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Role of SABP2 in Systemic Acquired Resistance Induced by Acibenzolar-S-Methyl in Plants.

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by

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ABSTRACT

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Plants have evolved an efficient mechanism to defend themselves against pathogens. Many biotic and abiotic agents have been shown to induce defense mechanism in plants. Acibenzolar-S-Methyl (ASM) is a commercially available chemical inducer of local and systemic resistance (SAR) response in plants. ASM functioning at molecular level is mostly unclear. This research was designed to investigate the mechanism of ASM action in plants. It was hypothesized that SABP2, a plant protein, plays an important role in ASM-mediated defense signaling. Biochemical studies were performed to test the interaction between SABP2 and ASM. Transgenic SABP2-silenced tobacco plants were used to determine the role of SABP2 in SAR induced by ASM. The expression of PR-1 proteins was used as a marker for SAR induction. Results showed that SABP2 converts ASM into acibenzolar that induces the expression of PR-1 proteins and develops the SAR response in ASM-treated plants.

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CHAPTER 1

INTRODUCTION

All animals including humans depend directly or indirectly on plants for food and nutrition. Plants are also the primary sources of timber, medicinal drugs, fibers, pesticides, fossil fuels, paper, pulp, and biofuels. Some organisms (known as pathogens) cause damage or diseases in plants. The infection caused by plant pathogens could impair the growth and reproduction of the plants that ultimately affect the productivity of food and non-food crop plants. This results in huge losses in crop production. The severity of this problem is even higher in many developing countries where vegetables and cereals are the main sources of traditional diet (Pinstrup-Andersen, 2000). Therefore, it is important to develop novel strategies to manage the diseases caused by pathogens.

To defend themselves, plants have limited resources as they lack mobile defender cells and a somatic adaptive immune system (Dangl & Jones, 2001 and ref. therein). Although various conventional breeding practices and a wide range of chemical pesticides are being used to make plants more capable to combat plant pathogens, there are some limitations. Lately, some of these pesticides have been found to reach our food and cause cancer and other harmful effects (Calaf & Roy, 2007; Pimentel et al., 2007; Zahm & Blair, 1992). Therefore, it is beneficial to explore alternatives to pesticide-based agriculture. One such alternative is to enhance a plant's own natural defense capacity using chemical inducers.

Recent advances in the field of molecular biology offer new opportunities for applied biological sciences that could be useful in exploring this approach to enhance a plant's own defenses. A shift in conventional research by the use of novel tools and resources of genomics and molecular biology has provided an impulse to studies in plant pathology and plant-pathogen interactions. These studies provide insight into the molecular basis of plant diseases and basic biology of the pathogen. This new area of research allows a comprehensive study of gene structure and function that offers the applications for protecting important crops from devastating diseases caused by pathogens.

Plant Defense Mechanism

Plants defense response depends on the innate immunity of each cell and on systemic signals initiating from the local infection sites (Dangl & Jones, 2001 and ref. therein). Plant-pathogen interaction initiates a sequence of early events that starts with the recognition between both partners that ultimately leads to the synthesis and transport of defense molecules to strategic sites (Benhamou, 1996 and ref. therein). The evidences suggest that both resistant and susceptible plants respond in the same manner. However, the development of disease by pathogen is caused by delayed plant response rather than nonexistence of defense mechanism (Dixon, 1994). Many evidences have suggested that most plant pathogens release an array of effector (virulence) molecules to suppress the host defenses machinery. These pathogen-derived molecules, known as pathogen associated molecular pattern (PAMP) or microbe-associated molecular pattern (MAMP), initiate a host immune response. These

molecules may include viral proteins, lipopolysaccharides, bacterial flagellin, yeast mannans, and peptidoglycans. MAMP molecules are recognized by a defined set of receptors known as pathogen or pattern-recognition receptors (PRRs) in host cells. Plants secrete many secondary metabolites such as phytoalexins and more specifically a family of intracellular receptors referred as nucleotide-binding leucine - rich repeat (LRR) domain (NBS-LRR) pathogen resistance proteins (R) that respond to pathogen encoded virulence-related factors (effector proteins) (Iriti & Faoro, 2007 and ref. therein).

In a successful resistance response, R protein from plant interacts with a particular pathogen's effector protein (avirulence (Avr) protein) that ultimately results in a localized resistance reaction known as the hypersensitive response (HR) (Hammond-Kosack & Jones, 1996; Heath, 1981). R- Avr interaction initiates a series of biochemical reactions in an infected host plant cell. Reinforcement of the plant cell wall by the deposition of callose, lignin, and phenolic compounds is one of the early steps in this series of reactions (Benhamou, 1996 and ref. therein). Other events may include secretion of small basic peptides 'defensins', production of reactive oxygen species (ROS) such as H_2O_2, production of oxylipin metabolites (oxidation products of polyunsaturated fatty acids), and programmed cell death (PCD) at and around the site of infection (localized resistance). Accumulation of plants' secondary metabolites such as phytoalexins, salicylic acid, jasmonic acid, and ethylene also occur with the late expression of pathogenesis-related genes (Gozzo, 2003 and ref. therein; Iriti & Faoro, 2007 and ref. therein).

Many times localized infection by pathogens induces resistance in other parts of the plant directed at a number of widely different pathogens classes. In nutshell a multistep defense response in plants starts with the perception of signal by the plant cell is transduced intracellularly leading to the synthesis, accumulation, and transport of various defense molecules to strategic sites (Benhamou, 1996 and ref. therein).

Depending upon the type of elicitors and the pathways involved, two kinds of induced resistance have been described (Kloepper et al.,1992; Vallad & Goodman, 2004 and ref. therein). Induced Systemic Resistance (ISR) is activated by plant growth promoting bacteria (PGPR) and is mediated by jasmonic acid and ethylene (Kloepper et al., 1992; Pieterse et al., 1996). On the other hand, systemic acquired resistance (SAR) results from pretreatment of the plant by pathogens, salicylic acid (SA), or SA-like compounds, and it involves SA mediated defense signaling (Kloepper et al.,1992; van Loon, 1987). These induced resistance responses have been shown to be effective against a broad range of pathogens and parasites including fungi, bacteria, viruses, parasitic plants, nematodes, and even insect herbivores (Metraux, 2002 and ref. therein; Vallad & Goodman, 2004 and ref. therein).

SAR and Its Components

Ross (1961) challenged tobacco plants with tobacco mosaic virus (TMV) and observed an enhanced state of resistance to secondary infection in uninfected, distal parts of the plants that was termed systemic acquired resistance (SAR) (Reviewed in Durrant & Dong, 2004). This study showed that SAR offers a kind of acquired immunity in which a series of translocated signals from the local infection process activate the

defense mechanism in the distal, uninfected parts (Mauch-Mani & Metraux, 1998 and ref. therein). SAR has been described in more than 30 di- and monocotyledonous plant families (Metraux, 2002 and ref. therein; Sticher et al., 1997 and ref. therein). At the molecular level, SAR has been shown to develop with a coordinated expression of a large number of pathogenesis-related (PR) gene families in both local and systemic tissues (van Loon & van Kammen, 1970). This expression of the low molecular weight heterogeneous group of PR proteins is induced in plants by pathogen infection as well as by exogenously applied chemicals. These proteins were first detected in *Nicotiana tabacum* cv. Xanthi nc and *N.t.* cv. Samsun NN by comparing extracts, made at pH 8.0, of healthy and TMV-infected leaves using polyacrylamide gel electrophoresis (PAGE) (Antoniw & White, 1983). Although the precise role of PR proteins is not well defined in plant defense, their coordinated expression with SAR indicates their use as molecular markers of SAR response in plants. These PR-mediated defense responses have been characterized in tobacco, *Arabidopsis,* and cucumber plants (Durrant & Dong, 2004 and ref. therein).

In addition to the expression of a set of defense gene *(PR-1, PR-2, PR-5)* families, SAR also involves the accumulation of SA (Durrant & Dong, 2004 and ref. therein). During SAR, SA level increases locally and systemically in infected host tissues (Ryals et al., 1996; Yalpani et al., 1991). SA activates the expression of two groups of genes. The activation of one group of genes (*PR*) is a part of the late event of SA-mediate pathway, while some other groups of genes are activated early in the pathway (Qin et al., 1994; Uknes et al., 1993). The genes coding for glutathione S-transferases (GSTs) are early SA-activated genes that play a role during the normal

metabolism of plant secondary products and in plant disease resistance by communicating defense signal between the species (Marrs, 1996; Xiang et al., 1996).

In addition, SA strongly stimulates the release of secondary metabolites such as phytoalexins and coumarins (antimicrobial) and alkaloids (chemical defense of plants) (Zhao et al., 2005 and ref. therein). The roles of biochemicals downstream of SA in SAR pathway are not very clear but significant efforts have been made to elucidate the role of a positive regulator of SAR, NPR-1/NIM1 (nonexpresser of PR genes / noninducible immunity) (Durrant & Dong, 2004 and ref. therein). Genetic analyses in *Arabidopsis* plants nonresponsive to SA showed mutants having mutations in NPR-1/NIM1 (Cao et al., 1994; Delaney et al., 1994; Delaney et al., 1995; Shah et al., 1999).

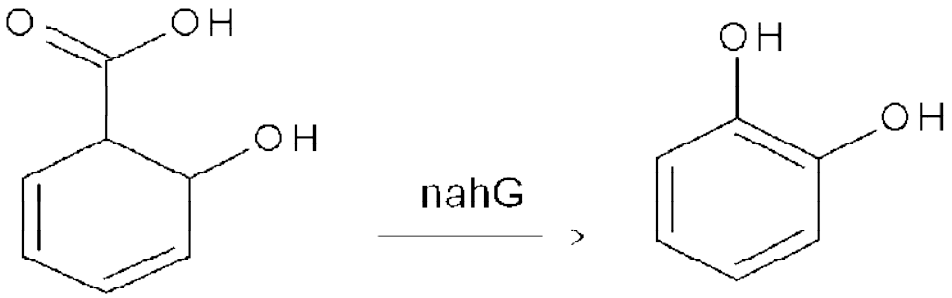
It is now well established that besides biological agents, exogenous application of various chemicals such as SA and its synthetic analogs activate induced defense responses with or without SA accumulation (Walters et al., 2005 and ref. therein)**.** Induction of systemic resistance by pathogens and chemical inducers can either lead to direct defense activation or to the priming of plant cells (Walters et al., 2005 and ref. therein). Priming is an augmented capacity to mobilize cellular responses also referred as 'Primed' (Katz et al., 1998) state of the plant. A pretreatment with low doses of salicylic acid (SA), β-aminobutyric acid (BABA), dichloroisonicotinic acid (INA), or benzothiadiazole (ASM) has been shown to prime the cells to react more quickly and efficiently to subsequent chemical treatment or pathogen attack by inducing either the same or another set of defense genes (Conrath et al., 2002 and ref. therein). The primed cells also protect the plants against abiotic stresses. The dual role of ASM to induce SAR and to prime potentiated expression of defense genes has been suggested

by various studies (Conrath et al., 2002 and ref. therein).

Salicylic Acid and Its Importance in Plant Defense

Salicylic acid was discovered from the extracts of willow (salix) tree bark and has been used as anti-inflammatory drug since the 18th century (Weissmann, 1991; White, 1979). It is a hydroxyl group bearing phenolic compound. Phenolic compounds including SA play important roles in lignin biosynthesis, act as allelopathic compounds, and regulate plant responses to abiotic stimuli and pathogen attacks (Vlot et al., 2009 and ref. therein). Additionally, SA helps in seedling establishment, seed germination, cell growth, respiration, senescence-associated gene expression, stomatal closure, basal-thermo tolerance, nodulation in legumes, thermogenesis, and fruit yield (Vlot et al., 2009 and ref. therein).

In 1897 Bayer Company introduced a drug, aspirin, with antiinflammatory properties of acetylsalicylic acid. Use of salicylates by humans was known for a long time but its effect on plants was first shown in 1979. Treatment of tobacco plants with aspirin enhanced their resistance to subsequent infection by tobacco mosaic virus (TMV) (Antoniw et al., 1980; White, 1979). Later, it was documented that the resistance to TMV was due to accumulation of PR proteins. This observation established a connection between SA and PR proteins (Gaffney et al., 1993; Malamy et al., 1990; Metraux et al., 1990). The importance of SA in SAR signaling was shown by subsequent experiments using transgenic plants over expressing a bacterial salicylate hydroxylase gene (*nahG*) that effectively reduced the level of endogenous SA and made the plant susceptible to diseases (Figure 1) (Delaney et al., 1994).



Salicylic acid Catechol

Figure 1. Transgenic *nahG* Plants Convert Salicylic Acid into Catechol. This conversion results in the susceptibility of plants against pathogens (Delaney et al., 1994).

For a long time SA synthesized through the phenylalanine ammonia lyase (PAL) mediated pathway was thought to be responsible for disease resistance. In 2001 mutation analysis in *Arabidopsis* plants showed that the phenylalanine ammonia lyase (PAL) mediated pathway is responsible for the rapid production of SA associated with local cell death, whereas the isochorismate synthase (ICS) mediated pathway is more important for sustained SA synthesis during development of SAR (Figure 2)(Wildermuth et al., 2001) .

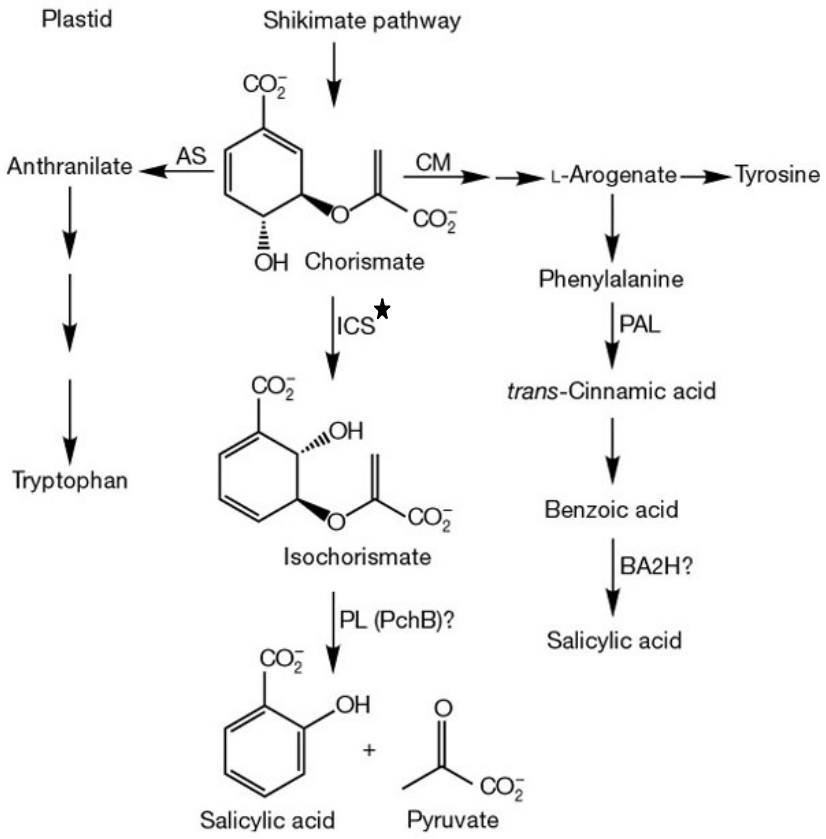


Figure 2. Biosynthesis of Salicylic Acid from Chorismate via Isochorismate Synthase (ICS). Chorismate pathway was shown to be involved during defense response in *Arabidopsis*. *SA mutant defective in the expression of isochorismate synthase (ICS) was found unable to induce local and systemic resistance. (Figure adapted by permission from Macmillan publishers' Ltd: Nature (Wildermuth et al., 2001) copyright.

Almost the entire SA produced in plants is converted into a salicylic acid *O-β*-glucoside (SAG) by a pathogen-inducible SA glucosyltransferase (SAGT). Some other SA derivatives like salicyloyl glucose ester (SGE) and methyl salicylate (MeSA) or its glucosylated derivative methyl salicylate *O-β*-glucoside (MeSAG) also accumulate in lesser amount (Figure 3) (Vlot et al., 2009 and ref. therein).

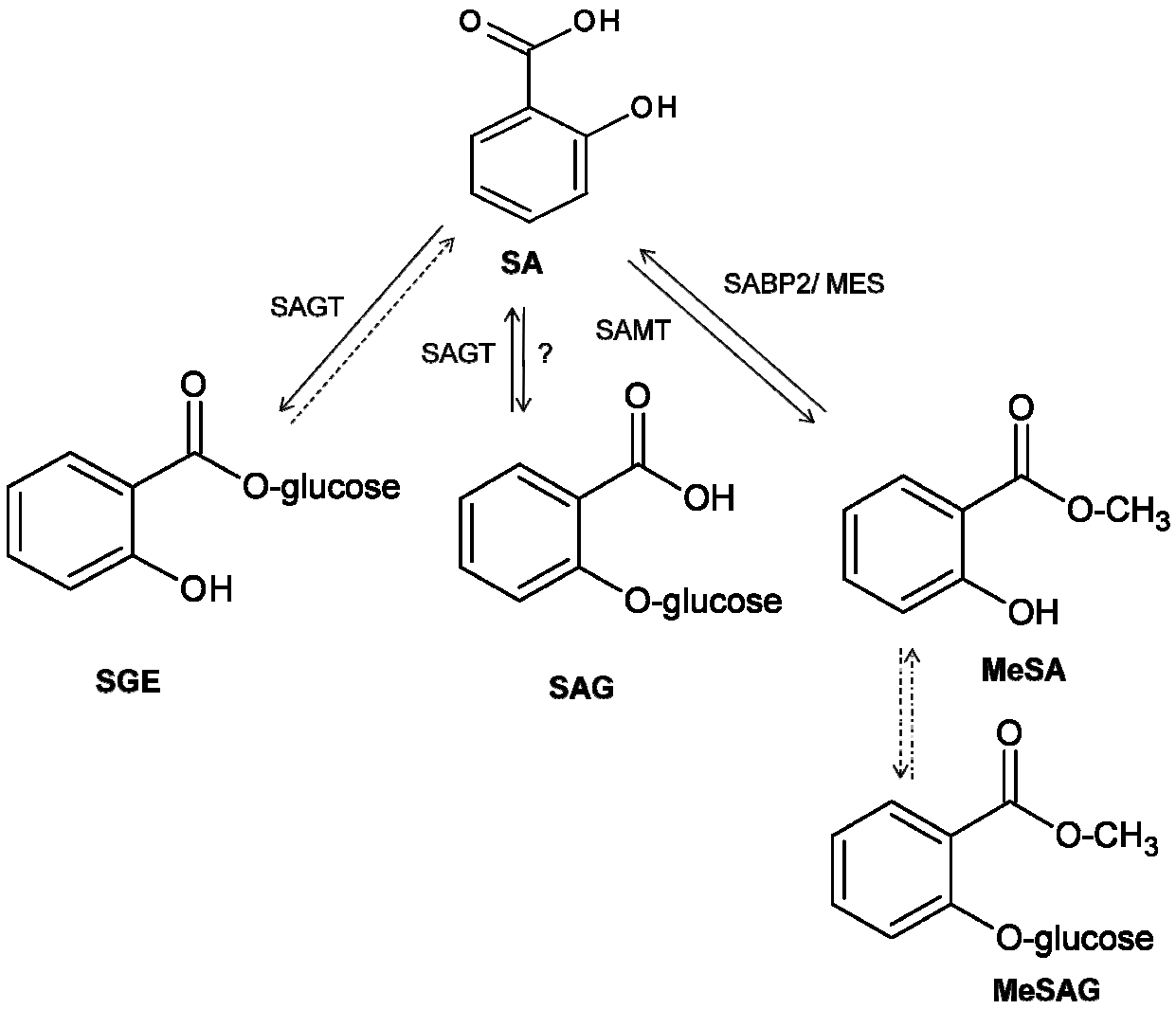


Figure 3. Synthesis of Salicylic Acid and Its Conjugates via ICS and PAL Pathways. These derivatives are SA *O-B*-glucoside (SAG),SA glucosyltransferase (SAGT), salicyloyl glucose ester (SGE), methyl salicylate (MeSA) or its glucosylated derivative MeSA *O-B*-glucoside (MeSAG).(Vlot et al., 2009).

