly mixed by vortexing and then centrifuged at 1606 x g at 4°C for 10 minutes. The resulting supernatant was transferred to a new 1.5 mL Eppendorf tube. An equal volume of isopropanol was added to the supernatant, mixed by inverting tube several times and kept at room temperature for 30 minutes. The mixture was centrifuged at 18,800 x g at room temperature for 30 minutes. The supernatant was discarded by pouring and 70% ethanol was added to wash the pellet. Pellet with ethanol was centrifuged at 18,800 x g at room temperature for 5 minutes. Ethanol was discarded, and the pellet was placed under a heated lamp to dry for 5 – 10 minutes. The pellet was resuspended in 50 µL of sterile water and the concentration

was measured using a Nanodrop spectrophotometer. The plasmid DNA was stored at -20°C for future use.

Sequencing of pSITE-2CA-SIP470

Purified recombinant plasmid (~1000 ng) and 1 µL of DK677 (forward primer) and 1 µL DK675 (reverse primer) was added to a PCR tube was mixed. This sample was sent to the DNA Analysis Facility on Science Hill at Yale University for sequencing using the Sanger method.

Preparation of *Agrobacterium tumefaciens* strain *GV3101* Chemically Competent Cells

Agrobacterium tumefaciens strain *GV3101* was streaked on LB agar plates containing rifampicin (20 µg/mL) and gentamycin (30 mg/mL). This plate was incubated for 48 hours at 28°C. A single isolated colony was collected using a sterile pipette tip and grown in 5 mL LB broth containing rifampicin (20 µg/mL), and gentamycin (30 mg/mL) in a shaker (250 rpm) at 28°C overnight. One-hundredth dilution of the overnight culture was added to LB broth with a final volume of 100 mL containing the appropriate antibiotics previously mentioned. The bacterial culture was grown until OD_{600}=1.0 was achieved. The bacterial suspension was poured into a sterile Falcon tube and centrifuged at 3000 x g for 5 minutes at 4°C. The resulting supernatant was discarded and then suspended in 5 mL $CaCl_2$ (20 mM). This suspension was centrifuged at 3000 x g for 5 minutes at 4°C. Again, the supernatant was discarded and the pellet was resuspended in 1 mL $CaCl_2$ (20 mM). The bacterial suspension was kept on ice and the pellet was gently mixed by slow pipetting and swirling. A glycerol stock was made as previously described and stored at -80°C.

Transformation of *Agrobacterium tumefaciens GV3101* in pSITE-2CA-SIP470

A. tumefaciens strain *GV3101* competent cells were thawed on ice. To this, 1000 ng of pSITE-2CA-SIP470 was added and mixed by gentle swirling. This bacterial mixture was flash frozen in liquid nitrogen and then allowed to thaw at 37°C for 5 minutes. One millilter of SOC media was added to the cells and incubated for 3 hours at 28°C. After incubation 50 µL, 100 µL and 150 µL aliquot of the transformed culture was spread onto LB plates containing and rifampicin (20 µg/mL), spectinomycin (100 µg/mL) and gentamycin (30 mg/mL) and incubated for two days at 28°C.

Transient Expression of eGFP-tagged SIP470 in *Nicotiana benthamiana*

A single isolated colony of pSITE-2CA-SIP470 in *A. tumefaciens* strain *GV3101* was grown in 5 mL LB broth containing spectinomycin (100 mg/mL), rifampicin (20 µL/mL) and gentamycin (30 mg/mL) for 20 hours at 28°C in shaker at 250 rpm. Five hundred microliters of the culture were added to 50 mL fresh LB containing 20µM acetosyringone and appropriate antibiotics for 20 hours at 28°C in shaker at 250 rpm. The overnight culture was centrifuged at 3000 x g for 5 minutes at 4°C and the supernatant was discarded. The resulting pellet was completely resuspended in 5 mL of infiltration medium (10 mM $MgCl_2$, 10mM MES; adjusted pH to 5.7 with HCl) and centrifuged at 3000 x g for 5 minutes at 4°C. The supernatant was discarded and the pellet was again washed by resuspension in 5 mL infiltration medium and centrifuged at 3000 x g for 5 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 20 mL infiltration medium in a sterile conical flask. The bacterial culture OD_{600} = 0.6 was maintained and 150 µM acetosyringone was added. The conical flask was wrapped in aluminum foil and incubated at room temperature on a shaker (250

rpm) for two hours. Likewise, the same treatment was done for HCPro (helper component proteinase) as this was co-infiltrated into the leaves of the plants. HCPro is a proteinase that suppresses the plant's silencing system so that a foreign protein (SIP470) could have an extended and enhanced expression. The bacterial suspension was then infiltrated on the abaxial side of two to three mature leaves of *N. benthamiana*. Co-infiltration of pSITE-2CA-SIP470 and HCPro was also completed. The plant was kept at room temperature under continuous lighting for 48 hours until leaf samples were collected for confocal microscopy and subcellular fractionation.

Confocal Microscopy of pSITE-2CA-SIP470

The leaf samples from transiently expressing pSITE-2CA-SIP470 without and with HCPro were used for confocal microscopy analysis. The confocal analysis was performed at the microscopy core facility James H. Quillen College of Medicine, East Tennessee State University. Forty-eight-hour post-infiltrations samples of *N. benthamiana* leaf were visualized under Leica TCS SP2 Pectral confocal & multiphoton system.

Co-localization of SIP470-eGFP with mCherry tagged organelle marker proteins

Confocal microscopy of SIP470-eGFP HCPro was co-infiltration with mCherry fluorescent protein in *N. benthamiana* as described earlier. mCherry fluorescent protein is found in pBIN vector. Two-day post infiltration samples were used for confocal analysis. Co-localization of SIP470-eGFP + mCherry will be expressed as a yellow color in the images. Specific marker proteins used include golgi bodies, tonoplast, plasma

membrane, plastids and peroxisomes. SIP470-eGFP+HCPro was observed without mCherry as a control.

Subcellular Fractionation

Two to three grams of transiently expressing pSITE-2CA-SIP470 *N. benthamiana* leaves at 48 h were deveined, rinsed in distilled water, blotted dry and cut into smaller pieces. At 4°C, leaf pieces were ground using cold, sterile mortar and pestle in 10 mL cold grinding buffer. Four layers of pre-chilled cheesecloth were used to filter the remaining liquid from the leaf homogenate. The filtrate was collected in a sterile 50 mL Falcon tube and centrifuged at 100 x g for 2 minutes at 4°C to remove unbroken cells.

The resulting supernatant was transferred to a new tube and centrifuged at 600 x g for 10 minutes at 4°C. The supernatant was collected in a new tube and used for the chloroplast fraction and the pellet was used for the nuclei fraction. The nuclei pellet was resuspended in 1 -2 mL grinding buffer and centrifuged at the same conditions stated. This washing was repeated three times. The resulting supernatant was centrifuged at 2420 x g for 5 minutes at 4°C to collect the chloroplast fraction. The supernatant was transferred to a new tube and the pellet was washed three times in grinding buffer as described above. The transferred supernatant was centrifuged at 16,000 x g for 15 minutes at 4°C to collect the mitochondrial fraction. The pellet was washed 2 – 3 times with grinding buffer as described previously.

The supernatant was centrifuged at 100,000 x g in the ultracentrifuge for 1 hour at 4°C to collect the plasma membrane and cytosolic fractions. The supernatant was transferred to a new centrifuge tube and centrifuged again at 100,000 x g for 1 hour at

4°C to collect the cytosolic fraction. The plasma membrane fraction was separated by differential centrifugation using a sucrose gradient. The pellet was dissolved in 700 µL grinding buffer and added to an ultracentrifuge tube containing the sucrose step gradient: 3 mL of 50%, 2 mL each of 40%, 38%, 25% and 18% sucrose solution. The mixture was centrifuged at 100,000 x g for 1 hour at 4°C. The pellet from the 40% - 50% interphase was collected and dissolved in 5 mL grinding buffer and centrifuged at 100,000 x g for 1 hour at 4°C. The supernatant was discarded and the pellet was collected as the plasma membrane fraction.

Isolation of Apoplastic Proteins

The localization of pSITE-2CA-SIP470 was predicted to be an extracellular protein so to test this, the apoplastic fluid was isolated following infiltration of *N. benthamiana* leaves with the pSITE-2CA-SIP470 plus HCPro construct. The protocol of the isolation of apoplast was adapted from Andreasson et al. (2017) with slight modifications. For leaf infiltration, leaves were rolled and placed into a 20 cc needless syringe filled with 1X phosphate buffer. Negative pressure was created by plugging the tip of the syringe and slowly pulling the plunger once inch from the end of the barrel.

SDS-PAGE

Leaf disc samples taken from infiltrated leaves served as the total sample was ground using a mechanical grinder and 100 µL 1X SDS dye containing 2.5% β-mercaptoethanol was added. One hundred milliliters of each fraction (chloroplast, nuclei, mitochondria, plasma membrane, cytosol) was added to an Eppendorf tube and 100 µL of 2X SDS dye containing 2.5% β-mercaptoethanol was. Each sample was

boiled in a water bath was 5 minutes and then centrifuged at 16,200 x g for 10 minutes at room temperature. Nine microliters of each sample were loaded to a 12% SDS PAGE gel. The gel was run in 1X SDS buffer for about 1 hour at 20 A, 200 V. The gel was processed and used for Western blot analysis.

Western Blot Analysis

PVDF membrane was prepared soaking membrane into 100% methanol for 15 seconds, rinsing with water for 2 minutes and incubating the membrane in transfer buffer for 5 – 10 minutes before use. The gel from SDS was transferred to the PVDF membrane using 1X Transfer buffer (Appendix B) for 1 hour at 94 V at 4°C. After the transfer was complete, the membrane was placed in 100% methanol for 10 seconds then taken out and left to dry on Whatman paper. Once dried, the membrane was again placed into methanol for 10 seconds. The methanol was discarded and Ponceau-S stain was added to the membrane. The membrane was stained for two minutes at room temperature. The stain was discarded and the membrane was rinsed with deionized water 2 – 3 times and a picture of the membrane was taken. The membrane was then washed with deionized for 5 minutes. After washing, the membrane was treated with anti-eGFP primary antibody (1:2500) and incubated at 4°C overnight. After incubation, the membrane was washed with 1X PBS buffer for 5 minutes, twice followed by washing with 1X PBS plus 0.03% Tween 20 for 5 minutes twice and then again with 1X PBS for 5 minutes twice. After washing the membrane was treated with anti-rabbit (1:5000) secondary antibody and 1 – 2 hours at room temperature. Following incubation, the membrane was washed in the same manner as stated previously. The membrane was incubated in ECL substrate accordingly to the manufacturer's

instructions and developed in Li-COR ECL scanner. The membrane was stained with Coomassie brilliant blue to further visualize the detected protein.

CHAPTER 3

RESULTS

Hypothesis 1: Tobacco SIP470 has a role in biotic and abiotic stress signaling

Screening of SIP470 RNAi-silenced lines

To investigate the possible role of tobacco SIP470 in abiotic and stress conditions, RNAi lines were generated (Audam 2016), and the T2 generation seedlings were grown for screening. RNA was isolated from the plants as described in the methods, and the expression of SIP470 was analyzed using RT-PCR. In Figure 3, the screening results indicated that in plant #10 (lane #12), showed some degree of silencing (reduced intensity of SIP470 bands). Seeds from this transgenic tobacco line were collected for analysis (T3 generation). A T3 generation screening of plant #10 was carried out (Figure 4). There were no obvious SIP470 silenced plants in the T3 generation (Figure 4).

Further screenings was carried out by first germinating the seeds on antibiotic (kanamycin) selection media. Three-week old seedlings growing on antibiotic selection media were transferred to autoclaved soil. These plants were analyzed for SIP470 expression. In Figure 7, lane #3, this plant showed a higher degree of silencing compared to the wild-type and this plant is currently being grown for seeds. Also, in Figure 9, lane #6 this plant expressed a higher silencing of SIP470 compared to the wild-type and this is currently being grown for seeds. Figures 5, 6 and 8 did not have any plants that showed a substantial degree of silencing compared to the respective wild-types.

