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Effect of Pesticides on Salicylic Acid Binding Protein 2 (SABP2) and Plant Defense

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ABSTRACT

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Tobacco SABP2 has been shown to display high affinity for salicylic acid (SA) and methylsalicylate (MeSA) and plays an important role in SAR signal development. Using biochemical approach, SABP2 has been shown to demonstrate strong esterase activity in converting MeSA to SA. Recent study shows that tetra fluoroacetophenone, a synthetic analog of SA, competitively inhibits SABP2 esterase activity as well as suppresses SAR signal development in tobacco mosaic virus (TMV)-infected tobacco plants. Not much has been studied on the effect of pesticides on plant defenses. Because both AChE and SABP2 are esterase-like proteins belonging to α/β hydroxylase superfamily, we hypothesize that pesticides may inhibit the MeSA esterase activity of SABP2 and block SAR development. Biochemical and molecular biology techniques were used to test this hypothesis. SAR in tobacco-TMV plant-pathogen system is measured by significant decrease in TMV-induced lesion sizes in secondarily inoculated distal leaves.

DEDICATION

To my mother Chia Julie Awoh, my dad Simon Awasum, brothers Joannes Paulus, Chia Killian, Chia Albert, Babila Awasum, Michael Awasum, my sisters Brunhilda Gama, Prisca Anderson, Linda Awasum and Vivian Awasum. Also to my uncle Jobain Cosmas and aunt Celine Jobain.

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CHAPTER 1

INTRODUCTION

Plants form the basic staple food for all forms of life. They are major source of oxygen and food from the process of photosynthesis because animals are not able to provide all these essential components. The cattle (source of beef) that feed on grass and the fish that consume algae are all dependent on plants for food. Other plant uses include: providing shelter and materials for clothing (e.g. cotton fibers), paper products, and medicines. Plants are constantly being challenged by various environmental factors; abiotic stresses (e.g. droughts, extreme temperatures, floods, and other natural disasters) as well as biotic factors such as insects, bacteria, and viruses (Walley et al. 2007) that may have adverse effects on plant yield and health and as well affect organisms that depend on them for survival such as insects, animals, and humans.

The presence of elicitors in the saliva of a chewing insect enables the plant to differentiate between general wounding and the feeding of an insect (). The plant, in response, may release volatile organic compounds (VOCs) including sesquiterpenoids, monoterpenoids, and homoterpenoids in response (Rose et al. 1996; Pare et al. 1999). These compounds help in repelling harmful insects or attracting predators that prey on these pests. For example, a wheat seedling infested with aphids will produce VOCs that will help repel other aphids (Ballhorn et al. 2009). Also, when lima beans and apple trees are damaged by spider mites, they produce substances that attract predatory mites

(Ballhorn et al. 2009). A cotton plant releases volatile substances that attract predatory wasps when damaged by moth larvae (Rose et al.1996; Pare et al. 1999).

A number of inducible defense mechanisms have evolved in plants that are triggered upon attack by microbial pathogens. These inducible defense mechanisms include biochemical, molecular, and morphological changes, such as expression of pathogenesis-related genes, production of antimicrobial compounds, programmed cell death in tomato, as well as oxidative burst (Thomas et al. 2000). Plants also protect themselves by using physical and chemical barriers such as cell walls, waxy epidermal cuticles, and bark that hinder pathogen entry (reviewed in Bari and Jones 2009). These substances protect the plant from pathogen invasion as well as provide the plant with rigidity and strength (reviewed in Bari and Jones 2009).

Recognition of a pathogen/effector molecule by a plant resistant (R) protein usually triggers a localized reaction called a hypersensitive response (HR) characterized by rapid cell death at the site of infection (Hammond-Kosack and Jones 1997; Durrant and Dong 2004). Hence, any single effector protein that is recognized by an R protein is called an avirulence factor (Dangl and McDowell 2006). Upon infection by an avirulent pathogen, a battery of defense responses is activated, accompanied usually by HR (Thomas et al. 2000). These responses are a result of recognition by ligand/receptor interactions (Hammond-Kosack and Jones 1997) specified by paired plant resistance and pathogen avirulence genes resulting in lesions at the infection site.

A complex signaling pathway including the phytohormones jasmonic acid, 1,2-oxophytodienoic acid, ethylene, or salicylate triggers the defense reaction of plants (Ryan and Pearce 1998 and ref. therein). Progress in identifying important components of these

pathways and understanding the role of jasmonates (JA), SA, and ethylene (ET) () in the plant's response to biotic stress has been enormous (e.g. cell surface receptors, elicitors) (Ryan and Pearce 1998 and ref. therein).

Plant Defense Signaling Pathways

Jasmonic Acid and Ethylene Pathways (JA/ET)

JA and ET are phytohormones usually associated with defense against necrotrophic pathogens and herbivorous insects (Bari and Jones 2009 and ref. therein). Even though SA and JA/ET pathways are mutually antagonistic, proof of synergistic interactions between them have also been shown (Schenk et al. 2000; Kunkel and Brooks 2002; Beckers and Spoel 2006). Besides plant defenses against insects and microbes, JAs are involved in various other processes such as seed germination, root growth, tuber formation, tendril coiling, and ripening of fruits (Bari and Jones 2009 and ref. therein).

A range of abiotic factors including wounding, osmotic stress, drought and exposure to elicitors (e.g chitins, oligogalaturonides, oligosaccharides, and yeast extracts) have been shown to induce JA signaling (Turner et al. 2002 and ref. therein). In *Arabidopsis,* JA biosynthesis is controlled through the activation of a JA biosynthetic pathway that regulates wound-induced JA biosynthesis (Turner et al. 2002 and ref. therein). Ryan et al. (2002) proposed the function of JA in plant defense and showed evidence for a link between wounding (caused by insect herbivores), JA formation (Figure 1), and gene induction for protease inhibitors that prevent feeding by insects.

Application of JAs exogenously was shown to induced expression of defense- related genes (Lorenzo and Solano 2005; Wasternack 2007).

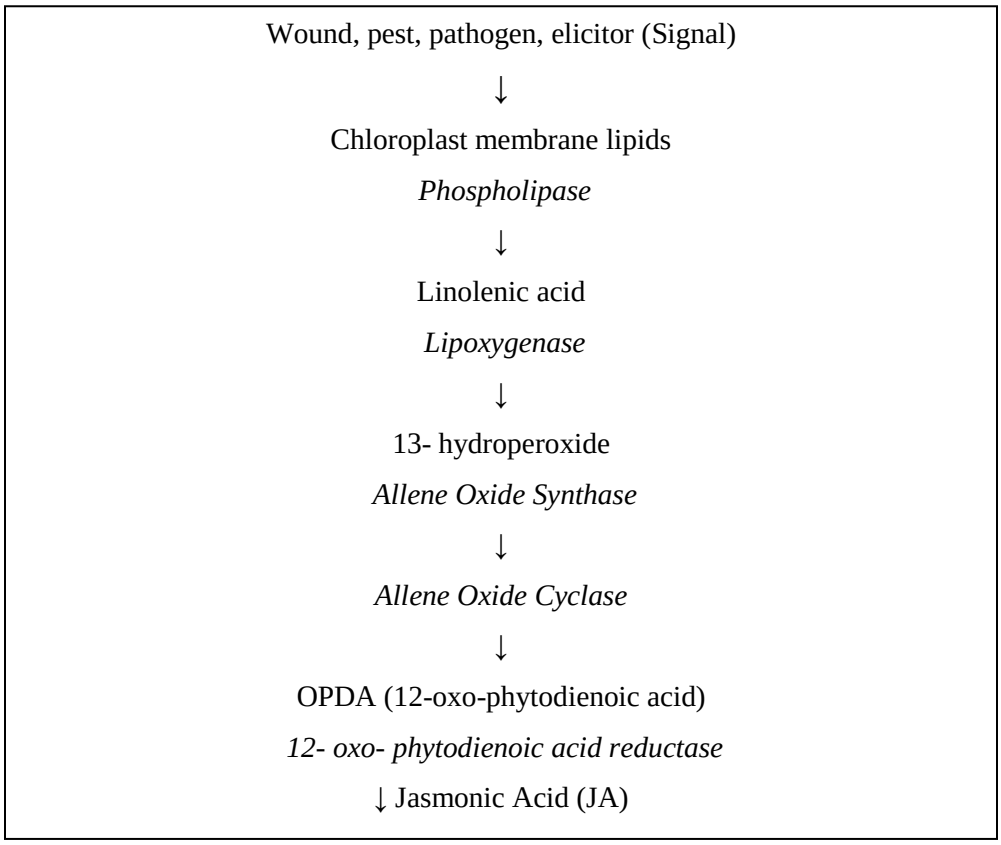


Figure 1**:** Schematic Diagram Showing JA Biosynthetic Pathway. Enzymes in this pathway are shown in italics. (Figure adapted from Creelman and Mullet 1997).

Salicylic Acid (SA) Pathway

SA is a phenolic compound containing a hydroxyl group and is synthesized by plants (reviewed in Vlot et al. 2009). Phenolics have been found to have several functions. They function in lignin biosynthesis, regulation of plant responses to abiotic stimuli, function in pigmentation, growth, reproduction, resistance to pathogens, and many other functions (reviewed in Lattanzio et al. 2006). They form one of the major classes of secondary metabolites. SA also function in seed germination, cell growth,

seedling establishment, respiration, stomata closure, responses to abiotic stress, senescence-associated gene expression, basal thermotolerance, nodulation in legumes, and fruit yield (reviewed in Vlot et al. 2009).

SA is involved in both local and systemic induced disease resistance responses. Advances in our understanding of plant defense signaling have shown that a network of signal transduction pathways are employed by plants some of which are dependent while others are independent of salicylic acid (reviewed in Pieterse and Van Loon 1999). Examples include signal transduction pathways mediated by phytohormones such as auxins, ethylene, cytokinin, gibberellins, jasmonates, and peptide hormones (reviewed in Bari and Jones 2009). SA is a key signal in thermogenesis regulation and disease resistance (Pieterse and Van Loon 1999). SA also activates defense responses against biotrophic and hemi-biotrophic pathogens as well as plays a role in systemic acquired resistance (SAR) development (reviewed in Bari and Jones 2009).

SA in plants is synthesized via 2 pathways, one mediated by phenylalanine ammonia lyase (PAL) while other is mediated by isochorismate synthase (ICS). In the PAL-mediated pathway, phenylalanine serves as precursor. Phenylalanine also serves as a precursor for the biosynthesis of other plant compounds such as phytoalexins, phenolics, and flavonoids that may provide physico-chemical barriers, hence preventing pathogen invasion (reviewed in Vlot et al 2009). In tobacco, PAL converts phenylalanine to cinnamic acid, which is further converted to benzoic acid which serves as a precursor of SA (Figure 2). Through the shikimate pathway, chorismate is converted to isochorismate by ICS. Isochorismate is then converted to SA by isochorismate pyruvate lyase (Figure 2).

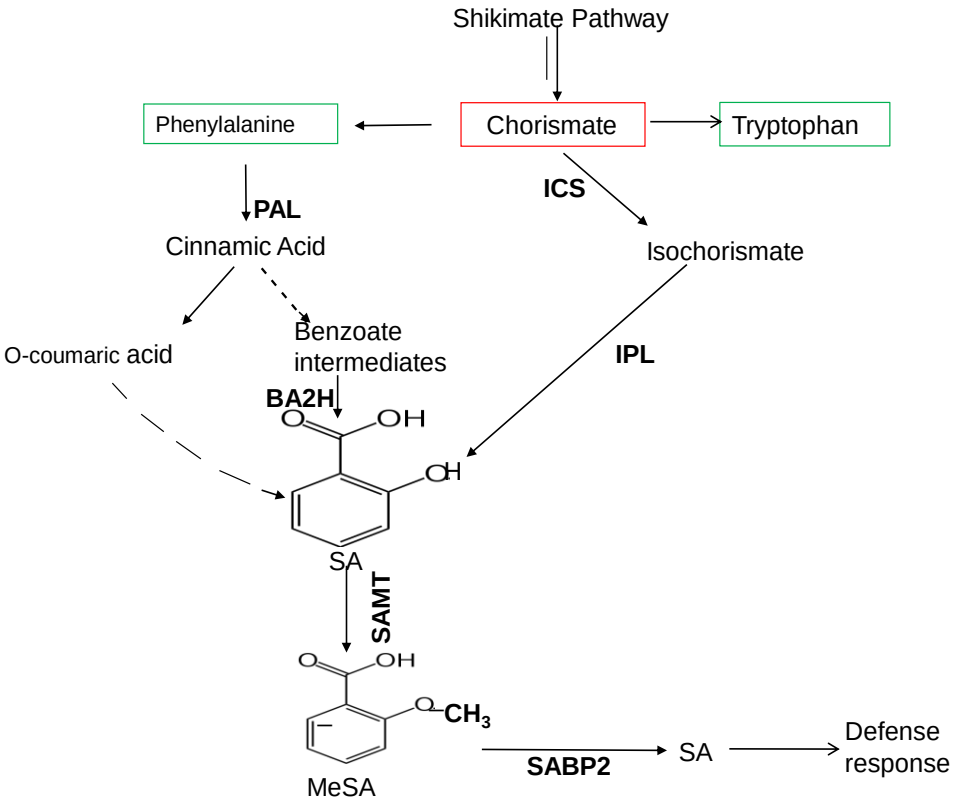


Figure 2**:** Simplified Pathway for SA Biosynthesis and Disease Resistance. Enzymes in this pathway are shown in bold. Abbreviations: PAL, phenylalanine ammonia lyase; ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; BA2H, benzoic acid-2-hydroxylase; SAMT, SA methyltransferase; (Figure adapted from Vlot et al. 2009).

One of the best signal studied transduction pathways in which salicylic acid is a key component is SAR (Pieterse and Van Loon 1999). In the 1960s, Ross showed that TMV (tobacco mosaic virus)-challenged tobacco plants developed increased resistance to secondary infection on distal tissues (Ross 1961). This spread of resistance throughout plant tissues was termed SAR. Induction of SAR upon infection by a necrotizing pathogen coincides with an early increase in endogenous SA levels, not only at the primary infection site (local infection) but also systemically in uninfected tissues (Schneider et al. 1996).

The role of SA as a defense signaling molecule was demonstrated using transgenic Arabidopsis plants that express the *nah G* gene encoding bacterial salicylate hydroxylase, which converts SA to catechol (Delaney et al. 1994; Friedrich et al. 1995). These transgenic plants were not able to accumulate SA after pathogen infection and hence showed susceptibility to both avirulent and virulent pathogens. There was no expression of pathogenesis-related genes in their distal leaves and SAR development was compromised (Delaney et al. 1994).

The role of SA in basal defense and induced resistance to powdery mildew (*Oidium neolycopersia)* and grey mildew (*Botrytis cinerea*) in both tomato (*Lycopersicon esculentum)* and tobacco (*Nicotiana tabacum*) was investigated (Huang et al. 2000). When NahG transgenic tomato and tobacco plants were compared to their respective wild types plant lines, it was observed that SA is not involved in the basal defense against *O. neolycopersici* in tomato while NahG tobacco were more susceptible to *O. neolycopersici* infection with a greater effect in older plants (Achuo et al. 2004).