SAR Signaling Pathway

Although diverse routes of SAR signaling have been described to date, there is ample evidence to show the phloem as the pathway of alarm signal travel (Bel & Gaupels, 2004 and ref. therein). Early grafting experiments in cucurbits showed that systemic signal for SAR is generated in the noninfected scion grafted onto an infected

stock (Jenns & Kuc, 1977). Later, girdling experiments with cucurbits in which the induction of SAR was affected by blocking of phloem indicated that SAR signal was phloem mobile (Guedes et al., 1980). Shulaev and his coworkers (1995) suggested the SAR pathway in phloem by showing the systemic transport of radioactively labeled SA from inoculated tissues to systemic tissues. However, studies using [^{14}C] labeled photoassimilate distribution in *Arabidopsis* plants showed that the pattern of phloem translocation of [^{14}C] Suc did not correspond exactly with the induction of SAR, suggesting that the SAR signal might not be translocated exclusively through phloem (Kiefer & Slusarenko, 2003).

A better understanding of the SAR signaling pathway also assisted in the search for the systemic signal of SAR. Earlier labeling studies in TMV-infected tobacco and cucumber plants showed that SA is mobile during SAR and most of the SA accumulates systemically in upper noninfected leaves of infected plants (Molders et al., 1996; Shulaev et al., 1995). In addition, many studies have shown the presence of high level of SA in phloem sap in local and systemic tissues of infected plants, suggesting that SA is a mobile signal of SAR (Malamy et al., 1990; Metraux et al., 1990; Uknes et al., 1992; Yalpani et al., 1991). Further studies of SAR signaling revealed that SA is not a primary signal of SAR (Bel & Gaupels, 2004 and ref. therein). This was supported by grafting experiments between *nahG* (transgenic plants, unable to accumulate SA) and wild type tobacco plants and leaf excision experiment in cucumber in which higher induction of SAR was observed after removing the inoculated leaf (Rasmussen et al., 1991; Vernooij et al., 1994). Later, Shulaev and coworkers (1997) suggested that signaling might occur through the conversion of SA to its volatile derivative methyl salicylate (MeSA) that

could induce resistance in the uninfected parts of the same plant as well as in neighboring plants. Later, it was found that MeSA is normally absent in plants and only induced upon pathogen attacks (Huang et al., 2003; Seskar et al., 1998). Exogenous application of MeSA was shown to induce the expression of PR-1 proteins in tobacco plants (Seskar et al., 1998). Studies using biochemical and genomics approaches in *Arabidopsis* plants identified a gene *AtBSMT1* that encodes a protein both with benzoic acid (BA) and salicylic acid (SA) carboxyl methyltransferase activities and showed that MeSA is synthesized by SA carboxyl methyltransferase (SAMT) activity of *AtBSMT* that converts SA into inactive MeSA in infected tissues (Chen et al., 2003). MeSA is converted back to SA in systemic tissues to induce resistance. This conversion has been shown to be catalyzed by a methyl esterase with high affinity for SA, salicylic acid-binding protein 2 (SABP2) in tobacco plants (Forouhar et al., 2005). Further studies on SABP2 demonstrated that it is a very low abundance protein that belongs to the α/β hydrolase superfamily and possesses the esterase and lipase activities. SABP2-silenced tobacco plants showed compromised local and systemic resistance to tobacco mosaic virus (TMV) and reduced expression of PR-1 protein (Kumar & Klessig, 2003). Later, grafted tobacco plants silenced in SABP2 expression in scions but not rootstocks showed attenuated SAR confirming that MeSA is a phloem mobile SAR signal, and it requires SABP2's esterase activity in the systemic tissues that converts biologically inactive MeSA to active SA (Park et al., 2007). These studies implicate MeSA as a mobile or volatile inducer of SAR. However, recent studies in *Arabidopsis* have shown the requirement of lipid signals such as JA-derived molecules for SAR (Nandi et al., 2004; Truman et al., 2007) and of a putative lipid transfer protein in challenged tissue to

initiate a mobile signal (Maldonado et al., 2002).

In pursuit of understanding downstream signaling components involved in SA - mediated signaling, mutant screening was performed leading to identification and characterization of NPR-1. Later studies confirmed its role in SAR as well as ISR (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1999). NPR-1 is normally present at low levels in plants. Its level increases two to three times following pathogen infection or treatment with SAR chemical inducers. Mutation studies suggested that NPR-1 expression is likely mediated by WRKY transcription factors. In addition, functional studies confirmed that *PR* gene induction results by due to migration of NPR-1 in nucleus after treatment with SAR inducers (Durrant & Dong, 2004 and ref. therein). Further studies in this direction have demonstrated that the monomer is the active form of NPR-1 for induction of PR-1 expression and suggested that SA accumulation triggers conversion of NPR-1 oligomer to monomer through changes in cellular redox status favoring reduction. This monomeric form of NPR-1 is then able to migrate to the nucleus where it interacts with TGA factors to induce *PR* gene expression (Durrant & Dong, 2004 and ref. therein; Mou et al., 2003 and ref. therein).

Chemical Inducers of SAR

SAR induced by biotic and abiotic agents involves multistep process that makes it intricate for pathogens to develop resistance. Besides pathogens, many chemicals including naturally occurring metabolites, inorganic compounds, and synthetic chemicals are known to induce resistance in plants that provides protection against future pathogen attacks. To qualify as a resistance inducer a chemical 1) should not

exhibit direct antimicrobial activity, 2) it should not be converted into antimicrobial compound, and 3) it should alter the plant-pathogen interaction from compatible to incompatible (including the expression of defense genes) (Reviewed in Sticher et al., 1997). Based on these criteria various chemical elicitors have been examined for their role in induction of defense response. This includes classes of carbohydrate polymers, lipids, and glycoproteins that are either secreted by micro-organisms or derived from the cell walls of fungi, bacteria, or plants such as elicitors derived from yeast cell walls (Walters et al., 2005 and ref. therein).

The role of fungal and bacterial components in plant defense was reviewed and it was reported that the oligomers of chitosan and polyunsaturated fatty acids provide protection against fungal pathogens (da Rocha & Hammerschmidt, 2005 and ref. therein). These compounds induce resistance that is not associated with the enhanced SA levels or SAR gene expression. In addition, the exogenous application of various plant components such as laminarin (β 1-3 glucan), brassinosteroides, gamma resorcylic acid, arachidonic acid, oxalic acid, jasmonates, and salicylic acid have been shown to induce resistance against various classes of pathogens in a wide range of crops (da Rocha & Hammerschmidt, 2005 and ref. therein; Daire & Mauch-Mani, 2007 and ref. therein).

Phosphate salts are known to induce resistance in cucumber, lettuce (*Lactuca sativa*), and pepper (*Capsicum annuum*). Phytogard (a crop protectans) containing 58% potassium phosphate has been shown to induce resistance against downy mildew in cauliflower plants (Bécot et al., 2000; da Rocha & Hammerschmidt, 2005 and ref. therein). A nonprotein amino acid, β- amino butyric acid (BABA) was shown as an

effective curative in many crops against fungal and bacterial pathogens (Cohen, 2002). Foliar application of this chemical protected tomato and potato foliage against *P. infestans,* protected broccoli against *Alternaria,* and protected lettuce against *Bremia lactucae* (da Rocha & Hammerschmidt, 2005 and ef. therein).

Silverman and his colleagues (2005) studied the structure and activity profiles of various mono and multisubstituted salicylates and related compounds using an induction of PR-1 protein as a marker for the induction of resistance. Among the 47 selected monosubstituted and multisubstituted salicylate derivatives, the eight derivatives that were fluorinated or chlorinated in the third and fifth position induced more PR-1 protein accumulation compared to SA with no substitutions.

Among the synthetic chemical inducers probenazole that contained oryzemate as active ingredient was found to be effective against rice blast disease, but it showed negative effects on other plants in field (Nakashita et al., 2002b). Some choroisonicotinamide derivatives have been shown to induce SAR in dicot and monocot plants. N-cynomethyl-2-chloro isonicotinamide (NCI) was characterized and reported to induce a broad range of disease resistance in tobacco and rice plants (Nakashita et al., 2002a).

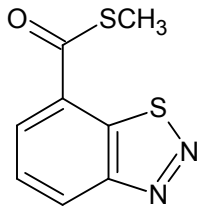
A recently identified chemical, 3-acetonyl-3-hydroxyoxindole (AHO) isolated from extracts of *Strobilanthes cusia,* shows induction of resistance. Tobacco plants treated with AHO accumulate higher levels of SA, express PR-1 proteins, and exhibit resistance towards TMV and *Erysiphae cichoracearum* (Li et al., 2008). Another study to identify inducers of LURP (Late/sustained Up-regulation in response to *Hyaloperonospora parasitica*) genes by screening a collection of 42,000 diversity-oriented molecules

resulted in identification of 114 candidate molecules. One of these 114 chemicals, 3,5-dichloroanthranilic acid (DCA) induced defenses against *H. parasitica and Pseudomonas syringae.* DCA activated defense in a transient manner in contrast to the long-lasting activation by ASM and INA (Knoth et al., 2009). Recently, synthetic cationic lipopeptides were shown to induce systemic defense responses in plants (Brotman et al., 2009) and hexanoic acid was shown to induce resistance against *Botrytis cinerea* in tomato plants (Vicedo et al., 2009).

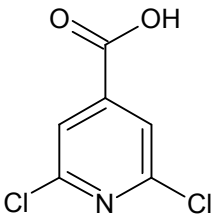
Functional Analogs of SA

Among all of the synthetic functional analogs of SA, two of the best known inducers were discovered in the 1990s. They mimic the pathogen induced SAR and are 2,6-dichloroisonicotinic acid (INA) and the benzo [1,2,3] thiadiazole-7-carbothiate acibenzolar-S-methyl (ASM) (Figure 4) (Friedrich et al., 1996; Lawton et al., 1996; Sticher et al., 1997 and ref. therein). SAR deficient *nim1* and *NahG* mutant plants developed SAR when treated with INA and ASM, which showed that these chemicals are the functional analogs of SA in SAR signaling (Kessmann et al., 1994; Lawton et al., 1996). These compounds were discovered before the role of SA in the biological induction of SAR was discovered (Sticher et al., 1997 and ref. therein). INA was among the first synthetic analog that produced similar SAR response as produced by pathogens. Later, INA associated phytotoxicity was reported in certain crops, which limited its use as commercial product for agricultural (Lyon & Newton, 1997). Although INA can induce the same resistance spectrum and the same biochemical changes as induced by pathogens in cucumbers and tobacco but due to insufficient crop tolerance

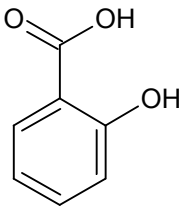
none of the INA derivatives were commercialized (Friedrich et al., 1996; Kessmann et al., 1994).



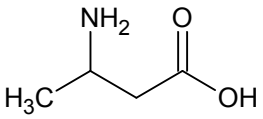
acibenzolar-S-methyl (ASM)



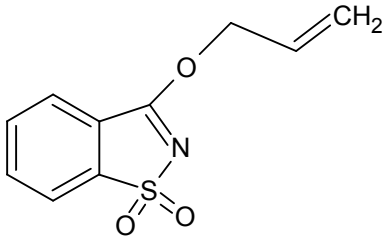
2, 6-dichlorosonicotinic acid (INA)



salicylic acid (SA)



β- amino butyric acid (BABA)



Probenazole

Figure 4. Chemical Structures of SAR Activators. ASM, INA, SA, BABA, and Probenazole have been shown to induce SAR in plants. (Gozzo et al., 2003).

Acibenzolar- S-Methyl and Its Action Spectrum

ASM belongs to the benzothiadiazole class of plant activators. It was discovered by special screening procedures to identify chemicals that activate defense response in plants. It was further tested biologically and chemically along with its other derivatives in a random screening process. Based on the screening results of the biological properties (no antimicrobial activity and induction of defense related PR proteins in plants) and overall field performance, ASM was chosen as the preferred chemical agent of disease control (Kunz et al., 1997). Figure 5 shows the synthesis of various benzo (1, 2, 3) thiadiazole derivatives.

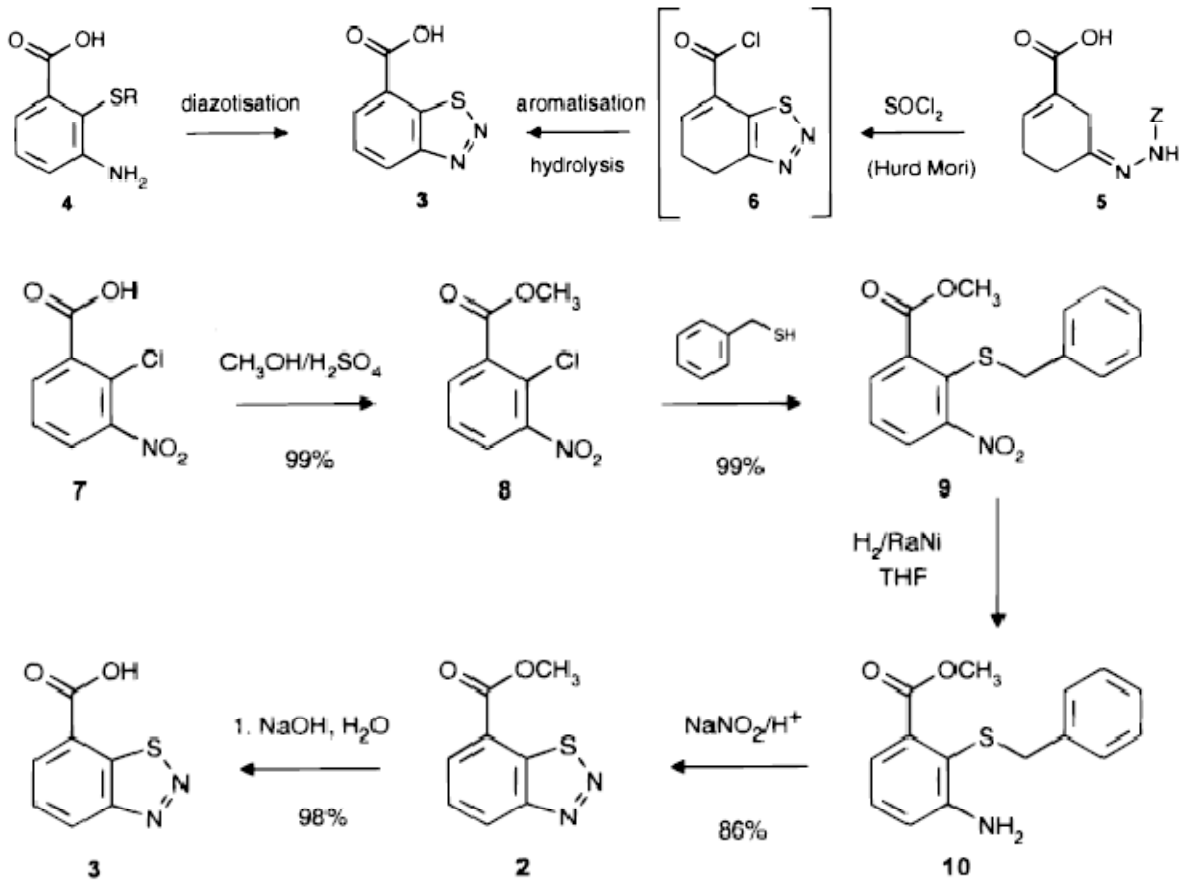


Figure 5. Synthesis of Various Benzo (1, 2, 3) Thiadiazole Derivatives. Compound 2 is Methyl benzo (1, 2, 3) thiadiazole-7-carboxylate and compound 3 is the desired carboxylic acid. (Figure adapted by permission from John Wiley and Sons: Pest Management Science (Kunz et al., 1999) Copyright.

Among chemical inducers ASM (EU patent # 0313-512, US patent # 4-931-581) (Kunz et al., 1997) is the most studied and first synthetic chemical developed and marketed as a SAR activator in Europe as BION and as ACTIGARD in the United States (Walters et al., 2005 and ref. therein). It was termed as a plant activator and a synthetic elicitor (Lyon & Newton, 1997). ASM was initially marketed for the control of powdery mildew on wheat and barley in Europe (Gorlach et al., 1996). Later, two different studies in 1999 showed that it reduced the mildew infection on wheat in field by between 64% and 77% (Walters et al., 2005 and ref. therein).

Cole (1999) tested the efficacy of ASM against wildfire and angular leaf spot diseases in tobacco. ASM provided 99% control of *Pseudomonas syringae* pv *tabaci,* 91% control of *Cercospora nicotiana,* and 89% control of *Alternaria alternate* in field studies (Cole, 1999; Perez et al., 2003). Later, Vallad and Goodman (2004) reviewed the field performance of ASM on 32 crops. ASM efficacy was found between 4-80% against a wide spectrum of diseases and a few studies showed even more than 80% diseases control (Walters et al., 2005 and ref. therein).

There are many other reports that have shown induction of SAR by ASM treatment. It was documented that ASM induces SAR against *Phytophthora palmivora* in papaya (Zhu et al., 2003), and it controls downy mildew in cauliflower seedlings (Godard et al., 1999). ASM induces resistance in tobacco against *O. neolycopersci*, but it was not effective in tomato (Achuo et al., 2004). ASM increased the activity of β-1, 3 glucanase (defense enzyme) against early blight (*Alternaria solani*) and powdery mildew (*Erysiphe cichoracearum*) in potato plants (Bokshi et al., 2003). A study on *Brassica napus* has shown that ASM induces SAR against fungal and bacterial pathogens (Hammerschmidt & Becker, 1997 and ref. therein). Moreover, it was documented that ASM reduces the lesions caused by tomato spotted wilt virus (TSWV) in tobacco plants (Mandal et al., 2008). Oostendorp et al. (2001) reveiwed the efficacy of ASM in monocots and dicots suggesting that monocots such as rice and bananas and dicots such as tobacco, tomatoes, some vegetables, and fruit crops are effectively protected by ASM. Histological observation suggested that like what is seen in dicots, multiple mechanisms operate in monocot such as wheat to stop powdery mildew infections and restrict the pathogen's ability to develop resistance in plants. ASM has a wide spectrum

of activity against fungal, bacterial, and viral pathogens in important crop plants (Table 1) (Oostendorp et al., 2001 and ref. therein).