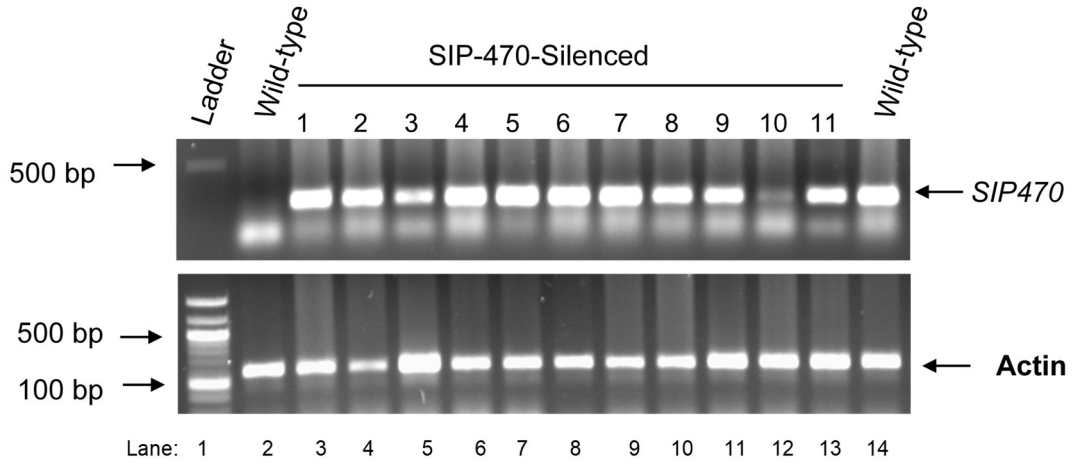


Figure 3: Screening of SIP470 RNAi-silenced lines. Ethidium bromide (EtBr) stained 1.5% agarose gels showing SIP470 at ~350 bp and actin (control). DNA ladder (100 bp) was used for both gels.

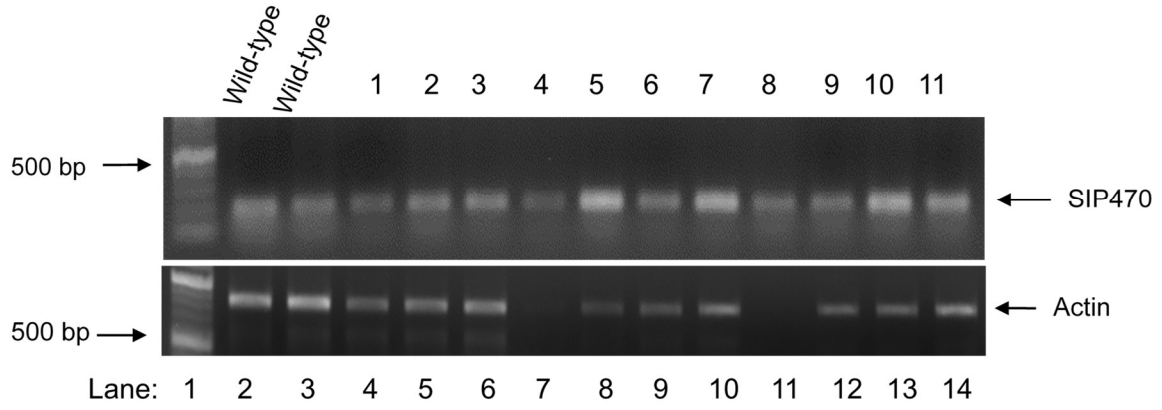


Figure 4*:* Screening of SIP470 RNAi-silenced lines. T3-generation screening of L10 line, T2 generation (Figure 3). Ethidium bromide (EtBr) stained 1.5% agarose gels showing SIP470 at ~350 bp and actin (control). DNA ladder (100 bp) was used for both gels.

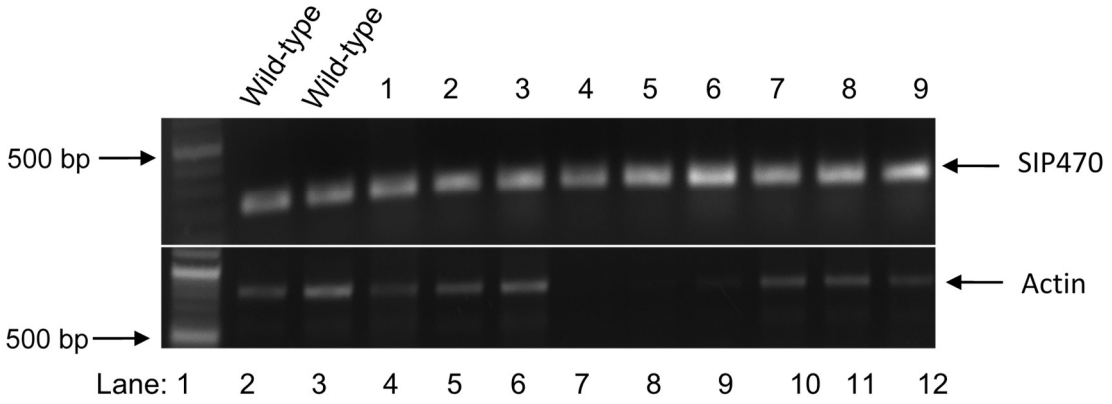


Figure 5 *:* Screening of SIP470 RNAi-silenced lines. Ethidium bromide (EtBr) stained 1.5% agarose gels showing SIP470 at ~350 bp and actin (control). DNA ladder (100 bp) was used for both gels.

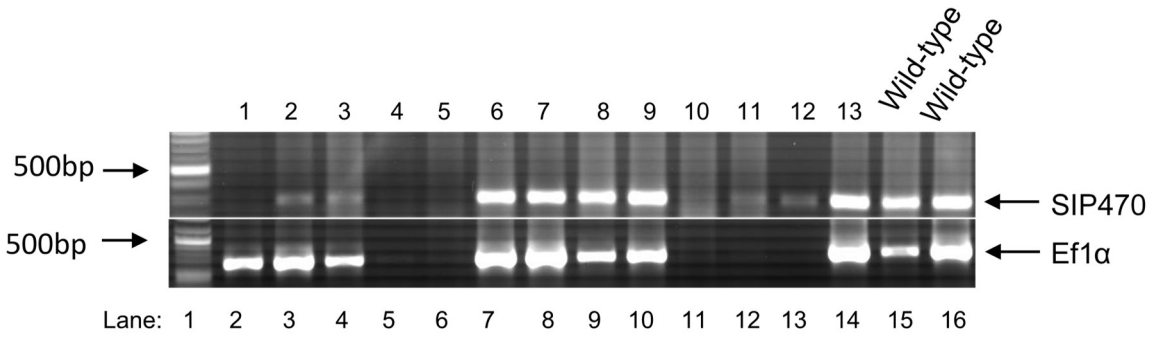


Figure 6: Screening of SIP470 RNAi-silenced lines. Ethidium bromide (EtBr) stained 1.5% agarose gels showing SIP470 at ~350 bp and Ef1α (control). DNA ladder (100 bp) was used for both gels.

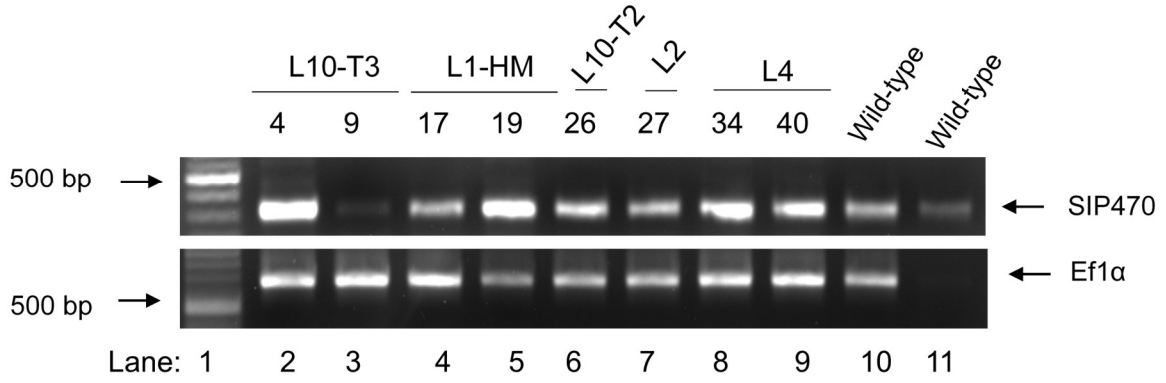


Figure 7: Screening of SIP470 RNAi-silenced lines. Ethidium bromide (EtBr) stained 1.5% agarose gels showing SIP470 at ~350 bp and Ef1α (control). DNA ladder (100 bp) was used for both gels.

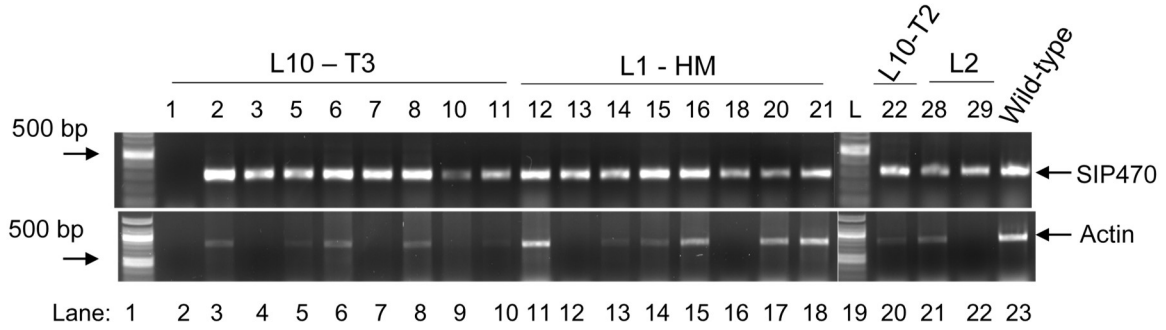


Figure 8: Screening of SIP470 RNAi-silenced lines. Ethidium bromide (EtBr) stained 1.5% agarose gels showing SIP470 at ~350 bp and actin (control). DNA ladder (100 bp) was used for both gels.

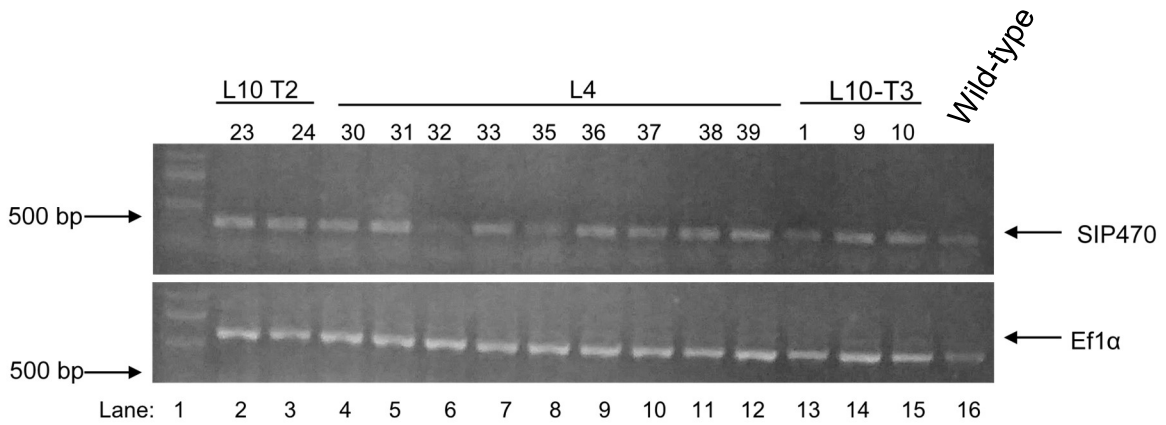


Figure 9: Screening of SIP470 RNAi-silenced lines. Ethidium bromide (EtBr) stained 1.5% agarose gels showing SIP470 at ~350 bp and Ef1α (control). DNA ladder (100 bp) was used for both gels.

Screening of SIP470 Overexpressing Lines

To investigate the possible role of SIP470 in abiotic and biotic stress conditions, β-estradiol inducible overexpressing lines were generated (Audam 2016). Analysis was performed using Western blot as described in the methods. Positive samples (+) were treated under the same conditions as SIP470. Three different screenings were carried out (Figures 10 – 12). No positively overexpressing SIP470 lines were identified.

