5-2020

Subcellular Localization of Tobacco SABP2 under Normal and Stress Conditions

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A Thesis

Presented to

The Department of Health Sciences

College of Public Health

East Tennessee State University

in Partial Fulfillment

of the Requirements for the

Honors-in-Discipline Scholars Programs

By

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April 2019

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Keywords: SABP2, Subcellular Localization, Confocal Microscopy

ABSTRACT

Subcellular Localization of Tobacco SABP2 under Normal and Stress Conditions

Salicylic acid (SA), a phytohormone, plays an important role in plant physiology. SA mediated innate immune pathway is an important pathway for plant immunity against pathogens. Plants resisting pathogen infection synthesize higher levels of Methyl Salicylate (MeSA), which is then converted to SA by the esterase activity of Salicylic Acid Binding Protein 2 (SABP2). The high level of the converted SA leads to enhanced pathogen resistance. The study of subcellular localization of a protein is critical in explaining its potential biochemical functions. SABP2 tagged with eGFP was expressed transiently in *Nicotiana benthamiana* leaves. The SABP2-eGFP expressing leaves were challenged with bacterial and viral pathogens and observed under confocal microscopy. Fluorescent signals were seen throughout the cell and more concentrated towards the cell periphery. To verify the localization, mCherry fluorescent organelle markers with specific targeting sequences were used. The results indicate that the SABP2 is likely a cytoplasmic protein, and there is no change in its localization upon infection by plant pathogens.

ACKNOWLEDGEMENTS

I would like to thank my thesis mentor Dr. Dhirendra Kumar for his unparalleled support, motivation, and encouragement throughout this research experience. Without his invaluable knowledge and unwavering guidance, I would not have been able to achieve this goal. Besides, I would like to thank Dr. Sean Fox for willing to read my thesis and provide me with valuable feedback. I am also grateful to Balkrishna Chand Thakuri, Saroj Chandra Lohani, Shantaya Andrews, Ryan Burgess, and Baylea Davenport for helping me with my project and answering several questions that I had. I am deeply indebted to the Honors College for the Student-Faculty Collaborative Research Grant, Department of Health Sciences, and Department of Biological Sciences for making this project feasible and providing me the platform to conduct and defend my thesis. I would also like to thank Rolf Fritz of the confocal microscopy core facility, Quillen College of Medicine, ETSU. I would also like to thank the Arabidopsis Biological Resource Center (ABRC) for providing plasmid vectors used in this research. This research was supported by a grant from the National Science Foundation (MCB#1022077) to Dr. Dhirendra Kumar and funds from the Department of Biological Sciences, ETSU. I cannot leave East Tennessee State University without thanking my family, friends, and all others who inspired and helped me. I would have never gotten this far without you all standing by me.

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INTRODUCTION

The Need of Research in the Field of Plants

Plants carry out photosynthesis to convert light energy into chemical energy. Not only do they provide food to organisms, they also maintain our atmosphere by absorbing carbon dioxide. Less carbon dioxide means less greenhouse gases and reduced global warming. Plants also provide various products that are used by humans, such as timber, medicines, oils, and rubber. Plants serve as habitat for many organisms. Many species of insects, mammals, reptiles, and bird live on the same tree.

The importance of plants to humans and other life forms on Earth is staggering, but plants are always under threat. Threats are of two types, biotic and abiotic stress. Fungi, bacteria, viruses, parasites, weeds, and insects are biotic stressors while drought (water deficit), excessive watering (water lodging/flooding), extreme temperatures (cold, frost, heat), and salinity (sodicity) are abiotic stressors. Plants interaction with other components of the ecosystem is necessary for survival, but sometimes those interactions result in death or yield loss, or quality loss. Biotic stresses from insects, diseases, and weeds cause a loss of 31-42 percent ($500 billion) out of the total US annual food production capacity of $1.3 trillion worldwide[1]. In developing countries, crop damage due to pathogens is more severe.

There are several ways how we can protect crops. The usage of pesticides is a very popular traditional approach. Though they protect our crops, chemicals from pesticides find their way into our food chain and cause significant life and health loss. Also, with the increasing resistance in the pathogen, the lethal dose is increased, which in turn increases the cost of production. In the last few decades, an increasing trend in molecular approaches has been made

to protect the crop and increase productivity. A clear and thorough understanding of a plant's own natural defense mechanism is important.

The Plant Immune System

Plants interact with microbes in several ways. There are interactions that are beneficial for the plants, interactions where the plants serve as a habitat for the microbes, and interactions where the plants suffer[2]. While plants deploy defense mechanisms, microbes try to subvert the defense systems of the plant.

Plants use pattern recognition receptors (PRRs) in response to slowly emanating pathogen-associated molecular patterns (PAMPs), for example, flagellin[3]. In another immune mechanism deployed mostly inside the cell, plants use the polymorphic NB-LRR protein products encoded by most R genes[4]. NB-LRR proteins get the name from their characteristic nucleotide-binding (NB) and leucine-rich repeat (LRR) domains. In step 1, PRRs recognize PAMPs and cause PAMP-triggered immunity (PTI) that prohibits further colonization. In step 2, thriving pathogens release effectors critical for virulence. Effectors intervene with PTI causing effector-triggered susceptibility (ETS). However, in step 3, NB-LRR proteins indirectly or directly specifically recognize a given effector and result in effector-triggered immunity (ETI). ETI is a fast and enhanced PTI response providing disease resistance to plants and causing hypersensitive cell death response (HR) at the site infection. In step 4, as a result of natural selection, pathogens avoid ETI shedding/changing effector gene or by acquiring extra effectors[4].

Another resistance phenomenon in plants is systemic acquired resistance (SAR). What makes SAR unique is its ability to strike over a broad and distinctive class of pathogens such as viruses, bacteria, oomycetes, and fungi[5,6]. SAR also induce long-lasting protection ranging from

weeks to month or even an entire season. Several studies have shown that SAR is one of the salicylic acids (SA) dependent responses. SA and its derivative methyl salicylate (MeSA) play an important role in the establishment of SAR, a long-distance signaling mechanism[7]. This unique feature of SAR makes it widely popular in crop production. Plants also possess the ability to transmit their inherited immunity against specific stresses to the next generation. Immune memory of SAR in *Arabidopsis* was passed onto the next generation in an experiment. When a virulent strain of *P. syringae pv tomato* was used for inoculations, SAR activation induced response to this bacterial species as well as with the oomycete *H. parasitica* in the F1 generation[8].

Phytohormones

Various physiological processes in plants are influenced by naturally occurring, organic substances known as phytohormones. Those processes are generally growth, differentiation, and development, but other phenomena such as stomatal movement may also be affected. The study of biosynthesis, signal transduction, and action of every hormone has garnered a lot of attention over the past few years[9]. It is important to understand that hormones do not act alone but in conjunction or opposition to one another.

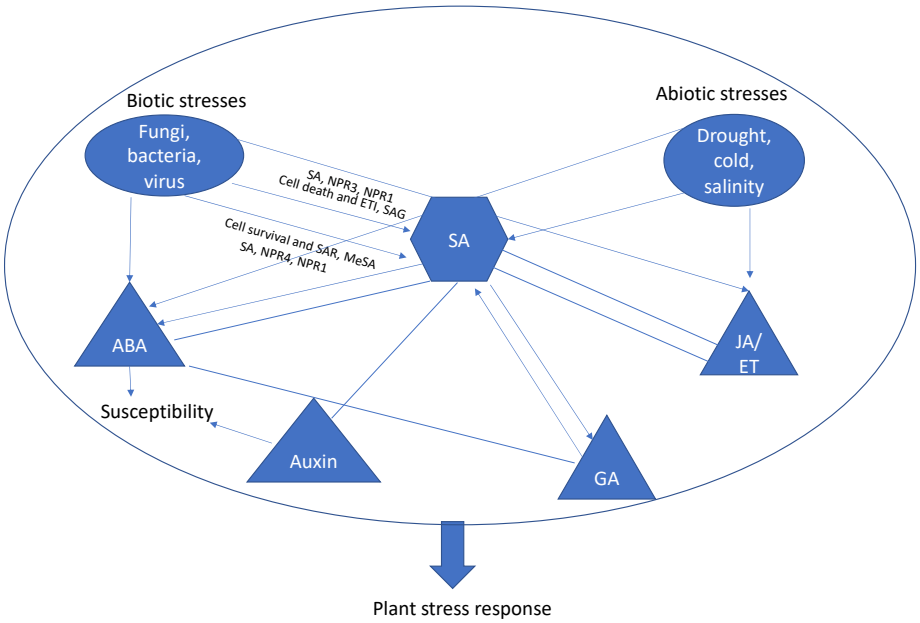

Fig 1: Mechanism of Plant Hormone Signaling under Stress. Salicylic acid (SA) and other phytohormones crosstalk to initiate defense responses under stress conditions. Adapted from Zhao et al[10].