Susceptibility of transgenic NahG *Arabidopsis* to several bacterial pathogens has been reported (Delaney et al. 1994). When compared with the wild-type tobacco (cv. Xanthi) plants, NahG tobacco plants were more susceptible to TMV, *Pseudomonas syringae* pv. *tabaci*, *Phytophthora parasitica*, and *Cercospora nicotianae* (Delaney et al. 1994), while NahG potato plants were less susceptible to *Phytophthora infestans* than the wild-type plants (Yu et al. 1997). SA showed no role in the basal defense of tobacco against *B. cinerea* but played a role in the basal defense of tomato against *B. cinerea* (Achuo et al. 2004).

Plants treated with chemical resistance inducers such as benzo (1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) have been shown to trigger the SA-dependent defense pathway (Lawton et al. 1996). Activation of this pathway via BTH resulted in an induced resistance against *O. neolycopersici* in tobacco but not in tomato (reviewed in Achuo et al. 2004). Using microscopic analysis, it was revealed that BTH treatment could prevent *Oidium* germ tube penetration through tobacco leaves while penetration was possible on tomato leaves regardless of BTH treatment (Achuo et al. 2004). Treatment of soil and leaf with BTH also induced resistance against *B. cinerea* in tomato and not in tobacco. To conclude, the SA dependent defense pathway is effective against various pathogens in tomato and tobacco (Lawton et al. 1996).

Several *Arabidopsis* mutants with defects in SA signaling (*eds1, pad4, eds5, sid2, and npr1*) remained resistant to *Pseudomonas syringae pv. phaseolicola* (*Psp),* while NahG *Arabidopsis* plants were defective in nonhost resistance to *Pseudomonas syringae pv*. p*haseolicola (Psp)* (van Wees and Glazebrook 2003). The lack of resistance to *Psp* in NahG plants is not due to SA absence but results from the production of catechol by the action salicylate hydrolxylase on SA (van Wees and Glazebrook 2003). Enhanced susceptibility of NahG *Arabidopsis* to *B. cinerea* was observed, but mutants *eds5, pad4, sid2,* and *npr1* had similar levels of infection compared to the wild type (Ferrari et al. 2003**)**. This enhanced susceptibility of NahG plants was not due to catechol accumulation because exogenous treatment with catechol showed enhanced rather than decreased resistance to *B. cinerea* (Ferrari et al. 2003). In systems where enhanced susceptibity was observed in NahG plants, BTH showed a significant suppressing effect, while no effect of BTH was observed in systems that lacked enhanced susceptibility of NahG plants (Achuo

et al. 2004). Consistency in data suggested that the increased susceptibility of *NahG* tomato to *Botrytis sp.* and of *NahG* tobacco to *Oidium sp.* is closely related to SA-dependent defense responses. Exogenous application of catechol had no effect on tobacco resistance to TMV and on SA-induced expression of pathogenesis-related genes (Friedrich et al. 1995). It has been shown that the SA-dependent defense pathway in tobacco is activated by BTH, SA, and TMV even though activation of the SA-dependent defense pathway by BTH is independent of SA accumulation (Lawton et al. 1996).

Defense Responses that do not Involve SA, JA or ET

Plants usually recognize an invading pathogen and trigger a defense response via direct or indirect interaction between the host resistant protein and its cognate pathogen-encoded effector protein termed effector-triggered immunity (ETI, formerly termed R gene mediated resistance) (Chisholm et al. 2006; Jones and Dangl. 2006). A lack of these corresponding genes by the plant or the pathogen results in the plant becoming susceptible to infection due to its inability to activate defense responses with sufficient intensity and (or) rapidity.

Plants also do have a pathogen-recognition system that is receptor based and is called pathogen associated molecular pattern (PAMP)-triggered immunity (PTI) (Chisholm et al. 2006; Jones and Dangl. 2006) that confers low-level resistance to virulent pathogens. A hypersensitive response is one of the most visible manifestations of ETI characterized by the development of necrotic lesions at the site of pathogen entry. ETI is associated with the accumulation of reactive oxygen species (ROS) and the

activation of diverse groups of defense genes including those that code for the various families of pathogenesis-related (PR) proteins (Dempsey et al. 1999).

Alkaloids, a class of nitrogen containing secondary metabolites, are found in some plants (approximately 20 % of vascular plant species) to protect themselves against pathogen attack (Hegnauer et al. 1988). Examples include caffeine, cocaine, morphine, and nicotine. Caffeine is present in plants such as coffee (*Coffea arabica*), cocoa (*Theobroma cacao*), and tea (*Camellia sinensis*). It is toxic to both insects and fungi (). The compound in plants that signals them to release these toxic substances is called systemin (Ryan and Pearce 1998), which activates genes and, in turn, produce proteinase inhibitors. Proteinases are used by insects to digest the ingested plant proteins. When these proteinase inhibitors are released by the plant, it prevents the insect's ability to digest the plant's proteins (Ryan and Pearce 1998). The activation of pathogenesis related genes extends to systemic plant parts conferring a broad-based and long lasting resistance to viral, bacterial, and fungal pathogens (Cao et al. 1998).

Systemic Acquired Resistance (SAR)

Movement of the signal generated in the infected leaf (local response) via the phloem to uninfected plant parts (systemic response) generates an SAR response (Durner et al. 1997; Dempsey et al. 1999**)**. SAR resembles the immune response in animals in several ways that include both innate and adaptive components (Hoffmann et al. 1999) with the innate response being immediate and nonspecific and mediated by chemical, humoral, and cellular barriers, whereas the adaptive immune system involves recognition

of specific "non-self" antigens in the presence of "self" allowing for the development of immunological memory (Aderem and Underhill 1999).

Initially, SA was thought to be the mobile signal because; (1) it induces defense responses when applied to plants, (2) it moves systemically to other plant parts, (3) it is found in phloem exudates of infected leaves, and (4) it is required in systemic tissue for SAR signal development (Vernooij et al. 1994). However, later studies using grafting experiments showed that SAR response in wild type scions was triggered by infected SA-deficient rootstocks (NahG) implying that SA is not the mobile signal for SAR (Vernooij et al. 1994; Pallas et al. 1996).

Methyl salicylate (MeSA), a methyl ester of SA that moves from primary infected leaf tissues (local response) through the phloem to systemic tissues, has recently been shown to be the key SAR signal (Park et al. 2007) in tobacco plants. SAR is characterized by an increased expression of a large number of pathogenesis-related (PRs) genes in both local and systemic tissues. TMV-infected tobacco plants showed enhanced resistance upon subsequent infection to TMV on the systemic tissues (Ross 1961). PR proteins were first described in the 1970s by Von Loon, when he observed the accumulation of novel proteins upon TMV infection of tobacco plants (Van Loon 1997). PR proteins such as chitinases and glucanases possess antimicrobial activity and are thought to contribute to a broad-based spectrum of resistance activated upon infection by pathogen (Pieterse and Van Loon 1999). The expression of *PR genes* induced by a pathogen is correlated with the development of SAR on uninfected tissue (systemic tissue), and can be mimicked by exogenous application of salicylic acid (Ward et al. 1991).

Salicylic Acid Binding Proteins (SABPs)

Several potential effector proteins have been identified in tobacco to elucidate the pathway through which SA signals disease resistance. The well-studied SA- binding proteins (SABP) include a catalase (Chen et al. 1993), ascorbate peroxidase (Durner and Klessig 1995), carbonic anhydrase (SABP3) (Slaymaker et al. 2002), and SABP2 (Du and Klessig 1997). SABP2 has been shown to display highest affinity for SA ((Kd of 90nM) among all the SABPs currently known (Du and Klessig 1997). SABP2 displays lipase/esterase activity and has been shown to be important for SAR activation (Kumar and Klessig 2003; Forouhar et al. 2005). The X-ray crystal structure of SABP2 suggests that it belongs to the α/β hydrolase super family of enzymes with Ser-81, His-238 and Asp-210 forming the catalytic site (Forouhar et al. 2005). Biochemical studies have shown that SABP2 possesses strong esterase activity with MeSA (substrate) and SA (product) inhibits this reaction (Forouhar et al. 2005). This suggests that SABP2 may be required to convert MeSA to SA, which is important for SAR activation as well as local defense responses. SABP2 is present in extremely low amounts in tobacco leaves (Du and Klessig 1997; Kumar and Klessig 2003).

Acetylcholinesterase (AChE) and AChE- Inhibiting Pesticides

A wide range of agricultural pesticides have been extensively used to control plant pests. A pesticide is a substance that may function in preventing, destroying, repelling, or mitigating a pest. Some of the most commonly used pesticides in the US, are malathion, paraoxon (currently not in use), and parathion (reviewed in Obare et al. 2010). These pesticides inhibit acetylcholinesterase (AChE) (Kiely et al. 2004). Most AChE-

inhibiting pesticides are divided into two categories, organophosphates and carbamates (Fukuto 1990). Carbamates inhibit AChE activity temporarily and the enzyme recovers within 30 minutes to several hours or days (O'Brien 1976; Fukuto 1990), while the organophosphate irreversibly bind AChE and its recovery is achieved only with the synthesis of new AChE (O'Brien 1967).

AChE has a very high catalytic activity (Attalla et al. 2010). The choline produced by AChE action is recycled (Figure 3) and transported into the nerve terminals where it is used in the synthesis of a new acetylcholine molecule and the process starts all over again (Attalla et al. 2010). Cholinesterase inhibitors inhibit the activity of AChE resulting in the accumulation of acetylcholine at the synaptic cleft (Attalla et al. 2010). This causes continuous neuromuscular contraction resulting in paralysis and eventual death of the organism (Attalla et al. 2010).

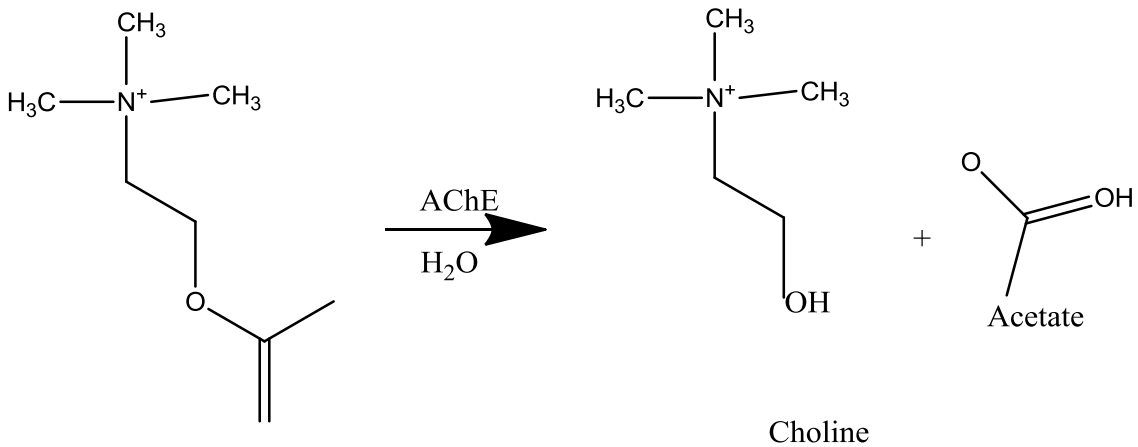


Figure 3: Conversion of Acetylcholine to Choline by AChE

X-ray structures of AChE co-crystallized with various ligands have provided information on the essential structural elements and motifs central to its catalytic

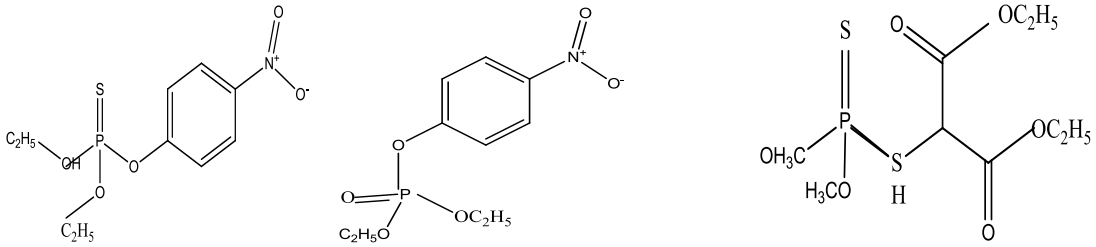
mechanism and mode of acetylcholine (ACh) processing (Lu et al. 2011). The presence of a narrow, long, and hydrophobic gorge approximately 20 Å deep was identified following the x-ray analaysis of AChE structure. The catalytic triad of AChE consists of Ser-203, His-447, and Glu-334 (Lu et al. 2011), while that of SABP2 is made up of Ser-81, His-238, and Asp-210 (Forouhar et al. 2005). Aspartate and glutamate are both negatively charged amino acids, further suggesting the similarities in their active sites and catalytic activities. The AChE catalytic triad is located in the active site of the narrow deep gorge whose lining consists mainly of aromatic residues that form a narrow entrance to the catalytic Ser-203 (Lu et al. 2011). Is has been postulated that a peripheral anionic site consisting of aromatic residues Tyr72, Tyr124, Trp286, Tyr341, and Asp74 is located at the rim of the gorge providing a binding site for allosteric modulators and inhibitors (Lu et al. 2011).

Esterase Activity of SABP2 and Acetylcholinesterase (AChE) Enzymes

Expression of putative esterase genes has been shown to change during plant development (Chandra and Toole 1977) and in response to stress and infection (Muarlidharan et al. 1996; Baudouin et al. 1997). The α/β hydrolase family of enzymes is rapidly becoming one of the largest groups of structurally related enzymes with diverse catalytic functions including esterases (reviewed in Holmquist 2000). Members in this family include AChE, dienelactone hydrolase, lipase, thioesterase, SABP2, and more (reviewed in Holmquist 2000).

Organophosphate Pesticides (OP)

Major uses of OP pesticides are in agriculture. OP pesticides are commonly applied on crop plants e.g. corn, cotton, wheat, potato, beet, tobacco, sunflower, sweet potatoe, and peanuts (U.S. Environmental Protection Agency). The target sites of most pesticides are mainly pest enzymes e.g., protox inhibitors (herbicides), ergosterol synthesis inhibitors (fungicides), and acetylcholinesterase inhibitors (insecticides) (reviewed in Hoagland et al. 2000). Some pesticides also interfere with enzymes present in nontarget organisms. For example, the insecticide carbaryl, a potent acetylcholinesterase inhibitor, inhibits esterases and amidases in plants (Frear and Still 1968) and microorganisms (Hoagland and Zablotowicz 1995). Carbaryl competitively inhibits acylamidase activity that is responsible for propanil metabolism in rice (Frear and Still 1968). Propanil has been shown to block electron flow through photosystem II (Frear and Still 1968). Carbaryl insecticides together with propanil acts to increase phytotoxicity. Studies showed that when leaf disc of both resistant and susceptible barnyardgrass, *Echinochloa crus-galli* are incubated for 5 h in a mixture of propanil and carbaryl, photosynthesis was completed inhibited (Frear and Still 1968). Commonly used OPs are parathion, malathion, chlorpyrifos, paraoxon, and azinphos methyl (see Figure 4) that degrade rapidly by hydrolysis on exposure to sunlight.