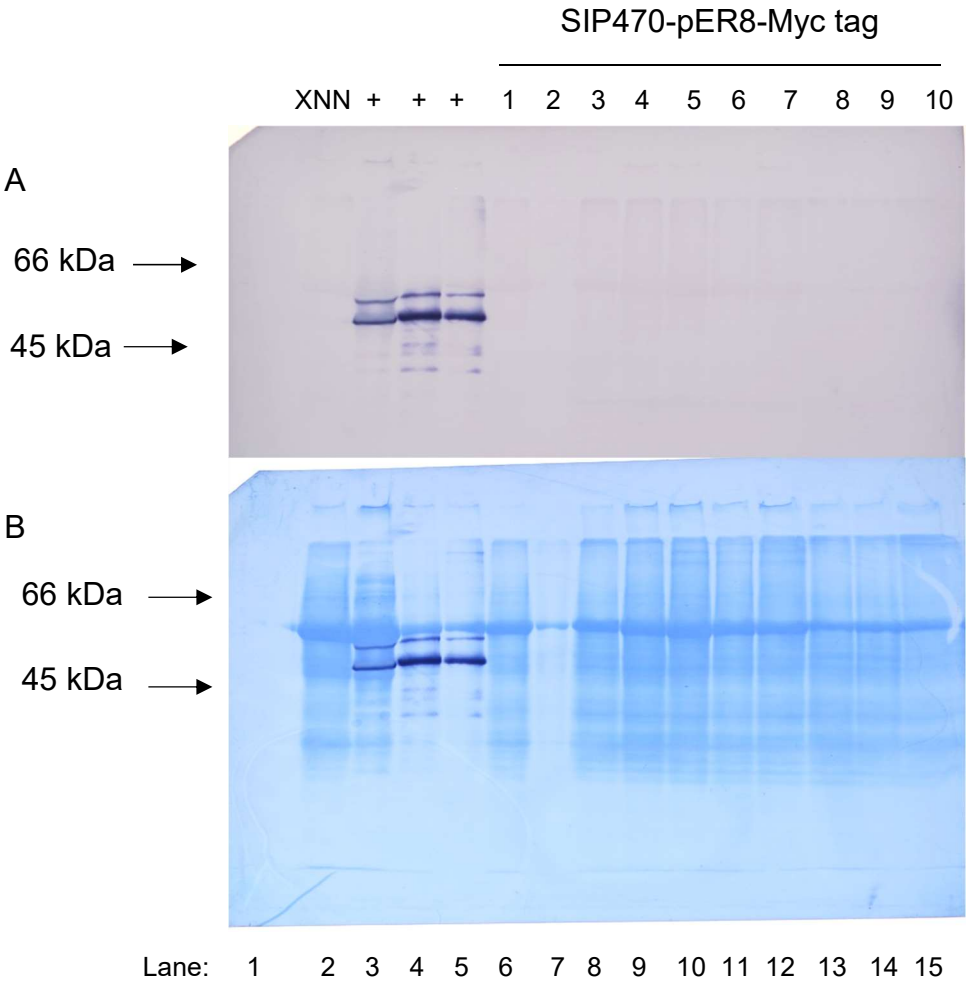


Figure 10: Screening of SIP470 overexpressing plants. Western blot analysis was used to detect β-estradiol induced protein. A shows positively known overexpression plant samples and SIP470-Per8-Myc. B shows a Coomassie blue stain of blot protein loading.

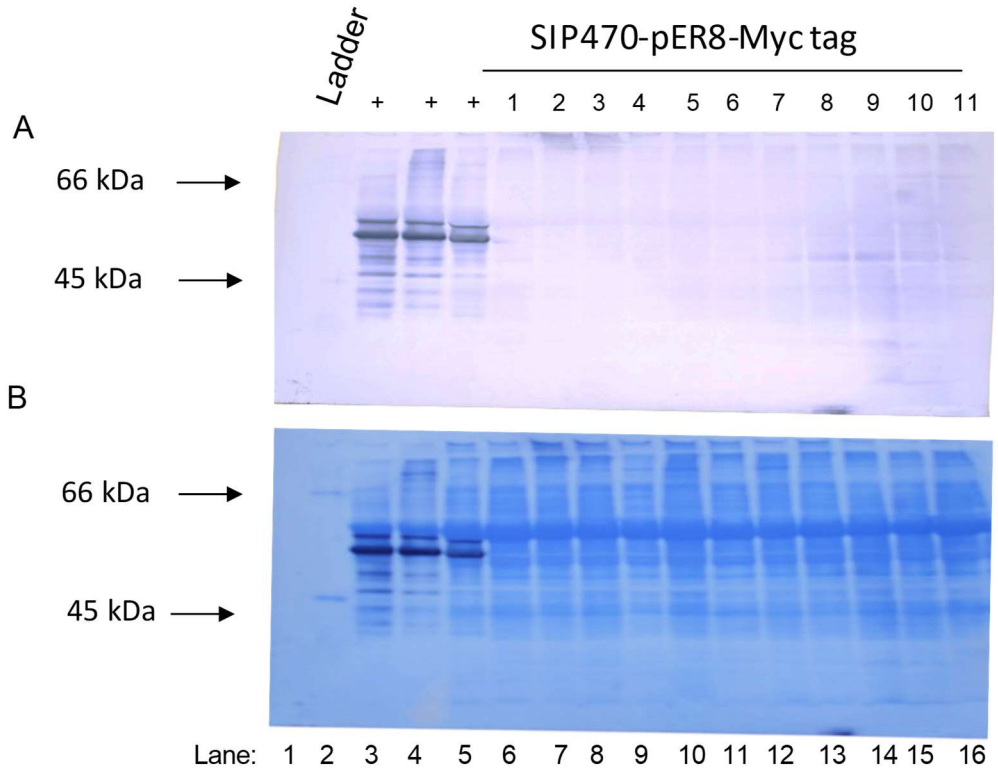


Figure 11: Screening of SIP470 overexpressing plants. Western blot analysis was used to detect β-estradiol induced protein. A shows positively known overexpression plant samples and SIP470-Per8-Myc. B shows a Coomassie blue stain of blot protein loading. Lanes # 3-5 loaded with positive control plant samples and SIP470-Per8-Myc.

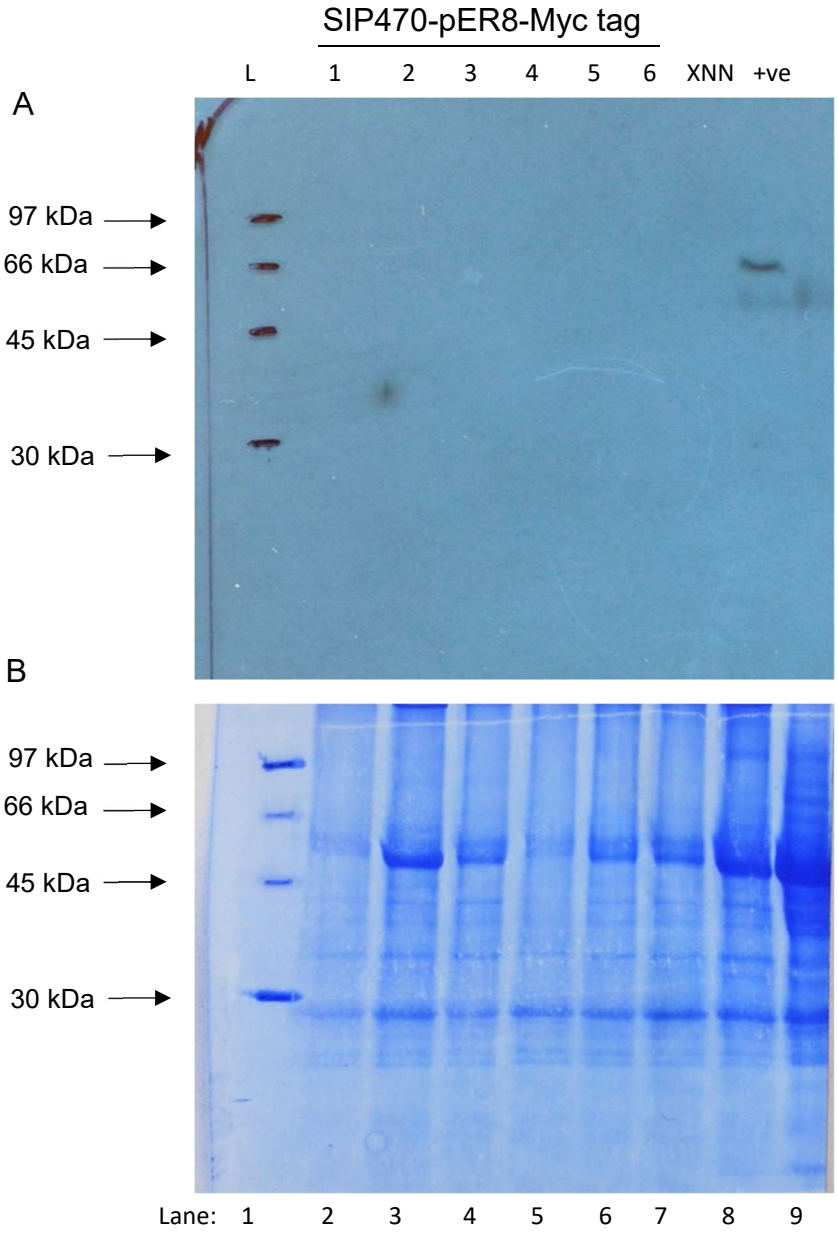


Figure 12: Screening of SIP470 overexpressing plants. Western blot analysis was used to detect β-estradiol induced protein. A shows positively known overexpression plant samples and SIP470-Per8-Myc. B shows a Coomassie blue stain of blot protein loading. Lane #9 loaded with a positive control.

Amplification of LTP12 in *Arabidopsis*

To complete the biochemical analysis in SIP470 homolog, LTP12, the aim was to clone LTP12 and then express it into *E. coli* and ultimately obtain purified protein for analysis. The amplification of LTP12 in *Arabidopsis* was unsuccessful. Attempts of amplifying LTP12 was done using various PCR conditions and different primer sets. In Figures 14 and 15, *Arabidopsis* leaves were infiltrated with $MgCl_2$ (mock) and *P. syringae avr DC3000* to induce expression of LTP12. In Figure 14, samples were taken at 0h, 2h, 6h, and 24h. LTP12 did not amplify as apparent primer dimers were visible in gel A. In Figure 15, different primer sets were used including a full length (DK736 and DK737) set. No bands were identified at the expected size ~356 bp. Unspecified bands were observed in 2h and 6h samples.

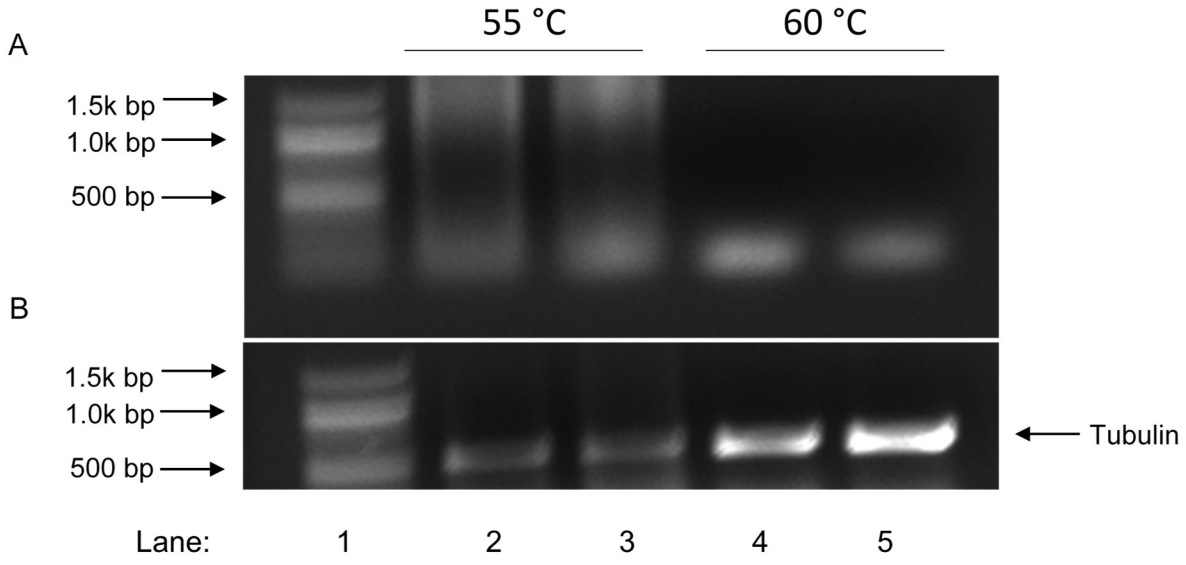


Figure 13: Amplification of LTP12 in *Arabidopsis*. Ethidium bromide (EtBr) stained 1.5% agarose gels failed to express LTP12. Samples were run at 55°C and 60°C. DNA ladder (100 bp) was used in A and B. Tubulin was used for quality control.

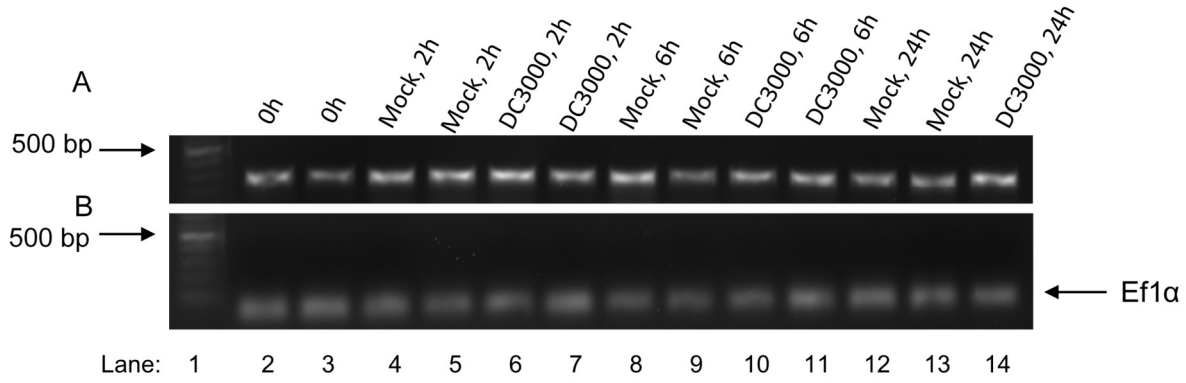


Figure 14: Amplification of LTP12 in *Arabidopsis*. Ef1α was used for quality control. Ethidium bromide (EtBr) stained 1.5% agarose gel.