Auxin

The main auxin in plants is indole-3-acetic acid (IAA). IAA is produced from tryptophan or indole, especially in leaf primordia, young leaves, and developing seeds. Auxin plays a role in several processes such as cell enlargement, cell division, vascular tissue differentiation, root initiation, apical dominance, and leaf senescence.

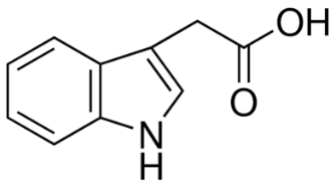

Fig 2: 3-Indoleacetic acid

Gibberellin (GA)

GA_1 is the most important GA in plants and mainly responsible for stem elongation. It is synthesized in the seed and young tissues of the shoot from glyceradehyde-3-phosphate via isopentyl diphosphate. GA causes stem growth, bolting, seed germination, and enzyme production.

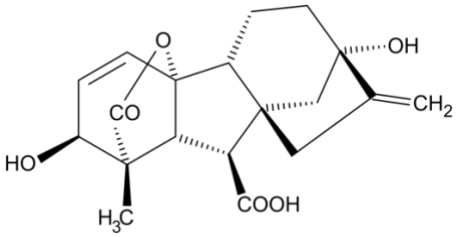

Fig 3: Gibberellic acid

Cytokinin (CK)

Zeatin is the most common CK in plants. Under the presence of auxin, CK, which are adenine derivatives, cause cell division in tissue culture. The effect of CK on plants is cell division, morphogenesis, growth of lateral buds, leaf expansion, and chloroplast development.

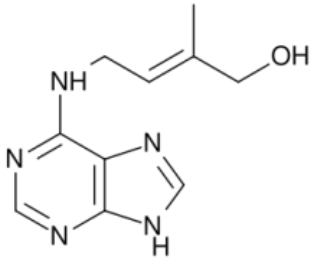

Fig 4: Cytokinin

Ethylene (ET)

Ethylene is known as the fruit ripening hormone. The synthesis of ET occurs in tissues in response to stress, such as tissues experiencing senescence or ripening. It easily diffuses from its site of synthesis because it is a gas. Some of the effects of ethylene are defense responses, post-

injury or disease, adventitious root formation, flower induction, flower opening, leaf senescence, and fruit ripening.

Abscisic Acid (ABA)

ABA is synthesized from glyceraldehyde-3-phosphate via isopentyl diphosphate and carotenoids. The synthesis takes place in roots and mature leaves in response to water stress. ABA promotes stomatal closure, maintains dormancy in seeds and buds, and produces response against environmental stresses such as drought, cold, soil salinity, heat, and heavy metal ion.

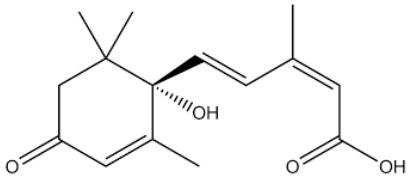

Fig 5: Abscisic Acid

Salicylic Acid (SA)

SA (2-hydroxy benzoic acid) is synthesized in plants from the amino acid phenylalanine. Salicylates, which are present in willow bark, act as potential regulatory compounds. SA protect plants from pathogens by producing pathogenesis-related (PR) proteins. It is involved in inducing the SAR in which the whole plant develops resistance when a part of the plant is attacked by pathogen. SA also causes thermogenesis, enhance flower longevity, inhibit seed germination, and counterattack effects of ABA. After synthesis, SA goes thorough different chemical modifications such as glycosylation, methylation, amino acid conjugation, hydroxylation, and sulfonation[11].

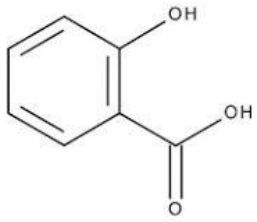

Fig 6: Salicylic Acid

Salicylic Acid and its Role in Systemic Acquired Resistance

Plants are constantly challenged by pathogens, and as a result, they employ basal and induced resistance mechanisms in their defense. SA confers resistance by triggering SAR in plants mainly against biotrophic and hemibiotrophic pathogens. When SA or its functional analog 2,6-dichloroisonicotinic acid was applied exogenously, SAR was induced in plants offering resistance against certain pathogens[5]. The transgenic plants expressing the bacterial gene salicylate hydroxylase (*nahG*) tend to avoid SA accumulation by readily converting it to catechol and become susceptible to pathogens[12]. Direct evidence supporting the role of SA in plant defense was obtained through the study of an *Arabidopsis* isochorismate synthase (ICS) *SA induction-deficient 2* (sid2) mutant, which is defective in the synthesis of SA[13]. The depletion of SA leads to the breakdown of SAR and gene-for-gene-gene resistance in *Arabidopsis*. In both cucumber and tobacco, SA levels were significantly higher in the phloem sap[14,15]. Likewise, in vivo SA-labeling experiments have shown that SA made in the leaves of TMV-infected tobacco or TNV-infected cucumber were also found in the uninfected tissues[16,17]. SA levels increased as much as 70% (tobacco) and 50% (cucumber) in pathogen-infected plants as a result of translocation from infected to uninfected leaves. This evidence suggests SA as the signal translocating from infected site to induce SAR elsewhere in the plant.

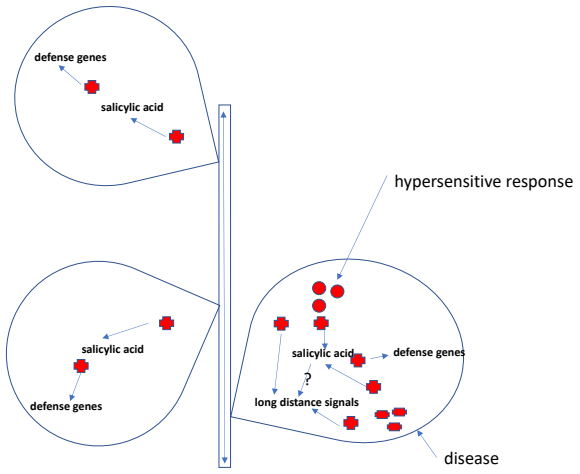

Fig 7: Systemic Acquired Resistance. SAR is induced in response to pathogen infection. Cell death caused either by hypersensitive response or disease promotes the synthesis of SA in infected leaves. SA, in turn, activates defense-related genes. Infected leaves transport long-distance signals causing SA synthesis and expression of defense-related genes in the remaining plant. Adapted from IntoBiology[18].

SA-Binding Protein 2 (SABP2)

SABP2 is a 29 kDa protein which is present in low quantities in tobacco leaves. This soluble protein, which binds SA with high affinity, belongs to the α/β fold hydrolase superfamily[19]. SABP2 is a methyl salicylate (MeSA) esterase enzyme. It cleaves off a methyl group from inactive MeSA, converting it into active SA, an end product inhibitor of the process[20]. When SABP2 is silenced using RNAi technique, both SA-induced expression of *PR1* genes and resistance level against the Tobacco Mosaic Virus (TMV) is lowered[21].

SABP2 has also been known for its ability to convert acibenzolar-S-methyl (ASM) an ester into acibenzolar, an acid. Acibenzolar-S-methyl effect is lost when SABP2 is silenced, thereby affecting *PR1* gene expression and induction of SAR[22]. ASM is an active analog of SA and is known to induce SAR responses in plants.

MeSA, which is produced by the activity of salicylate carboxyl methyltransferase (SAMT) is more hydrophobic than SA. It penetrates cell membranes quickly and is supposedly a mobile signal of the SAR along with molecules like pipecolic acid[23]. Infected cells are the site of MeSA production, but it travels to distant leaves through phloem transport. It is converted into active SA in theses systemic tissues by the methylesterase activity. Finally, SA triggers defense responses in these distal leaves, thus establishing SABP2 as a key enzyme linked to SAR development in tobacco[24,25].

Subcellular Localization

More than 10,000 proteins are synthesized by eukaryotic cells and transported to pre-determined target organelles[26]. Proteins tend to work optimally in a particular subcellular localization, so accurate transfer of a protein to its final destination is pivotal for its functioning. The functional characterization of every protein has been a challenge for molecular biologists/ biochemists since the post-genomic era. Large scale analysis of proteins can help us understand their interaction and functions in the cell. This is possible by determining the subcellular localization pattern of each protein. There are various subcellular compartments or organelles in a eukaryotic cell.

Transport into these organelles across the membrane is a regulated and complex process. When mRNA is translated into proteins in the cytoplasm, they can enter the secretory pathway (SP), non-secretory pathway (nSP), or persist in the cytoplasm. In fact, as much as half of the proteins that a cell synthesizes, have to be transported at least through one cellular membrane to reach to their target organelles[27]. In addition to protein function, subcellular localization is also important to achieve functional diversity and gain an advantage in protein design and synthesis[28].

Hypothesis

Plants produce high levels of SA when dealing with pathogens. SABP2, which carries out the conversion of MeSA into active SA, plays an important role in plant defense signaling. To fully understand the role of SABP2 in the defense signaling pathway, it is important to understand the subcellular localization of this protein. Subcellular localization experiments provide important clues about biochemical interaction and the function of proteins. In this study, we aim to understand the subcellular localization of SABP2 under normal conditions and see if the localization pattern changes in response to biotic stress. SABP2 is not known to have any target signal peptide, so it is possible that it may be localized in the cytoplasm.