Table 1.

ASM Activity in Important Crop Plants Against Various Classes of Pathogens

Crop	Bacteria	Viruses	Fungi	Nematodes	Insects
Cereals			+		
Rice	+		+		
Potato		+			+
Tobacco	+	+	+		
Tomato	+	+	+		+
Vegetables	+	+	+		+
Mango	+		+		
Citrus	+	+	+		
Grapes			+	+	
Banana			+	+	
Stone fruits	+				
Pome fruits	+		+		

Note. "+" represents effective resistance against the pathogens. (Oostendorp et al., 2001).

ASM induces resistance against many destructive diseases, especially blue mold, as well as against bacterial and virus diseases in tobacco (Figure 6).

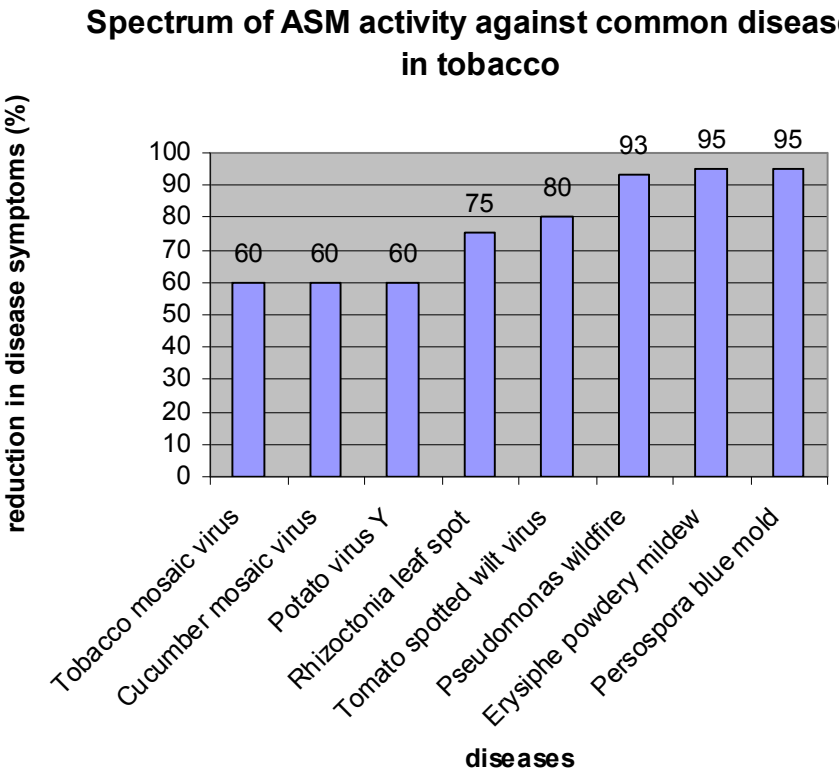


Figure 6. Broad Spectrum of ASM Activity in Tobacco Plants. ASM reduces symptoms of devastating diseases from 60% to 95% (Oostendorp et al., 2001).

The ASM induced response is crop specific such as ASM activates resistance against late blight *(Phytophthora infestans)* in tomato plants, while no reliable activation in potato was observed following ASM application (Oostendorp et al., 2001 and ref. therein). In some cases ASM could provide very high level of disease control, while in some plants the control is very low or absent (Miles et al., 2004). A field trial with barley cultivars showed that ASM did not induce resistance against barley yellow dwarf virus (Huth & Balke, 2002), *Phytophthora brassicae* in *Arabidopsis*, or *P. infestans* in potato (Si-Ammour et al., 2003). ASM induced resistance only against powdery mildew with side effects on growth against *Septoria* and leaf rust in wheat (Martinelli et al., 1993).

These studies showed that careful selection is required while using these chemicals to avoid negative effects on plant growth. Chemically induced resistance depends on several factors such as genotype, environment, as well as cultivar (Walters et al., 2005 and ref. therein). Studies on barley, winter wheat, and bell pepper suggested that ASM-induced resistance is specific to pathogen race (Romero et al., 2001; Walters et al., 2005 and ref. therein). Oostendorp and his colleagues documented that generally in monocots the resistance induced by ASM appears to be much longer lasting than that induced in dicots. The basis for this interesting difference is not known (Oostendorp et al., 2001 and ref. therein). Recently Romero and Ritchie (2004) suggested that chemical agents could be a durable source of genotype specific resistance induced by major *R* genes.

Significance of This Research

ASM is commercially available and marketed as a crop protection agent to farmers (Walters et al., 2005 and ref. therein). It can precondition the plants to fight pathogen infection without inducing them with pathogens for induced resistance. Ongoing research on disease resistance induced by commercially available chemical activators has provided a better alternative to conventional approaches for plant defense (Reviewed in Oostendorp et al., 2001). A better understanding of various defense-signaling pathways induced by the biotic and abiotic agents will be helpful in designing novel strategies for plant defense.

An induced resistance not only provides an enhanced capacity of resistance response but also involves low fitness cost to plants (less reduction in growth and seed

set) (Oostendorp et al., 2001 and ref. therein; Walters et al., 2005 and ref. therein). An improved knowledge of various pathways induced by chemicals will provide an efficient approach in protecting the field crops. A better understanding of chemically induced resistance and effects of these chemicals on the biomolecules in plants will help in solving complex interaction between plants and chemical activators.

Against viral and bacterial diseases where genetic approaches are not very useful, chemical activation provides an option to protect the plants. Additionally, in case of fungal pathogens that adapt resistance to fungicides very quickly, treatment of plants with fungicides and chemical activators such as ASM reduces the chances of developing resistance against fungicides (Oostendorp et al., 2001 and ref. therein). A complete and better understanding of molecular mechanisms of induced resistance and other associated issues-costs, sustainability, and different factors affecting defense responses is required for the effective resistance induced by the chemicals. Moreover, different modes of action of various chemicals suggest that they cannot be applied in the same way as fungicides. This presents a challenge to conventional marketing and agronomic practices. In addition, most inducers do not have curative properties, and they must be applied prior to infection. Therefore, there is a need to apply different combinations of these chemicals with fungicides to provide better resistance. A better understanding of the interactions occurring between plants, pathogens, and inducers will be helpful in finding new approaches of disease control.

Previous Studies on ASM-Induced Resistance

The mechanism of ASM action to induce resistance is largely unknown. ASM has been shown to be an effective inducer of SAR and *PR* gene expression. It induces a systemic resistance against a broad range of pathogen classes in a wide range of crops by inducing the same set of PR genes as induced by SA or pathogens. In addition ASM acts independently of plant hormones such as SA, jasmonic acid, and ethylene, which in high concentration could be toxic to plants (Friedrich et al., 1996; Lawton et al., 1996; Ward et al., 1991). Besides inducing expression of *SAR* genes, ASM also leads the accumulation of the secondary metabolites such as resveratrol and anthocyanins that are involved in plant defense mechanism (Iriti et al., 2004). It was demonstrated that ASM inhibits the activities of catalase and ascorbate peroxidase. By inhibiting the activities of these enzymes, ASM changes the H_2O_2 levels or the cellular redox status that might be involved in the activation of certain defense responses, mediated by ASM. In addition it induces the expression of defense related genes such as the acidic *(PR-1, PR-2, and PR-3)* with greater potency (Wendehenne et al., 1998). To determine the functional role of ASM (BTH), a BTH binding protein kinase (BBPK) was purified from tobacco (Pillonel, 2001). The substrate selectivity of this isolated enzyme suggested BBPK mediated regulation of NPR-1/ NIM1 downstream of SA. The effect of different SAR inducers on the inhibition of BBPK protein was measured. ASM inhibited BBPK activity to a lower extent suggesting that BBPK is not a substrate of ASM. ASM exhibited a direct, concentration-dependent inhibition of the NADH: Ubiquinone oxidoreductase activity of complex I of the mitochondrial electron transport chain in tobacco cells. The complex I activity was less sensitive to inhibition by SA compared to

ASM. SA, ASM, and the complex I inhibitor rotenone were shown to increase the production of reactive oxygen species in a concentration-dependent manner in a cell. The results indicated that both ASM and SA affect the mitochondria of treated plant cells and result in increased production of reactive oxygen species. It might be due to the inhibition of the NADH: Ubiquinone oxidoreductase activity of complex I that results in channeling of electrons via complex II, with concomitant higher levels of superoxide production (van der Merwe & Dubery, 2006).

SABP2 was shown to bind with ASM as determined by a competitive binding assay (Du & Klessig, 1997). The binding specificity of SABP2 with SA and its synthetic analogs (including ASM) was found to be 10-200 folds higher than the inactive analogs. ASM, which is much more prominent inducer of SAR genes, competed 15 folds better than SA for binding with SABP2. HPLC analysis was performed to detect and quantify the initial amount, translocation, and degradation of ASM and its acid derivative (acibenzolar) in the plants treated with ASM and acibenzolar. ASM was translocated from the primary treatment site (lower leaves) to systemic tissues and was degraded in the plant tissues after 72 hours from primary treatment. ASM treatment decreased the bacterial growth after 7 days of inoculation challenge that suggested that resistance was developed after degradation of ASM due to the activation of the plant‘s own defense mechanism (Scarponi et al., 2001). In search of a SA/ASM receptor, a SA-binding protein (SABP2) that converts nonfunctional methyl salicylate into functional salicylic acid in plants was purified and characterized by Kumar and colleagues (Forouhar et al., 2005; Kumar & Klessig, 2003). These studies suggested that SABP2 is a resistance signaling receptor of SA. Further biochemical studies confirmed that the esterase

activity of SABP2 was required for the conversion of nonfunctional methyl salicylic acid into functional salicylic acid and it is critical for the induction of the signal transduction pathway and SAR downstream of SA in plants. Recent studies suggested that SABP2 catalyzes the conversion of ASM ester into its acid form, acibenzolar (Enyong, 2008).

Purpose of This Study

The purpose of this study is to determine the role of SABP2 in Systemic Acquired Resistance induced by benzo [1, 2, 3] thiadiazole-7-carbothiate acibenzolar-S-methyl (BTH / ASM). Figure 7 shows a proposed pathway of SAR induced by ASM.

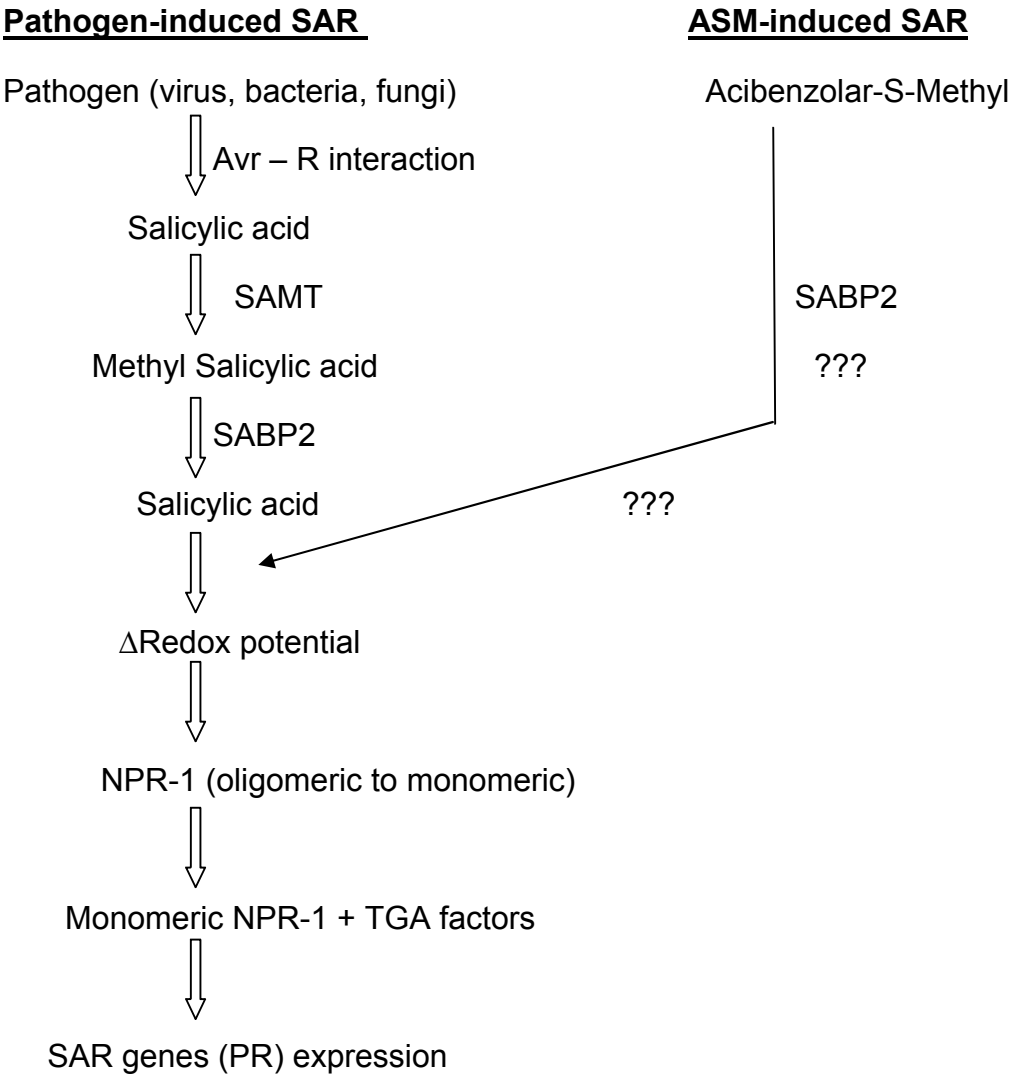


Figure 7. Proposed Pathway of ASM-Induced SAR.

Hypotheses

This thesis research was designed to analyze the defense pathway induced by ASM. Prior *in vitro* studies indicated that SABP2 converts ASM (ester) into acibenzolar, its acid form. This suggested that SABP2 might play a role in systemic resistance induced by ASM.

To test the relationship between SABP2 and ASM and further implications of this interaction in plants, the following hypotheses were developed and experiments were designed to test them.

1. SABP2 catalyzes the removal of the methyl group from ASM (ester) (Figure 8) and the resulting acid form is responsible for the induction of defense genes.

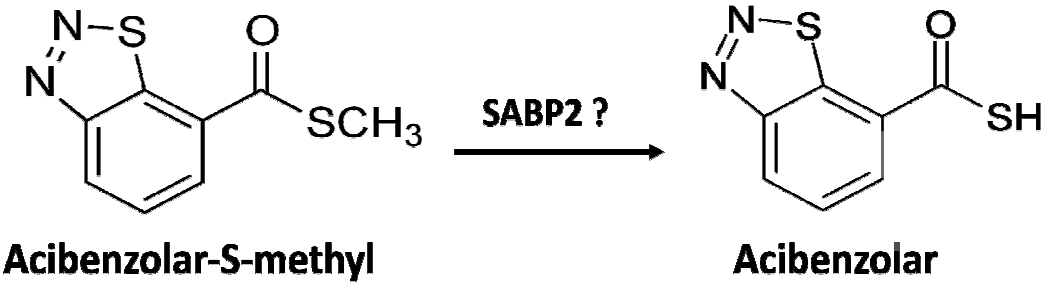


Figure 8. Requirement of SABP2 for the Conversion of ASM into Acibenzolar.

1. SABP2 catalyzes conversion of ASM ester into its acid form, acibenzolar that is required for induction of systemic acquired resistance (SAR).

As the expression of defense genes coordinates with induction of SAR, *PR-1* defense genes were used as a molecular marker of SAR response in tobacco model system. Transgenic SABP2- silenced plants were used to determine the role of SABP2 in SAR induced by ASM. To monitor the development of SAR, TMV-induced lesion sizes were measured and compared in control and SABP2-silenced plants.

CHAPTER 2

MATERIALS AND METHODS

Plant Materials

Two transgenic lines of tobacco plants (*Nicotiana tabacum* cv. Xanthi nc (NN) – control (C3) containing empty silencing vector (pHANNIBAL) and SABP2-silenced (1-2) lines (transgenic *N.t.* cv Xanthi nc in which SABP2 gene expression is silenced by RNA interference) (Kumar and Klessig., 2003) were used for this study. Soil containing peat moss (Fafard Canadian growing mix F-15, Agawam, MA) was autoclaved for 20 minutes prior to growing the plants. Seedlings were transferred into 4 x 4 inch flats after 14 days that were further transferred into pots after 30 days. The experiments were performed with 6- to 8-week old plants. All stages of plants were grown in a controlled growth chamber (PGW 36, Conviron, Canada) set at 16-h day cycle maintained at 22°C.

Chemicals and Reagents

Pure ASM was purchased from Chem Service (West Chester, PA). Analytical grade ASM was kindly provided by Syngenta Crop Protection (Greensboro, NC). β-mercaptoethanol (βMe), tetramethylethylenediamine (TEMED, electrophoresis grade), coommassie brilliant blue, ammonium persulfate (APS), ponceau-S, bovine serum albumin (BSA), TRIS base, ethylenediaminetetraacetic acid (EDTA), phenylmethanesulfonylfluoride (PMSF), sodium phosphate monobasic, sodium phosphate dibasic, tween 20, glycerol, methanol, acetonitrile, carborundum, trifluoroacetic acid (TFA), sodium chloride, protease peptone # 3, agar, sucrose, magnesium chloride, and all other standard chemicals were purchased from Fisher

Scientific (Pittsburgh, PA). Mini Protean 3 cell assembly for SDS-PAGE gel electrophoresis, 30% acrylamide, 10X SDS loading buffer , SDS dye, prestained low molecular weight marker, Bradford's reagent, and the Mini Trans Blot system for Western blotting were purchased from Bio-Rad (Hercules, CA). Polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Billerica, MA). PR-1, SABP2 polyclonal antibodies, anti-mouse, and anti-rabbit antibodies with HRP conjugate were either available in-house or were purchased from Sigma Aldrich (St. Louis, MO). Electrochemiluminescence (ECL) system for developing Western blots was purchased from GE Healthcare.

Synthesized Chemicals

SABP2 was expressed and purified from *E.coli* as described by Kumar and Klessig (2003). TMV was purified as described by Guo et al. (2000).

Buffers

Protein extraction buffer (pH 8.0), phosphate buffered saline (PBS) (pH 7.0), 20 mM sodium phosphate buffer (pH 7.2), SDS PAGE resolving buffer (pH 8.8), and stacking buffer (pH 6.8), Western transfer buffer, and blocking buffer were prepared as described in Appendix B.

Culture Media

King's B media was used to grow *Pseudomonas syringae.* It was prepared as described in Appendix B.

Other Materials

One ml syringes (BD syringes, NJ), sprayers (Sprayco, MI), cheesecloth, digital caliper, pestle grinder (Fisher Scientific), 0.2 µm filter unit (Nalgene, NJ), fast Prep 24 (MP Bio), spectrophotometer, eppendorf centrifuge (Fisher Scientific), high speed centrifuge (Sorvall RT6000 refrigerated centrifuge (DuPont) and HPLC (C-18 column, 250 x 4.6 mm, Microsorb MV- 100-5, Varian) were used to carry out this research.