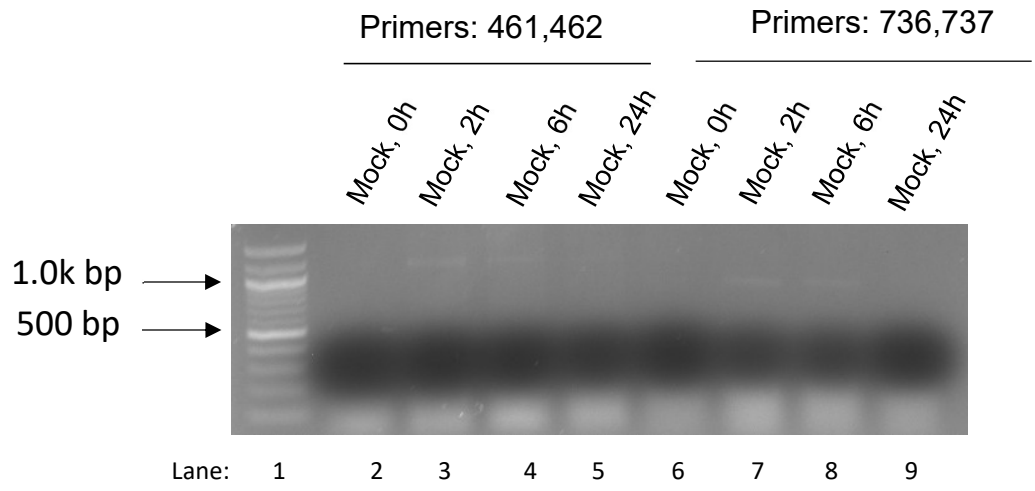


Figure 15: Amplification of LTP12 in *Arabidopsis*. Ethidium bromide (EtBr) stained 1.5% agarose gel.

Hypothesis 2: SIP470 is localized in the apoplast

Cloning of SIP470 into pSITE-2CA

The sequence-verified entry clone, pDONR221-SIP470 (Chapagai 2014) was used to clone SIP470 into the pSITE-2CA. Colony PCR was performed on isolated colonies and primers DK677 and DK675 were used to amplify the SIP470.

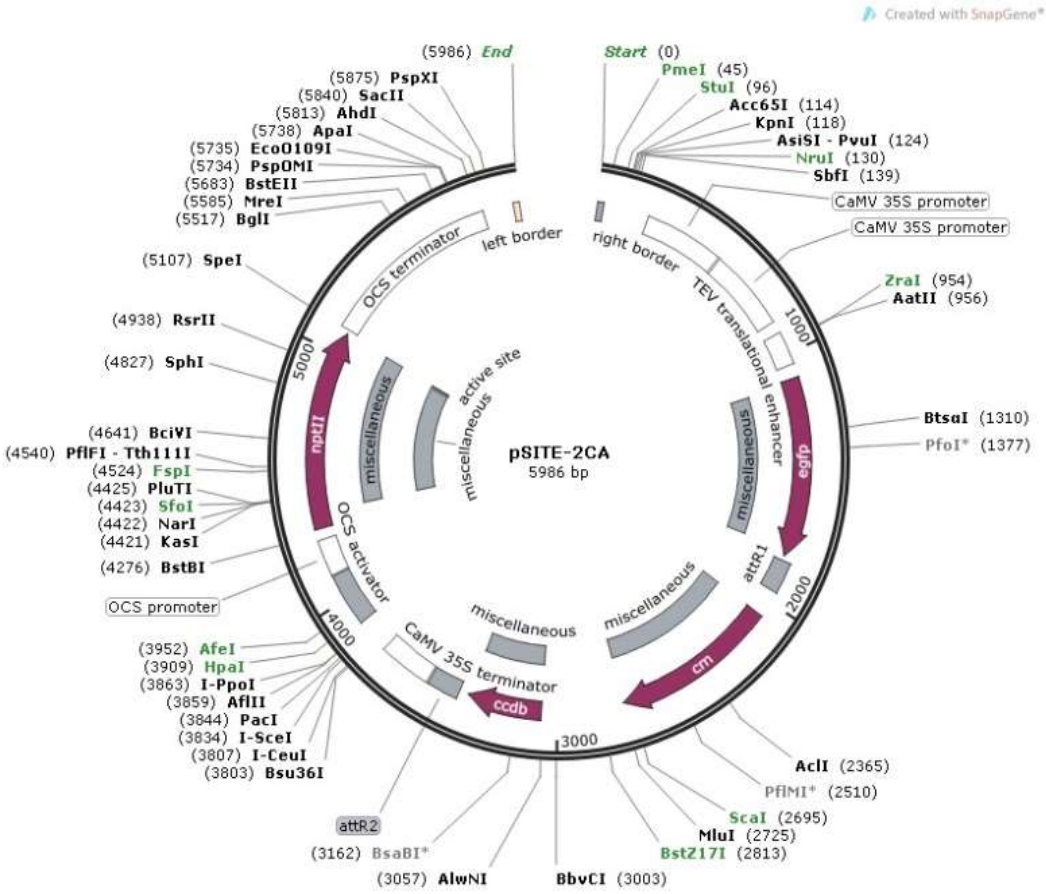


Figure 16: Vector map of pSITE-2CA used in confocal microscopy imaging and subcellular fractionation.

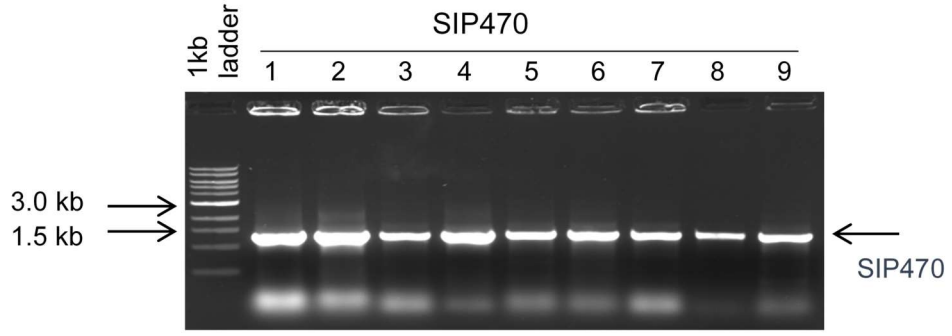


Figure 17: Colony PCR to detect SIP470 cloned in pSITE-2CA. Agarose gel 1.5%, stained with EtBr.

```
Colony#2          TTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCGTCATGGAAATGGTTGGCAAGATTG
SIP470            ------------------------------------ATGGAAATGGTTGGCAAGATTG
                                                       **********************

Colony#2          CGTGCTTTGTGTTACTTTGCATGGTGGTGGTTGCACCCCATGCAGAGGCACTGAGCTGCG
SIP470            CGTGCTTTGTGTTACTTTGCATGGTGGTGGTTGCACCCCATGCAGAGGCACTGAGCTGCG
                  ************************************************************

Colony#2          GCCAGGTTCAGTCTGGCCTGGCTCTTTGCCTCCCTTATCTGCAGAGTCGCGGCCCTCTAG
SIP470            GCCAGGTTCAGTCTGGCCTGGCTCTTTGCCTCCCTTATCTGCAGAGTCGCGGCCCTCTAG
                  ************************************************************

Colony#2          GGAGGTGTTGTGGCGGCGTTAAAGGTGTGTTGGGTGCTGCCCGGACCCCAGCTGACCGCA
SIP470            GGAGGTGTTGTGGCGGCGTTAAAGGTGTGTTGGGTGCTGCCCGGACCCCAGCTGACCGCA
                  ************************************************************

Colony#2          AGACTGCATGCACTTGCCTGAAATCAGCTGCTTATGCTATTAAGGGTATTAATATGGGCA
SIP470            AGACTGCATGCACTTGCCTGAAATCAGCTGCTTATGCTATTAAGGGTATTAATATGGGCA
                  ************************************************************

Colony#2          AAGCCGCTGGACTTCCTAGTGCTTGTGGCGTTAACATTCCCTACAAGATCAGTCCCTCTA
SIP470            AAGCCGCTGGACTTCCTAGTGCTTGTGGCGTTAACATTCCCTACAAGATCAGTCCCTCTA
                  ************************************************************

Colony#2          CTGACTGCTCCAGGGTCCAGTGATACCCAGCTTTCTTGTACAAAGTTGGCATTATAAGAA
SIP470            CTGACTGCTCCAGGGTCCAGTGA-----------------------------------
                  ***********************
```

Figure 18: Nucleotide sequence alignment of SIP470 cloned in pSITE2CA with the wild-type SIP470. The asterisk below the alignment indicates a positive match.

Plasmid from positive pSITE-2CA-SIP470 clone was isolated, analyzed on a 1.2% agarose gel and quantified.

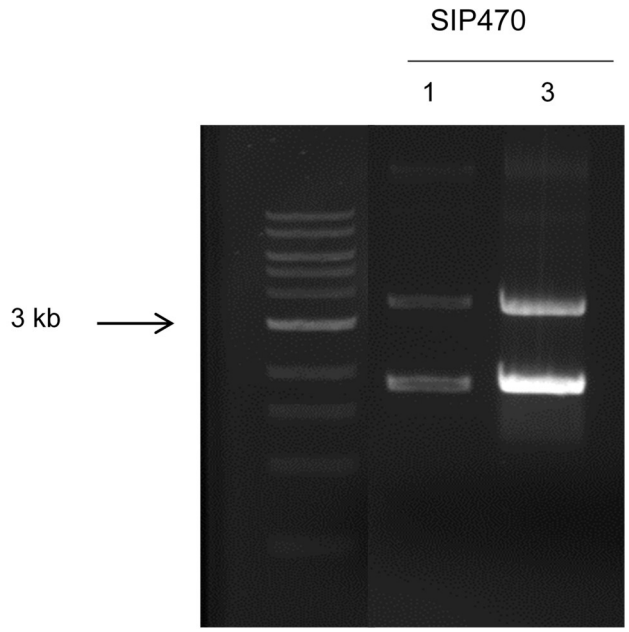


Figure 19*:* Plasmid extraction of SIP470 in pSITE-2CA colonies. EtBr stained 0.8% Agarose gel.

Transformation of pSITE-2CA-SIP470 into *Agrobacterium tumefaciens GV3101*

To the *Agrobacterium tumefaciens GV3101* competent cells, 1000 ng sequenced-verified pSITE-2CA-SIP470 (colony#2) was added and gently mixed as described earlier. The transformation mixture was flash frozen in liquid nitrogen, thawed and SOC media was added. The bacterial cells were plated on LB agar plates containing rifampicin (20 µg/mL), spectinomycin (100 µg/mL) and gentamycin (30 mg/mL).

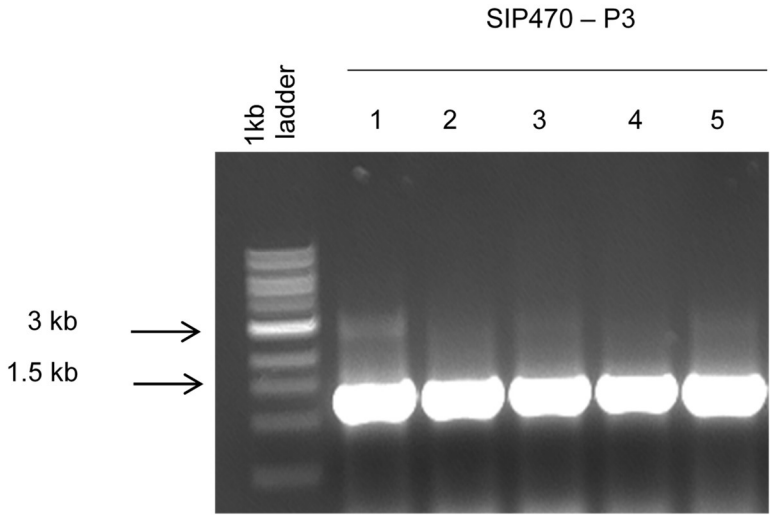


Figure 20: Transformation of pSITE-2CA-SIP470 into *Agrobacterium tumefaciens GV3101*. EtBr stained 0.8% Agarose gel. Size eGFP (762) + 35CaMV promoter (~300) + SIP470 (~345) = ~1400 bp

Transient expression of eGFP-tagged SIP470 in *Nicotiana benthamiana* leaves

The transient expression experiment was used to establish a timeline for the expression of eGFP-tagged SIP470. Expression was performed in absence and presence of HCPro. HCPro suppresses the natural gene-silencing mechanism of the plants to allow the expression of eGFP tagged SIP470. The results indicate that SIP470 expressed strongly at 2 days post infiltration (dpi) without the HCPro (Fig. 8). The SIP470 expressed strongly at 2 days in presence of HCPro in the plant (Fig. 7). *Agrobacterium GV3101* only and HCPro only was infiltrated into tobacco leaf as a control.