MATERIALS

Plants

The tobacco plant, *Nicotiana benthamiana* (NB), was primarily used in this study. Besides NB, *Nicotiana tabacum* cv Xanthi NN (XNN), NahG transgenic tobacco, SABP2-silenced transgenic tobacco (1-2), and the susceptible tobacco (susceptible) were also used in this SABP2+eGFP transient expression study. The plants were grown from seeds inside the growth chamber maintained at 22°C and 14 hrs of light. Autoclaved soil was used to fill up 4x4 inch plastic containers, and roughly 20 seeds were sown per container. The containers were placed in a tray filled with water to provide moisture to the germinating seeds. The tray was covered with a plastic cover and placed in the growth chamber. Once the seeds started germinating, the cover was removed, and seedlings were allowed to grow further. After two weeks, one seedling was transferred into each 4x4 inch container containing autoclaved soil saturated with water. The plants were watered regularly for three weeks and finally transferred to 8-inch pots. Water and fertilizers were added to the plants regularly to keep them healthy and readily available for experimental use.

Chemicals and Reagents

The chemicals and reagents used in this study include, Agarose, Agar, Luria Bertani (LB) broth, Taq polymerase, Deoxynucleoside triphosphate (dNTP), Platinum Pfu DNA polymerase, 100 bp DNA ladder, Ethidium bromide, Ethylenediaminetetraacetic acid (EDTA), Triton X-100, Methanol, Ethanol, Liquid nitrogen, Sodium dodecyl sulfate (SDS), Acrylamide, Tris-HCl, Ammonium persulfate (APS), Tetramethylethylenediamine (TEMED), β-mercaptoethanol

(BME), Acetosyringone, Magnesium chloride ($MgCl_2$), Calcium chloride ($CaCl_2$), Tween 20, Ponceau S, Polyvinylidene fluoride (PVDF) membrane, Low molecular weight (LMW) protein ladder, Western Blot (WB) enhanced chemiluminescence (ECL) substrates, anti-SABP2 primary antibody, and anti-rabbit secondary antibody.

Antibiotics

The antibiotics used in this study include Spectinomycin, rifampicin, kanamycin, gentamycin, ampicillin, and carbenicillin.

Instruments

The common laboratory instruments used in this study include, Biorad Polyacrylamide gel electrophoresis apparatus, UV-visible spectrophotometer, NanoDrop™ 1000 spectrophotometer, UV imaging system, Microcentrifuge, Biorad Thermocycler for PCR, Airegard laminar flow work station, pH meter, Growth chamber, Autoclave, Incubator, Shaker, SDS-PAGE gel apparatus, WB apparatus, 1 mL syringe, Cork borer, Leica TCS SP8 confocal microscope.

Primers

Table 1: List of oligonucleotides with their sequence

Primer	Sequence (5'-3')	Purpose
DK549-Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGGAAGGAAAACACTTTG	Cloning SABP2 into pDONR221
DK550-Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAGTTGTATTTATGGGCAAT	Cloning SABP2 into pDONR221
DK571-Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTGTATTTATGGGCAATTTC	Cloning SABP2 with C-ter-GFP/no stop

Plasmids

Table 2: List of plasmids with their source

Vector	Purpose	Source
pDONR-221	Gateway cloning entry vector	Invitrogen
pSITE-2CA	Gateway destination vector	ABRC

Markers

For the subcellular localization study, mCherry fluorescence protein markers were used. mCherry constructs for endoplasmic reticulum (ER), mitochondria, Golgi apparatus, peroxisome, plastid, tonoplast, and plasma membrane were obtained from ABRC stock center.

Cell Strain

Agrobacterium tumefaciens LBA4404 cells, Top 10 *Escherichia coli* cells

METHODS

Cloning of Full-Length SABP2 in pDONR221

In a sterile microcentrifuge tube, 5 µl of 10X Pfu amplification buffer, 1.5 µl of 10 mM dNTP mixture, 0.5 µl of 50 mM $MgSO_4$, 1.5 µl of primer mix (10 µM each), 2 µl of template DNA (10 pg – 200 ng), 0.4 µl of platinum Pfu DNA polymerase, and autoclaved distilled water was added to make 50 µl as the final volume. PCR reaction for 35 cycles with two minutes extension time and melting temperature (Tm) of 68 °C was performed.

Agarose Gel Electrophoresis

0.5 gm of agarose salt was mixed with 50 ml of 1X TAE buffer in a conical flask and microwaved for 1 minute. Agarose solution was allowed to cool down to 50 °C and 2.5 µl (stock 10 mg/ml) of ethidium bromide (EtBr) was added. Agarose was poured into a gel tray containing well comb and left to solidify at room temperature for 10 mins. The gel was placed in the electrophoresis system and filled with 1X TAE buffer. 2 µl of the ladder was loaded in the first lane and samples in the additional lanes. The gel was run at 80 V for 1.5 hours, and DNA fragments were visualized on a UV transilluminator photographed.

Gel Extraction and Purification

Following electrophoresis, the expected DNA band was cut out of the agarose gel using a clean surgical blade. Qiagen's gel extraction kit was used to extract the DNA by following the manufacturer's instruction manual. The extracted DNA was quantified using Nanodrop.

Cloning of SABP2 into Gateway Entry Vector pDONR221

In a 1.5 ml microcentrifuge tube, 1 µl of purified SABP2 (attB-PCR product), 1 µl of pDONR221 (donor vector), 1 µl of BP clonase enzyme mix, and 7 µl of TE buffer was added and mixed. After incubating the reaction for 2 hours at 37 °C, 1 µl of the proteinase K solution was added and incubated for 10 minutes at 37 °C in order to end the reaction. The reaction mixture was centrifuged and stored on 4 °C ice for transformation into chemically competent *E. coli* cells.

Preparation of Chemically Competent Top10 *E. coli* cells

An LB plate was streaked with frozen glycerol stock of Top10 *E. coli* cells and grown overnight at 37 °C. On day 2, a single colony was taken from the LB plate and inoculated in 50 ml LB broth. The culture was grown at 37 °C in a shaker until the OD_{600} reaches 0.4-0.6. In a pre-chilled 50 ml falcon tube, the culture was transferred and centrifuged for 10 mins at 3000 x g maintained at 4 °C. The supernatant was decanted, and the pellet was resuspended in ice-cold 0.1 M $CaCl_2$. Centrifugation was carried out once again to harvest the cells. After discarding the supernatant, the pellet was resuspended in 2 ml of 0.1 M ice-cold $CaCl_2$. Sterilized glycerol was mixed with the cells to make 25% final concentration glycerol stock, snap-frozen with liquid nitrogen, and store in the -80 °C freezer.

Transformation of pDONR221-SABP2 into Chemically Competent Top10 *E coli* cells

Two µl of the reaction mixture (plasmid DNA) was added to 100 µl of Top10 *E. coli* and mixed without vortexing. Then it was placed in an ice bath for 30 minutes. Following incubation, the mixture was heat shocked for 45 seconds at 42 °C water bath and allowed to cool on ice for 2

minutes. To the transformation mixture, 800 µl of room temperature SOC media was added in the vial, mixed and shaken vigorously at 250 rpm at 37 °C for 60 minutes. 50 µl, 100 µl, 150 µl of transformed cells were spread on selection plates (LB agar plates containing 100 µg/ml kanamycin) and incubated at 37 °C.

Screening of Transformed pDONR221-SABP2 Clone Using Colony PCR

A single colony was picked with the help of a sterile toothpick, streaked on a fresh LB plate containing antibiotics (50 µg/ml kanamycin), and the tip was rinsed in 30 µl of sterile water in a PCR tube. Only 10 µl of the solution was utilized for PCR amplification and 4.8 µl of water, 2 µl of 10X PCR buffer, 2 µl of dNTP, 0.4 µl of Taq polymerase, forward and reverse primer each of 0.4 µl were added to make final volume 20 µl. The thermocycler for PCR reaction was programmed for 94 °C, 5 minutes (initial denaturation), 94 °C, 30 sec (denaturation), T_m-55 °C, 45 sec (primer annealing), 72 °C, 1 min per kb (extension), and 72 °C, 5 min (final extension) for 30 cycles. 0.8 % agarose gel electrophoresis was conducted to analyze the results of the PCR reaction.

Plasmid DNA Isolation of Recombinant pDONR221-SABP2

Positive colonies verified by PCR were inoculated in a test tube containing 5 ml LB broth and 50 µg/ml of kanamycin. The plasmid DNA from the overnight culture was extracted using QIAprep Spin Miniprep Kit as per manufacturer's instructions. Nanodrop spectrophotometer was used for DNA quantification, and the DNA was stored at -20 °C.