Parathion Paraoxon Malathion

Figure 4: Some Organophosphate Pesticides that Bind AChE. They have différent chemical structures but perform the same function, i.e inhibiting AChE activity. Structures were drawn using the ChemDraw Std software.

Significance of Research

Pesticides are chemical compounds used to kill pests, weeds, insects, and plant pathogen (viruses, bacteria, fungi, nematodes). About three-quarters of all pesticides used in the U.S., (i.e. over 5 times the amount used by industry and government agencies and about 7 times the amount for home use in gardening) is in the agricultural sector (www.centerforfoodsafety.org). In 2001, about 675 million lbs. of pesticides were used in the U.S. agricultural sector (www.centerforfoodsafety.org). Herbicides make up nearly two-thirds of agricultural pesticide used (433 million lbs. in 2001). A significant trend in the use of pesticides involves herbicides. USDANASS (USDA National Agricultural Statistics Service) data showed that the rise in herbicide use on field crops such as cotton, soybeans, and corn started in 2002 and 2003 (Center for Food Safety, 2008). This is important for the proposed research because these pesticides are applied on plant crops to kill pests so as to make the plants healthy and increase crop yield. If applied pesticides suppress the plant's defense mechanism, it could make plants more susceptible to microbial pathogens and could eventually reduce crop yield.

Hypotheses

This thesis research was designed to determine the effects of pesticides on SABP2 enzymatic activity and plant defenses. SABP2 is critical for catalyzing the conversion of MeSA, a phloem mobile SAR signal, to SA that is required to induce defense responses in tobacco and other plants (Kumar and Klessig 2003, Forouhar et al 2005, Park et al 2007). Inhibition of SABP2 activity by pesticides may make plants more susceptible to microbial pathogens leading to disease and loss in productivity. The following hypotheses were developed and experiments were designed to test them.

Hypothesis 1: Organophosphate pesticides inhibit the enzymatic activity of SABP2

Hypothesis 2: Organophosphate pesticides block SAR development in TMV infected tobacco plants

Hypothesis 3: Pesticide treatment blocks PR-1 protein production on systemic leaves of TMV infected tobacco plants.

Alternate Hypothesis: Agricultural pesticides have no effect on SABP2 activity and plant defenses.

CHAPTER 2

MATERIALS AND METHODS

Plant Materials

Tobacco plants (*Nicotiana tabacum* cv. Xanthi nc (NN) (wild type) and SABP2-silenced (1-2J) lines (SABP2 gene expression stably silenced by RNA interference) (Kumar and Klessig 2003) were used for this study. Prior to sowing seeds, soil containing peat moss (Fafard F15, Agawam, MA) was autoclaved for 20 minutes. The seedlings were transferred to 4 x 4 inch flats after 10-14 days and were grown for 4 weeks. Later individual plantlets were transferred to 8 inch pots. Plants were all grown in a controlled PGW36 growth chamber (Conviron, Canada) set at 16 h of day/18 h of night cycle maintained at 22 °C. Experiments were carried out using 6 to 8 week old plants.

Chemicals and Reagents

Organophosphate pesticides (paraoxon, malathion) were purchased from Chem Service (West Chester PA). Chemicals purchased from Fisher Scientific (Pittsburgh, PA) were phenylmethanesulfonylfluoride (PMSF), ammonium persulfate (APS), bovine serum albumin (BSA), acetonitrile, β- mercaptoethanol (βME), coommassie brilliant blue-R250, ponceau-S, TRIS Base, ethylenediaminetetraacetic acid (EDTA), sodium phosphate monobasic and sodium phosphate dibasic, Tween 20, glycerol, methanol, carborundum, tetramethylethylenediamine (TEMED), sodium chloride, protease inhibitor cocktail, protease, sucrose, magnesium chloride, and dimethyl sulfoxide (DMSO). Mini Trans Blot system for Western Blot, acrylamide (30 %), SDS dye, a low molecular weight prestain protein marker, and Bradford's reagent were all purchased from Bio-Rad

(Hercules, CA). Polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Billerica, MA). Monoclonal anti-rabbit IgG and anti- Mouse IgG peroxidase conjugate were purchased from Sigma Aldrich (St. Louis, MO). Monoclonal anti-PR-1 (tobacco) and rabbit polyclonal SABP2 were available in-house. The electrochemiluminescence (ECL) system for developing western blots was purchased from GE Healthcare (Piscataway, NJ). Para-nitrophenyl acetate and methylsalicylate were purchased from Sigma. BCA protein assay Kit (Pierce). Recombinant SABP2 was expressed and purified from *E.coli* (Kumar and Klessig 2003). TMV purification was carried out as described by (Guo et al. 2000).

Buffers

Various buffer solutions were prepared for the different experiments carried out. Amongst them were; protein extraction buffer (pH 8.0), phosphate buffered saline (PBS) (pH 7.0), 20 mM sodium phosphate buffer (pH 7.2), the SDS PAGE resolving buffer (pH 8.8), and stacking buffer (pH 6.8), transfer buffer for Western Blot analysis, Tris-HCl buffer (pH 8.0), 10 mM and 250 mM immidazole in 1X Ni-NTA buffers, 0.01 % Tween 20, 10 mM Bicine buffer (pH 8.0). Composition of these buffers are provided in Appendix B.

Other Materials

Other materials used in this research were one ml syringes (obtained from BD syringes, NJ), spray bottles and cheesecloth (Fisher Scientific), electronic digital caliper, pestle grinder (Fisher Scientific), assay plates (96 Well Round bottom, nontreated sterile

Polystyrene) (Corning Incorporated, NY), Multi-Mode microplate reader (BioTeK Synergy HT), SigmaPlot Software. WX Ultra centrifuge (Sorvall) RT6000 refrigerated centrifuge (Sorvall), UV-Visible spectrophotometer, table top centrifuge (Eppendorf, NA), Beckman Model J21 centrifuge, Type 50 rotor ultra centrifuge, French Press-Cell Disrupter (Thermo Electron Corporation), Mettler Toledo Weighing balance, Sonic Dismembrator Model 500 (Fisher Scientific).

Methods

Expression and Purification of SABP2 (6x histidine tag at C-terminal)

A single bacterial colony containing pET21-SABP2 plasmid grown on an LB-ampicillin (Amp) (100 µg/ml) plate was inoculated into 3 ml of LB containing Amp (100 µg/ml). This was incubated overnight at 37 ºC on a shaker (250 rpm). The overnight culture (500 µl) was inoculated into a 50 ml of LB containing Amp (100 µg/ml). The culture was then incubated at 37 ºC on a shaker (250 rpm) until the OD reached 0.5-0.6. From the 50 ml total culture, 1 ml (uninduced) was pipette out into a 1.5 ml tube, centrifuged at 5000 rpm for 10 mins at 4 ºC. Pellet was saved as uninduced control. Rest of the culture solution was induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) (1mM final concentration) and incubated on a shaker (250 rpm) overnight at 18 ºC. From the IPTG induced culture, 1 ml was pipetted into a 1.5 ml tube and again centrifuged at 5000 rpm for 10 mins at 4 ºC and pellet was saved as induced pellet. The rest of the culture solution was transferred into a 50 ml tube and centrifuged at 5000 rpm for 20 mins at 4 ºC. The supernatant was discarded and tubes containing the pellets were kept on

ice. Ni-NTA binding buffer (1X) was prepared and 200 µl of it was added to both induced and uninduced pellets and resuspended by vortexing. Resuspended bacteria cells were then sonicated on ice, 3 times for 10 secs each at 20 % amplitude at 15 sec intervals using a Sonic Dismembrator Model 500 (Fisher Scientific). The sonicated bacterial extract was centrifuged at 12,000 rpm at 4 ºC for 10 mins. The supernatant contains the soluble proteins for both uninduced and induced samples while the pellet contains the insoluble proteins. Each pellet (induced and uninduced) was resuspended in 200 µl of 1X Ni- NTA binding buffer. Protein samples (7.5 µl supernatant + 7.5 µl 2x SDS-PAGE dye) were boiled, centrifuged, and supernatant loaded on a 12 % SDS PAGE gel. Following electrophoresis at constant current of 20 mA, gel was stained with coomassie brilliant blue and photographed.

For affinity purification of SABP2, 6 ml of Ni-NTA resin was mixed with 24 ml 1X Ni-NTA binding buffer in a 50 ml tube. The resin was allowed to settle and 24 ml buffer was removed by pipetting. The 1X Ni-NTA buffer (6 ml) was added to the pellet from the 50 ml bacteria culture kept on ice, resuspended, and sonicated for 10 sec with 15 sec intervals (process was repeated 6 times). The sonicated extract was centrifuged at 12,000 rpm at 4 ºC for 10 mins. The supernatant containing soluble proteins was mixed with the resin and the mixture incubated at room temperature (25 ºC) for 1 hr.

The empty chromatographic column (Biorad) was rinsed with milli Q water then followed by washing buffer (1× Ni-NTA). The resin mixture with protein extract was loaded into the column with outlet cap at the bottom closed. The cap column was opened when the resin had settled down. Flow-through was collected into 1.5 ml tubes. About 40 ml washing buffer was added to the column and collected as wash. SABP2 was eluted

with 6 ml of 250 mM imidazole and fractions were collected into 1.5 ml tubes (1ml fractions). Eluted fractions were mixed with 2x SDS dye (7.5 µl proteins + 7.5 µl dye), boiled, centrifuged, and separated on an SDS PAGE gel. Gel was stained with coomassie brilliant blue dye. Fractions containing purified SABP2 were pooled (8 ml total) and precipitated with ammonium sulfate (80 % saturation; 560g/1000ml). Precipitated protein was centrifuged at 12,000 rpm for 20 min at 4 ºC. The pellets were resuspended in 2.5 ml of 10 mM Bicine, pH 8.0. The resuspended protein were desalted on a PD10 column (GE Healthcare) equilibrated with 10 mM Bicine buffer pH 8.0 (Appendix B) and loaded on a Q sepharose column equilibrated with 10 mM Bicine, pH 8.0. Bound proteins were eluted with a gradient of 0-500 mM ammonium sulfate in 10 mM Bicine, pH 8.0. Fractions were collected and run on a 12 % SDS PAGE Gel at 20 mA and stained as described earlier.

Determining the Effect of Paraoxon on SABP2 Activity

SABP2 converts para-nitrophenyl acetate (pNAc) into para-nitrophenol (pNP) (Figure 5). The effect of paraoxon on SABP2 activity was monitored using a spectrophotometer or microwell plate reader (Synergy HT). Stock solution of 5 mM pNAc (MW 181.15) was prepared in 100 % acetonitrile. For reaction, assay mixtures were prepared in a total volume of 300 µl in 50 mM Tris-Cl (pH 8.0) containing various pNAc concentrations (50, 100, 200, 300 µM diluted from the 5 mM stock) and recombinant purified SABP2 (1 µM in 10 mM Bicine, pH 8.0). A stock solution of 2 mM paraoxon was prepared in 100 % DMSO (Stock is 98.7 %; 3.586 M). Various paraoxon dilutions (10 and 100 µM) were then prepared from the 2 mM stock solution and added to the reaction mixtures. Using a multichannel pipette, the following components were

added into wells of a 96 well plate. Tris-Cl buffer was first added followed by SABP2 and then pesticide (paraoxon) (Total reaction volume was 300 µl). The reaction mixture was incubated for 30 mins at room temperature and the volumes corresponding to the various para-nitrophenylacetate concentrations were then added, mixed, and absorbances read at 405 nm at 30 sec intervals for 10 mins using a multiwell plate reader. Experiments were all carried out at room temperature (25 ºC)**.** Data obtained were corrected for spontaneous hydrolysis of para-nitrophenylacetate. The amount of product formed at each substrate concentration was calculated using Beer Lambert's Law (A = εCL.), ε = molar extinction coefficient of pNP =17500 M^{-1} cm^{-1} in Tris-Cl buffer), C = concentration of pNP formed, A= absorbance and L= path length of 1 cm. Initial velocities (*Vi*) obtained at each concentration of pNAc were calculated by dividing the concentration of product (pNP) formed by a total incubation time of 1 min for all conducted experiments**.**

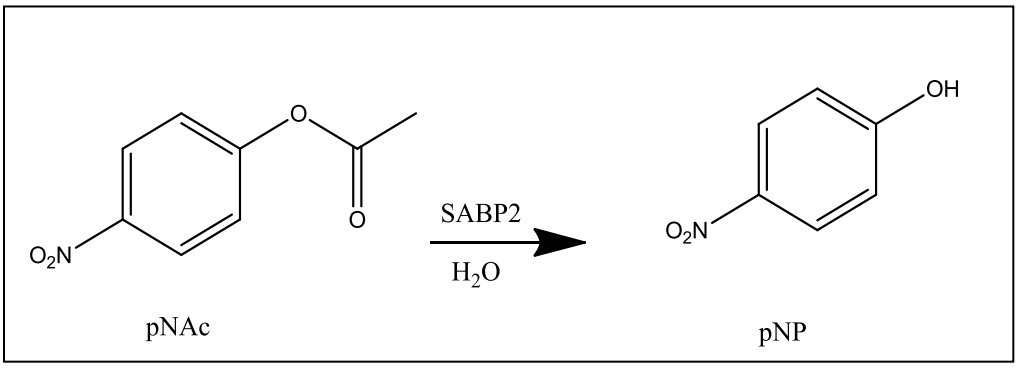


Figure 5: SABP2 Catalyzed Conversion of pNAc to pNP

Determining the Effect of Malathion on SABP2 Activity

Para-nitrophenylacetate (5 mM) was prepared in 100 % acetonitile as previously described. Enzyme assay mixtures were prepared in 50 mM Tris-Cl buffer, pH 8.0 and a total reaction volume of 300 µl solution containing various substrate concentrations (50, 100, 200, 300 µM) plus 1 µM recombinant SABP2 were prepared. Purified SABP2 was quantified using the Pierce BCA Protein assay kit. A stock concentration of 2 mM malathion was prepared in 100 % DMSO (98.7%; 2.987 M stock solution). Various concentrations of malathion (10, 100 µM) were made from the 2 mM stock solution and also added into the reaction mixtures. All subsequent experimental procedures were carried out as previously described for the paraoxon experiment.

Determining the Effect of Increasing Pesticide Concentrations on Tobacco Leaves

Wild type *Nicotiana tabacum* cv Xanthi-nc (NN) tobacco plants (6-8 weeks old) were used for this experiment. Various pesticide concentrations in 0.01 % Tween 20 were infiltrated on a fully developed leaf to determine the appropriate concentration that could be used for the experiment without adversely affecting the leaves. According to Reinecke (2007), azinphos methyl (0.15 kg), an organophosphate pesticide was applied at a recommended rate of 450 µM.