Methods

HPLC Analysis of Chemical Conversion of ASM by SABP2

HPLC was used to examine the enzymatic activity of SABP2 on ASM. The enzymatic reaction was analyzed as described by Scarponi et al. (2001) with minor modifications. Briefly, a C-18 column was equilibrated with 80% methanol containing 0.3% TFA. Flow rate was set at 0.7 ml/min and peaks were monitored at 255 nm. Pure ASM (1mM) and pure acibenzolar (1mM) were diluted in 20mM sodium phosphate buffer (pH 7.2) (6 µl ASM / acibenzolar + 14 µl buffer) and incubated at 25°C for 30 minutes. ASM (0.4 mM) was diluted in 20mM sodium phosphate buffer (pH 7.2) and incubated with purified SABP2 (6 µl ASM + 10 µl buffer + 4 µl SABP2) for 10, 20, and 40 minutes at 25°C. After incubation 20 µl of each reaction mixture was injected into the column and the flow rate was maintained at 0.7 ml/min. Retention times and peak heights of pure acid, pure ester (controls), and the product of SABP2 with ASM reactions were measured and compared.

Analysis of SABP2 Requirement in ASM-Induced Expression of PR-1 Protein

ASM Treatment of Plants

Three lower leaves of control(C3) and SABP2-silenced (1-2) plants were spray-treated with 0.1mM ASM (available as 50% active ingredients in wettable powder formulation) dissolved in 20 mM sodium phosphate buffer (pH 7.2). For control treatment, plants were spray treated with only the buffer. All types of plants were treated in the same manner. Treated plants were kept at 22°C on light controlled bench stations. The same ASM treatment method was used for most of the experiments. Plants were treated with lower concentrations of ASM for priming experiments (< 5 µM).

Testing Expression of PR-1 Protein

After 48 hours of ASM or buffer treatment as described earlier, samples (two leaf discs with cork borer # 7) were collected from the systemic leaves and homogenized in 0.1 ml protein extraction buffer (Appendix B) using Fast Prep 24 and centrifuged at 4°C at 15,871 x g for 10 minutes. The protein content of the supernatant was determined using Bradford reagent (following manufacturer's instructions). To the 50 µl of supernatant 50 µl of 2X SDS loading dye containing β -Mercaptoethanol was added and mixed. Each protein sample and prestained low molecular weight marker (Bio-Rad) were incubated in boiling water bath for 5 minutes and centrifuged at 21,130 x g for 10 minutes at room temperature. Supernatant equal to 20 µg protein was loaded onto 15% SDS- PAGE gel. Gel electrophoresis was performed at 20 mA for 1 hour. All the buffers and gels were prepared as described in Appendix B.

Transfer of the proteins from the gel to the membrane was carried at 4°C. Transfer membrane (PVDF) was prepared by first treating it with 100% methanol for 10-15 seconds, followed by washing with distilled water twice for 1 minute each, and stored in 1X transfer buffer (Appendix B) containing 10% methanol for 10 minutes. Whatman filter papers (3mm) and sponges were soaked in transfer buffer (Appendix B) for 10-20 minutes. The SDS-PAGE gel equilibrated in transfer buffer was placed onto the equal size PVDF membrane. The gel and membrane were sandwiched between 3 mm Whatman filter paper and sponge and clamped tightly together after ensuring no air bubbles have trapped between the gel and membrane. The sandwich was placed along with the cooling module and 1X transfer buffer (containing 10% methanol) was added. Transfer was carried for 1 hour at 100V. After 1 hour the PVDF membrane was stained with ponceau -S stain (Appendix B) for 1 minute and destained with distilled water 2-3 times and photographed to verify equal loading of proteins that was done by assessing the intensity of large subunit (LSU) of ribulose bisphosphate carboxylase or oxygenase. The blot was washed with 1 X PBS buffer (3 times for 1 minute each) and blocked with the blocking buffer (Appendix B). The blot was probed with PR-1 antibodies (1:1000) in 5 ml blocking buffer for overnight at 4°C. After which the blot was sequentially washed with 10 ml of 1X PBS (2 times for 5 minutes each), 10 ml of 1X PBS-T (2 times for 5 minutes each), and finally, with 10 ml of 1X PBS (2 times for 5 minutes each). After washing, the blot was probed with Goat Anti-Mouse HRP-conjugated secondary antibody (1:5000) for 30 minutes at 25°C. Washing was performed again as described earlier with three additional rinses using PBS to remove excess PBS-T. The blot was visualized using ECL system (GE Healthcare) as per manufacturer's instructions.

Assessment of the Level of ASM-Induced SAR

Pathogen Inoculation

For viral (tobacco mosaic virus) inoculation three lower leaves of C3 and 1-2 plants were treated with 0.1mM ASM. Seven days after ASM treatment the inoculation with tobacco mosaic virus (TMV) was carried out as described in Guo et al. (2000). Briefly, carborundum (a chemical abrasive) was evenly dusted on the surface of three leaves, and TMV at a concentration of 2 µg/ml in 20 mM sodium phosphate buffer (pH 7.2) was rubbed onto the carborundum dusted leaves of using prewashed cheese cloth soaked in diluted TMV solution. As a control carborundum dusted leaves were treated with buffer only. Plants were kept at 22ºC on light controlled bench stations for 7 days.

For bacterial (*Pseudomonas syringae*) Inoculation ASM treatment of C3 and 1-2 plants was performed as described earlier. Seven days following ASM treatment, the upper untreated leaves were inoculated either with *P. syringae* pv *tabaci* (10^4 cfu/ml) or with *P. syringae* pv *tomato* (10^5 cfu/ml). For inoculation a single colony of *P. syringae* pv *tabaci* (*Pst*) and *P. syringae* pv *tomato* (*Pstm*) was grown in King's B medium (Appendix B) at 28°C with shaking for 1-2 days. The culture was centrifuged (Sorvall RT 6000) for 10 minutes at 1,877 x g. The bacterial pellet was resuspended in 10 ml filtered sterile 10 mM $MgCl_2$ (2 times) and finally suspended in 20 ml of 10 mM $MgCl_2$. Optical density (OD) of bacteria was measured at 600 nm using spectrophotometer. Each bacterial culture was diluted in 10 mM $MgCl_2$ to obtain a final concentration of 10^4 cfu/ml or 10^5 cfu/ml (calculated as 0.2 OD_{600} = 10^8 cfu/ml). This diluted bacterium was injected into leaves using a 1 ml needleless syringe. One leaf disc from each leaf was collected 2 or 7 days after secondary inoculation and used to determine the growth of bacteria.

For this one leaf disc was ground in1 ml of 0.1 M sucrose solution (filter sterile). Dilution series from 10^{-1} to 10^{-5} were made so that colonies could be counted easily and 20 µl was spotted on a King's B media (Appendix B) containing plate. The plates were incubated at 28° C for 1-2 days. The number of colonies was counted in each dilution. Bacterial count in buffer-and ASM-treated plants were compared in control (C3) and SABP2-silenced (1-2) plants.

Assessment of SAR Induced by ASM

For the assessment of SAR level against TMV, the diameter of 15 TMV-induced lesions on systemic (upper) leaves were measured after 5-7 days post-TMV inoculation using a digital caliper. The average diameter of 15 lesions was plotted for different treatments.

For the assessment of SAR level against bacteria, the final bacterial count was performed by the following method-

Total number of colonies present in 1 ml solution = colonies present in 20 µl solution X 50 X dilution factor. Bacterial colonies were counted at various times after secondary inoculation.

Analysis of the Induction of SABP2 by ASM

For analysis of ASM induced expression of SABP2, C3 plants were treated with 0.1mM ASM, and samples were collected 24 and 48 hours after ASM treatment as described earlier. Samples were ground and protein content was determined as described earlier and were loaded (20 µg) on the 15 % SDS-Polyacrylamide gel and

Western blotting was performed as descried earlier using SABP2 primary antibodies and Goat Anti-Rabbit HRP-conjugated secondary antibody.

Analysis of Acibenzolar-Induced Expression of PR-1 Protein

Three lower leaves of C3 and 1-2 plants were spray treated with 0.1 mM acibenzolar. Leaf samples were collected after 48 hours of treatment from the same leaves. Samples were processed for Western analysis of PR-1protein expression as described earlier.

Analysis of Defense Signal in SAR Induced by ASM

Making of Chimera Plants

Chimera plants were generated and used for analysis of defense signal movement. For generating chimera plants grafting was performed using C3 and 1-2 chimera plants as either rootstock or scion. Chimeras were made as follows: scions from 5- to 6-week-old C3 plants were grafted onto rootstocks from 6-week old 1-2 plants and vice versa (denotes as C3/1-2 and 1-2/C3 respectively). Scions were cut below the fourth or fifth leaf from the apex, the rootstocks were cut above the fourth leaf from the root, and cut parts were soaked in water. The axillary buds on rootstocks were removed using a razor blade. The stem of scions was then cut into a V-shape and inserted into a slit made on the cut stem of rootstocks. The graft junction was stabilized with parafilm. The whole plant was covered with a transparent plastic bag for a week to avoid moisture loss and kept in a light (16-hour), and temperature (22°C) controlled growth chamber for experiments. Figure 9 shows a representation of making chimeric grafts.

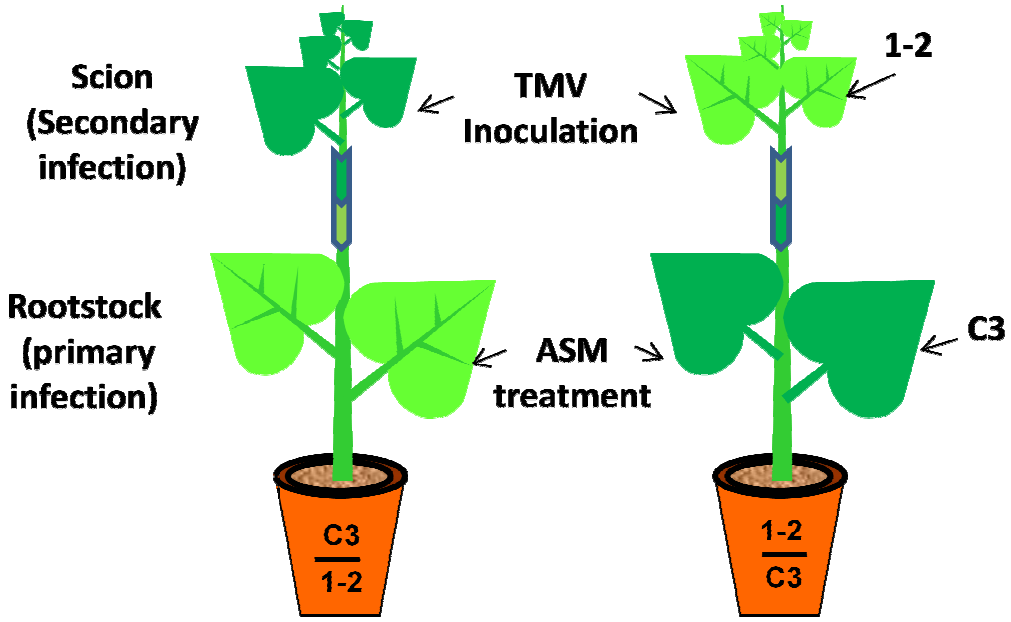


Figure 9. A Representation of Making Chimera Plants. Chimera plants were used for the determination of signal in SAR induced by ASM. Control (C3) scions were grafted on SABP2-silenced (1-2) rootstocks and vice-versa.

Analysis of Expression of PR-1 Protein in Chimera Plants

The rootstock leaves of 1-week-old chimera plants were treated with 0.1 mM ASM (as described earlier). After 48 hours scion leaf samples were collected and processed for PR-1 expression using Western analysis as described earlier.

Assessment of SAR in Chimera Plants

Three rootstock leaves of chimera plants were treated with 0.1mM ASM. Seven days later the scion leaves of ASM treated chimera plants were inoculated with TMV at a concentration of 2 µg/ml in 20 mM sodium phosphate buffer (pH 7.2) as described earlier. Plants were kept at 22°C on a light controlled bench stations for 7 days. Seven

days after TMV inoculation, diameters of the lesions were measured on the systemic (scion) leaves as described earlier.

Molecular Analysis of ASM-Induced Priming

Three lower leaves of both C3 and 1-2 plants were treated with various low concentrations (< 5 mM) of ASM to induce priming. Two days later the upper leaves were challenged with TMV (2 µg/ml in 20 mM sodium phosphate buffer, pH 7.2) as described earlier. Control treatment was performed with buffer only. Leaf discs were collected from the upper leaves after 0, 24, and 48 hours of TMV inoculation and processed for PR-1 analysis as described earlier to test the induction of defense genes during ASM-induced priming. After 7 days lesion sizes on the systemic leaves were measured to assess the level of SAR as described earlier.

CHAPTER 3

RESULTS

Enzymatic Conversion of ASM to Acibenzolar

The enzymatic conversion of ASM to acibenzolar by SABP2 was monitored using HPLC. Firstly, the optimal reaction conditions (buffer, pH, and temperature) were determined for the SABP2 activity. Finally, the reactions were performed at room temperature (25°C) with sodium phosphate buffer (pH 7.2). All the reactions were set up as described earlier. Retention times of pure ASM and acibenzolar in HPLC column were determined. Acibenzolar eluted at 5.2 minutes after injection (Figure 10).

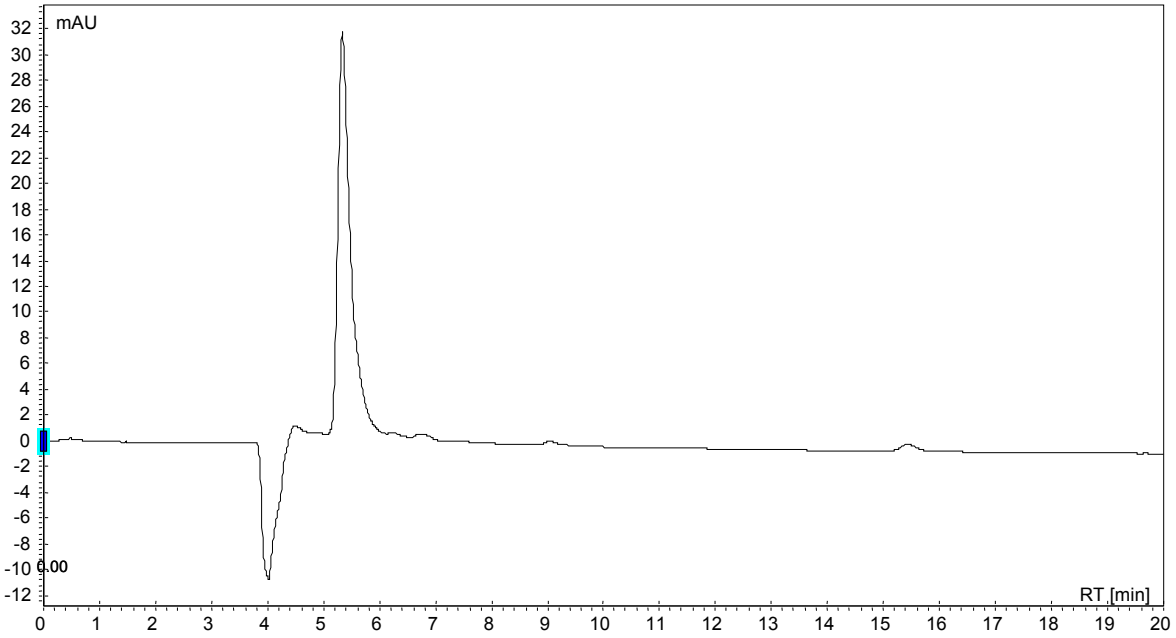


Figure 10. HPLC Histogram Showing the Retention Time of Acibenzolar in C-18 Column. Pure acibenzolar (Final concentration = 0.4 mM, 6 µl) was prepared in 20 mM sodium phosphate buffer, pH 7.2 (14 µl) and total 20 µl volume was injected.

Figure 11 shows the retention time of ASM. It was retained in C-18 column for longer time compared to acibenzolar. ASM eluted at 9.0 minutes after injection.

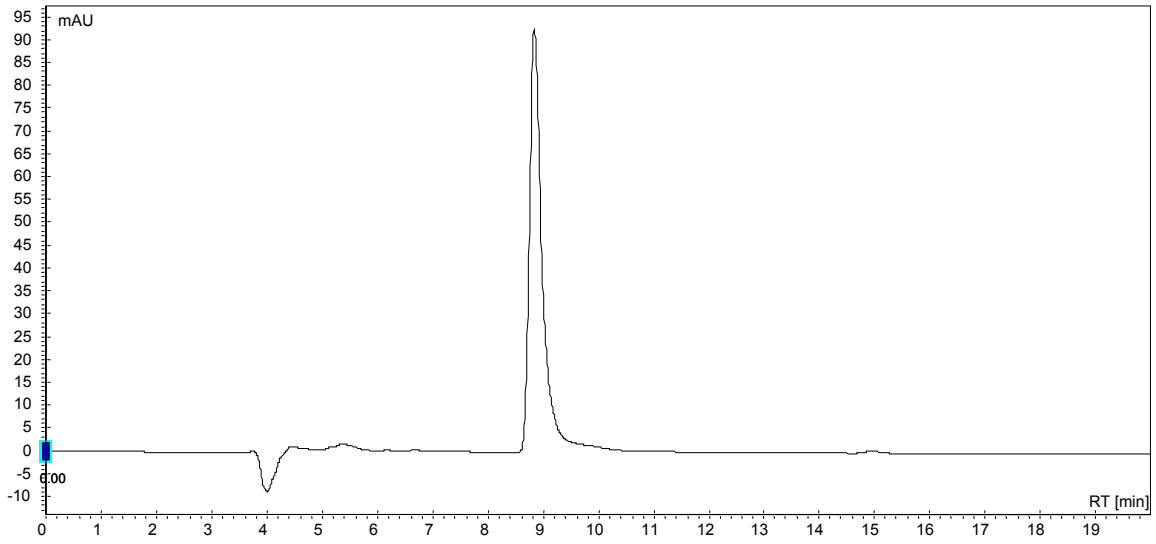


Figure 11. HPLC Histogram Showing the Retention Time of ASM in C-18 Column. Pure ASM (Final conc. = 0.4 mM, 6 µl) was prepared in 20 mM sodium phosphate buffer, pH 7.2 (14 µl) and total 20 µl volume was injected.

ASM and acibenzolar peaks were monitored in the reaction catalyzed by SABP2. ASM (0.4 mM) was diluted in 20 mM sodium phosphate buffer (pH 7.2) and incubated with purified SABP2 (5 µM). Total volume of 20 µl (6 µl ASM + 10 µl buffer + 4µl SABP2) was injected into the column after 10, 20, and 40 minutes of the reaction.

Figure 12 shows the histogram of the change in the peak heights of ASM and acibenzolar in HPLC column. Peak height of ASM decreased while that of acibenzolar increased on longer incubation of SABP2 with ASM.