DNA Sequencing of pDONR221-SABP2 Clones

To prepare 10 µl of DNA sequencing mixture, 1000 ng of purified recombinant plasmid was mixed with 1 µl primer. DNA sequencing was performed at DNA analysis facility – Yale University using Sanger method.

Cloning of SABP2 into Destination Vector pSITE-2CA

In a 1.5 ml tube, 1 µl of entry clone (50-150 ng) and 1 µl of destination vector (150 ng/µl) were added with pH 8.0 TE buffer to 8 µl. After thawing the LR clonase enzyme mix on ice for 2 minutes, 2 µl was added to the reaction and mixed well by vortexing. The reaction was incubated at 25 °C overnight. One µl of Proteinase K solution was added to terminate the reaction by incubating at 37 °C for 10 minutes. The reaction mixture was stored at 4 °C before the transformation experiment was carried out. Then the transformed cells were grown on a LB agar plate with selection antibiotic spectinomycin (100 µg/ml). Colony PCR was performed to screen successfully transformed colonies, and the boiling method was used to extract plasmid from the verified clone. DNA sequencing was performed at the DNA analysis facility mentioned earlier.

Plasmid DNA Extraction

After growing verified colonies in 5 ml LB broth containing selection antibiotics overnight, centrifugation was performed at 18,800 x g for 60 seconds to pellet the bacterial cells. Ice-cold STE solution (500 µl) was added and mixed well with the pellet after discarding the supernatant. Again, centrifugation was carried out at 4 °C at 13, 684 x g for 60 seconds, and the supernatant was discarded. 500 µl of ice-cold STE solution was mixed well with the pellet, then

freshly prepared lysozyme solution (10 mg/ml) in 10 mM Tris-HCl, pH 8.0 was added and introduced to the water bath for 45 seconds. The bacterial solution was allowed to cool at room temperature then centrifuged at 13, 684 x g for 15 minutes at 4°C. A sterile toothpick was used to remove any cell debris and Phenol:Chloroform: Isoamyl alcohol (25:24:1) was added in equal volume. It was mixed well by vortexing then centrifuged at 1606 x g for minutes at 4°C. Following centrifugation, the supernatant was mixed with an equal volume of isopropanol in a 1.5 ml Eppendorf tube and stored at room temperature for 15 minutes. Centrifugation was done at room temperature at 18, 800 x g for 30 minutes. The supernatant was discarded, and ice-cold 70% ethanol was used to wash the pellet by centrifuging at 18,800 x g for 10 minutes. Again, the supernatant was discarded, and tubes were dried under a lamp. The pellet was mixed with 50 µl 55 °C sterile water in order to measure concentration by the use of Nanodrop spectrophotometer. The extracted DNA was stored at -20 °C for future use.

Preparation of Chemically Competent Agrobacterium tumefaciens *LBA4404* Cells

The glycerol stock (stored at -80 °C) of *Agrobacterium tumefaciens* cells were streaked on a LB agar plate containing rifampicin (20 µg/ml) and spectinomycin (100 µg/ml). Following incubation at 28 °C for two days, a single colony was picked and grown in 5 ml LB broth with antibiotics at 250 rpm at 28 °C. One ml overnight culture was inoculated into 100 ml LB broth and incubated until OD_{600} = 1.0 was achieved. Then the bacterial suspension was centrifuged for 10 minutes at 3,000 x g at 4 °C to get the cells. The pellets were resuspended with 5 ml pre-cooled $CaCl_2$ (20mM), centrifuged at 3,000 x g, 4 °C for 5 minutes to get the cells. The cells were again resuspended in 1 ml pre-cooled $CaCl_2$. The prepared glycerol stock was stored at -80 °C freezer.

Transformation of *Agrobacterium tumefaciens LBA4404* Cells with pSITE-2CA-SABP2

The freeze/thaw shock method was employed to transform sequence-verified pSITE-2CA-SABP2 plasmid DNA into *LBA4404* cells. Competent cells from -80 °C were revived by thawing on ice and mixed with 1000 ng of pSITE-2CA-SABP2 gently by tapping. The tube was submerged in a container containing liquid nitrogen to flash freeze, then thawed for 5 minutes at 37 °C. 1 ml SOC media was added to the cells and incubated at 28 °C shaker, 250 rpm for 3 hrs. 50 µl, 100 µl, and 150 µl of cultures were spread on antibiotic-containing (rifampicin 20 µg/ml and spectinomycin 100 µg/ml) LB agar plates. After incubating plates for two days at 28 °C, PCR screening using primers was performed as described earlier.

Agrobacterium-Mediated Transient Expression of pSITE-2CA-SABP2 in *Nicotiana benthamiana* Leaves

Day 1: *Agrobacterium* stock stored at -80 °C was used to streak LB plates containing antibiotics and grown in 28 °C incubator for two days.

- pSITE-2CA-SABP2: Spectinomycin (100mg/ml), Rifampicin (20 mg/ml)
- HcPro: 20 mg/ml Rifampicin

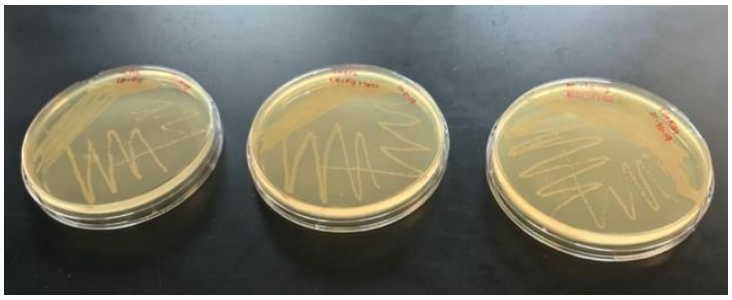

Fig 8: Plasmid Constructs Grown on LB Plates

Day 3: Individual *Agrobacterium* colonies were picked using microtip and grown in 3-ml LB broth in 28 °C shaker, 250 rpm for 20 hrs.

- pSITE-2CA-SABP2: 3 µl of Spectinomycin (100mg/ml), 3 µl of Rifampicin (20 mg/ml)
- HcPro: 3 µl of Rifampicin (20 mg/ml)

Day 4: 200 µl of subculture was transferred to 20-ml LB media with 20 µM Acetosyringone (100 mM stock) and incubated in 28 °C shaker, 250 rpm for 16-20 hrs.

- pSITE-2CA-SABP2: 20 µl of Spectinomycin (100mg/ml), 20 µl of Rifampicin (20 mg/ml), 4 µl of Acetosyringone
- HcPro: 20 µl of Rifampicin (20 mg/ml), 4 µl of Acetosyringone

Day 5: The bacterial culture was transferred into two sterile 50 ml falcon tubes and centrifuged for 5 mins at 3,000 x g at 4 °C and supernatant were discarded. The pellet was resuspended in 5-10 ml of sterile infiltration medium and centrifuged again for 5 minutes. After discarding the supernatant, the pellet was resuspended in 10 ml of the infiltration medium. OD was measured using water as blank (9:1 i.e., 900 µl sterile water:100 µl SABP2/HcPro suspension). The final OD_{600} was adjusted to 0.6. 150 µM Acetosyringone was added from a stock of 100 mM. Conical flasks were incubated at room temperature for 2-4 hrs. at 250 rpm after wrapping with aluminum foil. Leaves were infiltrated with and without HcPro using 1 mL needleless syringe. This typically required 1-4 infiltration sites per leaf. Following *Agrobacterium* infiltration, plants were maintained in the laboratory under continuous fluorescent lighting for at least 72 hrs. After 24, 48, and 72 hrs. post infiltration, leaf samples were collected using cork-borer size #6 and flash-frozen in liquid nitrogen. The samples were stored in -80 °C freezer.

Fig 9: *Nicotiana benthamiana* Plants Exposed under Continuous Lighting Conditions Post-Infiltration

Sample Preparation and SDS-PAGE Gel

To determine the time course expression of SABP2+eGFP in the various plant models, leaf samples were taken out from -80 °C freezer and put in a container filled with liquid nitrogen. An Eppendorf tube containing leaf sample was held by forceps, and cell disintegration was carried with the help of a mechanical tissue grinder. To the powdered leaf sample, 100 µl of 2X SDS dye-containing 5% of β-mercaptoethanol were added to each sample. After adding the reagents, the samples were stored at room temperature for 10 minutes and then vortexed. Now the samples were boiled in a water bath for 5 minutes and then cooled for 10 minutes. Finally, centrifugation was carried out at 16,200 x g for 10 minutes at room temperature. Ten µl of supernatant samples were run on a 12% SDS-PAGE gel. For two gels, voltage and current were set up to 200 V and 25 µAmp respectively and run for 45 minutes to an hour.