Purification of Tobacco Mosaic Virus (TMV)

TMV purification was carried out and used for *in vivo* experiments on tobacco plants. TMV infected *Nicotiana tabacum* cv Xanthi-nc (nn) leaves (50 g) were homogenized in 100 ml of 0.5 M sodium phosphate, pH 7.2 containing 14 mM β-mercaptoethanol in a kitchen blender for 4 mins in the cold room (4 ºC). The homogenate

was filtered through cheese cloth. While stirring at room temperature, 8 ml of n-butanol/100 ml was added to the filtrate to coagulate the chloroplasts. After the chloroplasts had coagulated, stirring of the filtrate continued for an additional 15 mins. Using the Beckman Model J2-21 Centrifuge, the mixture was centrifuged at 10,000 x g for 10 mins at room temperature. The supernatant was decanted into a flask taking care not to decant the butanol layer. While stirring the supernatant, 4.0 g of solid polyethylene glycol, (PEG)/100 ml was added to the supernatant (average molecular weight of PEG-3350). After the PEG had dissolved, the suspension was centrifuged at 10,000 x g for 10 mins at room temperature. The supernatant was poured off and discarded. Tubes were drained and the pellets containing the virus resuspended in 20 ml of washing buffer (0.01 M EDTA, pH 7.2/100 ml of original homogenate (i.e. 1/5 original volume). The resuspended particles were centrifuged at 10,000 x g for 10 mins at 4 ºC to prepare for the second PEG precipitation. While stirring the supernatant, 0.4 g of NaCl + 0.4 g of PEG/10 ml of virus suspension was added. For 20 ml, 0.8 g of NaCl + 0.8 g of PEG were used. The suspension was centrifuged at 10,000 x g for 10 min 4 ºC. The supernatant was discarded, tubes drained, and pellets stored at 4 ºC. The pellets were resuspended in 10 ml of 0.01 M EDTA buffer/100 ml original homogenate. The mixture was clarified by centrifugation at 10,000 x g for 10 mins at 4 ºC. The solution was put in Type 50 rotor, ultra centrifuge, tubes filled to shoulder mark with EDTA buffer, and centrifuged using the WX Ultra centrifuge (Sorvall) for 1 hr at 28500 x g to separate the virus from low molecular weight molecules such as soluble proteins. The Supernatant was discarded, tubes drained, and pellets resuspend in 5 ml of 0.01 M EDTA buffer/100 ml original homogenate and stored at 4 ºC. The TMV was quantified using the Pierce BCA kit.

Various dilutions of this stock solution were prepared in 20 mM phosphate buffer and applied (by gentle rubbing) on wild type tobacco leaves to determine the number of lesions produced for each concentration. The dilution corresponding to a protein concentration of 0.45 µg/ml produced distinct lesions (total 240/leaf) and was used in subsequent TMV based experiments.

Effect of Pesticides on SAR Development in Tobacco Plants

The experiments described below were carried out with both wild type (*N.t.* Xanthi NN) and 1-2J (SABP2 silenced plants). For the negative control, 3 lower leaves of each tobacco plant were treated with 20 mM phosphate buffer, pH 7.2. Distal (upper) leaves, 3 each, were then sprayed with 100 µM paraoxon (in 0.01% Tween 20) at 48, 72, and 96 hp1°i (hours post primary inoculation) respectively. These leaves were later challenged with 2.0 µg/ml TMV at 144 hp1°i. TMV inoculation was carried out as follows: Carborundum was dusted onto the leaf and a cheese cloth soaked in the buffer or TMV solution was rubbed gently on the leaf. Carborundum helps to wound the leaf surface so that the virus can penetrate easily. Sizes (diameter) of TMV induced lesions on the distal leaves (15 lesions/leaf) were measured after 6-7 days of TMV inoculation using an electronic digital caliper. Only distinct lesions were measured. Some smaller necrotic spots that start to appear later and other lesions that are larger in size and appear to be due to overlapping of 2 or more lesion were not measured.

For the positive control experiment, 3 lower leaves of a tobacco plant were inoculated with 2.0 µg/ml TMV (primary infection). Distal (upper) leaves, 3 each, were then sprayed with phosphate buffer containing 0.01 % Tween 20 at 48, 72, and 96 hp1°i

respectively. These leaves were later challenged with 2.0 µg/ml TMV at 144 hp1°i. Sizes of TMV- induced lesions on these distal leaves were measured using an electronic digital caliper after 6-7 days of secondary TMV inoculation.

For the pesticide (paraoxon) treatment, 3 lower leaves of a tobacco plant were first inoculated with TMV (primary infection). Distal (upper) leaves, 3 each, were then sprayed with 100 µM paraoxon (in 0.01 % Tween 20) at 48, 72, and 96 hp1°i respectively. These leaves were later challenged with TMV at 144 hp1°i. TMV induced lesion sizes were then measured after 6-7 days of secondary TMV inoculation as previously described. Based on the results from these experiments (see results section), some changes were made with subsequent experiments. A paraoxon concentration of 450 µM was used in subsequent experiments with a single pesticide treatment on distal leaves at 48 hp1°i.

Pesticide concentrations were prepared as follows: 1.5 µl of 2.987 M stock Malathion (MW: 330.36) was dissolved in 200 µl of DMSO and then diluted in 10 ml of 0.01 % Tween 20 to give a final concentration of 450 µM. From the 3.586 M paraoxon stock (MW: 275.22), 1.25 µl was dissolved in 200 µl of DMSO and then diluted in 10 ml of 0.01 % Tween 20 to give a final concentration of 450 µM.

For the negative control experiment, 3 lower leaves of a tobacco plant were first inoculated with 20 mM phosphate buffer, pH 7.2. Distal (upper) leaves, 2 each, were then sprayed with 450 µM pesticide at 48 hp1°i. These leaves were later challenged with 0.45 µg/ml TMV at 144 hp1°i (Figure 6). Sizes of TMV induced lesions (lesions that appeared early enough were included in the study) on the distal leaves were measured using a caliper after 6-7 days of TMV inoculation as previously described.

For the positive control experiments, 3 lower leaves of tobacco plant were first inoculated with TMV (primary infection). distal (upper) leaves (2) were then sprayed with 20 mM phosphate buffer (in 0.01 % Tween 20) at 48 hp1°i. These leaves were later challenged with TMV (secondary infection) at 144 hp1°i (Figure 6). Sizes of TMV induced lesions (lesions that appeared early enough) on these distal leaves were measured using a digital caliper after 6-7 days of TMV inoculation as previously described.

For the pesticide (paraoxon) treatment, 3 lower leaves of a tobacco plant were first inoculated with TMV (primary infection). Distal leaves (2) leaves were then treated with 450 µM Pesticide (in 0.01 % Tween 20) at 48 hp1°i. These leaves were later

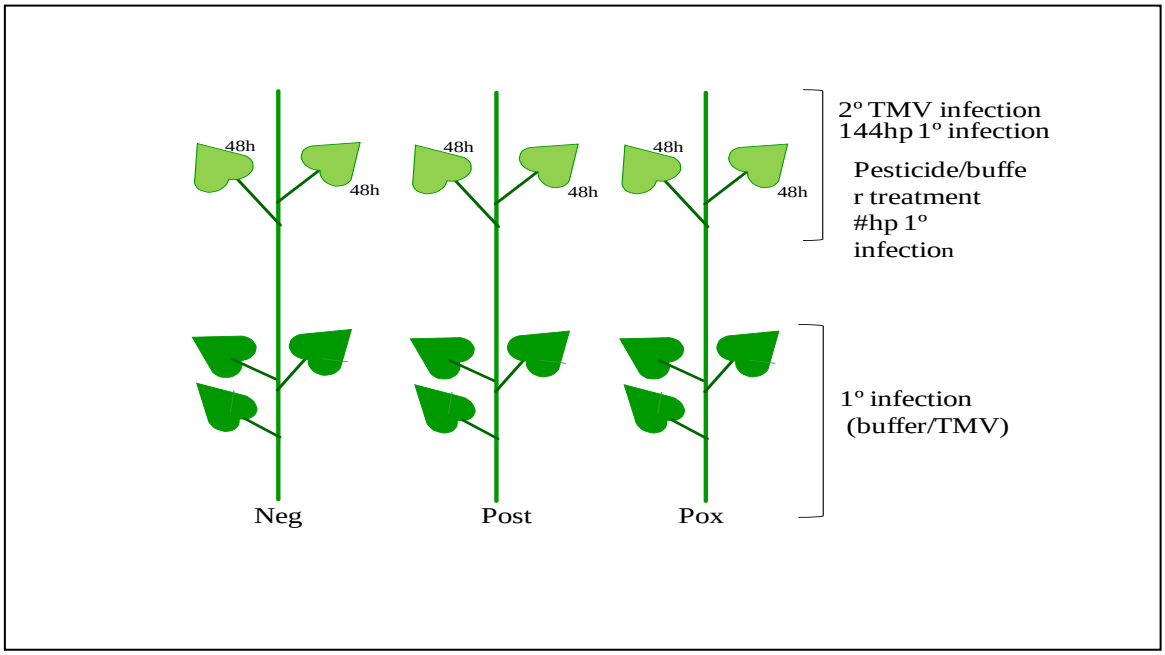


Figure 6: Experimental Design to Test if Pesticide Treatment Blocks SAR. Three different treatments: Neg (negative control), Post (positive control), Pox (pesticide treatment). At 48 hp1°i, 2 distal leaves from each treatment were treated with pesticide/buffer. At 144 hp1°i, distal leaves were all challenged with TMV and lesion sizes measured after 6-7 days.

challenged with TMV (secondary infection) at 144 hp1°i (Figure 6). Sizes of TMV induced lesions on these distal leaves were measured using a digital caliper after 6-7 days of TMV inoculation.

Determining the Effect of Pesticide on PR-1 Protein Production

A total of 6 plants were used for this experiment, 2 plants for each treatment. At time 0 hr**,** 2 leaf discs from the bottom lower leaf of each treatment (Plant 'A'-negative control, plant 'B'-positive control and plant 'C'-pesticide treated) were collected for RT-PCR analysis and for Western Blot analysis. The leaf discs were frozen in liquid nitrogen and stored at -80 ºC until ready for RNA isolation. Lower leaves (3) of plants B and C were TMV-treated while plant A was mock (buffer) treated. At 48 hp1ºi, 2 distal leaves of plants A and C were sprayed with either paraoxon or malathion while distal leaves of plant B were sprayed with buffer. After 48 hr of treatment with pesticide/buffer on distal leaves, 2 discs from distal leaves were again collected and stored at -80 ºC. Also at 96 hp1ºi(hours post primary inoculation), leaf discs from lower (primary) leaves were collected and stored at -80 ºC. At 144 hp1ºi, all the distal leaves of each treatment were challenged with TMV and at 48 hp2 ºi (hours post secondary TMV inoculation) or 192 hp1ºi, leaf discs were collected and stored at -80 ºC. RT-PCR and Western blot experiments were then carried out on the different samples to analyze PR-1 expression.

RT-PCR Analysis of *PR-1* Genes on Tobacco Distal leaves

Total RNA was purified using the NucleoSpin RNA Kit (Clontech) following manufacturer's instructions (see Appendix B). The amount of RNA in solution (ng/µl)

was determined using the NanoDrop ND-1000 spectrophotometer and the A260/A280 ratio was determined. Purified RNA was used for cDNA synthesis with reverse transcriptase. One microgram of total RNA plus 1 µg of dT-14 (1 µg/µl) primer (5'TTTTTTTTTTTTTTTTTTTTV3') were added into a sterile RNase-free micro centrifuge and the total volume made up to 10 µl with diethylpyrocarbonate (DEPC)-treated water. The reaction was heated to 70 ºC for 5mins to melt secondary structure of the template. The reaction was immediately cooled on ice to prevent secondary structure from reforming. The following components were added to the annealed primer/template in the following order; M-MLV 5X reaction buffer (4 µl), 10 mM dNTP (1 µl), 40 U/µl RNAsin (1 µl), M-MLV RT (1 µl), nuclease-free water (2 µl) to make up the final volume to 10 µl. The sample was gently mixed and incubated for 60 mins at 42 ºC followed by 70 ºC for 10 mins. Samples were then stored at -20 ºC for RT-PCR analysis. For the PCR amplification of PR-1, 1µl cDNA from each sample, 1 µl of 10X Taq buffer, 1 µl of 2.5 mM dNTP, 0.2 µl of 10 U/µl Taq, 0.8 µl of 10 µM Fwd and Rev PR-1 primers (Fwd: 5'ATGGGATTTGTTCTCTTTTCA3' and Rev: 5'TTAGTATGGACTTTCGCC3') and 6 µl of DEPC-treated water added to make up a total 10 µl volume. PER mix was subjected to 35 cycles (94 ºC for 2 mins, 94 ºC for 30 sec, 55 ºC for 30 sec, 72 ºC for 45 sec and 72 ºC for 5 mins) of amplification. From the PCR products, 5 µl of each was then mixed with DNA gel loading buffer containing dye and loaded on a 1 % agarose gel containing ethidium bromide. A 100 basepair ladder (100 ng total) was used. The gel was run at 100 volts.

Effect of Pesticides on PR-1 Protein Expression in Systemic Leaves

Frozen leaf discs were ground in 200 µl of protein extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 % glycerol, 0.1 % triton X-100, β- mercaptoethanol (1 µl/ml), Protease Inhibitor Cocktail (Sigma-Aldrich, 10 µl/ml). The homogenate was centrifuged at 11,700 x g for 15 mins at 4°C. The supernatant containing total soluble proteins was transferred into 1.5ml tubes and kept at -20°C for further analysis. The protein samples were quantified using the Bio-Rad Protein Assay Dye Reagent Concentrate (based on the method of Bradford) (Bradford 1976). Two microgram of total protein was loaded onto a 12% SDS- PAGE gel. Gel electrophoresis was performed at constant current of 20 mA for 1 hour. All the buffers and gels were prepared as described in Appendix B.

Protein transfer from the gel to the membrane was carried out at 4 °C. The transfer membrane (PVDF) was soaked in 100% methanol for 15 sec, then washed with distilled water followed by 5-10 min wash in 1X transfer buffer containing 10 % methanol. Whatman filter papers (3 mm) and sponges were soaked for 20 minutes in transfer buffer. The SDS-PAGE gel equilibrated in transfer buffer was placed on the PVDF membrane. Both the gel and membrane were sandwiched between the Whatman filter paper and sponge then clamped tightly together after ensuring that no air bubbles have been trapped between the gel and membrane. Transfer was carried out at 4 ºC for 1 hour at 100 V. After the transfer, the membrane was taken out and placed in 100 % methanol for 10 sec and placed on a 3mm Whatman paper for 15 mins and allowed to dry. The membrane was again placed in methanol for 10 sec and washed with 1X PBS buffer followed by staining with ponceau-S for 1 min to verify for equal loading of

proteins. A picture was taken and washed again with distilled water to clear off the stain. The blot was blocked with the blocking buffer (see Appendix B) and incubated with mouse PR-1 antibodies (1: 1000) in 5 ml blocking buffer overnight at 4 °C. After overnight incubation, the blot was washed with 10 ml each of the following buffers in this order, 1X PBS , 1X PBS-T, and finally, with 1X PBS (2 times for 5 minutes each). After washing, the blot was probed with secondary antibodies (Goat Anti-Mouse HRP-conjugated antibodies) (1:5000) for 30 mins at 25° C. Washing was again carried out as previously described. Equal parts of the ECL development solution "stable peroxide solution" and "luminol solution" were mixed together at room temperature and solution poured onto the blot and kept for 1 min. The blot was removed and wrapped in a plastic wrap and put on the screen of an x-ray film cassette. In the dark room, the blot was exposed (1 to 30 min) to the x-ray film and developed. The blot was visualized using instructions from the ECL system. The film was then aligned on the x-ray film cassette to determine the molecular weights of the observed protein bands. The position of the molecular weight markers was marked on the autorad.