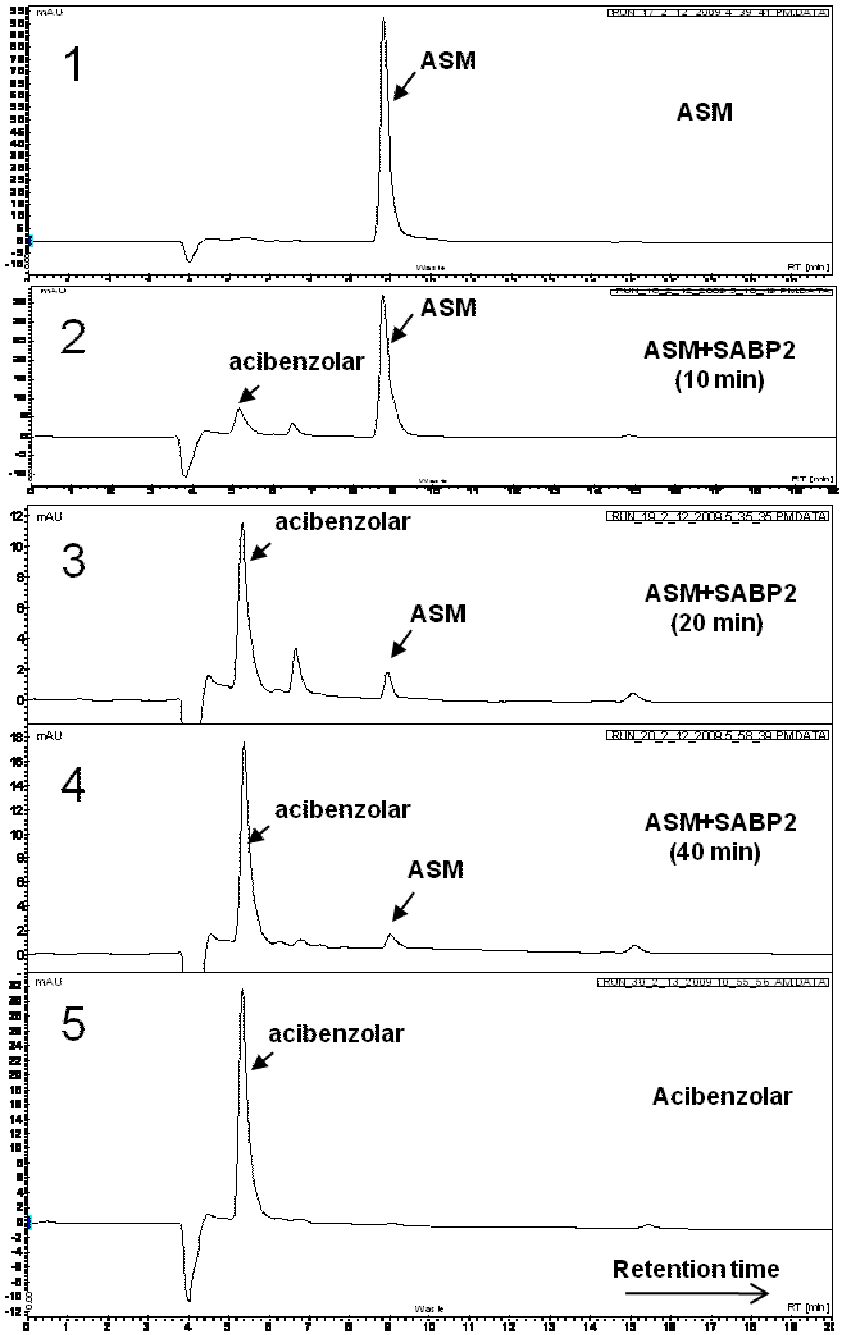


Figure 12. HPLC Histogram Showing the Conversion of ASM Overtime into Acibenzolar on Incubating SABP2 with ASM (Final conc. = 0.4 mM). Panel 1 and 5 show the peaks of pure ASM and acibenzolar respectively. Panel 2, 3, and 4 show the decrease in ASM peak with simultaneous increase in acibenzolar peak on increasing incubation time of SABP2 with ASM.

The conversion of ASM into acibenzolar was found 60% , 95%, and 99% after 10, 20, and 40 minutes respectively of incubation with SABP2 (Figure 13).

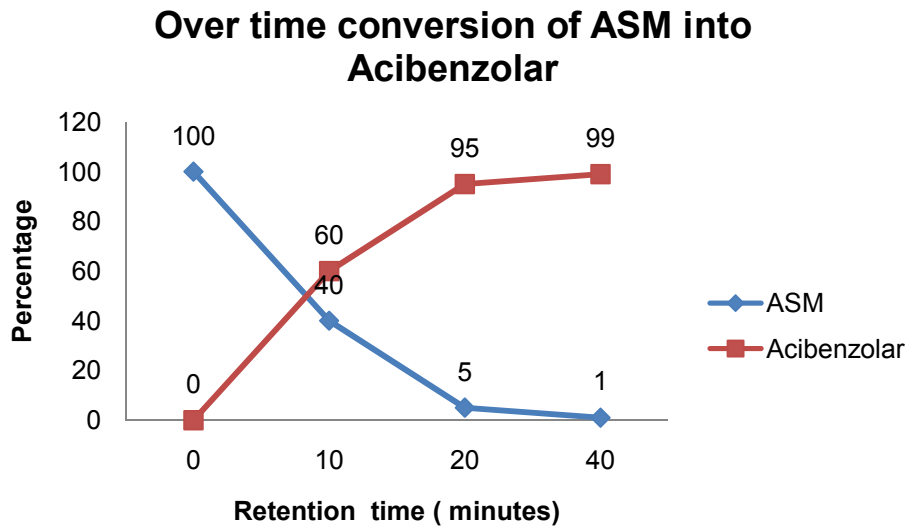


Figure 13. SABP2-Mediated Conversion of ASM into Acibenzolar. On incubating ASM with SABP2 for 10, 20 and 40 Minutes, acibenzolar percentage increased with a simultaneous decrease in ASM percentage.

Testing Expression of PR-1 Protein in ASM-Treated Plants

For testing expression of PR-1 proteins induced by ASM, three lower leaves of C3 and 1-2 plants were treated with various concentrations of ASM as described earlier. Preliminary experiments were designed to determine the concentration of ASM required for inducing robust expression of PR-1 in tobacco plants. The concentrations used were 25 µM, 50 µM, and 100 µM. In addition, ASM-treated leaf samples were harvested after various time intervals for PR-1 analyses to determine the optimal time required for the expression of PR-1.

Samples used were from 48 and 96 hours post ASM treatment. Optimal concentration of ASM was determined as 0.1mM that induced abundant production of PR-1 protein after 48 hours of treatment. Later all the experiments were conducted using this concentration of ASM (0.1 mM) and PR-1 protein expression was monitored after 48 hours. ASM-treated and nontreated (buffer-treated) plants were analyzed for PR-1 protein expression. Figure 14A and 14B show results of Western blotting following development using ECL system. Results show that control (C3) plants treated with ASM induced abundance of the PR-1 protein (thick band in Figure 14B, lane 3) compared to weak expression of PR-1 protein in SABP2-silenced (1-2) plants.

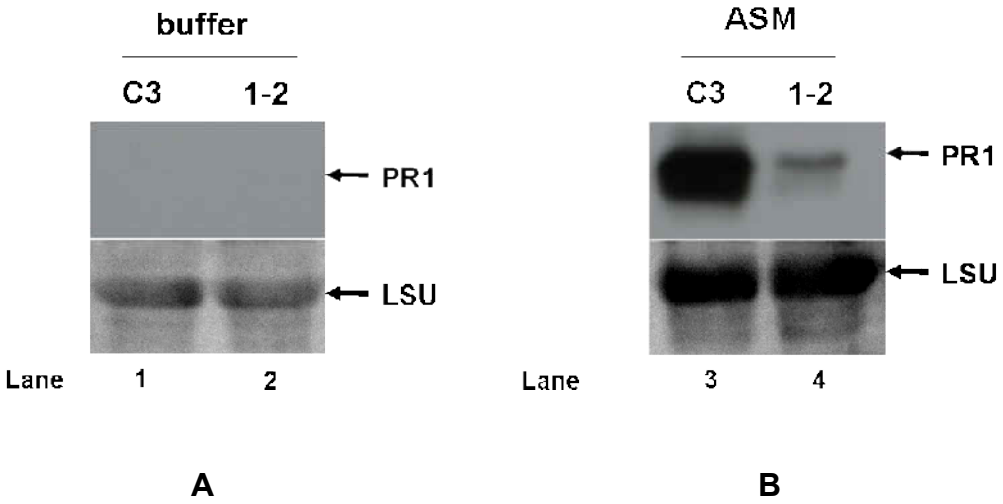


Figure 14 A & B. Western Blots Showing the Expression of PR-1Protein Induced by ASM. C3 and 1-2 plants were treated with buffer and ASM. Leaf samples were collected from the upper nontreated leaves and processed for PR-1 analysis after 48 hours of treatment. Lanes 1 and 2 show the expression of PR-1 induced by buffer (Figure 14 A). Lanes 3 and 4 show the expression of PR-1 Induced by ASM. As shown, PR-1 expression is compromised in 1-2 plant (Figure 14 B, Lane 4). Lower panels of 14 A and B show the equal loading verified by staining of the large subunit of RUBISCO with ponceau S.

Determination of Lesion Sizes (Degree of Infection)

Three lower leaves of control (C3) and SABP2-silenced (1-2) plants were treated with 0.1 mM ASM by foliar spray. Seven days later the upper leaves were inoculated with TMV as described earlier. After 5 -7 days of TMV inoculation challenge the diameters of TMV induced lesions (#15) on the systemic leaves were measured. Figure 15 shows the leaves of both C3 and 1-2 plants with TMV-induced lesions.

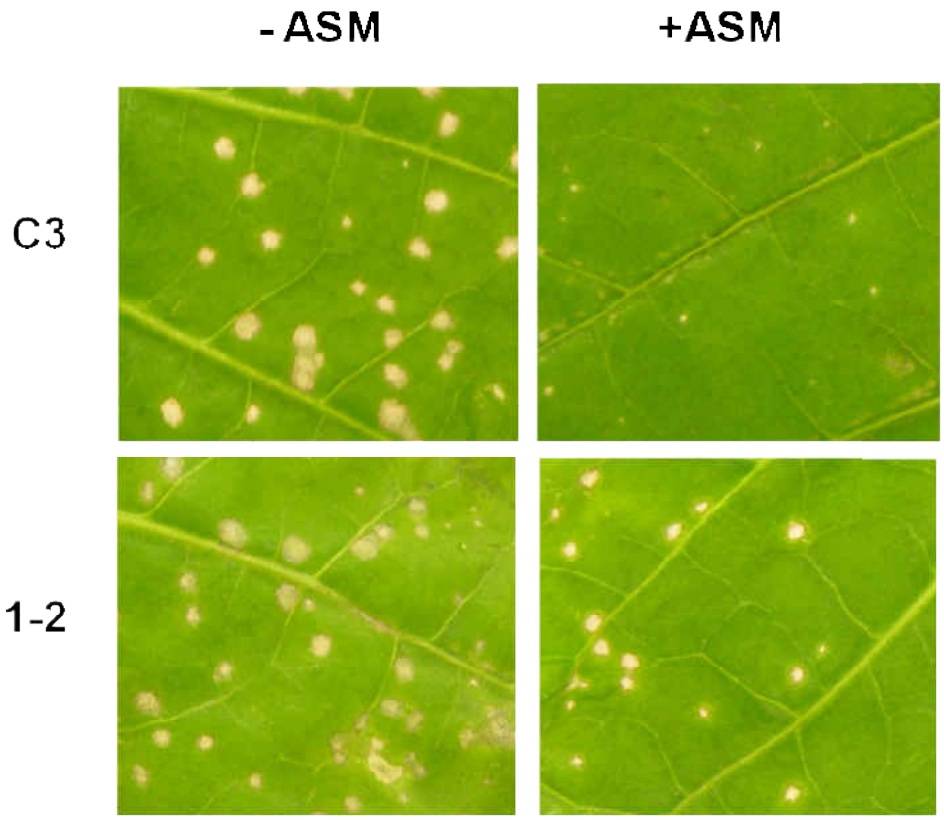


Figure 15. ASM-and Buffer-Treated C3 and 1-2 Plants Showing the Differences in Lesion Sizes. Lesion sizes in ASM-Treated plants were compared with those in nontreated (buffer-treated) plants. No significant difference in lesion sizes was observed in ASM- and buffer-treated 1-2 plants, while significant reduction in lesion sizes was observed in ASM-treated C3 plants compared to buffer-treated C3 plants.

The average of lesion sizes in the control plants treated with buffer and ASM was found to be 1.96 mm and 0.59 mm respectively, while those in 1-2 plants the average of lesion sizes was found to be 2.11 mm and 1.84 mm respectively (Table 2).

Table 2.

TMV-Induced Lesion Sizes and SAR Response in ASM-Treated C3 and 1-2 Plants

	Lesion size on systemic leaves Average diameter ± SD (mm)			
Plants	**+ Buffer**	**+ ASM**	**% Reduction**	**SAR**
C3	1.96 ± 0.12	0.59 ± 0.05	70	+
1-2	2.11 ± 0.07	1.84 ± 0.07	12	-

Figure 16 shows the graphical representation of the lesion sizes in C3 and 1-2 plants treated with buffer and ASM.

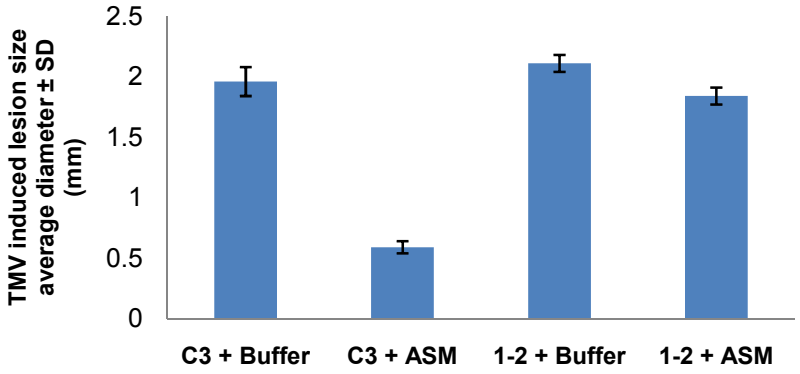


Figure 16. Graph Showing TMV-Induced Lesion Sizes in ASM- and Buffer-Treated C3 and 1-2 Plants. The systemic leaves of ASM- and buffer-treated plants were challenged with TMV after 7 days of treatment. Diameters of lesions were measured 7 days after TMV challenge. Data are average lesion diameters ± SD.

Analysis of ASM-Induced Expression of SABP2 Protein

The lower leaves of C3 plants were treated with 0.1 mM ASM as described earlier and samples were collected after 24 and 48 hours of ASM treatment from the upper untreated leaves. Samples were processed for the analysis of SABP2 protein expression. Western analysis was performed using SABP2 antibodies as described earlier. As shown in the Figure 17, ASM treatment did not induce expression of SABP2 in plants. The same blot was stripped and reprobed using anti PR-1 antibodies to show that ASM treatment did work and induced the expression of PR-1 protein.

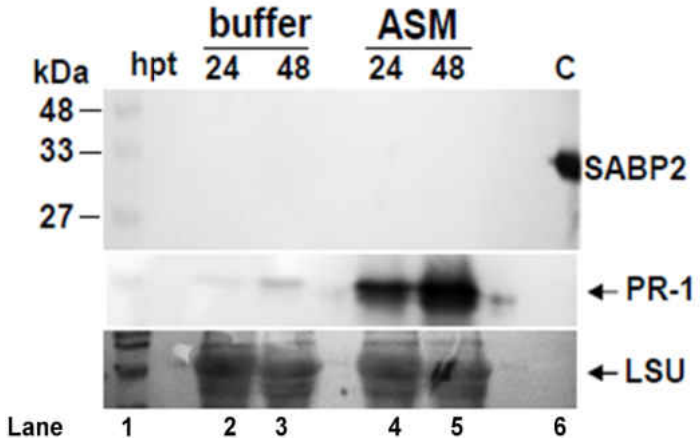


Figure 17. Western Blot Showing the Expression of SABP2 Protein Induced by ASM. Top panel shows the blot probed with SABP2 antibodies. Middle panel shows the blot probed with PR-1 antibodies. Bottom panel shows the equal loading verified by staining of the large subunit of RUBISCO (LSU) with ponceau S. Lane 1 in each panel shows the prestained marker. Lanes 2 and 3 show the expression of SABP2 (top panel), PR-1(middle panel) and LSU (bottom panel) 24 and 48 hours after buffer treatment. Lanes 4 and 5 show the expression of SABP2 (top panel); PR-1 (middle panel) and LSU (bottom panel) 24 and 48 hours post ASM treatment. Lane 6 in top panel shows the positive control of SABP2 expression.

Analysis of PR-1 Protein Induction by Acibenzolar

Plants were treated with acibenzolar (0.1 mM-in 20 mM sodium phosphate buffer) to induce defense proteins. Samples were collected after 48 hours of ASM treatment and used for Western analysis of PR-1 protein expression. Results show that acibenzolar treatment induced the expression of PR-1 proteins in both C3 and 1-2 plants (Figure 18), while ASM did not induce PR-1 in 1-2 plants (Figure 14B).

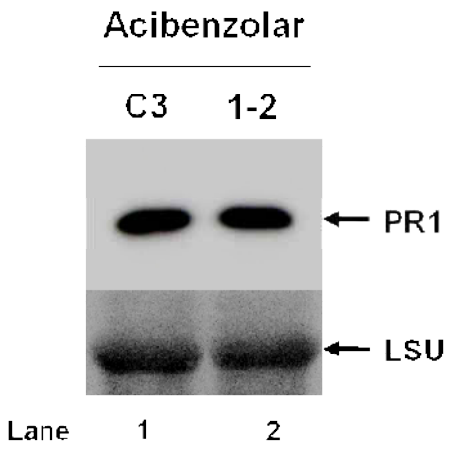


Figure 18. Western Blot Showing the Expression of PR-1 Protein Induced by Acibenzolar. Lane 1 and 2 show the same level of PR-1 expression after treating the plants with 0.1mM acibenzolar. Lower panel shows the equal loading verified by staining of the large subunit of RUBISCO with ponceau S.

SAR Assessment in Chimera Plants

The chimera plants have been described as scion as top half over the rootstock as bottom half of plant, denoted as scion (sc) / rootstock (rs) (sc/rs: C3/C3, C3/1-2, 1-2/1-2, 1-2/C3). The rootstock leaves of chimera plants were treated with ASM, samples were collected from scion leaves, and Western analysis was performed to analyze PR-1 protein expression. The results showed the expression of PR-1 in C3 /1-2 chimera plants, while it was compromised in 1-2 / C3 plants (Figure 19).

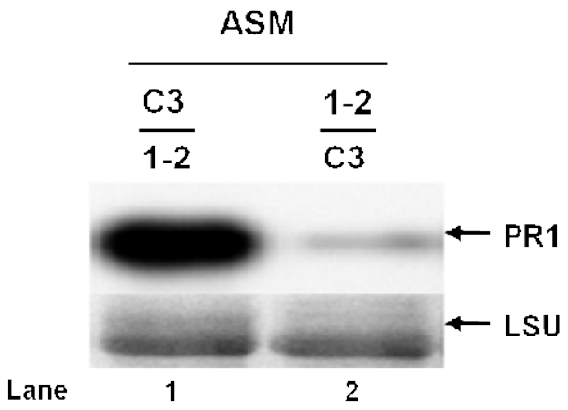


Figure 19. Western Blot Showing the Expression of PR-1 Protein in Chimera Plants. Lane 1 shows the expression of PR-1 in chimera plant with C3 Scion and 1-2 rootstock, while Lane 2 shows the compromised expression of PR-1 in chimera plant with 1-2 scion and C3 rootstock. Lower panel shows the equal loading verified by staining of the large subunit of RUBISCO with ponceau S.