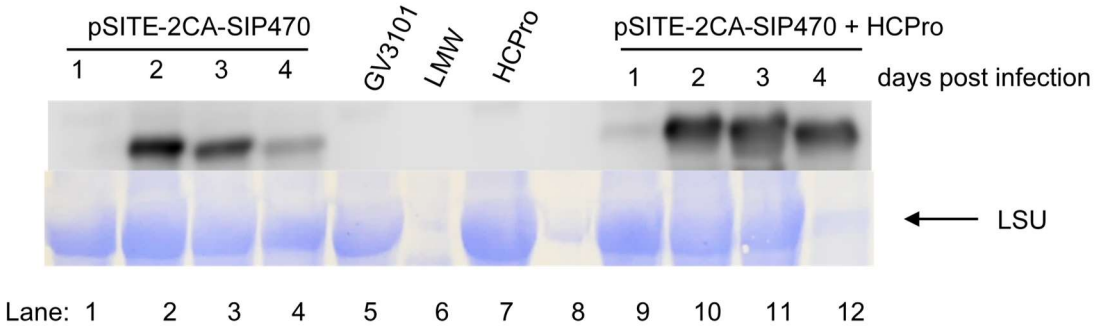
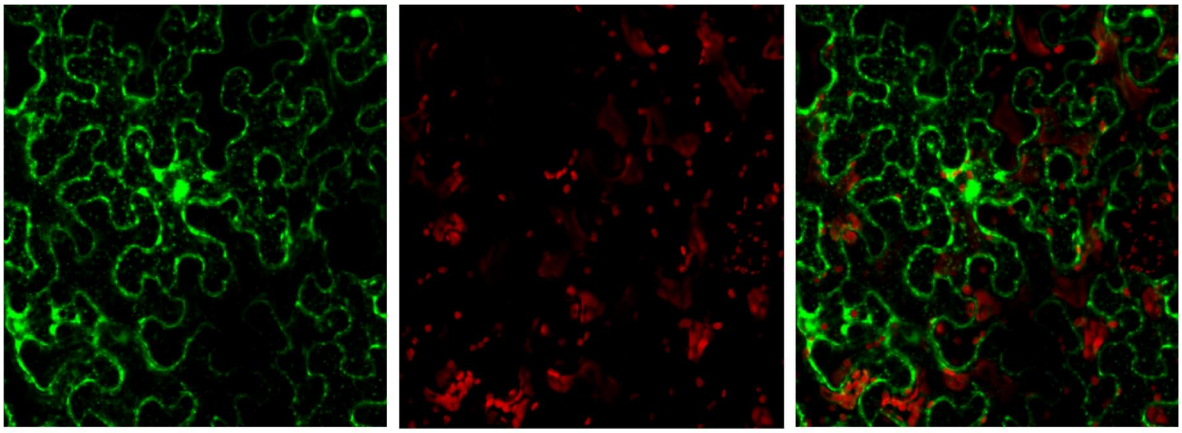


Figure 21: Time course experiment of the expression of eGFP-SIP470 in *N. benthamiana*. Lane #5 only contains the GV3101 construct and sample was taken on day 4 post infiltration. Lane #7 has only HCPro construct and sample was observed on day 4 post infiltration. LSU (large subunit of RuBisCO) is the loading control.

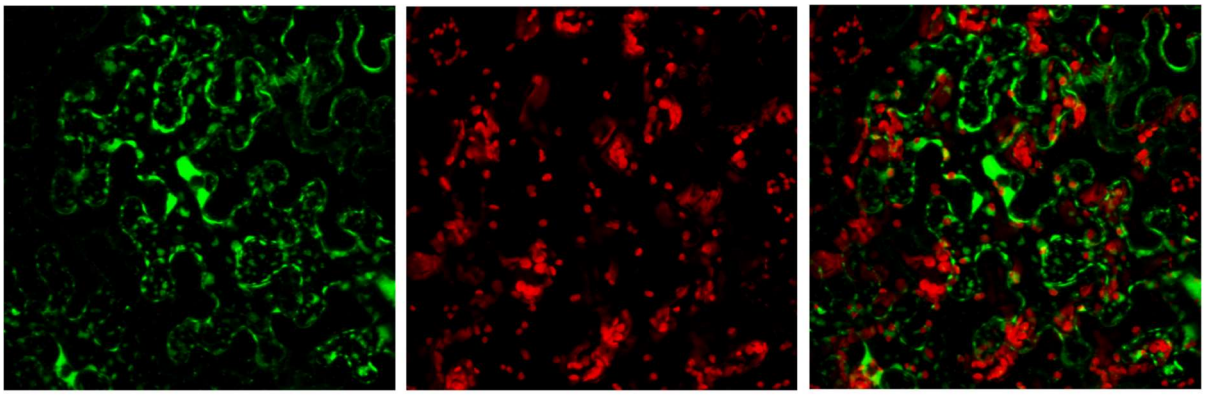
Confocal Microscopy of SIP470+eGFP tagged protein

To observe the expression of SIP470-eGFP, confocal microscopy was used. SIP470 in presence of HCPro expressed strongly at 2 days (Figure 6) and was chosen for confocal microscopy. On confocal analysis, chloroplast displayed autofluorescence and observed throughout. The SIP470-eGFP fluorescence was observed primarily around the periphery of the cells in a discontinuous pattern (Figures 22 and 23).



A. SIP470+eGFP+HCPro B. Chlorophyll C. Merged Images

Figure 22: Confocal microscopy of pSITE-2CA-SIP470 + HCPro expression in Tobacco leaf. A. shows pSITE-2CA + HCPro present through GFP detection. B shows autofluorescence of chlorophyll in leaf. C shows a merger or images A and B.



A. SIP470 + eGFP B. Chlorophyll C. Merged Images

Figure 23: Confocal microscopy of pSITE-2CA-SIP470 expression in Tobacco leaf. A. shows pSITE-2CA present through GFP detection. B shows autofluorescence of chlorophyll in leaf. C shows a merger or images A and B.

Confocal Microscopy of SIP470+eGFP+mCherry tagged protein

Specific proteins markers labeled with mCherry fluorescent protein were co-infiltrated with SIP470-eGFP in presence of HCPro into *Nicotiana bethamiana* leaves and observed using confocal microscopy. Co-localization of SIP470-eGFP + mCherry will be expressed as a yellow color in the images. Specific marker proteins used include golgi bodies, tonoplast, plasma membrane, plastids and peroxisomes (Figures 25 – 29). SIP470-eGFP+HCPro was observed without mCherry as a control (Figure 24).

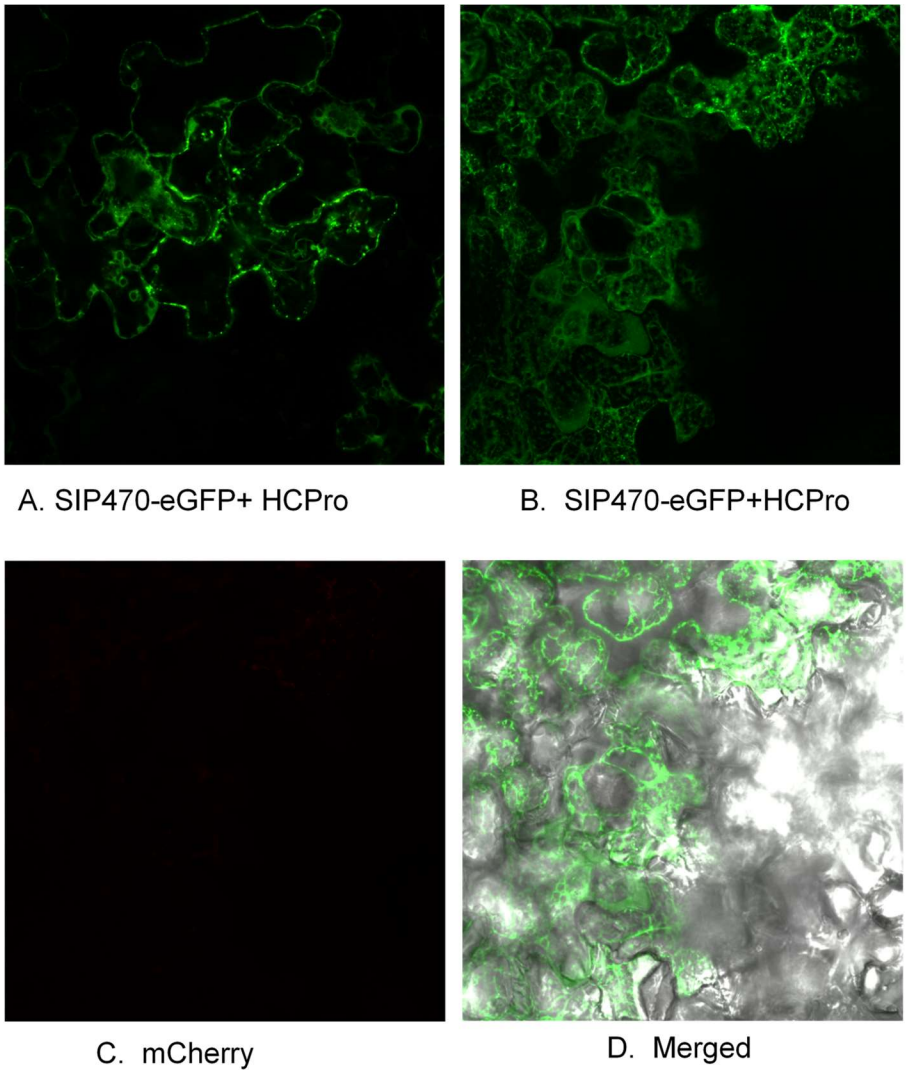
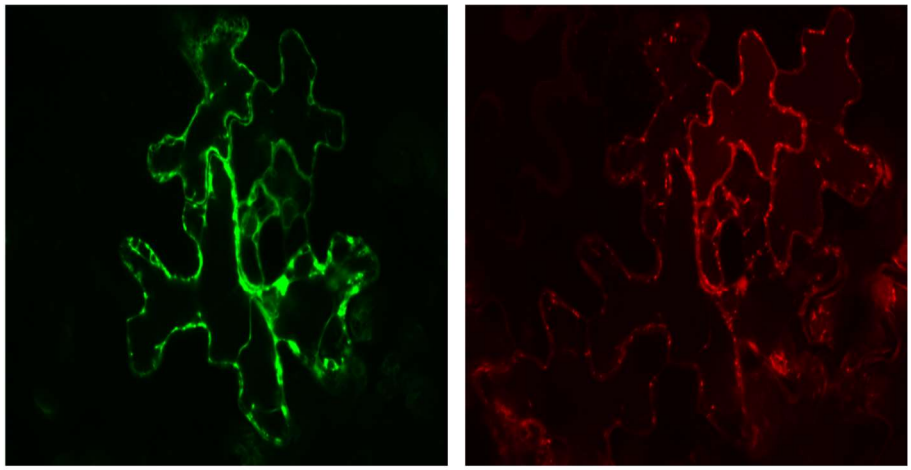
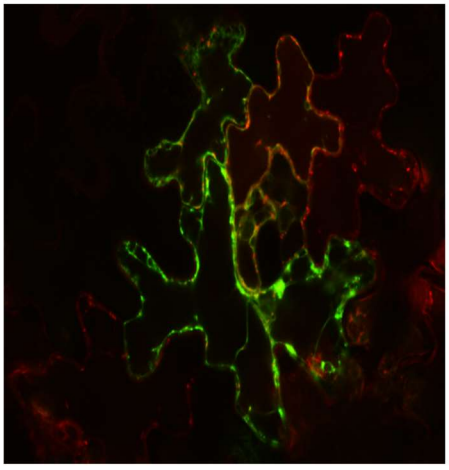


Figure 24*:* Confocal microscopy of SIP470-eGFP in presence of HCPro expression in Tobacco leaf. A. shows pSITE-2CA through GFP detection. B shows autofluorescence of chlorophyll in leaf. C shows a merger or images A and B.