CHAPTER 3

RESULTS

SABP2 Expression and Purification

To determine if the SABP2 was expressed as a soluble or insoluble (inclusion bodies) protein, supernatant and pellet following sonication were run on a 12 % SDS PAGE gel and stained with coomassie (Figure 7). Results in Fig. 7 show that majority of SABP2 protein was expressed as insoluble inclusion bodies while some protein was still in soluble fractions. The soluble fraction was used for purification using Ni-NTA column chromatography.

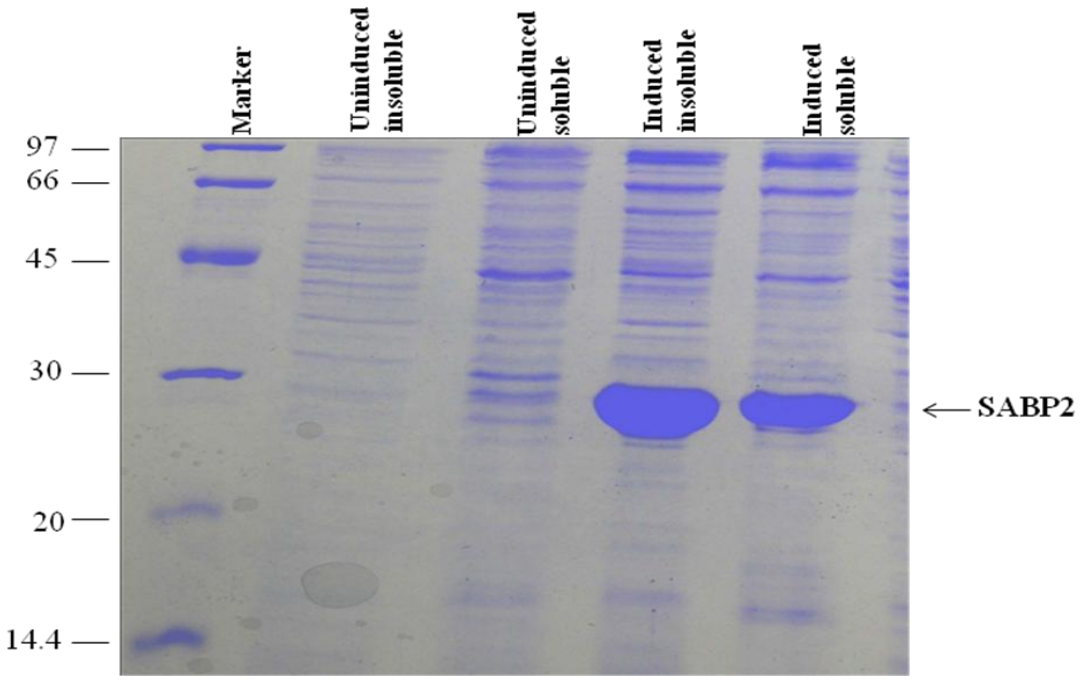


Figure 7: SDS PAGE Gel Showing Recombinant SABP2 Expression. SABP2 is expressed as a ~29 kDa protein in the soluble fraction and insoluble fraction.

Eluted protein fractions (after concentration with ammonium sulfate) that were obtained at the end of the SABP2 expression and purification procedure earlier discussed in the methods section (7.5 µl protein + 7.5 µl 2x SDS dye) were run on an SDS PAGE gel and stained with coomassie. Samples were run alongside a LMW marker (Figure 8). The protein bands from the different eluted fractions corresponded to a MW of 29kDA, the size of SABP2. Fractions 2, 3, and 4 were pooled to test for SABP2 esterase activity.

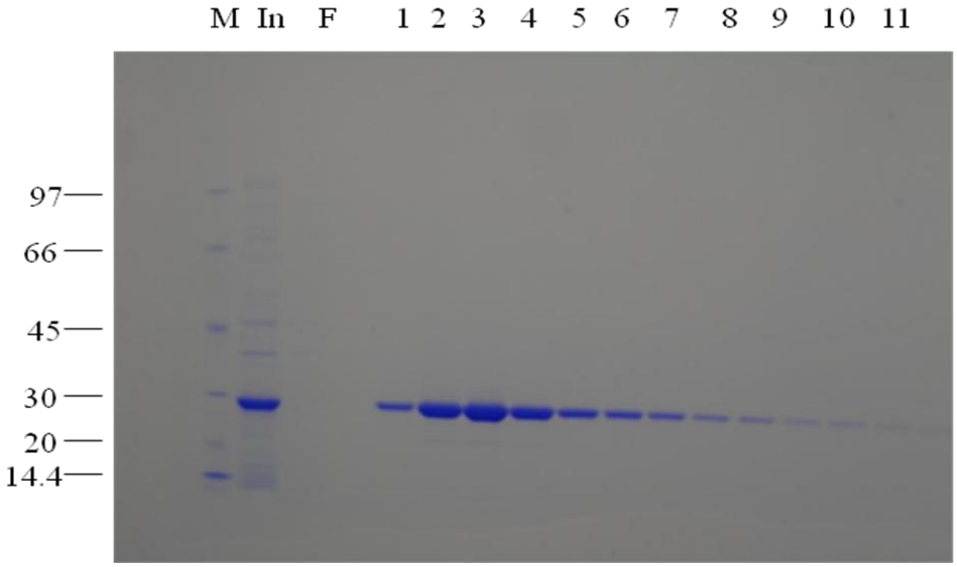


Figure 8: SDS PAGE Gel Showing Eluted SABP2 Fractions. (M=LMW marker, In = input, F= Flow through, fractions 1-11=eluted SABP2 fractions. Note: Band at 29 kDa corresponding to size of recombinant SABP2

Effect of Paraoxon on the Enzymatic Activity of SABP2

The amount of para-nitrophenol product formed (in the presence of increasing paraoxon concentration) at various para-nitrophenylacetate concentrations was monitored at 30 sec intervals for 10 mins at a wavelength of 405 nm. Figure 9 shows a representation of results obtained after an enzymatic reaction performed in a multiwell plate. Fig 9A shows absorbance curve (in red) of conversion of pNAc into para-

nitrophenol (pNP). Each reaction was carried out in triplicate. Expanded view of well #F6 (B) and E2 (C) are show as a representation.

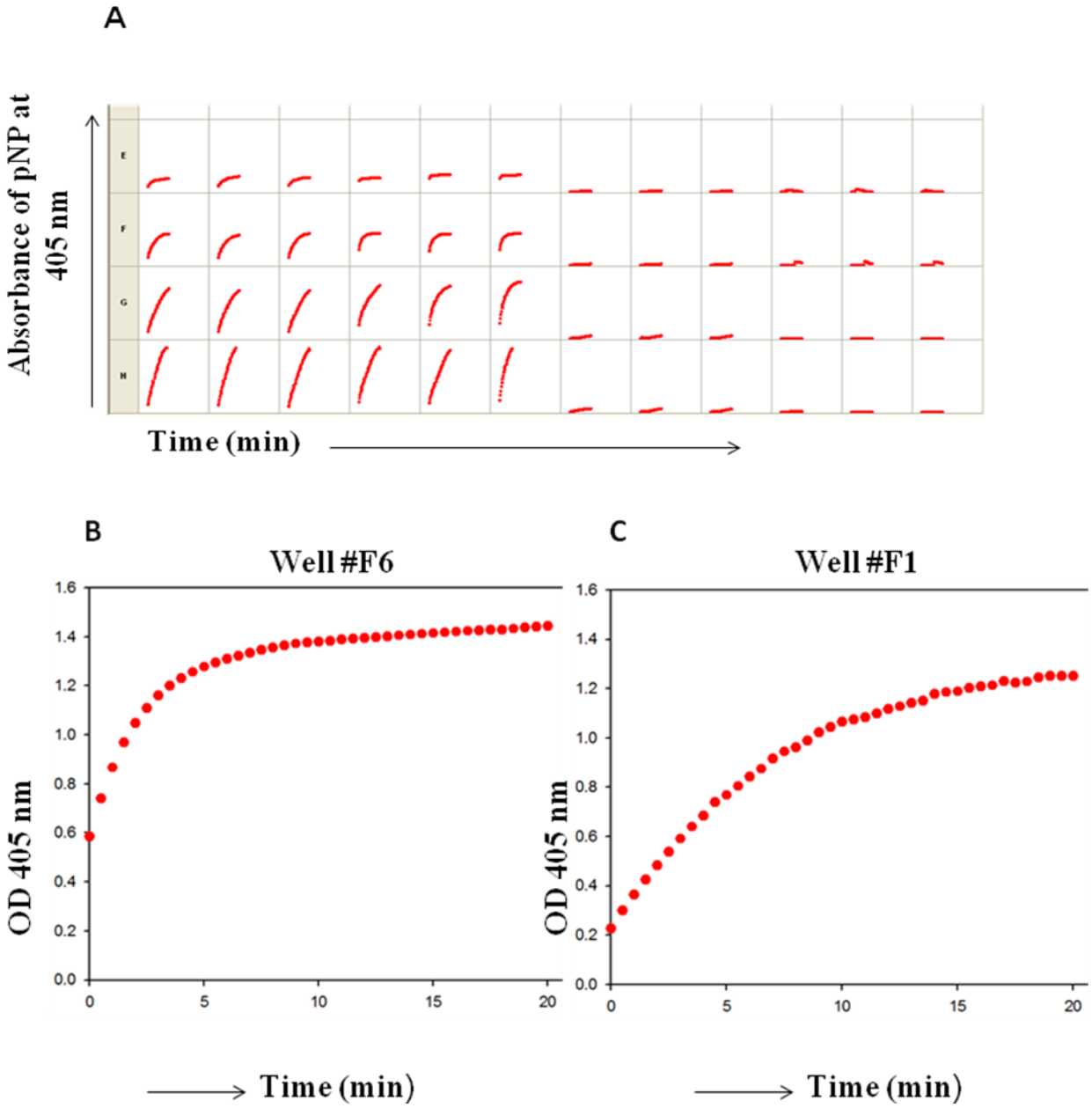


Figure 9: Graphical Representation of Results Obtained After a Kinetic Run On a 96 Well Plate. (A) Results obtained after a kinetic run conducted in triplicates. Well #E1-3 to H1-3 (paraoxon (10 µM)+SABP2+pNAc), E4-6 to H4-6 (SABP2+pNAc), E7-9 to H7-9 (paraoxon+pNAc) while wells #E10-12 to H10-12 (pNAc alone). (B) A representation of results obtained from well #F6 (SABP2+pNPAc). (C) A representation of results obtained from well #F1 (SABP2+paraoxon+pNPAc).

The time course graph was linear for 1.5 mins (Figure 10). Calculations of the kinetic constants (Vmax and Km) were done with data obtained for 1 min. Data obtained were corrected for spontaneous hydrolysis of para-nitrophenylacetate (Table 1).

Table 1: SABP2 mediated Conversion of Para-nitrophenolacetate (pNAc) to Para-nitrophenol (pNP). Conversion of various starting concentrations (0-300 µM) of pNAc monitored for 5 min at 25°C. n=3 (data shows averages of 3 readings each)

Time	50 µM	100 µM	200 µM	300 µM
0.0	0.000	0.000	0.000	0.000
0.5	0.030	0.159	0.186	0.207
1.0	0.070	0.272	0.351	0.397
1.5	0.100	0.367	0.497	0.575
2.0	0.127	0.443	0.631	0.739
2.5	0.128	0.505	0.757	0.894
3.0	0.132	0.556	0.810	1.044
3.5	0.138	0.596	0.976	1.183
4.0	0.142	0.633	1.074	1.569
4.5	0.148	0.660	1.163	1.685
5.0	0.151	0.685	1.249	1.793

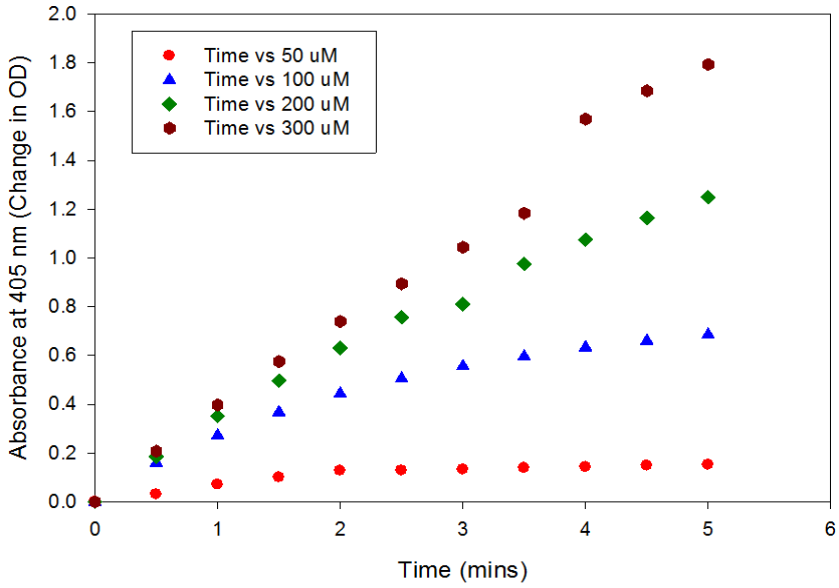


Figure 10: Absorbance-Time Course Showing the Absorbance of Para-nitrophenol formed at Various Starting Substrate (pNAc) Concentrations. 50 µM (Red), 100 µM (Blue), 200 µM (Green), 300 µM (Brick Red).

Data from Table 2 suggest a Michaelis–Menten like kinetic in which the reaction rate increased with increasing substrate concentration and a point was reached where the rate of reaction stayed almost constant (Figure 11).

Table 2: Initial Velocities (*Vi*) Obtained at Various Substrate Concentrations in the Presence of Varying Paraoxon Concentrations.

pNPAc (µM)	*Vi-1* 0 µM Pox (µM)	*Vi-2* 0 µM Pox (µM)	*Vi-1* 10 µM Pox (µM)	*Vi-2* 10 µM Pox (µM)	*Vi-3* 10 µM Pox (µM)	*Vi-1* 100µM Pox (µM)	*Vi-3* 100µM Pox (µM)
0	0.0	0.0	0.0	0.00	0.0	0.0	0.0
50	24.6	23.1	11.5	14.00	14.0	10.0	8.0
100	36.8	34.0	14.2	14.50	17.8	12.4	11.1
200	40.0	35.0	15.6	18.20	18.6	13.4	12.0
300	45.0	35.5	17.0	18.50	16.4	12.5	11.2

In the presence of paraoxon (10 or 100 µM), there was a significant decrease in the reaction rate in comparision to reactions carried out in absence of paraoxon (0 µM). For example, at 100 µM pNPAc, *Vi* with 100 µM paraoxon was lower than that with 0 µM paraoxon (Figure 11).