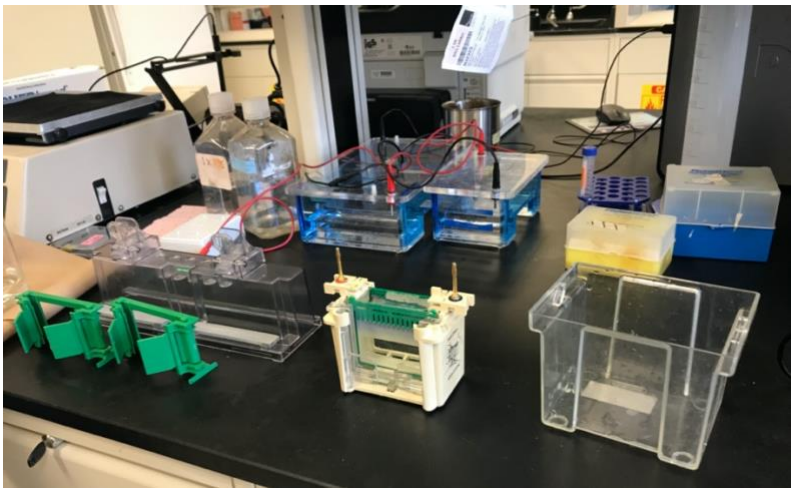

Fig 10: SDS-PAGE Gel Apparatus

Western Blot Analysis

The gel was separated by using a plastic spatula in a glass pan filled with Milli-Q water. The western transfer cast was arranged in the order as the black part of cast>>black foam>>Whatman paper>>gel>>PVDF membrane>>Whatman paper>>black foam>>white part of cast. The PVDF membrane was prepared by placing in methanol for 15 seconds and then rinsing with water for at least 2 minutes, followed by 5-10 minutes in transfer buffer. The protein transfer was run in 1X transfer buffer for ~1 hrs at 95-100 V at 4 °C. The membrane was removed from the sandwich and placed in methanol for 10 seconds. Then it was dried on Whatman paper for about 10-15 minutes and again placed in methanol for 10 seconds. To verify the successful transfer of proteins, the membrane was stained in Ponceau S for 2 minutes and then rinsed with Milli-Q water. After verification, the membrane was de-stained with water and 1X PBS until bands disappeared. Five ml of anti-SABP2 primary antibody and blocking buffer were added to the blot and incubated overnight at 4 °C with gentle shaking. The next day, primary antibody solution was decanted, and the membrane was washed twice with 1X PBS

buffer for five minutes, each followed by twice with 1X PBS+0.3% Tween and finally twice with 1X PBS to remove any non-specific binding. The membrane was incubated in 5 ml of anti-rabbit secondary antibody in blocking buffer for 30 minutes at room temperature with gentle shaking. Washing steps were repeated. The blot was incubated in ECL developing reagents and visualized using a LICOR C-DIGIT ECL scanner or developed directly with TMB developing solution.

Confocal Microscopy

Post infiltration, plants were taken to the confocal microscopy facility located in the James H. Quillen College of Medicine. High-resolution images of leaf disc samples were taken with the help of the Leica TCS SP8 confocal microscope.

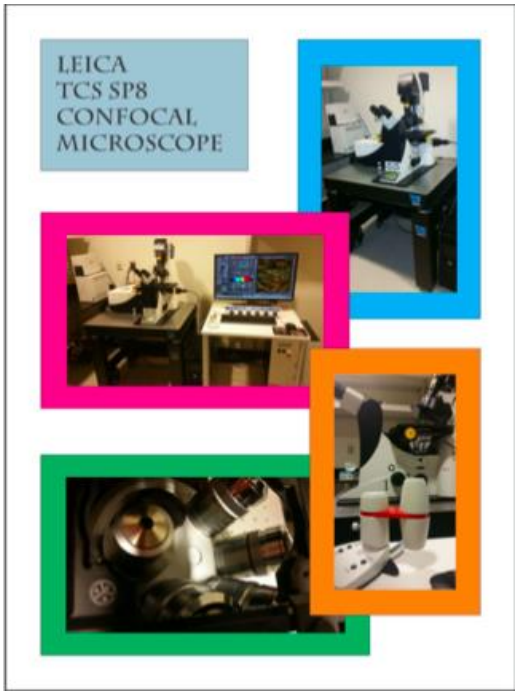

Fig 11: LEICA TCS SP8 Confocal Microscope. Source: DBMS Microscopy Core Facility

RESULTS

Time Course Expression of SABP2+eGFP in Various Plant Models

Agrobacterium containing pSITE-2CA-SABP2 plasmid construct was transiently expressed in NB, XNN, NG, 1-2, and susceptible plants, and exposed under lighting conditions. Post infiltration, leaf samples were collected at 24 hrs., 48., and 72 hrs., and flash-frozen in liquid nitrogen. The samples were later disintegrated by ultrasonic probe sonicator, and SDS-PAGE gel was run. Protein from the gel was transferred to the membrane by western blot. Protein detection was carried out using the ECL western blotting substrate. Western blot analysis confirmed that SABP2+eGFP is best expressed in NB plants at 48 hrs. post-infiltration, as seen in Figure 12.

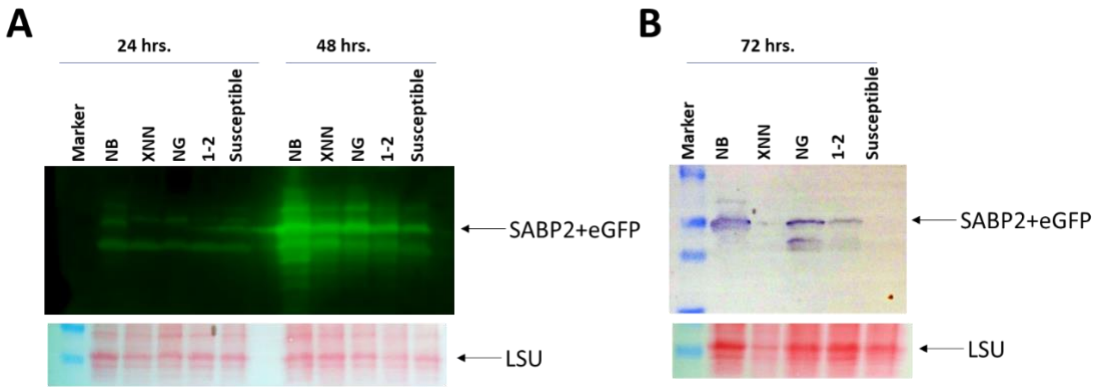

Fig 12: Western blot Analysis of Transiently Expressed eGFP+SABP2. The leaf samples contained *Nicotiana benthamiana* (NB), *Nicotiana tabacum* cv Xanthi NN (XNN), NahG transgenic tobacco, SABP2-silenced transgenic tobacco (1-2), and the susceptible tobacco (susceptible). Prestained protein size markers were used to determine the size of the expressed proteins. Ponceau staining was used to determine the equal loading of the samples showing the large subunit of RUBISCO (LSU).

Confocal Microscopy of SABP2+eGFP Expression

Nicotiana benthamiana leaves treated with and without HcPro were visualized under the confocal microscope to detect SABP2+eGFP expression. The unique capability of a confocal microscope lies in obtaining optical section of tissues and creating 3D reconstructions from it[29]. The green fluorescent signal showing the expression of SABP2+eGFP is seen throughout the cell and is more concentrated towards the cell periphery. The epidermal cells in plant leaves have a large central vacuole contributing to a very high vacuole to cytoplasmic volume ratio. The large central vacuole pushes the cell contents towards the cell periphery, thus creating a thin layer of cytoplasm between the vacuolar membrane (tonoplast) and cell membrane[30]. Fluorescent signals observed in the epidermal cells can be explained by a few possible options. The signals could be coming from tonoplast, or plasma membrane, or cytoplasm. The in-depth study indicated that the signals were diffused and most likely coming from the cytoplasm. A HcPro construct was used to reduce plant's native gene silencing activity and allow enhanced and prolong the transient expression of pSITE-2CA-SABP2[31]. Leaves inoculated with HcPro showed intense fluorescent signals.

Expression of eGFP+SABP2 under Normal (non-stress) Conditions

SABP2 tagged with eGFP was expressed transiently in tobacco leaves with and without HcPro. The mCherry organelle markers specific for the plasma membrane, plastids, Golgi apparatus, vacuole, peroxisome, mitochondria, and endoplasmic reticulum were used as an intracellular probe. Confocal microscopy studies revealed that SABP2+eGFP signals are localized throughout the cell and more prominently towards the cell periphery. The large central vacuole in epidermal cells pushes the cell contents towards the periphery, so SABP2 is localized

in the cytoplasm. SABP2+eGFP signals as seen in Figure 13(A) is a characteristic of cytoplasmic proteins.