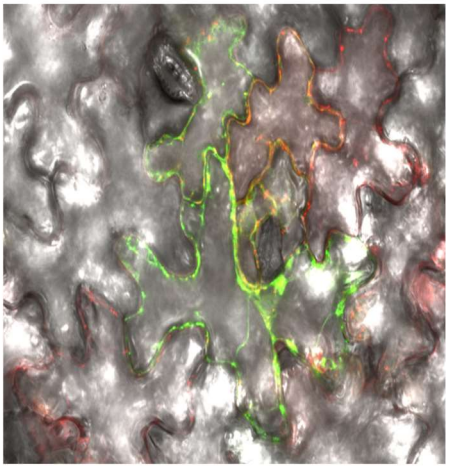


A. SIP470-eGFP+HCPro

B. mCherry

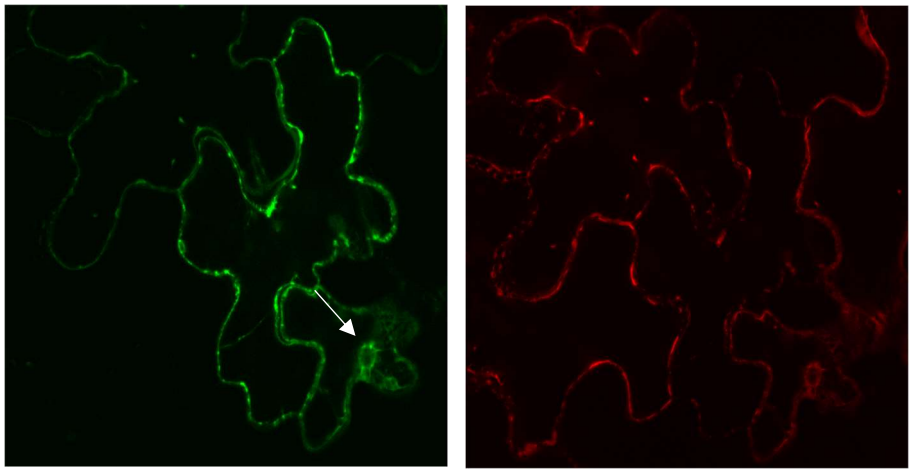


C. SIP470-eGFP+ HCPro+ mCherry



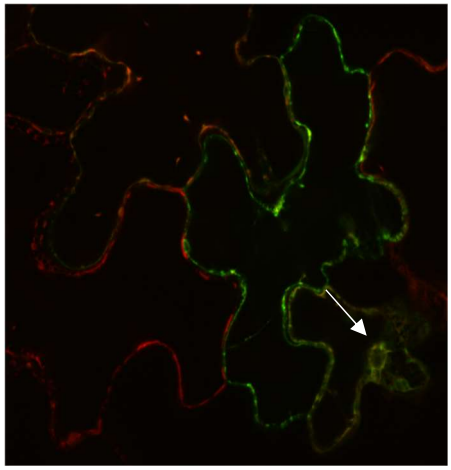
D. Merged

Figure 25: Co-localization of SIP470-eGFP with golgi bodies protein marker. A. shows SIP470-eGFP+HCPro present through GFP detection. B shows mCherry expression. C shows co-infiltrated constructs. D shows a merged image.

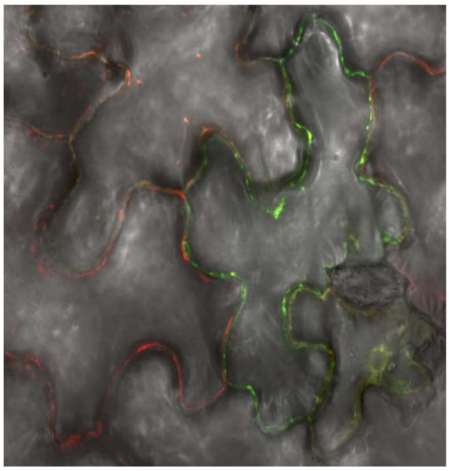


A. SIP470-eGFP+ HCPro

B. mCherry



C. SIP470-eGFP+ HCPro + mCherry



D. Merged

Figure 26: Co-localization of SIP470-eGFP with tonoplast protein marker. A. shows SIP470-eGFP+HCPro present through GFP detection. B. shows mCherry expression. C. shows co-infiltrated constructs. D. shows a merged image.

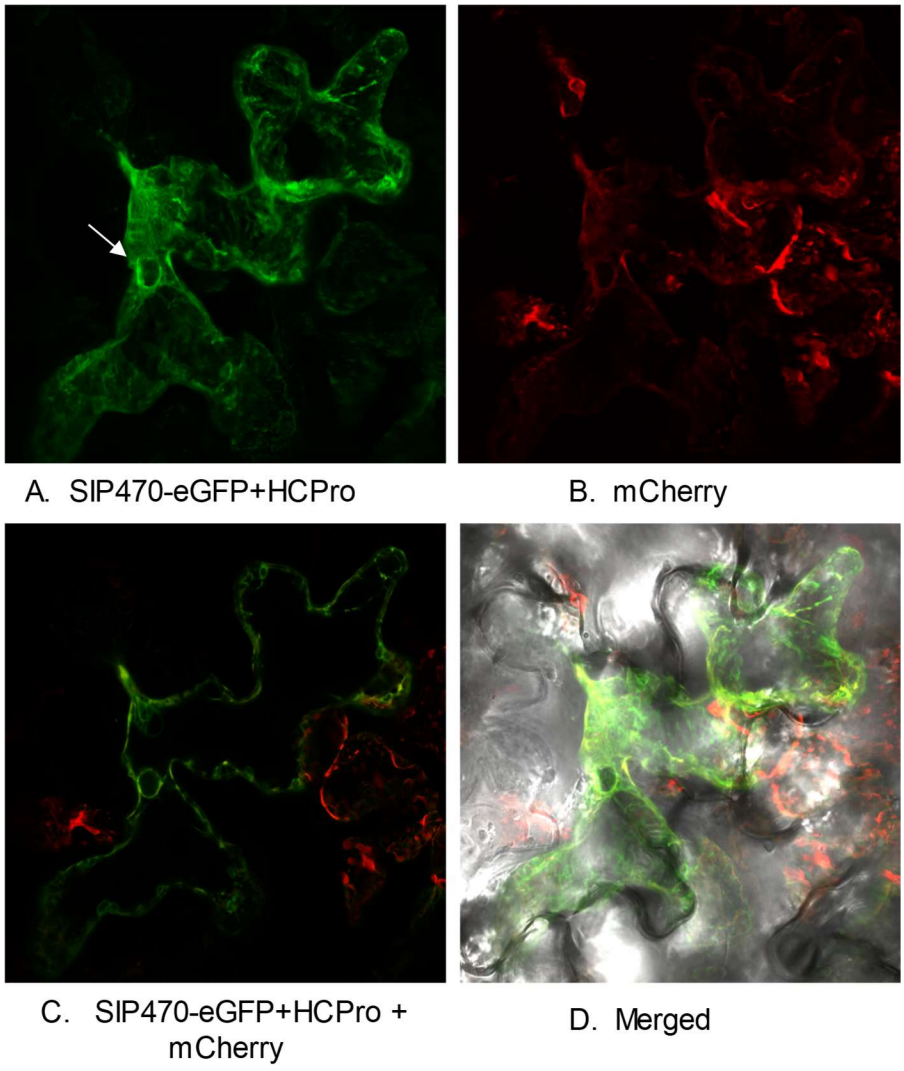


Figure 27: Co-localization of SIP470-eGFP with plasma membrane protein marker. A. shows SIP470-eGFP+HCPro present through GFP detection. B. shows mCherry expression. C. shows co-infiltrated constructs. D. shows a merged image.

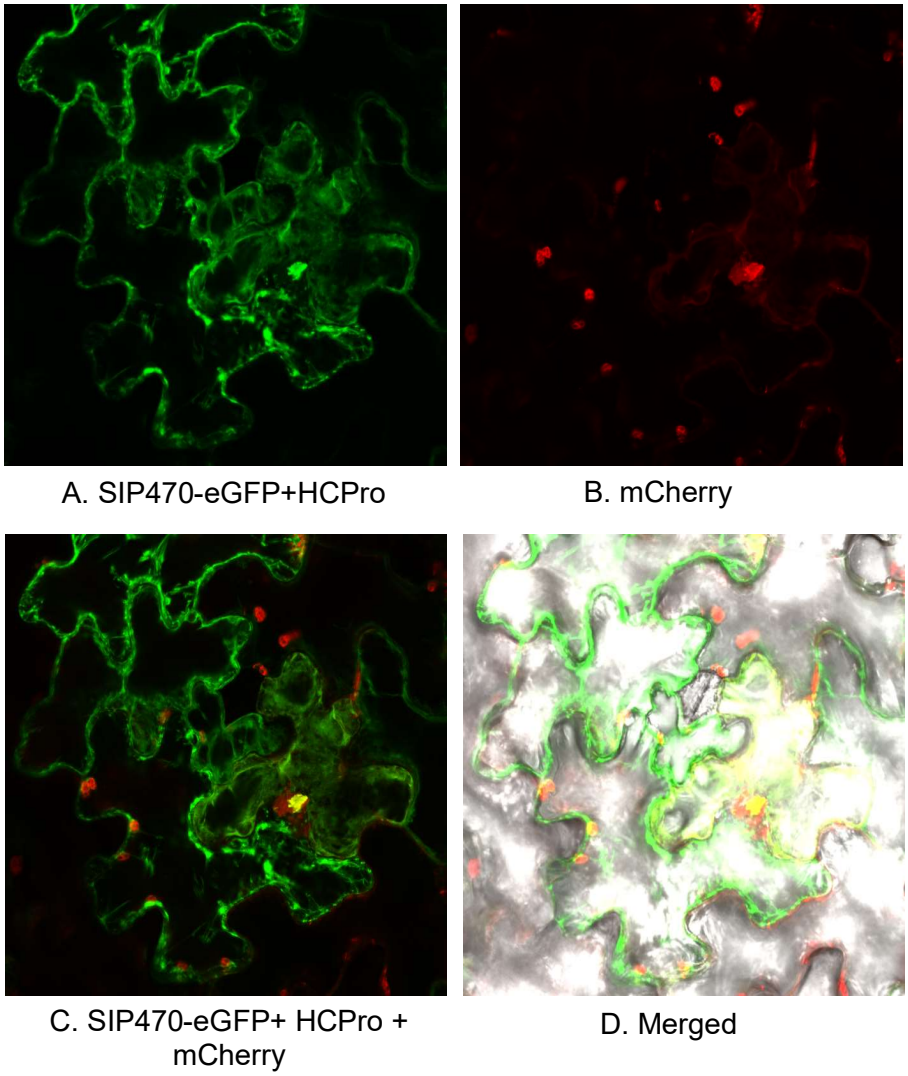
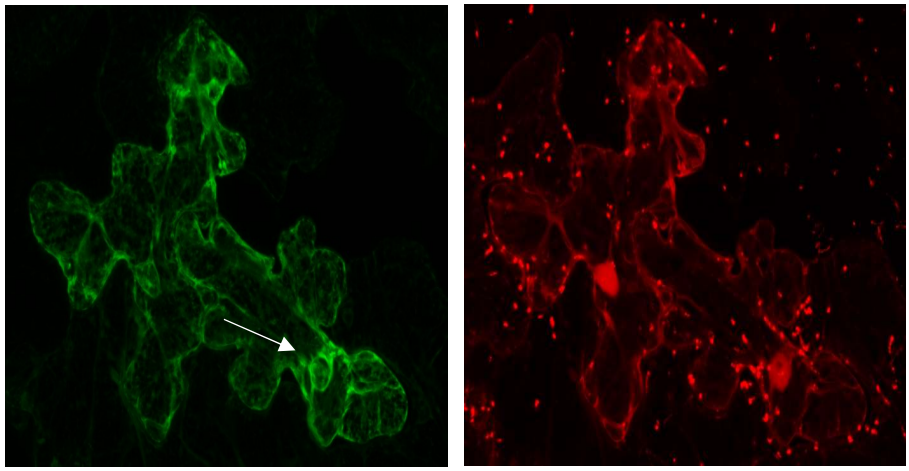
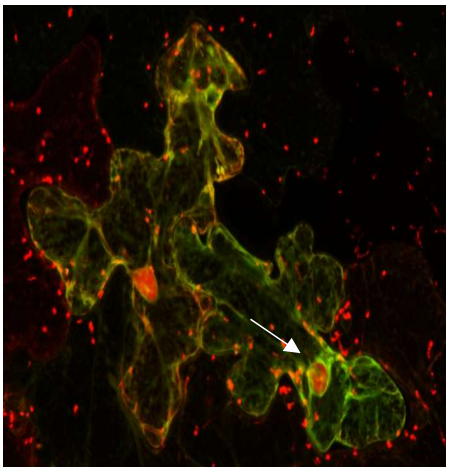


Figure 28: Co-localization of SIP470-eGFP with plastid protein marker. A. shows SIP470-eGFP+HCPro present through GFP detection. B. shows mCherry expression. C. shows co-infiltrated constructs. D. shows a merged image.