Data from Table 2 were used to plot the *Vi* vs [S] graph using SigmaPlot and the Vmaxapp, Kmapp values were obtained directly from the graph. At 0 µM paraoxon, Vmaxapp decreased from 46 µM/min (Red circles- 0 µM paraoxon) to 18.9 µM/min (Blue triangles- 10 µM paraoxon) and to 13.5 µM/min (Green hexagon- 100 µM paraoxon). Kmapp values decreased from 40 µM (at 0 µM Pox) to 21.5 µM for (10 µM paraoxon) and 21.4µM (100 µM paraoxon).

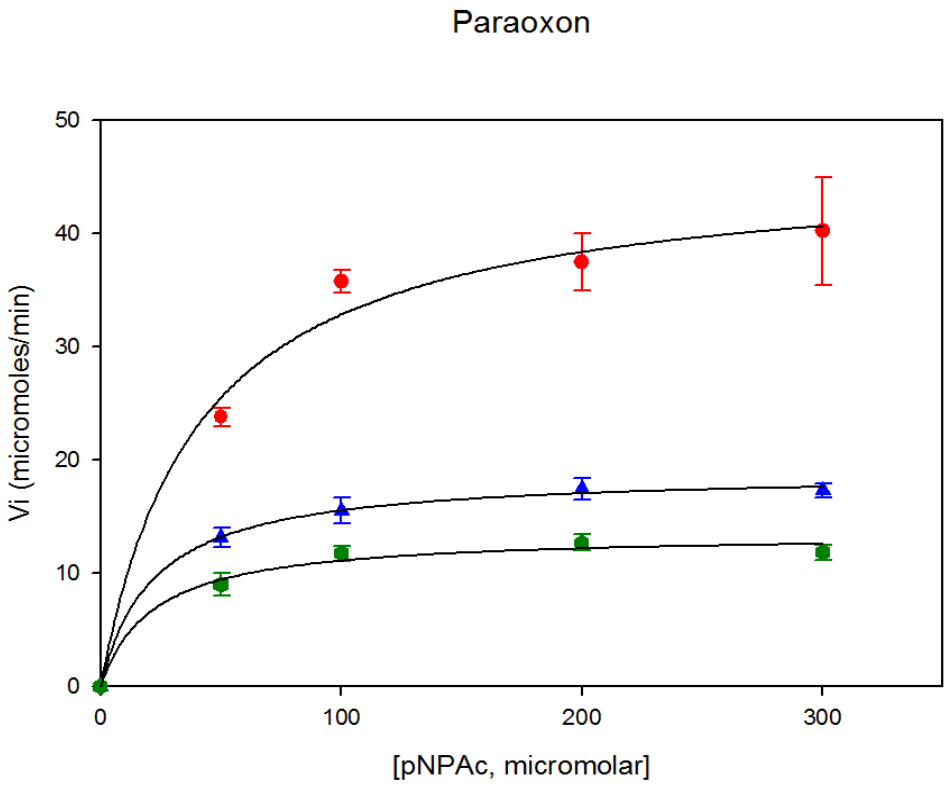


Figure 11: Effect of Paraoxon on SABP2 Catalytic Activity. A plot of initial velocity (*Vi*) versus various pNPAc concentrations obtained with increasing paraoxon concentrations. 0 µM Paraoxon (Red circles), 10 µM paraoxon (blue triangles) and 100 µM paraoxon (green hexagons) n = 3.

Because results suggested a Michaelis–Menten like kinetics, a double reciprocal plot (Figure 12) was plotted using data from Table 3 to determine if the inhibition was of competitive, noncompetitive or uncompetitive type.

Table 3: Reciprocals of Both *Vi* and Various Substrate Concentrations in the Presence of Varying Paraoxon Concentrations.

1/[pNPAc]	1/*Vi-1* 0 µM Pox (µM)	1/*Vi-2* 0 µM Pox (µM)	1/*Vi-1* 10 µM Pox (µM)	1/*Vi-2* 10 µM Pox (µM)	1/*Vi-3* 10 µM Pox (µM)	1/*Vi-1* 100 µM Pox (µM)	1/*Vi-3* 100 µM Pox (µM)
0.020	0.040	0.043	0.086	0.071	0.071	0.100	0.125
0.010	0.027	0.028	0.070	0.068	0.056	0.080	0.090
0.005	0.025	0.028	0.064	0.055	0.054	0.075	0.083
0.003	0.022	0.028	0.058	0.054	0.061	0.080	0.089

A linear regression analysis (Figure 12) on the data from Table 3 was performed using SigmaPlot. The y-intercept and slope of the regression line were calculated (Figure 12). These parameters were used to calculate both Vmaxapp and Kmapp, respectively. Using the equation 1/Vmax = y-intercept, Vmaxapp was calculated. Kmapp values (substrate concentration at half Vmax) were calculated by using the equation slope = Km/Vmax. Both Vmaxapp and Kmapp values were similar to those obtained in Figure 11. In both cases, Vmaxapp and Kmapp decreased with increasing paraoxon concentration (0-100 µM), which is a characteristic of uncompetitive inhibition. Because the reciprocal plots are not parallel to each other, the inhibition cannot be of uncompetitive type. It appears that paraoxon mediated inhibition of SABP2 may be of mixed type.

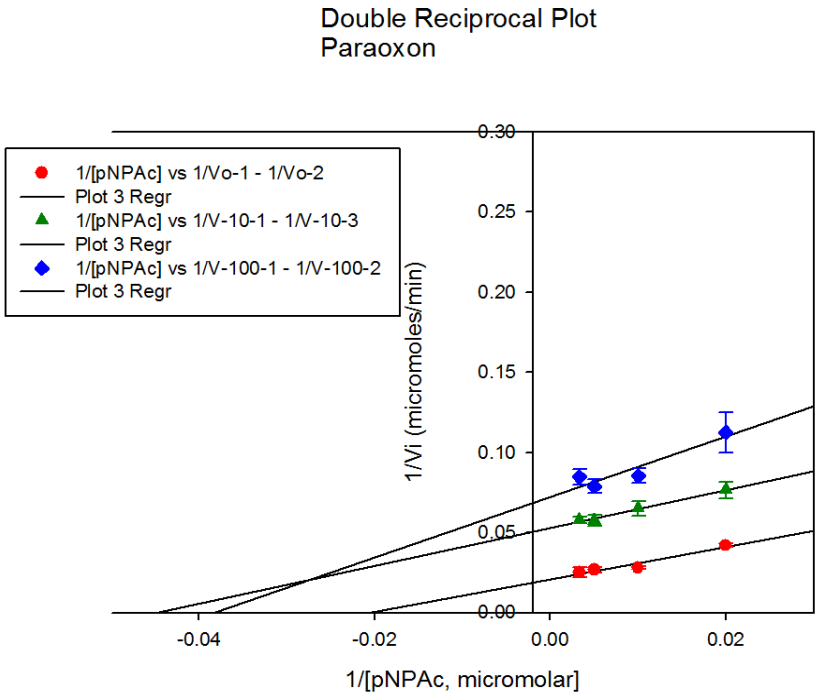


Figure 12: Double Reciprocal Plot Showing the Effect of Paraoxon on the Hydrolysis of pNAc to pNP by SABP2. 0 µM Paraoxon (Red), 10 µM paraoxon (Green) and 100 µM paraoxon (Blue) n=3.

Effect of Malathion on the Enzymatic Activity of SABP2

The amount of SABP2 catalyzed para-nitrophenol formation in the presence of increasing malathion concentration at various substrate concentration was monitored at 30 sec intervals for 10 mins at a wavelength of 405 nm. Initial velocities (*Vi)* were obtained at a 1 min time point (Table 4).

Table 4: Initial Velocities Obtained at Various Substrate Concentrations in the Presence of Varying Malathion Concentrations.

pNAc (µM)	Vi-1 0 µM Mal	Vi-2 0 µM Mal	Vi-1 10 µM Mal	Vi-2 10 µM Mal	Vi-1 100µM Mal	Vi-2 100µM Mal	Vi-3 100µM Mal
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
50	34.70	33.40	18.42	19.23	12.98	14.00	12.90
100	45.00	48.60	27.02	28.73	15.72	17.30	15.60
200	55.80	51.00	32.00	30.58	23.42	23.64	22.00
300	45.50	44.00	35.84	34.01	24.00	25.00	25.50

Results showed that in the presence of increasing malathion concentrations, the reaction rate decreased suggesting that pesticide (malathion) affects the enzymatic activity of SABP2 (Figure 13).

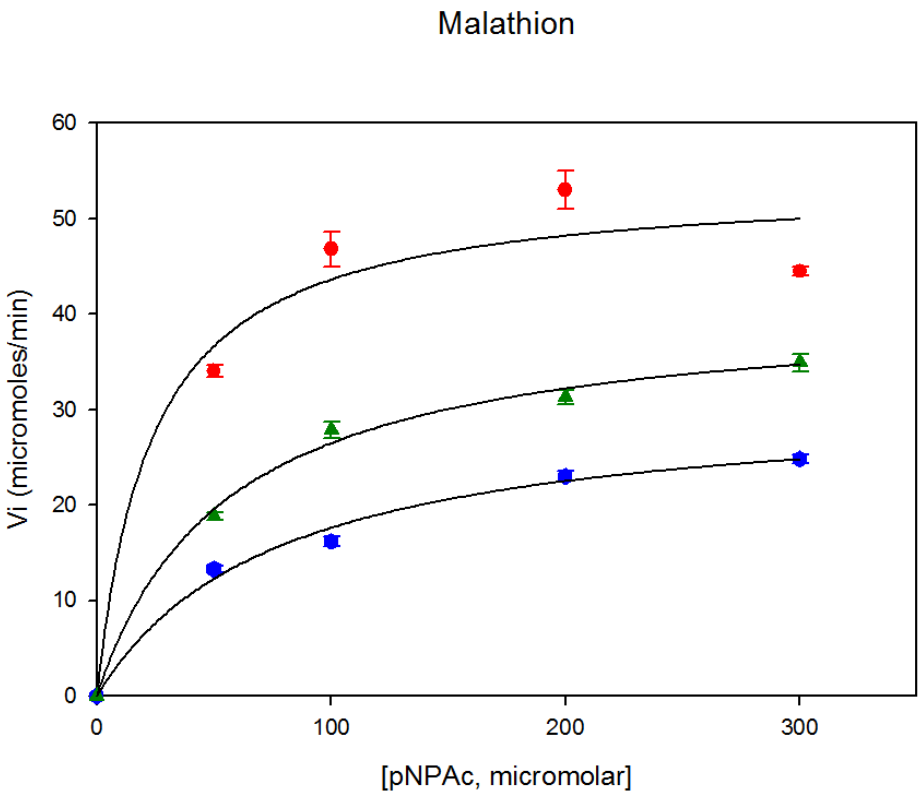


Figure 13: Effect of Increasing Malathion Concentrations on SABP2 Enzymatic Activity. 0 µM Malathion (Red), 10 µM Malathion (Green) and 100 µM malathion (Blue) n=3.

A double reciprocal plot with 1/Vo against 1/ [S] was plotted using data from Table 5.

Table 5: Reciprocals of Both *Vi* and Various Substrate Concentrations in the Presence of Varying Malathion Concentrations.

1/[pNAc]	1/Vi-1 0 uM Mal	1/Vi-2 0 uM Mal	1/Vi-1 10 uM Mal	1/Vi-2 10 uM Mal	1/Vi-1 100uM Mal	1/Vi-2 100uM Mal	1/Vi-3 100uM Mal
0.020	0.029	0.03	0.054	0.052	0.074	0.071	0.077
0.010	0.022	0.021	0.037	0.034	0.063	0.057	0.064
0.005	0.018	0.02	0.031	0.032	0.042	0.042	0.045
0.003	0.022	0.022	0.027	0.029	0.041	0.040	0.039

Vmaxapp, and Kmapp values were calculated as previously described. Vmaxapp decreased from 54 µM/min (Red circles- 0 µM malathion) to 41 µM/min (Blue diamonds-10 µM malathion) and to 31 µM/min (Green triangles-100 µM malathion) (Figure 14). Kmapp values increased from 23 µM (Red- 0 µM malathion) to 54 µM (Blue- 10 µM malathion) and to 76 µM (Green- 100 µM malathion) (Figure 14). . Based on the above data and double reciprocal plot, the type of inhibition of SABP2 demonstrated by malathion is possibly of a mixed type.

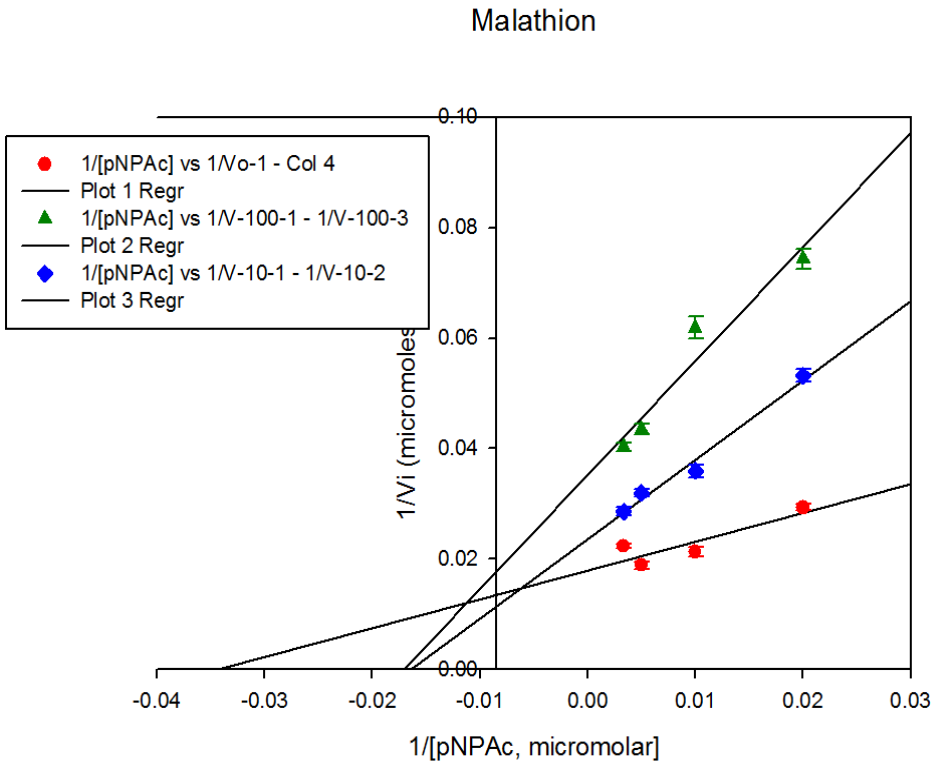


Figure 14: Double Reciprocal Plot Showing the Effect of Malathion on the Hydrolysis of pNPc to pNP by SABP2. 0 µM malathion (Red circles), 10 µM malathion (Blue diamonds) and 100 µM malathion (Green triangles) n=3.