Chloroplast

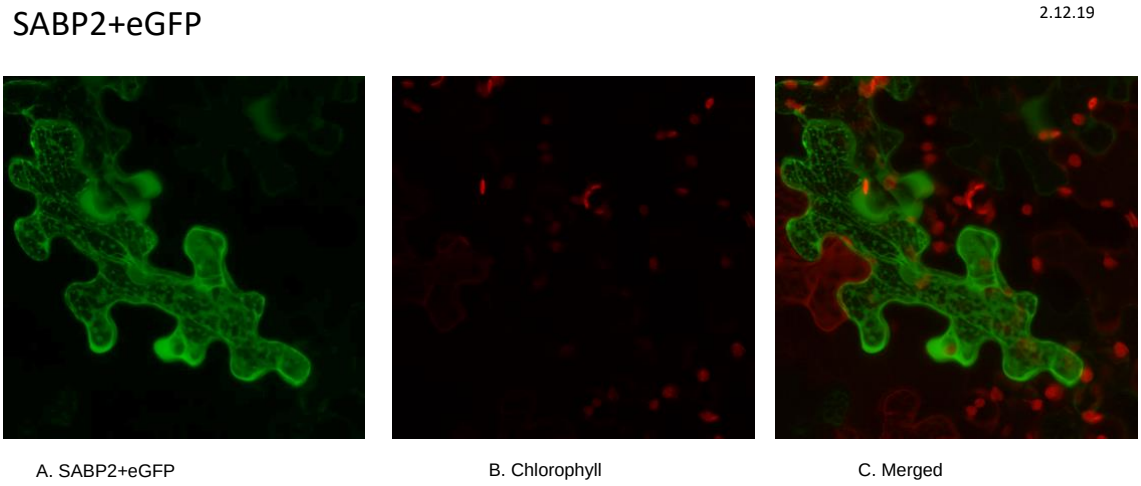

Fig 13: Confocal Microscopy Image of SABP2+eGFP and Auto Fluorescence Protein in Tobacco Leaf. A. SABP2+eGFP expression in tobacco cell. B. Chlorophyll autofluorescence in red channel. C. SABP2+eGFP and chlorophyll fluorescing merged.

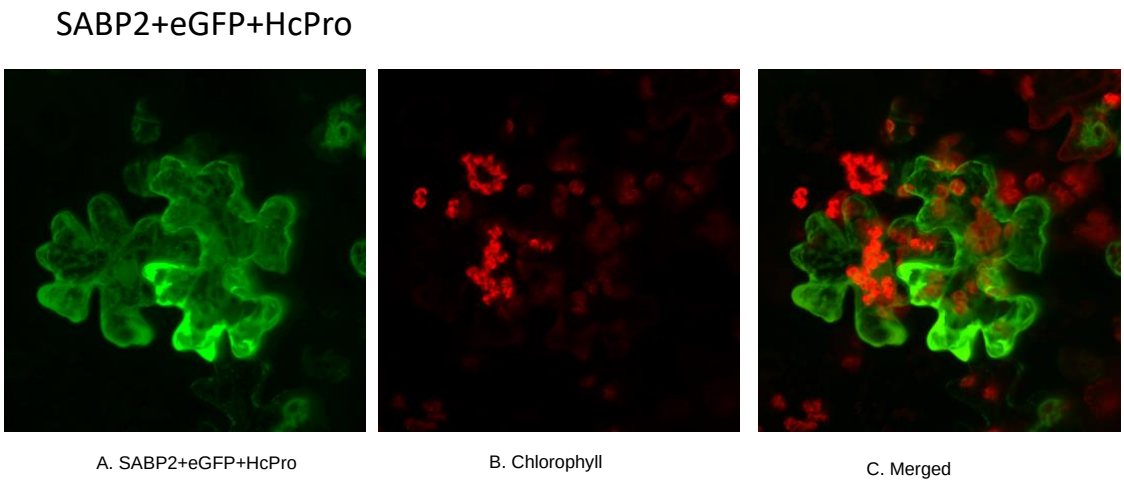

Fig 14: Confocal Microscopy Image of SABP2+eGFP+HcPro and Auto Fluorescence Protein in Tobacco Leaf. A. SABP2+eGFP expression in tobacco cell. B. Chlorophyll autofluorescence in red channel. C. SABP2+eGFP and chlorophyll fluorescing merged.

Plasma Membrane

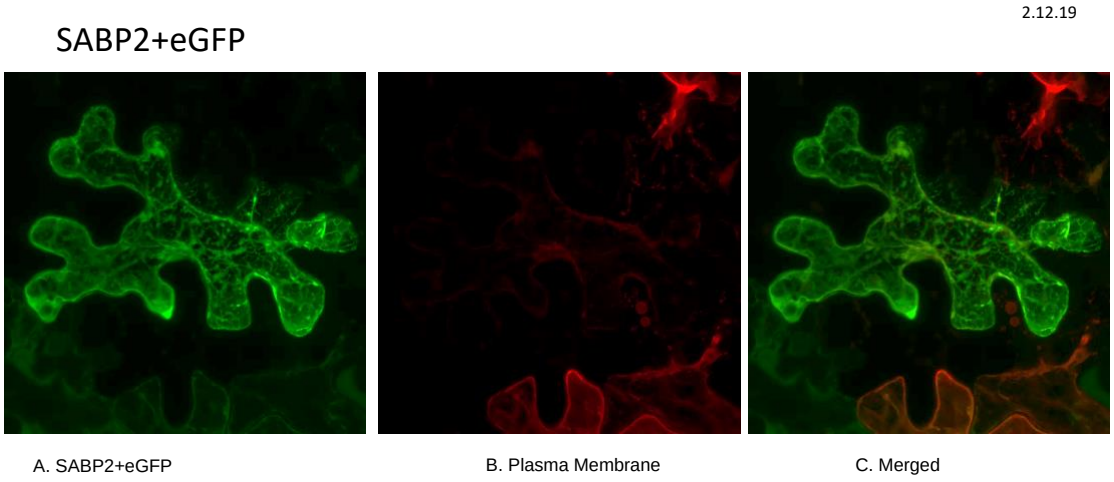

Fig 15: Confocal Microscopy Image of SABP2+eGFP and Auto Fluorescence Protein in Tobacco Leaf. A. SABP2+eGFP expression in tobacco cell. B. Expression with mCherry marker. C. SABP2+eGFP expression merged with marker.

Biotic Stress

N. benthamiana is susceptible to many plant pathogens making it widely popular in host-pathogen research. Its use in the context of plant innate immunity and defense signaling is more appealing. In this experiment, leaves inoculated with pSITE-2CA-SABP2 construct were subjected to biotic stress 24 hrs before confocal microscopy. Biotic stress was applied by inoculating the treated plants with bacterial and viral plant pathogens, namely, *Pseudomonas syringae DC3000*, *Pseudomonas maculicola*, and the Tobacco Mosaic Virus (TMV). When plants are infected, the expression of pathogenesis-related (PR) proteins is upregulated to provide resistance. SABP2 converts inactive MeSA into active SA, which is a key to signal transduction pathway and activation of plant defenses in response to pathogen attack. We were keen to determine how pathogen infection will affect the localization of SABP2+eGFP. SABP2+eGFP signals are seen throughout the cell, and more towards the cell periphery as the large central

vacuole in epidermal cells pushes everything towards the periphery. Fluorescent signals observed in Figure 16, 17, and 18 (biotic stress) is similar to the signals observed in Figure 13, 14, and 15 (no stress condition). So, SABP2 is localized in the cytoplasm.

PS DC3000

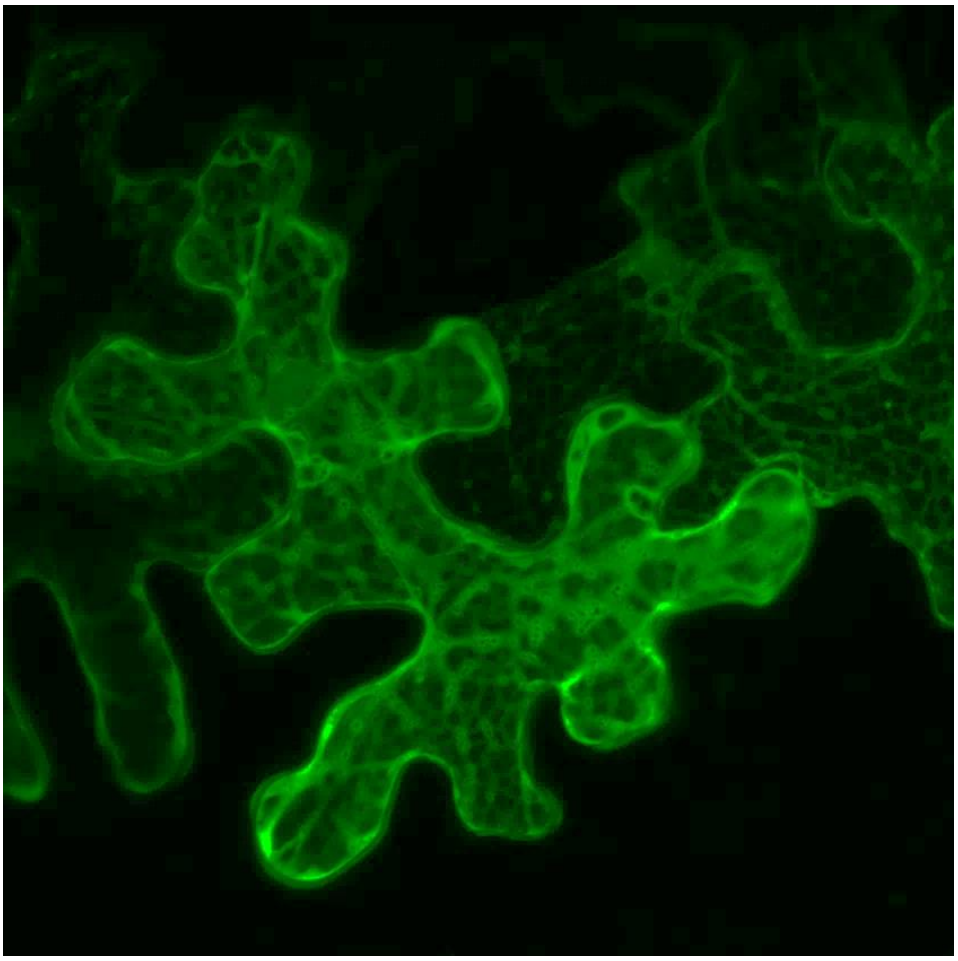

Fig 16: Confocal Microscopy of SABP2+eGFP in the presence of HcPro in Tobacco Leaf upon *Pseudomonas syringae* pv. *DC3000* infection.