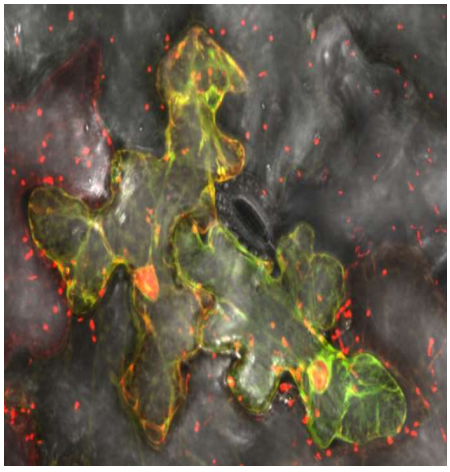


A. SIP470-eGFP+HCPro

B. mCherry



C. SIP470-eGFP+ HCPro + mCherry



D. Merged

Figure 29: Co-localization of SIP470-eGFP with peroxisome protein marker. A. shows SIP470-eGFP+HCPro present through GFP detection. B. shows mCherry expression. C. shows co-infiltrated constructs. D. shows a merged image.

Subcellular Fractionation of SIP470+eGFP

Subcellular fractionation of pSITE-2CA protein was performed to study the possible localization of SIP470. Two-day post infiltration leaf samples were collected, and subcellular fractionation was performed as described earlier. The different cellular fractions were processed with Western blot using anti-GFP primary antibody. Subcellular fractionation results show that eGFP tagged-SIP470 accumulates in the mitochondrial fraction. This experiment was repeated, and the same results were obtained (Figures 30 – 31).

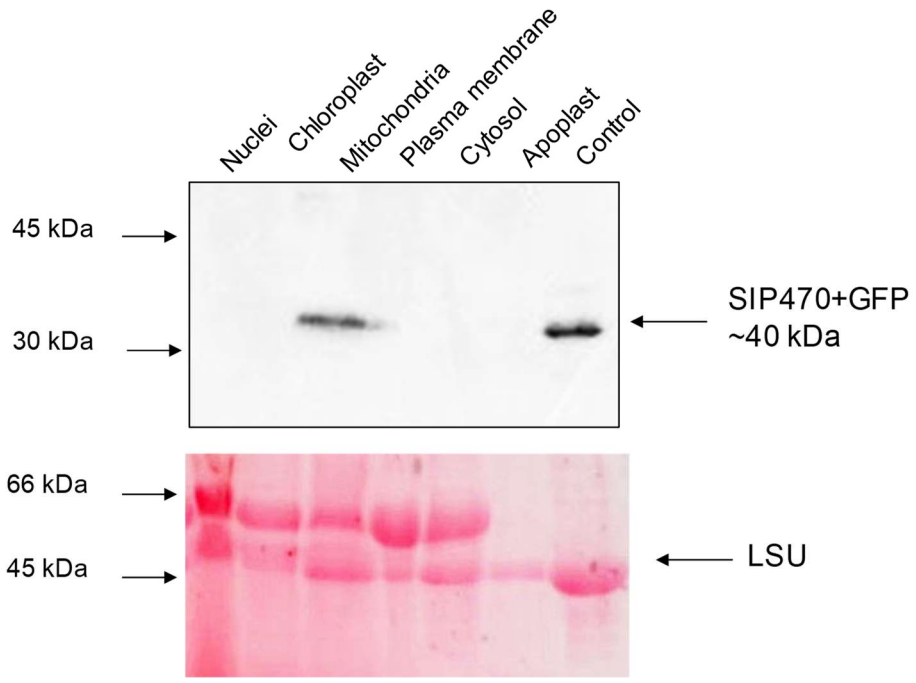


Figure 30: Subcellular Fraction of SIP470+eGFP. A shows Western blot analysis of SIP470+eGFP protein expression in various fractions. B shows ponceau stain showing RuBisCo large subunit (LSU) ~55 kDa.

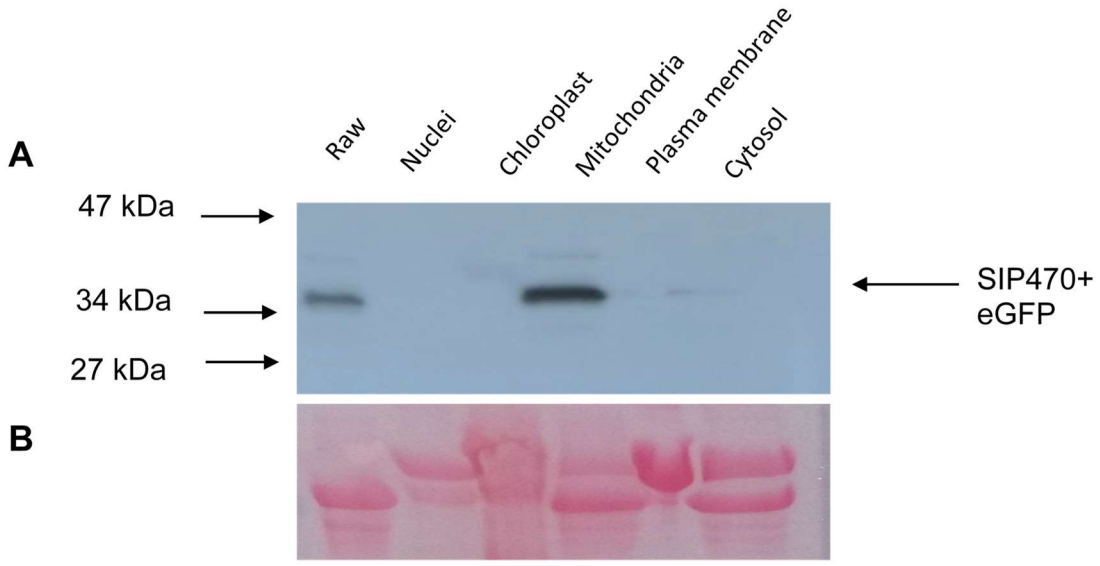


Figure 31: Subcellular Fraction of SIP470+eGFP. A shows Western blot analysis of SIP470+eGFP protein expression in various fractions. B shows ponceau stain showing RuBisCo large subunit (LSU) ~55 kDa.

CHAPTER 4

DISCUSSION

Plant immunity is a multi-faceted and intricate system which has many parallels to animal immune systems. Many plant hormones have been identified for their role in plant immunity; salicylic acid (SA) is one that has been studied extensively in disease resistance in plants. The complete biosynthesis of SA in plants is still elusive; the functions of this hormone are widespread and include disease resistance and thermogenesis (Vlot et al. 2009). During the pathogenic challenge, large quantities of SA is synthesized in the plastid (Schmid and Amrhein, 1995) and is subsequently converted to methyl salicylate (MeSA) and translocated throughout the plant via the phloem. MeSA is converted back to SA by the enzyme, salicylic acid binding protein 2 (SABP2), resulting in the activation of systemic acquired resistance (SAR) (Forouhar et al. 2005). SABP2 silenced plants were unable to mount SAR and were more susceptible to pathogenic attack by the tobacco mosaic virus (Kumar and Klessig 2003).

To discover more about the possible roles of SABP2, a yeast two-hybrid screening was performed with SABP2 as bait and tobacco total leaf proteins as prey. Several SABP2-interacting proteins (SIPs) were identified including SIP470. Bioinformatics has predicted SIP470 as being in the non-specific lipid transfer protein 1 (nsLTP1) subfamily (Chapagai 2014). To confirm this prediction, a lipid binding assay was performed using Ni-NTA column purified SIP470 and an artificial, fluorescent lipid binding substrate, TNS (*p*-toluidinonaphthalene-6-sulfonate) (Audam 2016).

Plant lipid transfer proteins are small, soluble proteins that include a hydrophobic cavity for lipid binding and transfer (Sodano et al. 1997) across the membrane (Kader 1996). This family has multiple roles in plants including wax accumulation, reproductive development, cell expansion, seed germination, abiotic and biotic stress (Salminen et al. 2016). LTP2 and LTP12 in *Arabidopsis* were identified as the closest homologs to tobacco SIP470 and knockout mutants were obtained and characterized for abiotic and biotic stress tests. Both *ltp12* knockout mutant was more susceptible to pathogenic infection compared to the wild-type, Col-0 (Chapagai 2014). In SAR response studies, *ltp2* and *ltp12* were not found to be compromised (Audam 2016). However, a recent study has shown that *ltp2-1* mutant is compromised in SAR compared to wild-type Col-0 (Carella et al. 2017). In abiotic tests, *ltp2* and *ltp12* were susceptible to oxidative stress test, salinity stress and drought recovery tests (Audam 2016).

To further understand the role of SIP470 in tobacco plants, transgenic tobacco SIP470 RNAi lines and overexpressing lines were generated (Audam 2016). The T2 generation SIP470 RNAi lines were screened multiple times with no detectable SIP470 silenced lines identified, except for #10 (Figure 3). Plant #10 was grown for seeds, and the T3 generation of Line #10 was screened for SIP470 silencing. The Line #10 in T3 generation did not show any reasonable silencing (Figure 4). It is likely that RNAi silencing of SIP470 is not heritable in the successive generations, which might indicate the importance of this protein in plant physiology. Also, a short coding sequence was used to generate the silenced lines in order to avoid the expression of other LTP homologs, which could have attributed to a decreased efficiency in silencing. Moreover, the estradiol-induced SIP470 overexpression T-1 transgenic lines were screened with

no positive lines (Figures 10-13). The seeds were grown in selective antibiotic resistance media, and therefore, only the transgenic seeds are able to grow in the media. An overexpression of SIP470 in plants may be deleterious and therefore, the plant suppresses the overexpression of this gene. The characterization of this SIP470's role in abiotic and biotic stress was unable to be completed in tobacco due to nonavailability of any acceptable transgenic lines.

The initial biochemical characterization of SIP470 in tobacco was completed, therefore, biochemical characterization of the *Arabidopsis* LTP12 homolog might yield answers as to its biochemical function. To clone the LTP12 cDNA from *Arabidopsis* leaves, total RNA was isolated, and first strand cDNA was synthesized and used for the amplification of LTP12 by PCR. *LTP12* cDNA failed to amplify at various temperatures or repeats of cycles (Figure 13). *LTP12* expression is highly induced during challenge with *Pseudomonas syringae DC3000* and *P.s. DC3000 avrRPT2* between 2 and 24 hours post leaf infiltration (Kilian et al. 2007). Mature Col-0 *Arabidopsis* leaves were treated with $MgCl_2$ and *P.s. DC3000* as described previously in the methods. The cDNA samples from 0h, 2h, 6h and 24h post infiltration was processed for LTP12 amplification by RT-PCR (Figure 14). LTP12 cDNA could not be amplified. Another attempt (Figure 15) was made to use various combination of LTP12 specific primers including the full-length amplification primers (DK736 and DK737). LTP12 is generally expressed in low quantities as it is primarily found in tapetum of the anther wall during reproduction (http://www.arabidopsis.org/portals).

To further characterize SIP470, determining where this protein is localized inside the cell might indicate the function. In silico analysis predicted that SIP470 is localized in

the extracellular region of the cell and also that it is most likely enters a secretory pathway following synthesis (Chapagai 2014). LTPs are generally considered apoplastic proteins and initial localization studies have supported extracellular localization along with an increasing number of intracellular localizations. Due to the N-terminal endoplasmic reticulum (ER) signal peptide, these proteins translocate to the ER membrane where the signal peptide is cleaved (Champigny et al. 2013). Once mature, these proteins are typically excreted to the extracellular regions of the plant cell. SIP470 has a predicted signal peptide, 1-25 amino acids (Chapagai 2014). Based on the initially identified function of lipid transfer as phospholipid transfer proteins and the in silico analysis, it was predicted that SIP470 likely localizes in the apoplast.

To investigate the localization of SIP470, a green fluorescent protein (GFP) was tagged to the N-terminal of SIP470 by cloning into pSITE-2CA vector. Transient expression of SIP470 was performed using pSITE-2CA-eGFP in the presence of HCPro. HCPro protein from Potyviridae group of RNA viruses is commonly used in transient expression experiments to suppress the host RNA silencing mechanism (Vallie et al. 2018). This allows for better expression of foreign proteins (SIP470) during transient expression in plants. The expression of SIP470 was observed over four days with (stronger expression) and without HCPro (weak expression of day 3 and 4) (Figure 21). In both constructs, a SIP470 specific signal was detected at two-day post infiltration. This time point was used for confocal microscopic analysis.