In vitro assay results suggest that both organophosphate pesticides, paraoxon and malathion, inhibit enzymatic activity of SABP2.

Effect of Pesticide (Paraoxon) on a Tobacco Leaf

This experiment was carried out to determine the pesticide concentration that when infiltrated into the tobacco leaf will not kill the plant. Various paraoxon concentrations were infiltrated into the leaf tissue using a syringe. Results showed that paraoxon concentration of 1 mM may cause death of leaf tissue (Figure 15). A paraoxon concentration of 100 µM had no effect on the leaf tissue. Results from prior experiments carried out with 100µM pesticide concentration did not block SAR development on distal leaves. A paraoxon concentration of 450 µM (an acceptable concentration used in the field) was later tested on the leaf by infiltration and did not cause death of leaf tissue (data not shown).

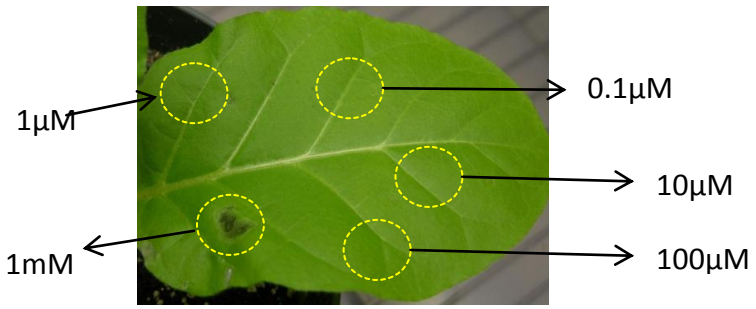


Figure 15: Effect of Increasing Paraoxon Concentrations on a Tobacco Leaf. Various paraoxon concentrations were infiltrated on different portions of the leaf and checked after 48 hrs.

Effect of Paraoxon on SAR Development

SAR was induced by treating the lower 3 leaves of wild type tobacco with TMV, while control (negative) plants received only buffer instead of TMV. At 48 hrs, 2 leaves immediately above the TMV/buffer treated lower leaves were treated with pesticide (paraoxon) or buffer (positive control). Seven days post-primary TMV inoculation, the

pesticide/buffer treated distal leaves of all plants were treated with TMV and lesions were allowed to develop. Sizes of lesion were measured and compared to assess SAR development. In the negative controls (NEG-1 and NEG-2), the lesion sizes were larger (2.87 ± 0.22 mm) because no inducer (TMV inoculation) of SAR was applied on the lower leaves (Figure 16). In comparison, the positive control treatment had an SAR inducer (TMV) on lower leaves; hence smaller lesion sizes (1.6 ± 0.16 mm, 62.5 % reduction compared to negative control) were observed (Figure 16). Robust SAR response was demonstrated by a reduction in lesion sizes. For the paraoxon treated plant, the lesion sizes were comparable to those of the negative control (2.65 ± 0.2mm, only 7.6 % reduction) even though SAR inducer (TMV) was inoculated on lower leaves of these plants (Figure 16). This suggests that paraoxon treatment blocked SAR development. Figure 16 also shows the mean lesion sizes and standard deviations obtained for each treatment and represented on a bar graph (n=45 lesions for each treatment). With the positive control experiment, the mean lesion sizes were smaller (62.5 % reduction) compared to the negative and paraoxon treatments (7.6 % reduction), suggesting SAR development (Figure 16). ANOVA analysis (using SPSS) of TMV induced lesion sizes of negative control and paraoxon treated plants gave p-value of 0.053 showing that difference is not significant. While a p-value of 0.02 was obtained when comparing the paraoxon treated and positive treatment controls showing a significant difference.

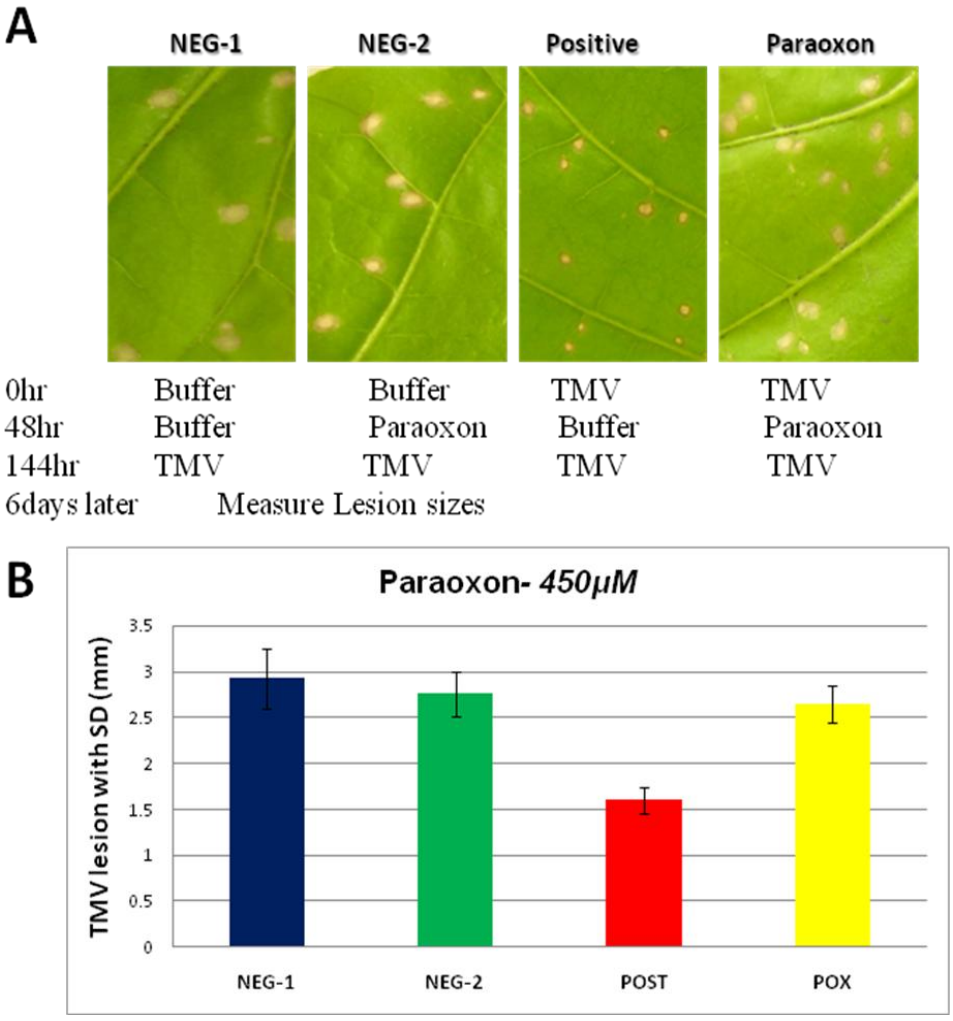


Figure 16: Effect of Paraoxon on SAR Development on TMV-Infected Tobacco Plants. TMV-induced lesion sizes were measured using a digital caliper after 144 hp1°i and pictures taken. Figure also shows the mean lesion sizes ± SD for each treatment represented on a bar graph. Neg (negative control), Post (positive control) and Pox (paraoxon treatment) n=45

Effect of Malathion on SAR Development

SAR was induced by treating the lower 3 leaves of wild type tobacco plant with TMV, while control (negative) plants received only buffer instead of TMV. At 48 hrs, 2 leaves immediately above the TMV/buffer treated lower leaves were treated with malathion or buffer (positive control). Seven days post primary TMV inoculations, the

pesticide/buffer distal treated leaves of all plants were treated with TMV. TMV induced lesions were allowed to develop. Sizes of lesion were measured and compared to assess SAR development. The negative controls (NEG-1 and NEG-2) produced larger lesion sizes (3.26 ± 0.37 mm) because there was no inducer (TMV inoculation) of the SAR response on the lower leaves (Figure 17). In comparison, the positive control treatment had an SAR inducer on lower leaves; hence smaller lesion sizes were observed (1.83± 0.15 mm, 44 % reduction) (Figure 17). SAR response was demonstrated by a reduction in lesion sizes to an average of 1.83 ± 0.15 mm. For the malathion treatment (3.07 ± 0.29 mm, 6 % reduction) the lesion sizes were comparable to those of the negative control even though there was an SAR inducer on lower leaves (Figure 17). This suggests that malathion treatment at 48 hr blocked SAR development. Figure 17 also shows the mean lesion sizes and standard deviations obtained for each treatment and represented on a bar chart (n= 45 lesions for each treatment). Comparing the malathion treatment control with the negative control treatments gave a p-value of 0.336 (not significant) and a p-value of 0.001 (significant) when comparing the malathion treated and positive treatment controls. This suggests a significant difference in the TMV induced lesion sizes on the plants treated with malathion and compared to plants with positive treatment for SAR.

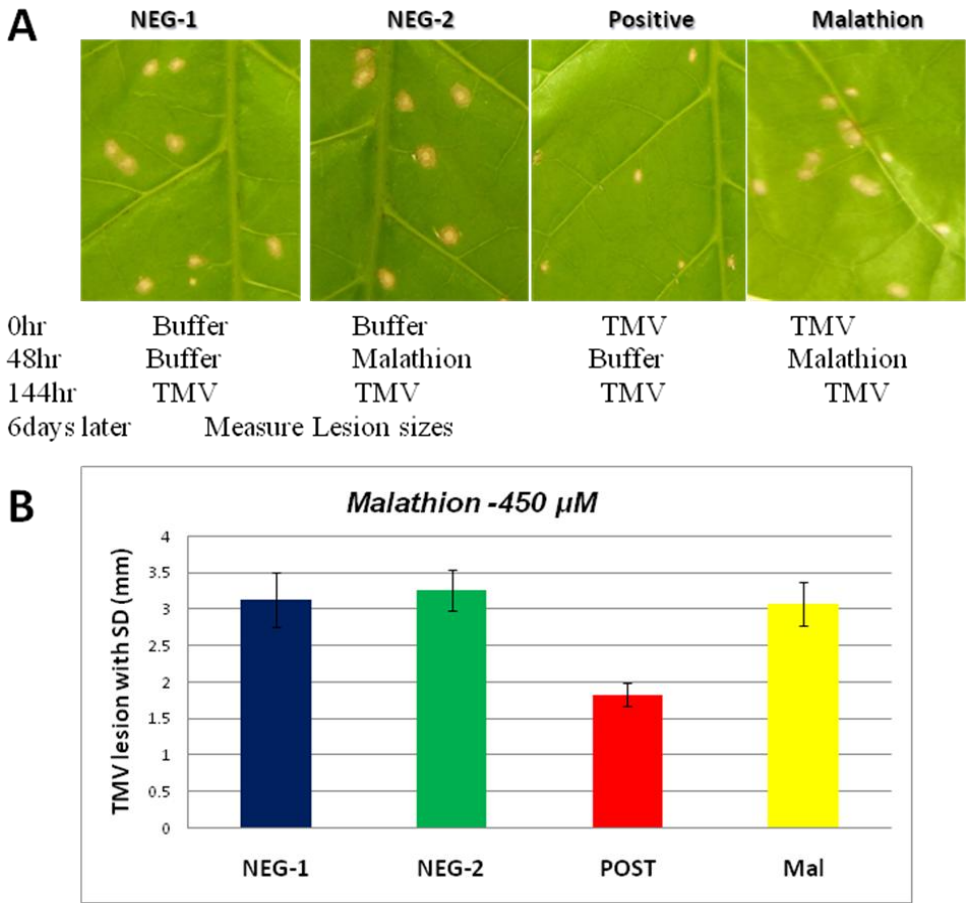


Figure 17: Effect of Malathion on SAR Development in TMV-Infected Tobacco Plants. TMV-induced lesion sizes were measured on distal leaves using a digital caliper after 144 hp1^{o}i and leaves were photographed. Figure also shows the mean lesion sizes ± SD obtained for each treatment represented on a bar graph. Neg (negative control), Post (positive control) and Mal (malathion treatment) n=45.

Effect of Pesticide Treatment on SAR Development in SABP2 Silenced Plants

Transgenic tobacco plants (1-2J) are stably silenced in SABP2 expression (Kumar and Klessig 2003). Experiments with SABP2 silenced plants (1-2J) showed no SAR development in any of the treatments. The mean lesion sizes for the 3 different treatments, Negative (2.01 ± 0.16 mm), Positive (1.92 ± 0.16 mm, 4.5 % reduction), and Paraoxon (1.99 ± 0.16 mm, 1 % reduction) were not significantly different from each

other. In the positive control experiment, where SAR development is expected with *N.t.* Xanthi NN plants, no SAR development (only 4.5 % reduction) was observed in 1-2J plants because they were SABP2 silenced (Figure 18). These results show that the lack of SAR development is due to SABP2 inhibition by paraoxon and not due to some other component.

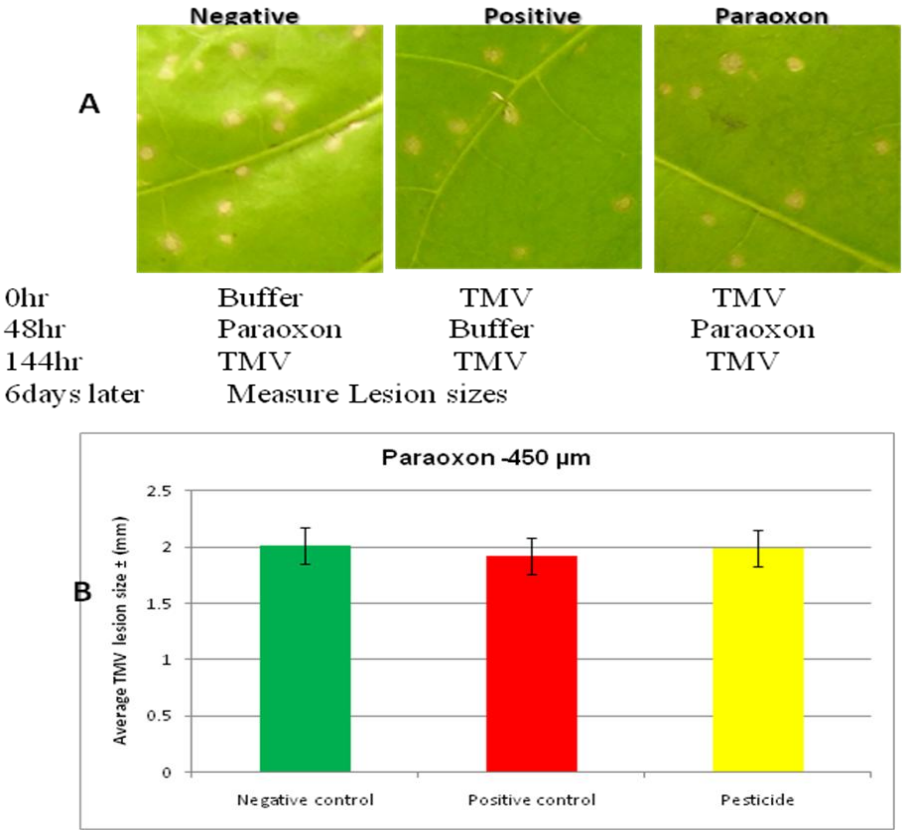


Figure 18: Effect of Paraoxon on SAR Development in SABP2 Silenced Plants. TMV-induced lesion sizes were measured on distal leaves using a digital caliper after 144 hp1°i and pictures taken. Figure also shows the mean lesion sizes ± SD obtained for each treatment represented on a bar graph. n=45.