Seven days after ASM treatment scion leaves were inoculated with TMV. Plants were allowed to develop lesions for 5-7 days after TMV inoculation. Figure 20 shows the comparison of TMV-induced lesion sizes on systemic leaves of 1-2/C3 (sc/rs) and C3/1-2 (sc/rs) plants.

C3 / 1-2 **1-2 / C3**

Figure 20. ASM-Treated Chimera Plants Showing the TMV-Induced Lesions. Diameters of lesions were found smaller in C3/1-2 plants compared to 1-2/C3 plants.

The comparison of average lesion sizes shows the significant reduction in lesion sizes and development of SAR in C3/1-2 plants compared to 1-2/C3 plants (Table 3).

Table 3.

Comparison of Lesion Sizes and Assessment of SAR in Chimera Plants

Grafts (sc/rs) *	Lesion size on systemic leaves Average diameter ± SD (mm) + Buffer	+ ASM	% Reduction	SAR
C3/C3	1.14 ± 0.06	0.74 ± 0.03	38	+
1-2/1-2	1.28 ± 0.04	1.20 ± 0.04	6	-
C3/1-2	1.89 ± 0.03	0.74 ± 0.04	60	+
1-2/C3	1.65 ± 0.04	1.39 ± 0.03	15	-

Note. *(sc/rs) – scion/rootstock

Assessment of ASM-Induced SAR Against Bacterial Pathogens

As described earlier, the lower leaves of C3 and 1-2 plants were treated with ASM (0.1mM) or buffer (20 mM sodium phosphate buffer, pH 7.2) (primary treatment) Seven days later systemic leaves were challenged with two different strains of bacteria in separate experiments. Two strains were chosen based on their virulence capacity. Tobacco is a host of *P. syringae* pv *tabaci* (virulent strain), while it is resistant to *P. syringae* pv *tomato* (avirulent strain). The bacteria were infiltrated into the systemic leaves of these plants (secondary inoculation) as described earlier. The growth of *P. syringae* pv *tabaci* was monitored after 2 days of secondary inoculation. Table 4 shows the effect of ASM treatment on the growth of *P. syringae* pv *tabaci (Pst)*.

Table 4.

Effect of ASM Treatment on the Growth of P. syringae pv *tabaci*

Number of *Pst* colonies (cfu)/milliliter				
Plants	**+ Buffer**	**+ ASM**	**% Reduction**	**SAR**
C3	2.5×10^5	4.5×10^4	81	+
1-2	1.3×10^5	4.0×10^4	69	+

In another experiment *P. syringae* pv *tomato* was used for secondary inoculation of ASM-treated plants. The growth of *P. syringae* pv *tomato* was monitored 2 days after secondary inoculation. Table 5 shows the effect of ASM treatment on the growth of *P. syringae* pv *tomato (Pstm)*.

Table 5.

Effect of ASM Treatment on the Growth of P. syringae pv *tomato*

Number of *Pstm* colonies (cfu)/milliliter				
Plants	**+ Buffer**	**+ ASM**	**% Reduction**	**SAR**
C3	9×10^4	1.5×10^4	83	+
1-2	6×10^4	2.5×10^4	58	+

Although ASM treatment reduced the growth of both *P. syringae* pv *tabaci and P. syringae* pv *tomato,* there was no significant difference seen in the reduction of bacterial growth in ASM-treated control (C3) and SABP2-silenced (1-2) plants.

Analysis of Molecular Mechanism of ASM Induced Priming

Low concentrations (< 5 µM) of ASM were used to induce priming in C3 and 1-2 plants. Various low concentrations of ASM were used to test a concentration that could induce a primed (enhanced) resistance response (PR-1 protein expression) in plants after secondary pathogen challenge. For this the lower leaves of control (C3) plants were treated with different concentrations of ASM (1° T). Two days later the upper leaves of C3 plants were mock- or TMV-inoculated (2° T) as described earlier. Leaf samples were collected 48 hours after secondary (2° T) inoculation and processed for PR-1 protein expression analysis. Figure 21 shows results of the direct and primed expression of PR-1 protein induced by buffer (0 µM ASM) and increasing concentrations of ASM (0.05, 0.25, 0.5, and 2.5 µM). As shown, only 2.5 µM ASM induced a primed expression of PR-1 protein.

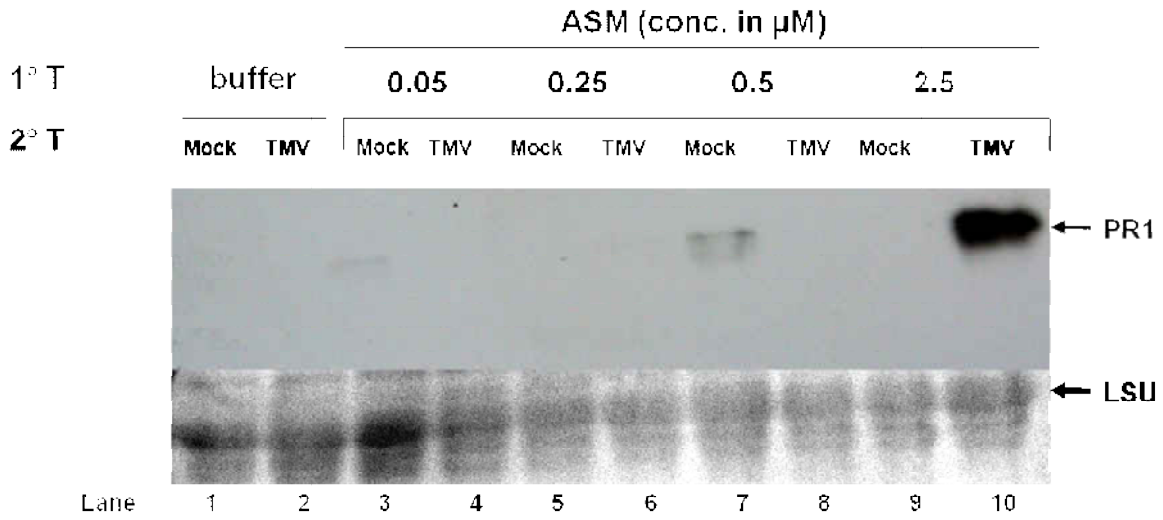


Figure 21. Western Blot Showing the Primed Expression of PR-1 Protein by Increasing Concentrations of ASM. Lane 7 shows a weak direct expression of PR-1protein induced by 0.5 µM and lane 10 shows a primed (enhanced) expression of PR-1 protein induced by 2.5 µm ASM. Lower panel shows almost equal loading verified by staining of the large subunit of RUBISCO with ponceau S.

Another experiment was designed to test the timing of priming response induced by 2.5 µM ASM. For this control plants were treated with 2.5 µM ASM (1° T) and challenged with TMV (2° T) after 48 hours as described earlier. Leaf samples were collected and processed for PR-1 protein expression analysis at 0 hour (just before 2° T) and after 24 and 48 hours of secondary challenge (2° T). Figure 22 shows the results of the expression of PR-1 protein induced by 2.5 µM ASM. As shown, ASM primed the expression of PR-1 after 48 hours of secondary TMV inoculation, while the same concentration of ASM did not induce direct expression of PR-1 protein as visible in secondary mock-challenged plants.

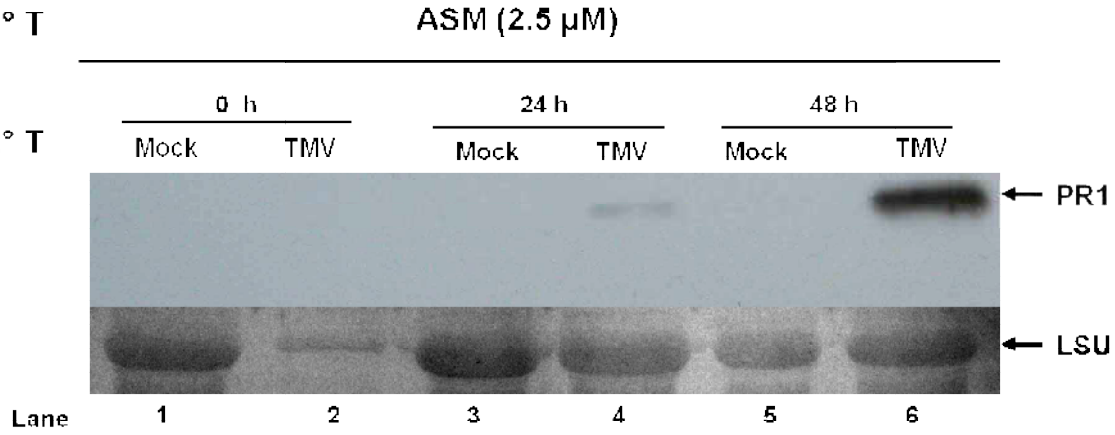


Figure 22. Western Blot Showing the Primed Expression of PR-1Protein by 2.5 µM ASM. Samples were processed from ASM-treated C3 plants after 0, 24, and 48 hours of secondary (mock / TMV) inoculation. Lanes 1, 3, and 5 show no direct PR-1 induction by ASM. Lane 2 shows no primed expression of PR-1. Lane 4 shows a weak and lane 6 shows an enhanced (primed) expression of PR-1, 24 and 48 hours after secondary inoculation respectively. Lower panel shows almost equal loading verified by staining of the large subunit of RUBISCO with ponceau S.

Preliminary experiment was conducted to assess the development of SAR in C3 and 1-2 plants treated with lower concentrations of ASM (< 5 µM). Results were plotted

using bar graph (Figure 23). C3 and 1-2 plants were first treated with various concentrations (0, 0.5, 1.25, and 2.5 µM) of ASM as described earlier. Two days later the upper leaves were challenged with TMV as described earlier. Diameters of lesions on the upper leaves were measured 7 days after secondary inoculation.

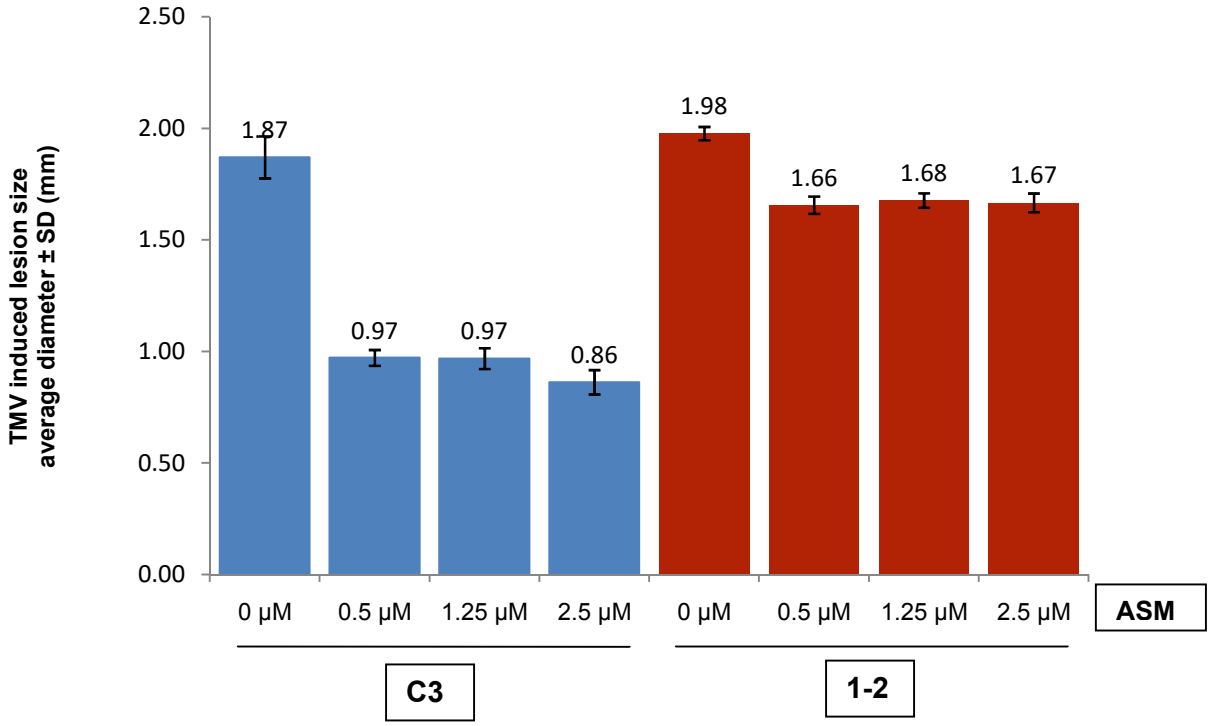


Figure 23. Graph Showing the TMV-Induced Lesion Sizes after Priming the Plants with Various Low Concentrations of ASM. ASM- and buffer (0 µM ASM) treated C3 and 1-2 plants were challenged with TMV after 2 days of treatment. Diameters of lesions were measured after 7 days of TMV challenge. As shown by blue bars, average lesion size decreased by ~ 50% in ASM (0.5,1.25, and 2.5 µM) treated C3 plants compared to buffer (0 µM ASM) treated C3 plants, while no significant difference (~15 %) was observed in lesion sizes of ASM- and buffer-treated 1-2 plants (shown by red bars). Data are average lesion diameters ± SD.

CHAPTER 4

DISCUSSION

SAR is induced in distal parts of plants in the response to necrotizing or avirulent pathogens. There are several published reports indicating that SA, a plant hormone synthesized using the Shikimate pathway, plays an indispensable role when plants are induced to resist pathogens during SAR response (Vlot et al., 2009 and ref. therein). The evidences also suggest that the SAR response is enhanced or induced by exogenous application of SA, synthetic chemicals, or functional analogs of SA. ASM is one such analog that is known to be the most potent activator of SAR. ASM has established its significance in inducing SAR in a wide range of crops. Because of its efficacy against a variety of pathogens, researchers have attempted to investigate the biochemical mode of action of ASM. It has been shown that ASM induces SAR in SA mutants plants (*nahG and sid2* mutants), suggesting that it acts downstream of SA in the SAR pathway and ASM-mediated pathway neither requires nor accumulates SA (Friedrich et al., 1996; Lawton et al., 1996). Although some evidence has suggested that ASM inhibits the activities of catalase and ascorbate peroxidase leading to the synthesis or decreased breakdown of reactive oxygen species (ROS), there is no conclusive evidence for this mechanism in all plants protected by ASM (Wendehenne et al., 1998). Recently it has been shown that ASM treatment causes the inhibition of the NADH: Ubiquinone oxidoreductase activity that might increase the production of superoxide (ROS) (van der Merwe & Dubery, 2006).

SA that is accumulated in the plants in higher amounts during stress conditions, including pathogenicity is converted into other derivatives in plants. Methyl Salicylate (MeSA) is one such derivative synthesized from SA, and this reaction is catalyzed by SA-methyl transferase (SAMT). MeSA is converted back to SA by esterase activity of salicylic acid binding protein 2 (SABP2) in systemic tissues. For successful development of SAR, SA is required as it induces the downstream signaling of both the local and systemic resistance responses (Vlot et al., 2009 and ref. therein). The biological and biochemical roles of SABP2 during resistance response have been described by structural analysis using X-ray crystallography (Kumar & Klessig, 2003). The binding and esterase assays were performed to determine potential natural substrates for SABP2. Among the tested substrates (methyl jasmonates, methyl indole acetic acid, and methyl salicylate) maximum esterase activity of SABP2 was found with methyl salicylate (MeSA). It was also observed that MeSA competed with SA for binding with SABP2 with same potency as SA in competition binding assay (Forouhar et al., 2005).

As ASM is an ester, it was hypothesized that it could be a potential substrate for SABP2. To test this hypothesis and better understand the biochemical mode of action of ASM, *in vitro* studies were designed using Thin Layer Chromatography (TLC) and High Pressure Liquid Chromatography (HPLC) to detect reaction products. In TLC studies ASM migrated farthest from the point of application while acibenzolar did not move much (data not shown). When ASM was incubated with SABP2 and spotted on TLC plate, it showed the similar response as shown by acibenzolar (did not migrate much).

This experiment showed that SABP2 catalyzes the conversion of ASM into acibenzolar (Enyong, 2008).

To validate the results of TLC experiments HPLC was used to analyze SABP2 reaction product measuring the absorbance and recording peak height (amount) and retention time (identity) of a compound. As the ester and acid have different retention times in a C-18 (hydrophobic) column, this study should help detect possible conversion from ester to acid in a reaction catalyzed by an enzyme. Pure ASM and pure acibenzolar showed different retention times and peak heights (Figures 10 and11). On incubating the ester (ASM) with pure SABP2, the amount of ASM decreased with a simultaneous increase in productivity of acibenzolar (Figures 12 and 13). These results indicate that SABP2 catalyzes the conversion of ASM into its acid form (acibenzolar). Based on the results from TLC and HPLC studies, it was logical to hypothesize that the similar conversion might be taking place in plants. Exogenous application of ASM induces a resistance response that could be due to conversion of ASM into acibenzolar catalyzed by SABP2. As documented earlier, that ASM induces the same set of PR proteins as induced by pathogens in SAR. PR-1 protein is the most abundant and most widely used marker of SAR response in tobacco plants. Expression of PR-1 protein was analyzed to verify the induction of defense response by ASM treatment.

Based on prior research that has shown ASM treatment induces the expression of PR-1protein in tobacco plants (Friedrich et al., 1996), our experiments were designed using SABP2-silenced and corresponding control (containing empty silencing vector) tobacco plants. SABP2-silenced plants were used to investigate the role of SABP2 in ASM-induced SAR. Results (Figure 14B) show that treatment of ASM on lower leaves

induced the expression of PR-1 protein in upper, untreated (systemic) leaves of control (C3) plants, while SABP2-silenced (1-2) plants did not show significant expression of PR-1 protein in systemic leaves. This suggests that in the absence of SABP2, ASM was not converted into acibenzolar. Therefore, it can be concluded that SABP2 is required for the proper functioning of ASM in inducing a defense response in plants. This acibenzolar acts downstream of SA and changes the redox potential of the cytoplasm that in turn allows migration of the monomeric form of NPR1 to the nucleus where it interacts with TGA class of transcription factors resulting in enhanced expression of defense related genes (Mou et al., 2003 and ref. therein).

ASM protects the tobacco plants from diverse classes of pathogens including tobacco mosaic virus (TMV) by developing a successful SAR response (Friedrich et al., 1996). Thus, TMV was used as a model pathogen to investigate the effect of ASM against TMV infection. Results of the analysis of lesion sizes (as a marker of disease severity) showed significant decrease in lesion size (69%) in systemic leaves of ASM-treated control plants as compared to buffer-treated control plants. A significant decrease in lesion size was not observed in ASM-treated 1-2 plants (13%), compared to buffer-treated plants (Figures 15 and 16; Table 2). A significant decrease in lesion size in ASM-treated control plants suggests that the SAR response was successfully developed by ASM, while in 1-2 plants the absence of SABP2 restricted the ASM activity in inducing a successful SAR response. Therefore, it can be concluded that SABP2 catalyzed conversion of ASM into acibenzolar is critical for the development of full SAR.