Confocal analysis was used in subcellular localization to visualize live cells in plant leaves. *N. bethamiana* leaves were infiltrated with pSITE-2CA-SIP470 with and without HCPro and after two days, leaf samples were collected and used for confocal

analysis. The images obtained showed localization of SIP470 near the periphery of the epidermal cells. In addition, there was a discontinuous pattern of fluorescence that appears as punctate structures throughout (Figure 22 and 23). This similar pattern was observed in another LTP, DIR1 (Carella et al. 2017). DIR1, a known LTP protein in *Arabidopsis* necessary for SAR. It is suggested to be localizated at the plasma membrane and endoplasmic reticulum (Carella et al. 2017). In sunflower seeds the LTP, Ha-AP10 is localized in the apoplast and upon imbibition, it relocalizes in intracellular organelles (Buhot et al. 2004). In *Arabidopsis*, LTP 1 is localized in the cell wall (Thoma 1994) and LTP3 is found in the cytoplasm (Guo et al. 2013). nsLTP are pre-proteins that become functional once they have targeted their appropriate domain (Liu et al. 2015).

To further analyze the localization of SIP470 in tobacco, co-localization of SIP470-eGFP+HCPro and mCherry was co-infiltrated tobacco leaves and observed using confocal microscopy. mCherry is a fluorescent protein expressed under the control of a 35S promoter in pBIN20 binary vector. Co-localization studies can be a useful tool that is commonly used to understand protein functions and identify transport pathways with the use and a marker of a particular organelle (Dunn et al. 2011). SIP470-eGFP in presence of a HCPro pattern of localization was similar to what was observed in the previous analysis with punctate structures around the periphery (Figure 24).

mCherry-tagged Golgi marker protein fluoresced in the transformed cells near the periphery (Figure 25). Golgi appears as individual round disks, or it may show ER labeling and appear as network extension throughout (Nelson et al. 2007). There was

no obvious co-localization with SIP470 in the same cell using this particular marker protein.

SIP470-eGFPand mCherry –tagged tonoplast marker was observed, and there was no apparent co-localization with eGFP-tagged SIP470 (Figure 26). The tonoplast is the membrane of the cell's vacuole, and the mCherry pattern was continuous around the periphery in transformed cells.

mCherry marker protein for the plasma membrane was observed and showed a diffused pattern around the periphery of the cell (Figure 27). The plasma membrane marker did not co-express with eGFP-tagged SIP470.

Plastids are described as the largest punctate organelle in fully developed chloroplasts (Nelson et al. 2007). In the mCherry marker protein for plastids, rounded shaped punctate structures were observed (Figure 28). SIP470-eGFP+HCPro+mCherry did not express co-localized in transformed cells. The yellow color observed in the merged picture is primarily attributed to overlapping of cells rather than co-localization.

Lastly, peroxisomes were observed in mCherry construct, and it showed a high saturation in punctate structures and in the nuclei of transformed and non-transformed cells (Figure 29). Co-localization was not apparent in the observable cells.

In Figure 29, the arrow points to the perinuclear region of the cell where SIP470-eGFP is expressed. In ER localization, there is a continuous, uniform tag with the perinuclear region which is clearly seen by arrows in Figures 26, 27 and 29 (Nelson et al. 2007). If the GFP expression was isolated to the vacuolar side or plasma membrane side, then it likely indicated one of those localizations as opposed to the ER (Nelson et

al. 2007). LTPs traffic lipids through a secretory pathway in the (ER) and plasma membrane (known as non-vesicular transport) or by vesicles (Wong et al. 2018). SIP470 could possibly be localized in the ER. Co-localization with an ER organelle marker could possibly make that distinction clearer. Alternatively, the SIP470 pattern of localization suggests that it is in punctate organelles which could suggest plastids, mitochondria, peroxisomes and golgi bodies (Nelson et al. 2007).

The subcellular fractionation results, which showed localization in the mitochondria, could not be confirmed due to the failure of the mCherry expression. Figures 27, 29, 30, 32 all showed a similar pattern of expression around the nucleus in both eGFP and mCherry proteins. The nuclear envelope allows for diffusion of molecules up to 50 kDa to pass between the cytoplasm and nucleus (Cooper 2000). Alternatively, this pattern could also be indicative of the ER localization, which is expressed in the perinuclear region as a continuous band (Nelson et al. 2007). Co-localization with mitochondria and ER marker proteins might give a more conclusive response for the localization of SIP470.

To further investigate precisely where the localization of SIP470 might be within the cell, differential centrifugation and sucrose gradient method were used to identify cellular fractions, and this was analyzed by western blot using anti-GFP antibody. Results showed that SIP470+eGFP was primarily localized in the mitochondrial fraction and a very faint band was seen in the cytoplasmic fraction. Although most of the research does not support plant LTP localization is the mitochondria, there is recent research in the mammalian system for LTPs that are localized in the mitochondria (Gao and Yang 2018; Scharwey et al. 2013). Mitochondria are rich in lipids as it shuttles lipids

between the inner and outer mitochondrial membranes (Scharwey et al. 2013). A lipid transfer protein, VPS13 in mammalian cells was localized to ER-mitochondria contact sites (Gao and Yang 2018). SIP470 could be a novel protein that has a particular function related to the mitochondria or more studied are needed for verification of subcellular localization.

Future Directions

Subcellular localization of SIP470 could be studied under abiotic and biotic stress conditions to determine if the localization changes under stress. LTPs are well known to contribute to plant defense systems and their adaptability to environmental stress. Hence, it would be interesting to observe any possible changes. The virulent pathogen such as *Pseudomonas syringae pv. tabaci* could be used for this study. Similarly, salinity (NaCl) or osmotic (mannitol) or drought conditions could also be used to study any changes in the localization of SIP470.

Lipid binding assay has determined that SIP470 is a lipid transfer protein using the lipophilic fluorescent substrate, TNS (Audam 2016). The actual in-planta substrate for SIP470 is unknown. A competitive lipid binding assay could be done using various saturated and/or unsaturated lipids. For this reaction, TNS will be incubated with SIP470 and the fluorescence measured. A lipid will be added to this mixture and if the lipid displaces the TNS, there will be a decrease in fluorescence indicating a preference to binding the lipid.

To further characterize SIP470 in tobacco, new silenced and overexpression lines could be generated to determine how this protein can affect biotic and abiotic stress in the plant. Generation of new silenced lines could be done using CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Cas9 system. This system can be used considering that the entire genome for *N. tabacum* is available. It would be important to compare the results to that of the homologs in *Arabidopsis*.

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APPENDICES

APPENDIX A: Abbreviations

APS: Ammonium Persulfate

DEPC: Diethylpyrocarbonate

DTT: Dithiothreitol

eGFP: enhanced Green Fluorescent Protein

EtBr: Ethidium Bromide

ETI: Effector-Triggered Immunity

ETS: Effector Triggered Susceptibility

HCPro: Helper Component Proteinase

ICS: Isochorismate Synthase

IPL: Isochorismate pyruvate lyase

IPTG: Isopropyl β-D-1-Thiogalactopyranoside

KDa – Kilo Dalton

LMW: Low Molecular Weight

MeSA: Methyl Salicylate

MS: Murashige Skoog

MES: Morpholinoethanesulfonic acid

NB-LRR – Nucleotide Binding Leucine Rich Repeat

Ni-NTA – Nickel-Nitrilotriacetic Acid

NPR1: Nonexpresser of Pathogenesis-Related protein 1

OD: Optical Density

PAL: Phenylalanine Ammonia Lyase

PAMPs: Pathogen Associated Molecular Patterns

PCR: Polymerase Chain Reaction

PMSF: Phenylmethylsulfonyl Fluoride

PTI: PAMP-Triggered Immunity

PVDF: Polyvinylidene Fluoride

ROS: Reactive Oxygen Species

RNAi: RNA Interference

RT-PCR: Reverse Transcriptase- Polymerase Chain Reaction

SA: Salicylic Acid

SABP2: Salicylic Acid Binding protein 2

SAMT: Salicylic Acid Methyl Transferase

SAR: Systemic Acquired Resistance

SDS: Sodium Dodecyl Sulfate

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SIP: SABP2 Interacting Protein

SIP470: SABP2 Interacting Protein-470

TEMED: Tetramethylethylenediamine

mA: Milli-ampere

β-ME: β-mercaptoethanol

µg – Microgram

µM: Micromole

µL – Microliter

mg/mL: Milligram/Milliliter

ng/µL – Nanogram/Microliter

APPENDIX B: Buffers and Reagents

1.5% Agarose Gel1x TAE Buffer= 50 ml

0.75 g Agarose

Agarose was added to 1XTAE buffer and microwaved for 70 seconds.

Mixture was placed in 55°C water bath for 15 minutes.

2.5 µL (10 mg/mL) ethidium bromide was added to the mixture and this was poured into the cast, a comb was added and allow to solidify.**20% APS (Ammonium persulfate) (0.5 mL)**

0.5 mL distilled water

0.1g Ammonium persulfate

0.1% DEPC treated water (100 mL)

0.1 mL Diethyl pyrocarbonate

100 mL Water

Completely dissolve mixture and incubate for 12 – 16 hours at 37°C. Autoclave at 121°C at 15 psi atmosphere pressure for 20 minutes.

SDS-PAGE Loading Dye, 2X (100 mL)

0.4 g SDS final concentration 0.4% (v/v)

0.2 g Bromophenol blue, final concentration 0.2% (v/v)

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

Glycerol = 20 ml, final concentration 20% (v/v)

Add 5% βME before use

10X SDS-PAGE Running Buffer (1 L)

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

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

10 g SDS

1X SDS-PAGE Running Buffer (1 L)

Add 100 mL of 10X SDS-PAGE Running Buffer in 900 ml of distilled water

10X Phosphate Buffer Saline (PBS) (1 L)

4.1 g Sodium Phosphate monobasic (M.W: 119.96 g/mol)

10 g Sodium Phosphate dibasic (M.W: 141.96 g/mol)

76 g Sodium Chloride (M.W: 58.44 g/mol)

1X Phosphate Buffer Saline (PBS) (1 L)

Add 100 ml of 10X PBS in 900 ml of Milli-Q Water

1X PBS plus 0.3% Tween Twenty (1 L)

Combine 100ml of 10X PBS in 897 ml of distilled water followed by 3 mL of Tween twenty

10X Western Blotting Transfer Buffer (1 L)

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

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

1X Western Blotting Transfer Buffer (1 L)

10X Western Blotting Transfer Buffer = 100 mL

Methanol = 100 mL

Cold distilled Water = 800 mL**Ponceau S Stain (50 ml)**

2.5 mL Acetic acid, final concentration = 5%

0.05 g Ponceau S, final concentration = 0.1%

47.5 mL distilled Water

Coomassie Brilliant blue de-staining solution (1 L)

Distilled Water = 500 mL

Acetic acid = 100 mL

Methanol = 400 mL

Coomassie Brilliant Blue staining solution (1 L)

Distilled Water = 400 mL

Acetic Acid = 100 mL

Methanol = 500 mL

1 g Coomassie Brilliant Blue was added to the solution

1X TAE Buffer (1 L)

Dissolve 20 ml of 50X TAE buffer in 980 ml of distilled water.**50X Tris Acetate EDTA (TAE) Buffer (1 L)**

Glacial acetic acid = 59.10 mL

0.5 M EDTA (pH 8.0) = 100.0 mL

Tris base (M.W: 121.1 g/mol) = 242.0 g

Add distilled water to make final volume 1000 mL

STE Buffer (100 mL)

EDTA = 0.029 g

Tris = 0.121 g

NaCl = 0.584 gAdjust final pH to 8.0 with HCl and store in 4 ^{o}C.

STET solution (100 ml)

To STE buffer add 5% Triton X-100 and store in 4 ^{o}C.

Grinding Buffer (1 L)

0.42 g EDTA (M.W. = 416.20g/mol), final concentration = 1 mM

60.12 g Mannitol (M.W. = 182.17g/mol), final concentration = 0.33 M

4.19 g MOPS (M.W. = 209.26g/mol), final concentration = 20 mM

0.75 g Glycine (M.W. = 75.07g/mol), final concentration = 10 mM

Adjust pH to 7.2 with NaOH

To sterilize, autoclave solution at 121°C, 15 psi atmospheric pressure for 20 minutes. Cool at 4°C and add 5 g PVP and 2 g BSA. Mix well.

Add β-mercaptoethanol (0.02% final concentration) and PMSF (1 mM final concentration) prior to use.

Guanine HCl buffer

4 M Guanine thiocyanate

5 mM Sodium Citrate, pH 7.0

0.5% Sarkosyl

LB Broth (100 mL)

Dissolve 2.5 g of LB media into distilled water for a final volume of 100 mL. To sterilize, autoclave the solution at 121°C, 15 psi atmospheric pressure for 20 minutes.

LB agar Media (100 mL)

Dissolve 2.5 g of LB media and 1.5 g agar in distilled water for a final volume of 100 mL. Autoclave solution at 121°C, 15 psi atmospheric pressure for 20 minutes.

Half Strength MS Media (1 L)

MS Salt = 2.155 g

Sucrose = 1 g

Adjust pH with 1 M KOH to 5.9

To solution, add 0.9% agar and make final volume to 1L with distilled water. Sterilize by autoclaving solution at 121°C, 15 psi atmospheric pressure for 20 minutes.

VITA

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