Effect of Pesticide on PR-1 Protein Production in Tobacco Distal Leaves

Experiments were carried out to determine the effect of pesticide treatment on PR-1 Protein production on distal leaves. Leaf discs were collected at 48 hrs following pesticide treatment on distal leaves and a Western Blot was carried out to detect the

levels of PR-1 Protein production. In all 3 treatments (i.e. positive, negative, and pesticide treatment), PR-1 proteins could not be detected in distal leaves at the 48 hr time point after pesticide/buffer treatments, while PR-1 proteins could easily be detected at the 96 hp1°i and at the 48 hp2°i (48 hrs after secondary TMV inoculation on distal leaves or 192 hp1°i) time points in both the positive and pesticide treatment controls, but not with the negative control treatment (Figure 19). Experiment was repeated with a longer film exposure (about 45 mins) so that any weak signal present could be detected, but no protein expression could be detected at the 48 hr time point (Figure 20). It could be concluded that this system was not sensitive enough to detect PR-1 protein expression under these conditions and therefore effect of pesticide on PR-1 protein expression could not be assessed.

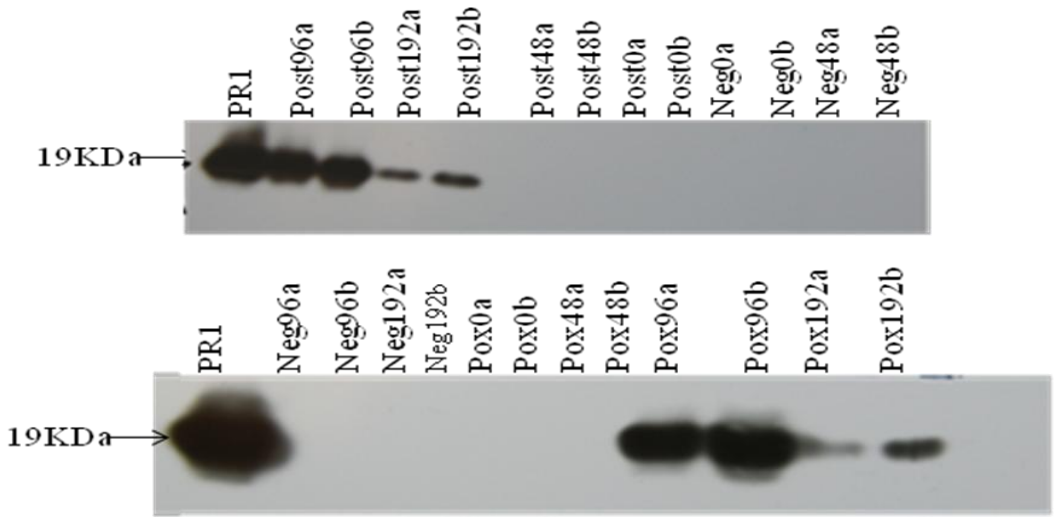


Figure 19: Effect of Pesticide (Paraoxon) Treatment on PR-1 Protein Production. Western Blot showing the expression of PR-1 proteins at different time points. Film was exposed for 25 mins. Post0a-b and Post96a-b (leaf disc was collected at 0 or 96hp1°i respectively after TMV inoculation on lower leaves of the positive control plant), Post48a-b and Post192a-b (leaf disc was collected on distal leaves after 48 hrs of pesticide treatment or collected at 192hp1°i with TMV on distal leaves). Same holds for Neg(negative control) and Pox (pesticide treated). PR-1 (positive control for PR-1 expression).

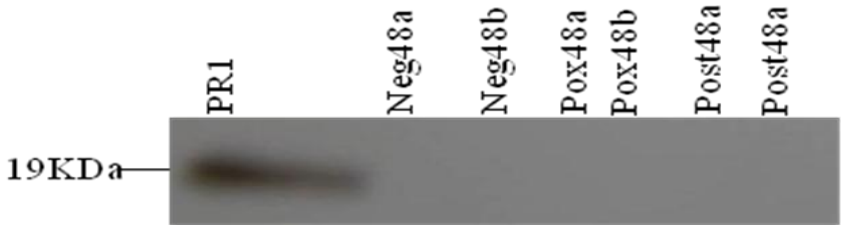


Figure 20: Effect of Pesticide Treatment on PR-1 Protein Production. Western Blot showing the production of PR-1 proteins at various time points. Neg-Pox-Post48a-b (leaf disc was collected on distal leaves 48 hrs after pesticide treatment.

Another approach (i.e. RT-PCR) was used to determine the levels of *PR-1* transcripts but still expression of *PR-1* could not be detected and experiments are being repeated to determine if this was due to some technical error or if PR-1 protein is not being expressed under these conditions.

CHAPTER 4

DISCUSSION

Because both SABP2 (which converts MeSA to SA) and AChE (which converts acetylcholine to choline) belong to the α/β hydrolase super family and have esterase activity, we hypothesized that pesticide inhibitors of AChE (organophosphates, inhibiting the conversion of acetylcholine to choline) may also inhibit the activity of SABP2.Comparing the structural features of AChE to SABP2 as revealed by X-ray crystallographic studies showed that AChE has a catalytic triad made up of Ser-203, His-447, and Glu-334 (Ordentlich et al. 1993), while X-ray crystallographic studies suggested that SABP2 catalytic triad is made up of Ser-81, His-238, and Asp-210 (Forouhar et al. 2005). Both AChE and SABP2 have identical amino acids (Ser and His) and a negatively charged amino acid (Glu for AChE and Asp for SABP2, both having similar properties) in their catalytic triad suggesting a similar mechanism of action in their enzymatic activity. Among the tested α/β hydrolase class of enzymes to which SABP2 catalytically and structurally belongs (Forouhar et al. 2005), organophosphate pesticides (e.g paraoxon and malathion) were able to inhibit the MeSA esterase activity of SABP2 *in vivo*.

Exogenous applications of organophosphate pesticides (paraoxon and malathion) were effective in blocking SAR development in TMV-infected tobacco distal leaves, where the MeSA esterase activity of SABP2 and MeSA are essential for SAR (Park et al. 2009) (Figures 17 and 18). Both TMV resistant wild type (*N.t.* Xanthi NN) tobacco plants and plants lacking SABP2 due to RNAi silencing, (1-2J plants) were used to test hypotheses. In the wild type (*N.t.* Xanthi NN) plants, pesticide treatment compromised

SAR development, while SAR was observed in plants not treated with pesticide i.e. buffer treated plants (Figures 17 and 18). ANOVA analysis of TMV induced lesion sizes showed a significant difference (p-value of 0.001 with malathion and 0.02 with paraoxon) in lesion sizes between the pesticide (malathion and paraoxon) treated and positive control treatments. SAR development is demonstrated by a reduction in TMV-induced lesion sizes. There was no significant reduction of lesion sizes in pesticide treated plants compared to negative control treatments (only 6-7 % reduction) but a marked reduction was observed in the positive control plants (44-62 % reduction) showing that SAR was compromised in pesticide treated plants.

Increased levels of SA cause a feedback inhibition of SABP2 activity and, as a result, the levels of MeSA increases and moves through the phloem to other parts of the plant where it is converted to SA by SABP2 upon pathogen attack (reviewed in Kumar and Klessig 2008). Results suggest that pesticide treatment blocks robust SAR development by inhibiting the MeSA esterase activity of SABP2 and these plants showed susceptibility to TMV (larger lesions). SAR development was observed in the positive control treatment and distal leaves became more resistant to pathogenic treatment (~44 - 62 % reduction in lesion size). SABP2 silenced plants showed no reduction in lesion sizes in any treatment (only 4.5 % reduction). This result shows that pesticides inhibited SABP2 to cause enhanced susceptibility in wild type plants expressing SABP2.

In vitro studies were also carried out to determine the effect of pesticides (malathion and paraoxon) on the activity of SABP2 by monitoring the rate of hydrolysis of the artificial substrate para-nitrophenylacetate to para-nitrophenol spectrophotometrically. As earlier mentioned, there was a significant decrease in the rate

of SABP2 catalysed hydrolysis of para-nirophenylacetate to para-nitrophenol in the presence of both increasing paraoxon and malathion concentrations (Figure 11 and 13). For example, at 100 µM paraoxon, the Vmaxapp decreased from 46 µM/min (0 µM paraoxon) to 13.5 µM/min (100 µM paraoxon). Similarly, at 100 µM malathion, the Vmaxapp decreased from 54 µM/min to 31 µM/min. This suggests that pesticides affect the rate of SABP2 catalyzed reaction. Results from the double reciprocal plot of both malathion and paraoxon did not suggest the inhibition to be of competitive, or noncompetitive, or uncompetitive type. For example, the paraoxon reciprocal plot showed a decrease in both Vmaxapp and Kmapp with increasing paraoxon concentrations, a characteristic of uncompetitive inhibition but because the reciprocal plots (lines) were not parallel to each other, the inhibition cannot be termed uncompetitive. The malathion reciprocal plot showed a decrease in Vmaxapp but an increase in Kmapp. Inhibition by both malathion and paraoxon was neither competitive, uncompetitive nor noncompetitive (reversible type inhibitions) suggesting that it is possibly a mixed type inhibition.

Para-nitrophenyl acetate (an artificial substrate for SABP2) was used because it provides a colorimetric assay. Para-nitrophenol is yellow in color whose absorbance could be monitored over time.

Summary

Based on the results, it could be concluded that both malathion and paraoxon reduce the rate of reaction catalyzed by SABP2. Also, because both pesticides show neither a competitive, uncompetitve, nor a noncompetitive type of inhibition, it could be suggested that inhibition by these pesticides is possibly of a mixed type. Treatments with paraoxon and malathion were able to suppress TMV induced SAR development in tobacco plants. The effect of pesticide on defense protein could not be assessed because Western Blot was not sensitive enough to detect PR-1 Protein expression under these conditions and a more sensitive procedure like RT-PCR analysis may be more suitable.

Directions for Future Research

Other pesticides that are currently being used in agriculture could be used to conduct further studies. Large numbers of pesticides could be screened to determine if they inhibit the enzymatic activity of SABP2. RT-PCR or microarray based analysis could be used to determine the effect of pesticides on defense genes/pathways.

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APPENDICES

APPENDIX A – ABBREVIATIONS

SAR - Systemic acquired resistance

SA - Salicylic acid

MeSA - Methyl salicylic acid

SABP2 - Salicylic acid binding protein 2

PR - Pathogenesis related

NPR-1 - Non- expresser of pathogenesis related 1 protein

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

TMV - Tobacco mosaic virus

βME - βeta mercaptoethanol

SDS PAGE - Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

APPENDIX B- BUFFERS

10X SDS-PAGE Buffer

Tris = 30 g, Glycine = 144 g

SDS = 10 g. Adjust the volume to 1 liter

10 mM Bicine Buffer

Bicine = 163g

Adjust pH to 8.0 with NaOH, and volume to 500ml

Protein Extraction Buffer (Final concentrations)

50 mM Tris base = 1.21 g

150 mM NaCl = 87.75g

10 % Glycerol = 20 ml

1 mM PMSF = 0.034 g

0.1 % Triton-X-100 = 200 µl

Protease inhibitor cocktail tablets = 2

Adjust the pH to 8.0 with HCl and volume to 200 ml with distilled water

βME (1 µl/ml) buffer

100 mM Sodium Phosphate Buffer contained 84 mM Na2HPO4 and 16 mM NaH2PO4 and pH adjusted to 7.5 with HCl

10X Phosphate Buffered Saline (Final Concentrations)

1.3 M NaCl = 76 g

70 mM Na2HPO4 = 10 g

30 mM NaH2PO4 = 4.1 g

Dissolve in 1000 ml distilled water. To make a 1X working solution, take 100 ml 10X PBS and dilute in 1000 ml distilled water**.**

1X Phosphate Buffered Saline + 5 % Tween 20

Pipette 50 ml in 1000 ml 1X PBS

4X SDS- PAGE Separating Gel Buffer (Final concentrations)

1.5 M Tris = 90.85 g

0.04 % SDS = 0.2 g

pH was adjusted to 8.8 and volume to 500 ml

4X SDS- PAGE Stacking Gel Buffer (Final concentrations)

0.5 M Tris = 30.28 g

0.04 % SDS = 0.2 g

pH was adjusted to 6.8 and volume to 500 ml

20 % APS

Ammonium per sulfate = 20 mg

Adjust the volume to 100 µl with distilled water

2X SDS-PAGE Gel Loading Dye (Final concentrations)

100 mM Tris - Cl (pH 6.8) = 10 ml

SDS = 4 g

Glycerol = 20 ml

0.2 % Bromophenol blue crystal ≤ 0.2 g

Volume was adjusted to 100 ml with distilled water and 5 ml per 100 ml added

Add 5 ml βMe / 100 ml dye.

10X Western Blot Transfer Buffer (Final concentration)

125 mM Tris base = 30.3 g

960 mM Glycine = 72.06 g

For 1X solution, 100 ml of 10X, 100 ml of methanol and 800 ml of distilled water was added together to make a 1 liter total volume.

15% SDS-PAGE Gel

Separating (Running) Gel Composition

Distilled Water (1.02 ml), 4X Separating (Running) gel Buffer (pH 8.8) = 1 ml

30 % Acrylamide

(acrylamide: bis-acrylamide, 29:1) = 1.98 ml

20 % APS = 8 µl is added just before pouring the gel, TEMED = 4 µl

The solution was mixed together and loaded on the assembled BioRad mini gel plates. Distilled water was added to the top of the gel solution. The gel will polymerize in about 20 mins.

Stacking Gel (5 %)

Distilled Water = 1.17 ml , 4X Stacking Buffer (pH 6.8) = 0.5 ml , 30% Acrylamide (acrylamide: bis-acrylamide, 29:1) = 0.66 ml. Before pouring the stacking gel, add 20 % APS = 4 µl, TEMED = 2µl was added. Water from the top of the separating gel was discarded and the stacking gel loaded carefully. The well comb was gently placed immediately and gel left to polymerize (20 minutes).

Blocking Buffer (final concentration): 3 % BSA=3g, 1 % Dry Milk = 1g and volume adjusted to 100 ml with 1X PBS buffer

Ponceau Stain

0.1 % Ponceau S = 100 mg

5 % Acetic acid = 5 ml and volume adjusted to 100 ml with distilled water.

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