The results consistently pointed toward the importance of SABP2 in converting ASM into acibenzolar and the importance of this conversion in the successful development of defense gene expression and SAR, indicating that SABP2 is a receptor of ASM.

To investigate the direct effect of ASM treatment on expression levels of SABP2, an experiment was performed using control (C3) tobacco plants. Leaf samples from ASM-treated plants were collected to analyze expression of SABP2 protein. Results showed that ASM treatment did not induce expression of SABP2, while it did induce expression of defense protein (PR-1) (Figure 17, Lanes 4 and 5). This suggests that ASM functions by inducing the production of PR-1 and inducing SAR without affecting SABP2 expression.

Because it was shown in earlier results (Figures 12,13, and 14B) that ASM conversion to acibenzolar is required for induction of PR-1 protein, it was logical to propose that direct treatment of acibenzolar could increase the PR-1 protein expression. Western blot (Figure 18) showed that acibenzolar induced PR-1 protein expression in both the control (C3) and SABP2-silenced (1-2) plants, while treatment by ASM induced the PR-1protein expression only in C3 plants. These results confirmed the importance of SABP2 for ASM-induced expression of defense molecules as well as the role of acibenzolar in induction of ASM-mediated defense pathway.

SABP2 is required in systemic tissue to process the defense signal in pathogen-induced SAR (Kumar & Klessig, 2008; Park et al., 2007). To investigate the role of SABP2 as a signaling compound in ASM- induced SAR, grafting experiments were performed to make chimera plants. Treatment of rootstock leaves with ASM induced

expression of PR-1protein in scion leaves of C3/1-2 (scion/rootstock) plants, while there was no significant induction of PR-1 protein observed in 1-2/C3 plants (Figure 19). This shows that C3/1-2 plants having SABP2 in scion leaves induced PR-1 protein expression despite the fact that the chimeras have SABP2 expression silenced in the rootstock. This result also implies that although SABP2 does not generate any signal upon ASM treatment in rootstock or local tissues, it is required in systemic tissues to process ASM. Results further suggest that ASM is translocated to other parts of the plants, and it induces resistance in systemic tissues after being converted into acibenzolar by SABP2. For the assessment of SAR in chimera plants the scion leaves were challenged with TMV. Significant decreases in lesion size were observed in the plants having SABP2 in systemic tissues (scion/rootstock: C3/C3 and C3/1-2 chimeras), while there were no significant decreases in lesion sizes in plants lacking SABP2 in the scion leaves (scion/rootstock: 1-2/1-2 and 1-2/C3 chimeras) (Figure 20; Table 3). These results indicate that the presence of SABP2 in systemic (scion) tissues, not in local (rootstock) tissues, is required for the successful development of the SAR induced by ASM.

SAR developed by ASM is effective against a broad range of pathogens including viruses, bacteria, and fungi (Metraux, 2002 and ref. therein). In addition to viral pathogen TMV, the effectiveness of ASM treatment on bacterial pathogen was also tested. Virulent (*P. syringae* pv *tabaci*) and an avirulent (*P.s. syringae* pv *tomato)* strains of *Pseudomonas syringae* were used. The systemic leaves of ASM-treated C3 and 1-2 plants were challenged with *P. syringae* pv *tabaci* or *P. syringae* pv *tomato* and growth of these two strains was monitored 2 days after secondary inoculation for *P. syringae* pv

tabaci and 7 days after secondary inoculation for *P. syringae* pv *tomato*. Results (Tables 4 and 5) show the decrease in growth of bacteria in ASM-treated plants. This decrease was more prominent against *P. syringae* pv *tomato* (Table 5). Because *P. syringe* pv *tomato* is an avirulent strain, this might be due to induction of SAR in plants. Thus, an enhanced expression of the SAR induced by ASM and *P. syringae* pv *tomato* resulted in less growth of this *Pseudomonas* strain compared to *P. syringae* pv *tabaci*. Although the reduction in bacterial count was observed in ASM-treated plants, there was no significant difference observed in the bacterial count between ASM-treated control (C3) and SABP2-silenced (1-2) plants. Possible explanations for this may be that (1) ASM response against bacteria is independent of SABP2 catalyzed conversion, (2) the reduced level of SABP2 in 1-2 plants is still sufficient to restrict bacterial growth, (3) treatment with bacterial pathogens increases the overall expression of SABP2 in 1-2 plants.

The induction of systemic resistance not only leads to direct activation of defense related genes but also leads to the priming of cells resulting in stronger defense responses following pathogen attacks (Conrath et al., 2002 and ref. therein). A better understanding of the molecular mechanism of ASM-induced priming could be useful in reducing the fitness cost of plants during stress and pathogenicity conditions. To understand the priming phenomenon various low concentrations of ASM were tested to optimize for the minimum concentration of ASM required to induce priming in plants. Priming was tested by analyzing expression of PR-1 protein in systemic tissues. As evident by the results (Figure 21), the concentration of ASM that induced priming without inducing, the direct defense (no PR-1 induction in mock-inoculated plants) was

found to be 2.5 μM. Further experiments suggested that a 2.5 μM ASM was low enough to induce the primed (enhanced) expression of PR-1 protein after 48 hours of secondary TMV inoculation (Figure 21, Lane 10; Figure 22, Lane 6) without inducing the direct expression of PR-1 after secondary mock inoculation (Figure 22, Lanes 1, 3, and 5). Lesion sizes were measured after ASM (1° T) and TMV (2° T) treatments in C3 and 1-2 plants as described earlier to assess the level of SAR development in ASM induced priming. Figure 23 shows that even the lowest concentration of ASM (0.5 μM) was able to reduce lesion sizes by ~50% in ASM-treated and TMV-inoculated C3 plants, while there was no significant difference observed in ASM-treated and TMV-inoculated 1-2 plants. This suggests that SABP2 has a potential role in ASM-induced priming. Further research in this direction may be helpful in elucidating the mechanism of priming.

Conclusions and Future Directions

This research was conducted to test the hypotheses that SABP2 catalyzes the demethylation of Acibenzolar- S-methyl to acibenzolar and this conversion is required for the successful expression of defense proteins and development of SAR. The results presented in this thesis support the hypotheses. This study established a relationship between SABP2 and ASM metabolism. Based on the results of this research, we propose a defense-signaling pathway induced by ASM in plants (Figure 24).

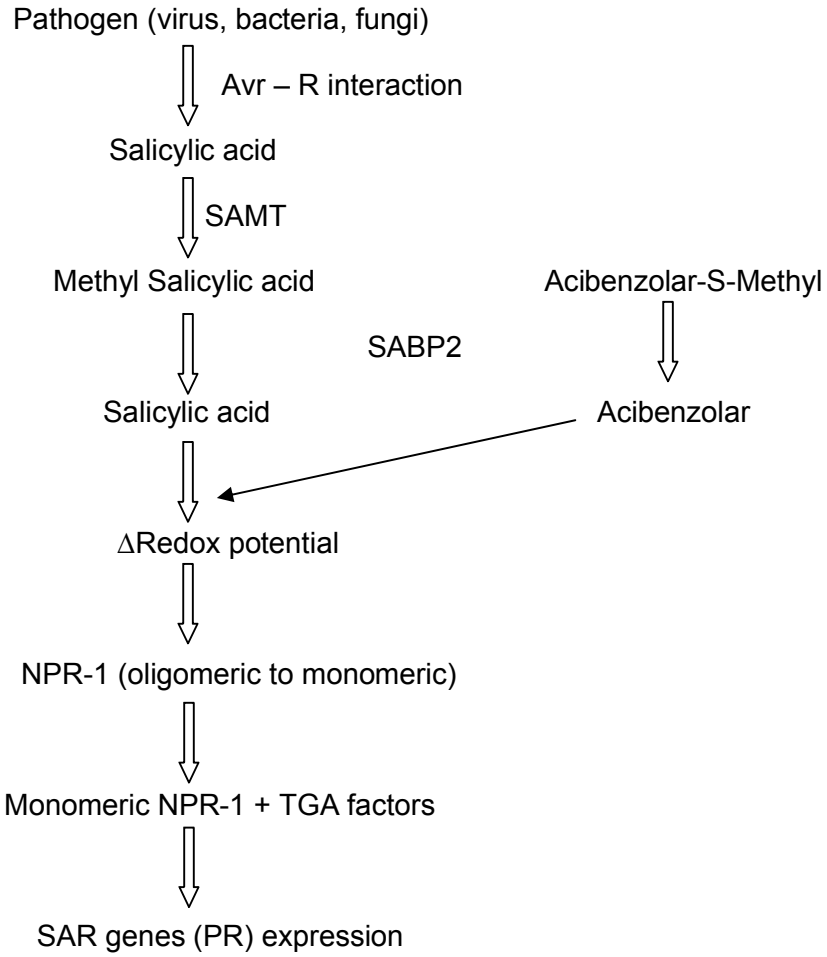


Figure 24. Signaling Pathway of SAR Induced by Acibenzolar-S-Methyl.

The SAR response induced by ASM was tested with a viral pathogen (TMV). Despite several attempts experiments with bacterial pathogens were not very successful as no significant difference was observed between the SAR induced in C3 and 1-2 plants against *Pseudomonas syringae*. The results suggest that ASM-induced defense against *Pseudomonas syringae* may not require SABP2. The other possibility could be that treatment with plant pathogenic bacteria induces expression of native SABP2 resulting in higher levels of SABP2 in 1-2 plants. These increased levels of

SABP2 may be sufficient to induce resistance response in 1-2 plants similar to that induced in C3 plants. Further research in this direction is required to make any conclusion. Other bacterial pathogens need to be tested and other methods need to be applied to monitor the growth of bacteria such as the measurement of diameters of bacterial spots.

Besides direct induction of PR-1 protein, plants also can be primed for a potentiated defense response when treated with ASM. Although preliminary results of the experiments designed to test the expression of PR-1 protein (Figure 21 and 22) and to assess the level of priming induced by ASM (Figure 23) have suggested that SABP2 might be required for ASM-induced priming, some inconsistency was observed in the results. The possible explanation for the inconsistency in priming results might be due to enhanced expression of different sets of PR genes or a combination of sets of PR-1 and other defense gene families in tobacco plants after treatment with low concentrations of ASM (Conrath et al., 2002 and ref. therein). Further research in this direction is required to make a conclusion. Priming experiments need to be repeated with proper controls and expression of other families of PR proteins needs to be tested in future.

The results of these findings could be used to develop better SAR inducing chemicals. The esterase activity of SABP2 plays an important role in understanding the functioning of ASM as a chemical inducer of plant defense. This information could be helpful in developing novel chemical inducers of SAR response.

REFERENCES

Achuo, E. A., Audenaert, K., Meziane, H., & Höfte, M. (2004). The salicylic acid-dependent defence pathway is effective against different pathogens in tomato and tobacco. *Plant Pathology, 53,* 65-72.

Antoniw, J., & White, R. (1983). Biochemical properties of the pathogenesis-related proteins from tobacco. *European Journal of Plant Pathology, 89,* 255-264.

Antoniw, J. F., Dunkley, A. M., White, R. F., & Wood, J. (1980). Soluble leaf proteins of virus-infected tobacco (*Nicotiana tabacum*) cultivars [proceedings]. *Biochemical Society Transaction, 8,* 70-71.

Bécot, S., Pajot, E., Le Corre, D., Monot, C., & Silué, D. (2000). Phytogard (K2HPO3) induces localized resistance in cauliflower to downy mildew of crucifers. *Crop Protection, 19,* 417-425.

Bel, A. J. E. V., & Gaupels, F. (2004). Pathogen-induced resistance and alarm signals in the phloem. *Molecular Plant Pathology, 5,* 495-504.

Benhamou, N. (1996). Elicitor-induced plant defence pathways. *Trends in Plant Science, 1*, 233-240.

Bokshi, A. I., Morris, S. C., & Deverall, B. J. (2003). Effects of benzothiadiazole and acetylsalicylic acid on β -1,3-glucanase activity and disease resistance in potato. *Plant Pathology, 52,* 22-27.

Brotman, Y., Makovitzki, A., Shai, Y., Chet, I., & Viterbo, A. (2009). Synthetic ultrashort cationic lipopeptides induce systemic plant defense responses against bacterial and fungal pathogens. *Applied and Environmental Microbiology, 75,* 5373-5379.

Calaf, G. M., & Roy, D. (2007). Human drug metabolism genes in parathion-and estrogen-treated breast cells. *International Journal of Molecular Medicine, 20,* 875-881.

Cao, H., Bowling, S. A., Gordon, A. S., & Dong, X. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell, 6,* 1583-1592.

Chen, F., D'Auria, J. C., Tholl, D., Ross, J. R., Gershenzon, J., Noel, J. P., et al. (2003). An *Arabidopsis thaliana* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach has a role in defense. *Plant Journal, 36,* 577-588.

Cohen, Y. R. (2002). β-aminobutyric acid- induced resistance against plant pathogens. *Plant Disease, 86,* 448-457.

Cole, D. L. (1999). The efficacy of acibenzolar-S-methyl, an inducer of systemic acquired resistance, against bacterial and fungal diseases of tobacco. *Crop Protection, 18*, 267-273.

Conrath, U., Pieterse, C. M., & Mauch-Mani, B. (2002). Priming in plant-pathogen interactions. *Trends in Plant Science, 7,* 210-216.

Daire, X. & Mauch-Mani, B. (2007). Chemical inducers of resistance in plants. *Chemistry Today, 25,* 15-20.

da Rocha, A. B., & Hammerschmidt, R. (2005). History and perspectives on the use of disease resistance inducers in horticultural crops. *Horttechnology, 15,* 518-529.

Dangl, J. L., & Jones, J. D. (2001). Plant pathogens and integrated defence responses to infection. *Nature, 411,* 826-833.

Delaney, T. P., Friedrich, L., & Ryals, J. A. (1995). *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc Natl Acad Sci U S A, 92,* 6602-6606.

Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., et al. (1994). A central role of salicylic acid in plant disease resistance. *Science, 266,* 1247-1250.

Dixon, R. A., Harrison, M. J., & Lamb, C. J. (1994). Early events in the activation of plant defense responses. *Annual Review of Phytopathology, 32,* 479-501.

Du, H., & Klessig, D. F. (1997). Identification of a soluble, high-affinity salicylic acid-binding protein in tobacco. *Plant Physiology, 113,* 1319-1327.

Durrant, W. E., & Dong, X. (2004). Systemic acquired resistance. *Annual Review of Phytopathology, 42,* 185-209.

Enyong, A. B. (2008). Synthesis and enzymatic reactions of novel plant agrochemicals catalyzed by SABP2 (Master's thesis). East Tennessee State University, Johnson city, TN.

Forouhar, F., Yang, Y., Kumar, D., Chen, Y., Fridman, E., Park, S. W., et al. (2005). Structural and biochemical studies identify tobacco SABP2 as a methyl salicylate esterase and implicate it in plant innate immunity. *Proc Natl Acad Sci U S A, 102,* 1773-1778.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, M. G., et al. (1996). A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant Journal, 10,* 61-70.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., et al. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. *Science, 261,* 754-756.

Glazebrook, J., Rogers, E. E., & Ausubel, F. M. (1996). Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. *Genetics, 143,* 973-982.

Godard, J.-F., Ziadi, S., Monot, C., Le Corre, D., & Silué, D. (1999). Benzothiadiazole (BTH) induces resistance in cauliflower (*Brassica oleracea* var *botrytis*) to downy mildew of crucifers caused by *Peronospora parasitica*. *Crop Protection, 18,* 397-405.

Gorlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K. H., et al. (1996). Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *Plant Cell, 8,* 629-643.

Gozzo, F. (2003). Systemic acquired resistance in crop protection: From nature to a chemical approach. *Journal of Agricultural Food Chemistry, 51,* 4487-4503.

Guedes, M. E. M., Richmond, S., & Kuc, J. (1980). Induced systemic resistance to anthracnose in cucumber as influenced by the location of the inducer inoculation with *Colletotrichum lagenarium* and the onset of flowering and fruiting. *Physiological Plant Pathology, 17,* 229-233.

Guo, A., Salih, G., & Klessig, D. F. (2000). Activation of a diverse set of genes during the tobacco resistance response to TMV is independent of salicylic acid; Induction of a subset is also ethylene independent. *Plant Journal, 21,* 409-418.

Hammerschmidt, R., & Becker, J. S. (1997). Acquired resistance to disease in plants. *Horticulture Review,18,* 247-288.

Hammond-Kosack, K. E., & Jones, J. D. (1996). Resistance gene-dependent plant defense responses. *Plant Cell, 8,* 1773-1791.

Heath, M. C., (1981). Non-host resistance. In R. C. Staples & G. H. Toenniessen (Eds.), *Plant disease control: Resistance and susceptibility*, (pp. 201-217). New York: John Wiley and Sons.

Huang, J., Cardoza, Y. J., Schmelz, E. A., Raina, R., Engelberth, J., & Tumlinson, J. H. (2003). Differential volatile emissions and salicylic acid levels from tobacco plants in response to different strains of *Pseudomonas syringae*. *Planta, 217,* 767-775.

Huth, W., & Balke, K. (2002). Bion- without effect on the development of BYDV infected plants of winter barley. *Journal of Plant Disease and Protection, 109,* 286-290.

Iriti, M., & Faoro, F. (2007). Review of innate and specific immunity in plants and animals. *Mycopathologia, 164,* 57-64.

Iriti, M., Rossoni, M., Borgo, M., & Faoro, F. (2004). Benzothiadiazole enhances resveratrol and anthocyanin biosynthesis in grapevine, meanwhile improving resistance to *Botrytis cinerea*. *Journal of Agricultural Food Chemistry, 52,* 4406-4413.

Jenns, A. E., & Kuc, J. (1977). Localized infection with tobacco necrosis virus protects cucumber against *Colletotrichum lagenarium*. *Physiological Plant Pathology, 11,* 207-212.

Katz, V. A., Thulke, O. U., & Conrath, U. (1998). A benzothiadiazole primes parsley cells for augmented elicitation of defense responses. *Plant Physiology, 117,* 1333-1339.

Kessmann, H., Staub, T., Hofmann, C., Maetzke, T., Herzog, J., Ward, E., et al. (1994). Induction of systemic acquired disease resistance in plants by chemicals. *Annual Review of Phytopathology, 32,* 439-459.

Kiefer, I. W., & Slusarenko, A. J. (2003). The pattern of systemic acquired resistance induction within the *Arabidopsis* rosette in relation to the pattern of translocation. *Plant Physiology, 132,* 840-847.

Kloepper, J. W., Tuzun, S., & Kuć, J. A. (1992). Proposed definitions related to induced disease resistance. *Biocontrol Science and Technology, 2*, 349-351.

Knoth, C., Salus, M. S., Girke, T., & Eulgem, T. (2009). The synthetic elicitor 3,5-dichloroanthranilic acid induces NPR1-dependent and NPR1-independent mechanisms of disease resistance in *Arabidopsis*. *Plant Physiology, 150,* 333-347.

Kumar, D., & Klessig, D. F. (2003). High-affinity salicylic acid-binding protein 2 is required for plant innate immunity and has salicylic acid-stimulated lipase activity. *Proc Natl Acad Sci U S A, 100,* 16101-16106.