PS maculicola

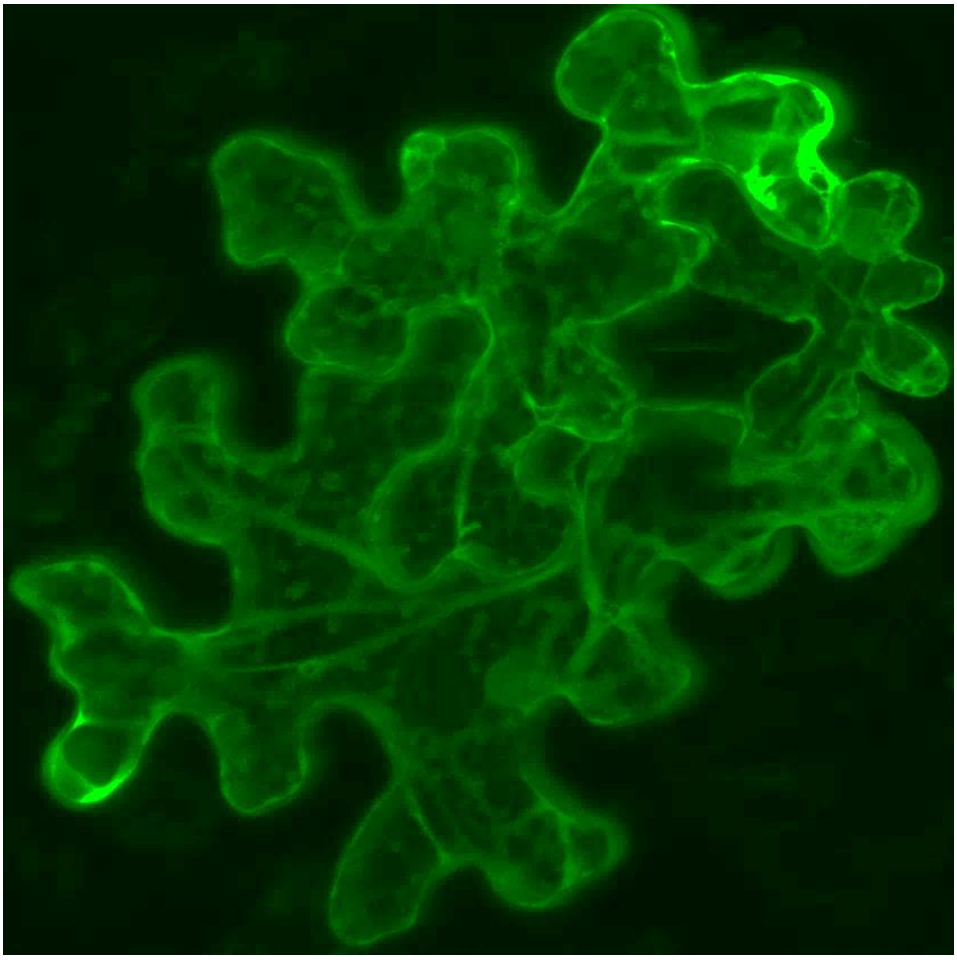

Fig 17: Confocal Microscopy Image of SABP2+eGFP in the presence of HcPro in Tobacco Leaf upon *Pseudomonas syringae* pv. *maculicola* infection.

Tobacco Mosaic Virus (TMV)

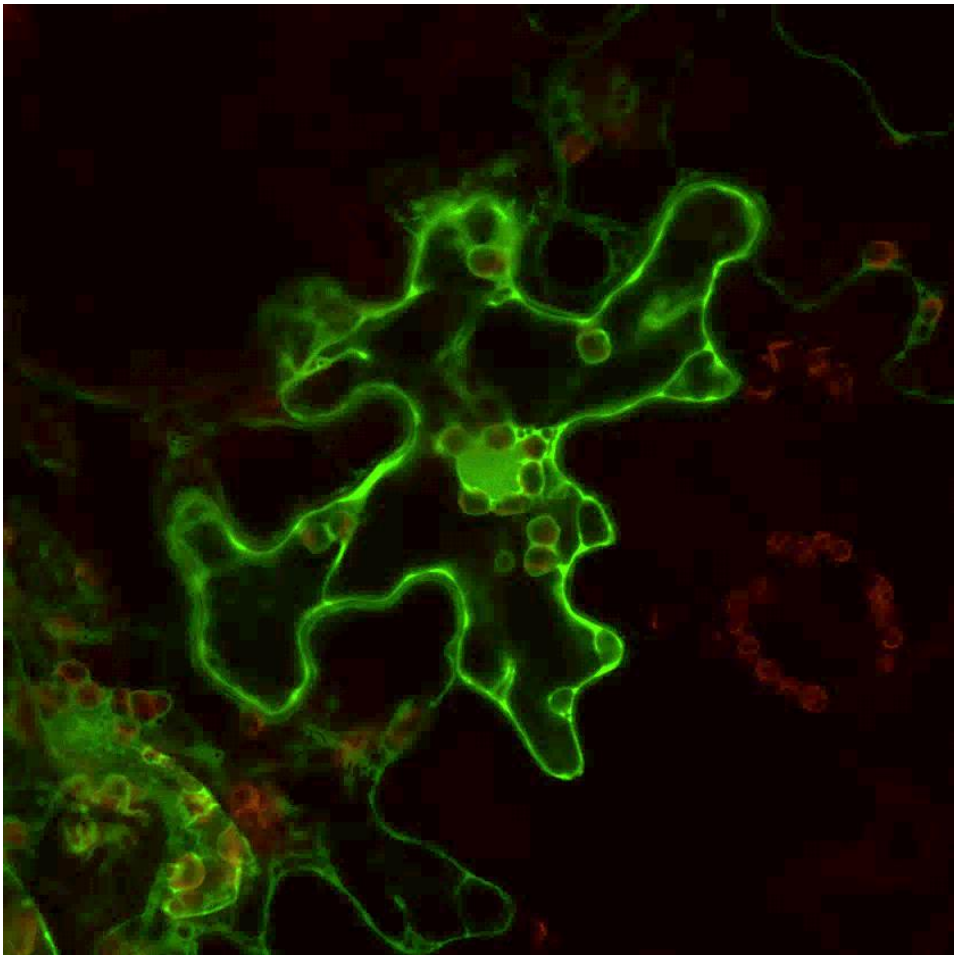

Fig 18: Confocal Microscopy Image of SABP2+eGFP expression in the presence of HcPro in Tobacco Leaf upon Tobacco Mosaic Virus infection.

DISCUSSION

Phytohormones play a major role in defense signaling in plants. Several plant hormones such as auxin, CK, GA, SA, JA, ET, and ABA employ synergistic and antagonistic actions to respond to stress[32]. The phenomenon is known as signaling crosstalk. Hormones work together to coordinate growth responses during stress.

Plants have acquired several defense mechanisms in response to biotic and abiotic stress. One of them is the SA-activated defense signaling pathway, which mediates defense by inducing expression of *PR* genes as well as SAR. SAR provides resistance to the whole plant after exposure to a pathogen locally. Endogenous levels of SA increase as much as 20-50-fold in TMV infected leaves while there is also a significant rise 5-10-fold in uninfected leaves[33]. It is evident that a high amount of SA is synthesized in plants resisting pathogen infection, subsequently leading to the activation of defense pathways. SA-mediated defense signaling pathways are yet to be fully understood. SABP2 is one of the many SA-binding proteins being studied with an aim to understand this metabolic pathway.

The methylesterase activity of SABP2 is known to catalyze the conversion of inactive MeSA into active SA, thereby inducing SAR[7,21]. In a study by Kumar and Klessig, the results suggested that SABP2 played an important role in containing viral replication in TMV-infected plants. In SABP2-silenced plants, the expression of *PR-1* gene was reduced[21]. Another study conducted by Tripathi et al. also suggested that SABP2-silenced plants not only failed to express *PR-1* gene, but those plants were also compromised in ASM induced SAR[22]. ASM is considered a synthetic analog of SA and has been widely used to induce SAR in crop plants.

In plants, mRNA is translated into proteins in the cytoplasm. After translation, the proteins either stay in the cytoplasm or leave the cytoplasm to enter secretory and non-secretory pathways. Based on their targeting sequences, proteins via the secretory pathway are transported to the ER membrane. Proteins from ER travel further to the Golgi apparatus, vacuole, plasma membrane, lysosome, or extracellular space. Subcellular localization studies are important in determining where a protein resides in the cell. To understand how the protein interacts and functions in the cell, subcellular localization study of each protein is vital. Computational methods focus on a short nucleic acid sequence, which is known as a targeting sequence to label a protein to a specific organelle[34]. This method is proven to be useful; however, it is susceptible to false-positive results. To verify results experimentally, cell fractionation and immunohistochemistry are two widely used approaches. In cell fractionation, differential centrifugation is followed by measuring the activity of unknown protein with a known protein marker for each organelle[34]. In immunohistochemistry, fluorescence, atom, or enzyme markers are used to label specific antibodies. This can be a time-consuming and expensive process, and results may not be specific for closely associated gene members.

In recent time, the fluorescent-tagged protein (FTP) approach is widely being studied to visualize protein localization in a cell. The FTP organelle markers are generated on the basis of well-defined targeting sequences. The visualization of the markers in their respective compartment has been noticed in several monocot and dicot plants. When SABP2 tagged with GFP and mCherry organelle set markers were transiently expressed in tobacco leaves and visualized under a confocal microscope, fluorescent signals were seen throughout the cell and more prominently towards the cell periphery. The large central vacuole in epidermal cells pushes cell contents towards the cell periphery creating a layer of cytoplasm between the vacuolar

membrane and plasma membrane. So, SABP2 is localized in the cytoplasm. The results were found to be similar in two batches of experiments. The signal is diffused, and the fact that SABP2 protein lack target signal peptide also suggests that it is a cytoplasmic protein. Tobacco leaves treated with HcPro showed intensified signal as expected.

We were interested to see how some selected pathogens behave in the *Nicotiana benthamiana* plant and whether it is going to change the subcellular location of SABP2. Upon pathogen infection, plants upregulate the *PR* gene expression and synthesize defensive compounds inducing local resistance and SAR. This defense signaling pathway is mediated by a plant phytohormone known as SA. *Agrobacterium* containing the pSITE-2CA-SABP2 construct was infiltrated in tobacco leaves and treated with bacterial and viral species 24 hrs. before confocal microscopy. The fluorescent signals visualized under the confocal microscope showed no significant difference under various biotic stress. SABP2+eGFP is seen in the entire cells and more concentrated towards the cell periphery. So, SABP2 is localized in the cytoplasm in normal and biotic stress conditions. The SABP2 localization study continues to provide a better view of the biochemical pathway involved in SA-mediated defense signaling defense in plants. Understanding the SA pathway can be used to strengthen plants' natural defense and reduce the cost and risk associated with the usage of a large quantity of pesticides in agriculture.

FUTRE DIRECTIONS

Many in silico prediction methods are in use to study the subcellular localization of proteins. The fluorescent-tagged protein (FTP) approach has added a lot to the understanding of SABP2 localization. The fluorescent signal observed under confocal microscopy could be coming from the vacuolar membrane, or plasma membrane, or cytoplasm. SABP2 has been hypothesized to localize in the cytoplasm. Biotic stress was applied to tobacco leaves in the form of bacteria and viruses to see if localization is affected. Confocal studies revealed no significant difference in the localization pattern of SABP2 under normal and stress conditions.

To further strengthen our result, an important technique is a subcellular fractionation. By using this process compartment, enriched fractions can be generated through the application of differential centrifugation. Many novel proteins and their function have been revealed by this process as it readily generates fractions of nucleus, cytosol, membrane, mitochondria, and other organelles by using subcellular fractionation kit.

The biochemical characterization of SABP2 is another aspect to explore. Subcellular localization of SABP2 is important to understand the SA-mediated pathway in plants. A combination of subcellular fractionation, immunological, and biochemical approaches will be implied to verify confocal microscopy results. SABP2 tagged with eGFP will be expressed transiently in *Nicotiana benthamiana* and observed under a microscope at least one more time. The knowledge obtained from this project can be used to cultivate disease-resistant crops and decrease our excessive dependence on harmful pesticides.

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APPENDICES

Appendix A: Abbreviations

ABA: Abscisic Acid

APS: Ammonium Persulfate

ASM: Acibenzolar-S-Methyl

CK: Cytokinin

ECL: Enhanced Chemiluminescence

eGFP: Enhanced Green Fluorescent Protein

ER: Endoplasmic Reticulum

ET: Ethylene

EtBr: Ethidium Bromide

ETI: Effector-Triggered Immunity

ETS: Effector-Triggered Susceptibility

FTP: Fluorescent-Tagged Protein

GA: Gibberellic Acid

HcPro: Helper Component Proteinase

HR: Hypersensitive Response

IAA: Indole-3-Acetic Acid

ICS: Isochorismate Synthase

JA: Jasmonic Acid

kDa: Kilodalton

LMW: Low Molecular Weight

MeSA: Methyl Salicylate

MES: Morpholinoethanesulfonic acid

NB-LRR: Nucleotide Binding Leucine Rich Repeat

nSP: Non-Secretory Pathway

OD: Optical Density

PAMPs: Pathogen Associated Molecular Patterns

PBS: Phosphate-Buffered Saline

PCR: Polymerase Chain Reaction

PR: Pathogenesis-Related

PRR: pathogen Recognition Receptor

PTI: PAMP-Triggered Immunity

PVDF: Polyvinylidene Fluoride

RNAi: RNA Interference

SA: Salicylic Acid

SABP2: Salicylic Acid Binding Protein 2

SAR: Systemic Acquired Resistance

SDS: Sodium Dodecyl Sulfate

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SP: Secretory Pathway

STE: Sodium Chloride-Tris-EDTA

TAE: Tris-Acetate-EDTA

TEMED: Tetramethylenediamine

TMV: Tobacco Mosaic Virus

WB: Western Blot

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

0.8 % Agarose Gel

100 ml of 1X TAE buffer is added to 0.8 gm of agarose and microwaved for 1 minute, swirling at 30 seconds. The solution is allowed to cool and 6 μl of EtBr is added, then poured in the gel dock with a comb and left to solidify

20% APS

0.2 gm APS dissolved in 1 ml of Milli-Q water

500 ml of 1X SDS-PAGE Running Buffer

50 ml of 10X SDS-PAGE running buffer dissolved in 450 ml of Milli-Q water

1 L of 1X PBS Buffer

100 ml of 10X PBS dissolved in 900 ml of Milli-Q water

1 L of 1X PBS, 0.3% Tween-20

100 ml of 10X PBS + 897 ml of Milli-q water + 3 ml of Tween-20

1 L of 1X Transfer Buffer

100 ml of 10X transfer buffer + 100 ml of methanol + 800 ml of Milli-Q water

100 ml of WB Blocking Buffer

3 gm BSA, final concentration 3% + 1 gm dry milk, final concentration 1% + 100 ml of 1X PBS

1 L of 1X TAE Buffer

20 ml of 50X TAE is dissolved in 980 ml of Milli-Q water

100 ml of Ponceau S Stain

5 ml glacial acetic acid, final concentration 5% + 0.1 gm of ponceau S, final concentration 0.1% + 95 ml Milli-Q water

100 ml of 1X STE Buffer

0.121 gm, 0.584 gm, and 0.029 gm of 100 mM NaCl, 10 mM Tris-Cl (pH 8.0), and 1 mM EDTA are added respectively and pH is adjusted with HCl to 8.0. Stored in 4 °C

100 ml of LB Broth

2.5 gm of LB media is dissolved in 100 ml of Milli-Q water and sterilized in autoclave for 20 minutes

100 ml LB Agar Media

2.5 gm of LB media and 1.5 gm of agar is measured and dissolved in 100 ml of Milli-Q water and sterilized in an autoclave for 20 minutes

SOC Media

2 gm of bacto tryptone (2%), 0.5 gm yeast extract (0.5%), 0.4 gm glucose (0.4%), 0.2 ml of 5M NaCl, 0.25 ml of 1 M KCl, 1 ml of 1M MgSO4, and 1 ml of 1 M $MgCl_2$ is added and QS to 100 ml with Milli-Q water. Then it is sterilized in an autoclave for 20 minutes and stored in 4 °C

100 ml of 0.5 M MES buffer

10.633 gm MES is dissolved in 75 gm Milli-Q water. pH is adjusted by adding low concentration HCL or NaOH, then volume is raised to 100 ml. The solution is autoclaved and stored in -20 °C freezer