Kumar, D., & Klessig, D. F. (2008). The search for the salicylic acid receptor led to discovery of the SAR signal receptor. *Plant Signal Behavior, 3,* 691-692.

Kunz, W., Schurter, R., & Maetzke, T. (1997). The chemistry of benzothiadiazole plant activators. *Pesticide Science, 50,* 275-282.

Lawton, K. A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., et al. (1996). Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *Plant Journal, 10,* 71-82.

Li, Y., Zhang, Z., Jia, Y., Shen, Y., He, H., Fang, R., et al. (2008). 3-Acetonyl-3-hydroxyoxindole: A new inducer of systemic acquired resistance in plants. *Plant Biotechnology Journal, 6,* 301-308.

Lyon, G. D., & Newton, A. C. (1997). Do resistance elicitors offer new opportunities in integrated disease control strategies? *Plant Pathology, 46,* 636-641.

Malamy, J., Carr, J. P., Klessig, D. F., & Raskin, I. (1990). Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. *Science, 250,* 1002-1004.

Maldonado, A. M., Doerner, P., Dixon, R. A., Lamb, C. J., & Cameron, R. K. (2002). A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*. *Nature, 419,* 399-403.

Mandal, B., Mandal, S., Csinos, A. S., Martinez, N., Culbreath, A. K., & Pappu, H. R. (2008). Biological and molecular analyses of the acibenzolar-S-methyl-induced systemic acquired resistance in flue-cured tobacco against Tomato spotted wilt virus. *Phytopathology, 98,* 196-204.

Marrs, K. A. (1996). The functions and regulation of glutathione s-transferases in plants. *Annual Review of Plant Physiology and Plant Molecular Biology, 47,* 127-158.

Martinelli, J. A., Brown, J. K. M., & Wolfe, M. S. (1993). Effects of barley genotype on induced resistance to powdery mildew. *Plant Pathology, 42,* 195-202.

Mauch-Mani, B., & Metraux, J. P. (1998). Salicylic acid and systemic acquired resistance to pathogen attack. *Annals of Botany, 82,* 535-540.

Metraux, J. P. (2002). Recent breakthroughs in the study of salicylic acid biosynthesis. *Trends in Plant Science, 7,* 332-334.

Metraux, J. P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., et al. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science, 250,* 1004-1006.

Miles, A. K., Willingham, S. L., & Cooke, A. W. (2004). Field evaluation of strobilurins and a plant activator for the control of citrus black spot. *Australasian Plant Pathology, 33,* 371-378.

Molders, W., Buchala, A., & Metraux, J. P. (1996). Transport of salicylic acid in tobacco necrosis virus-infected cucumber plants. *Plant Physiology, 112,* 787-792.

Mou, Z., Fan, W., & Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell, 113,* 935-944.

Nakashita, H., Yasuda, M., Nishioka, M., Hasegawa, S., Arai, Y., Uramoto, M., et al. (2002a). Chloroisonicotinamide derivative induces a broad range of disease resistance in rice and tobacco. *Plant Cell Physiology, 43,* 823-831.

Nakashita, H., Yoshioka, K., Yasuda, M., Nitta, T., Arai, Y., Yoshida, S., et al. (2002b). Probenazole induces systemic acquired resistance in tobacco through salicylic acid accumulation. *Physiological and Molecular Plant* Pathology, *61,* 197-203.

Nandi, A., Welti, R., & Shah, J. (2004). The *Arabidopsis thaliana* dihydroxyacetone phosphate reductase gene suppresssor of fatty acid desaturase deficiency1 is required for glycerolipid metabolism and for the activation of systemic acquired resistance. *Plant Cell, 16,* 465-477.

Oostendorp, M., Kunz, W., Dietrich, B., & Staub, T. (2001). Induced disease resistance in plants by chemicals. *European Journal of Plant Pathology, 107,* 19-28.

Park, S. W., Kaimoyo, E., Kumar, D., Mosher, S., & Klessig, D. F. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science, 318,* 113-116.

Perez, L., Rodriguez, M. E. M. E., Rodriguez, F., & Roson, C. (2003). Efficacy of acibenzolar-S-methyl, an inducer of systemic acquired resistance against tobacco blue mould caused by *Peronospora hyoscyami f. sp. tabacina*. *Crop Protection, 22,* 405-413.

Pieterse, C. M., van Wees, S. C., Hoffland, E., van Pelt, J. A., & van Loon, L. C. (1996). Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell, 8,* 1225-1237.

Pillonel, C. (2001). Identification of a 2,6-dichloroisonicotinic-acid-sensitive protein kinase from tobacco by affinity chromatography on benzothiadiazole-sepharose and NIM-metal chelate adsorbent. *Pest Managment Science, 57,* 676-682.

Pimentel, D., Cooperstein, S., Randell, H., Filiberto, D., Sorrentino, S., Kaye, B., et al. (2007). Ecology of increasing diseases: Population growth and environmental degradation. *Human Ecology, 35,* 653-668.

Pinstrup-Andersen, P. (2000). The future world food situation and the role of plant diseases. *Canadian Journal of Plant Pathology, 22,* 321-331.

Qin, X. F., Holuigue, L., Horvath, D. M., & Chua, N. H. (1994). Immediate early transcription activation by salicylic acid via the cauliflower mosaic virus as-1 element. *Plant Cell, 6,* 863-874.

Rasmussen, J. B., Hammerschmidt, R., & Zook, M. N. (1991). Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv *syringae*. *Plant Physiology, 97,* 1342-1347.

Romero, A. M., Kousik, C. S., & Ritchie, D. F. (2001). Resistance to bacterial spot in bell pepper induced by acibenzolar-s-methyl. *Plant Disease, 85,* 189-194.

Romero, A. M., & Ritchie, D. F. (2004). Systemic acquired resistance delays race shifts to major resistance genes in bell pepper. *Phytopathology, 94,* 1376-1382.

Ross, A. F. (1961). Systemic acquired resistance induced by localized virus infections in plants. *Virology, 14,* 340-358.

Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H. Y., & Hunt, M. D. (1996). Systemic acquired resistance. *Plant Cell, 8,* 1809-1819.

Scarponi, L., Buonaurio, R., & Martinetti, L. (2001). Persistence and translocation of a benzothiadiazole derivative in tomato plants in relation to systemic acquired resistance against *Pseudomonas syringae* pv *tomato*. *Pest Managment Science, 57,* 262-268.

Seskar, M., Shulaev, V., & Raskin, I. (1998). Endogenous methyl salicylate in pathogen-inoculated tobacco plants. *Plant Physiology, 116,* 387-392.

Shah, J., Kachroo, P., & Klessig, D. F. (1999). The *Arabidopsis* ssi1 mutation restores pathogenesis-related gene expression in npr1 plants and renders defensin gene expression salicylic acid dependent. *Plant Cell, 11,* 191-206.

Shulaev, V., Leon, J., & Raskin, I. (1995). Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? *Plant Cell, 7,* 1691-1701.

Shulaev, V., Silverman, P., & Raskin, I. (1997). Airborne signalling by methyl salicylate in plant pathogen resistance. *Nature, 385,* 718-721.

Si-Ammour, A., Mauch-Mani, B., & Mauch, F. (2003). Quantification of induced resistance against *Phytophthora* species expressing GFP as a vital marker: Beta-aminobutyric acid but not BTH protects potato and *Arabidopsis* from infection. *Molecular Plant Pathology, 4,* 237-248.

Silverman, F. P., Petracek, P. D., Heiman, D. F., Ju, Z., Fledderman, C. M., & Warrior, P. (2005). Salicylate activity. 2. potentiation of atrazine. *Journal of Agricultural Food Chemistry, 53,* 9769-9774.

Sticher, L., Mauch-Mani, B., Métraux, & JP. (1997). Systemic acquired resistance. *Annual Review of Phytopathology, 35,* 235-270.

Truman, W., Bennett, M. H., Kubigsteltig, I., Turnbull, C., & Grant, M. (2007). *Arabidopsis* systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. *Proc Natl Acad Sci U S A, 104,* 1075-1080.

Uknes, S., Dincher, S., Friedrich, L., Negrotto, D., Williams, S., Thompson-Taylor, H., et al. (1993). Regulation of pathogenesis-related protein-1a gene expression in tobacco. *Plant Cell, 5,* 159-169.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., et al. (1992). Acquired resistance in *Arabidopsis*. *Plant Cell, 4,* 645-656.

Vallad, G. E., & Goodman, R. M. (2004). Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop Science, 44,* 1920-1934.

van der Merwe, J. A., & Dubery, I. A. (2006). Benzothiadiazole inhibits mitochondrial NADH:Ubiquinone oxidoreductase in tobacco. *Journal of Plant Physiology, 163,* 877-882.

van Loon, L. C. (1987). Disease induction by plant viruses. *Advances in Virus Research, 33,* 205-255.

van Loon, L. C., & van Kammen, A. (1970). Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. "Samsun" and "Samsun NN". II. Changes in protein constitution after infection with tobacco mosaic virus. *Virology, 40,* 190-211.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., et al. (1994). Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plant Cell, 6,* 959-965.

Vicedo, B., Flors, V., de la, O. L. M., Finiti, I., Kravchuk, Z., Real, M. D., et al. (2009). Hexanoic acid-induced resistance against *Botrytis cinerea* in tomato plants. *Molecular Plant Microbe Interaction, 22*, 1455-1465.

Vlot, A. C., Dempsey, D. A., & Klessig, D. F. (2009). Salicylic acid, a multifaceted hormone to combat disease. *Annual Review of Phytopathology, 47*, 177-206.

Walters, D., Walsh, D., Newton, A., & Lyon, G. (2005). Induced resistance for plant disease control: Maximizing the efficacy of resistance elicitors. *Phytopathology, 95,* 1368-1373.

Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., et al. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell, 3,* 1085-1094.

Weissmann, G. (1991). Aspirin. *Scientific American, 264,* 84-90.

Wendehenne, D., Durner, J., Chen, Z., & Klessig, D. F. (1998). Benzothiadiazole, an inducer of plant defenses, inhibits catalase and ascorbate peroxidase. *Phytochemistry, 47,* 651-657.

White, R. F. (1979). Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. *Virology, 99,* 410-412.

Wildermuth, M. C., Dewdney, J., Wu, G., & Ausubel, F. M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature, 414,* 562-565.

Xiang, C., Miao, Z. H., & Lam, E. (1996). Coordinated activation of as-1-type elements and a tobacco glutathione S-transferase gene by auxins, salicylic acid, methyl-jasmonate and hydrogen peroxide. *Plant Molecular Biology, 32*, 415-426.

Yalpani, N., Silverman, P., Wilson, T. M., Kleier, D. A., & Raskin, I. (1991). Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. *Plant Cell, 3*, 809-818.

Zahm, S. H., & Blair, A. (1992). Pesticides and non-Hodgkin's lymphoma. *Cancer Research, 52*, 5485-5488.

Zhao, J., Davis, L. C., & Verpoorte, R. (2005). Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnology Advances, 23,* 283-333.

Zhu, Y. J., Qiu, X., Moore, P. H., Borth, W., Hu, J., Ferreira, S., et al. (2003). Systemic acquired resistance induced by BTH in papaya. *Physiological and Molecular Plant Pathology, 63,* 237-248.

APPENDICES

APPENDIX A – Abbreviations

1-2	- SABP2-silenced plants (transgenic *N.t.* cv Xanthi nc in which SABP2 gene expression is silenced by RNA interference
ASM	- Acibenzolar-S-Methyl
βME	- βeta mercaptoethanol
C3	- Control plants (*Nicotiana tabacum* cv Xanthi nc, a local lesion host of Tobacco Mosaic Virus and contains empty silencing vector
Cfu	- Colony forming unit
HPLC	- High-pressure liquid chromatography
ICS	- Isochorismate synthase
ISR	- Induced systemic resistance
KBM	- King's B medium
M	- Molar
MeSA	- Methyl salicylic acid
µg	- Microgram
mg	- Milligram
ml	- Milliliter
mM	- Millimolar
NIM-1	- Non- inducible immunity
NPR-1	- Non- expresser of pathogenesis related 1 protein
PAL	- Phenylalanine ammonia lyase
PR	- Pathogenesis related

RPM	- Revolutions per minute
SA	- Salicylic acid
SABP2	- Salicylic acid binding protein 2
SAMT	- Salicylic acid methyl transferase
SAR	- Systemic acquired resistance
SDS PAGE	- Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TLC	- Thin layer chromatography
TMV	- Tobacco mosaic virus

APPENDIX B - Buffers, Media, and Reagents

Acibenzolar-S-Methyl

For 1mM solution,

ASM = 0.02 g (analytical grade) (M.W. = 210.7)

Adjust the volume to 50 ml with 20 mM sodium phosphate buffer.

For 0.1mM working solution, take 5 ml of 1mM stock and dilute it to 50 ml with 20 mM sodium phosphate buffer

100 mM SODIUM PHOSPHATE BUFFER

1 M Na_2HPO_4 = 68.4 ml

1 M NaH_2PO_4 = 31.6 ml

Dilute the combined 1M stock solution to 1 liter with distilled water

Adjust the pH to 7.2 with HCl

PROTEIN EXTRACTION BUFFER

Tris base = 1.21 g (Final conc. = 50 mM)

NaCl = 87.75 (Final conc. = 150 mM)

Adjust the pH to 8.0 with HCl

Glycerol = 20 ml (Final conc. = 10%)

PMSF = 0.034 g (Final conc. = 1mM)

Triton-X- 100 = 0.2 ml (Final conc. = 0.1%)

Protease inhibitor cocktail tablets = 4

Adjust the volume to 200 ml with distilled water

Add 1 µl βME / 1 ml buffer

10X PHOSPHATE BUFFER SALINE

NaCl = 76 g (Final conc. = 1.3 M)

Na_2HPO_4 = 10 g (Final conc. = 70 mM)

NaH_2PO_4 = 4.1 g (Final conc. = 30 mM)

Add these chemicals in 1000 ml distilled water

For 1X working solution, Add 100 ml 10X PBS and dilute it to 1000 ml with distilled water.

1X PHOSPHATE BUFFER SALINE + 5 % TWEEN 20

Tween 20 = 50 ml in 1000 ml 1X PBS

4X SDS- PAGE (SEPARATING) GEL BUFFER

Tris = 90.85 g (Final conc. = 1.5 M)

SDS = 0.2 g (Final conc. = 0.04 %)

Adjust pH to 8.8

Adjust the volume to 500 ml

4X SDS- PAGE STACKING GEL BUFFER

Tris = 30.28 g (Final conc. = 0.5 M)

SDS = 0.2 g (Final conc. = 0.04 %)

Adjust pH to 6.8

Adjust the volume to 500 ml

10X SDS-PAGE TANK BUFFER

Tris = 30 g

Glycine = 144 g

SDS = 10 g

Adjust the volume to 1 liter

20 % APS

Ammonium per sulfate = 20 mg

Adjust the volume to 1 ml with distilled water

2X SDS-PAGE GEL LOADING DYE

1M Tris - Cl (pH 6.8) = 10 ml (Final conc. = 100 mM)

SDS = 4 g (Final conc. = 0.4%)

Glycerol = 20 ml (Final conc. = 20%)

Bromophenol blue crystal ≤ 0.2 g (Final conc. = 0.2%)

Adjust the volume to 100 ml with distilled water

Add 5 ml βMe / 100 ml dye just before use.

10X WESTERN BLOT TRANSFER BUFFER

Tris base = 30.3 g (Final conc. = 125 mM)

Glycine = 72.06 g (Final conc. = 960 mM)

For 1X solution, take 100 ml of 10X, 100 ml of methanol and 800 ml of distilled water.

Adjust the volume to 1 liter with distilled water

BLOCKING BUFFER

BSA = 3 g (Final conc. = 3%)

Dry milk = 1 g (Final conc. = 1%)

Adjust the volume to 100 ml with 1X PBS buffer

1M MAGNESIUM CHLORIDE

$MgCl_2$ = 95.21 g

Adjust the volume to 1 liter with distilled water

1M SUCROSE SOLUTION

Sucrose = 342 g

Adjust the volume to 1 liter with distilled water.

Filter the solution and store at -20°C

CHROMATOGRAPHY SOLVENT

Methanol = 800 ml (Final conc. = 80%)

TFA = 3 ml (Final conc. = 0.3 %)

Adjust the volume to 1 liter with distilled water

PONCEAU S STAIN

Ponceau S = 100 mg (Final conc. = 0.1%)

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

Adjust the volume to 100 ml with distilled water

15% SDS-PAGE GEL

Separating (Running) Gel Composition

Add the following solutions in a 15 ml tube (in order)

- Distilled Water = 1.02 ml
- 4X Separating (Running) Buffer (pH 8.8) = 1 ml
- 30 % Acrylamide (acrylamide: bis-acrylamide, 29:1) = 1.98 ml

Just before pouring the gel, add -

- APS 20% = 8 µl
- TEMED = 4 µl

Mix well by inverting or vortexing the tube

Add the above solution between the assembled BioRad mini gel plates. Immediately after pouring the gel, add water over the top of the gel solution.

Wait for 10-15 minutes for gel to polymerize.

Stacking Gel Composition (5 %)

Add the following solutions in a 15 ml tube (in order)

- Distilled Water = 1.17 ml
- 4X Stacking Buffer (pH 6.8) = 0.5 ml
- 30% Acrylamide (acrylamide: bis-acrylamide, 29:1) = 0.66 ml

Just before pouring the stacking gel, add -

- APS 20 % = 4 µl
- TEMED = 2µl

Discard the water from the top of the gel and carefully add the stacking gel solution without forming bubbles. Immediately place the comb gently and leave the gel to polymerize (20-30 minutes).

King's B MEDIUM

Protease peptone # 3 = 20 g

Potassium phosphate dibasic = 1.50 g

Magnesium sulfate = 1.50 g

Glycerol = 10 ml

Agar = 17.50 g (for solid medium)

Adjust the volume to 1 liter with distilled water

Autoclave for 30 minutes before use

VITA

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Presentations and Publications:

- Tripathi, D., Enyong, A.B., Jiang, Y.L., Kumar, D.(2009). SABP2, a Methyl salicylate esterase is required for the systemic acquired resistance induced by Acibenzolar-S- Methyl in plants (submitted to FEBS letters).
- Tripathi, D., & Kumar, D. (2009). Chemical inducers of plant defenses -Review (in preparation).
- Kumar, D., Tripathi, D., Jiang, Y.L., Enyong, A.B., Ingram, A., Manning, C.A. (2009). Characterization of the role of tobacco methyl salicylate esterase in the SAR induced by chemical elicitors. *Proceedings of plant biology, 327.*

- Tripathi, D., Jiang, Y.L., Kumar, D. (2009). Is SABP2 a receptor for Acibenzolar-S-Methyl: a chemical inducer of systemic acquired resistance in plants? Paper presented at *Phytochemical Society of North America annual meeting*.
- Tripathi, D., Jiang, Y.L., Kumar, D. (2009). Role of SABP2 in systemic acquired resistance induced by Acibenzolar-S-Methyl. Paper presented at *Appalachian Research Forum, ETSU.*
- Tripathi, D., Jiang, Y.L., Kumar, D. (2010). Functional analysis of defense responses induced by Acibenzolar-S-Methyl in plants. Talk presented at *Appalachian Research Forum, ETSU.*
- Tripathi, D., Jiang, Y.L., Kumar, D. (2010). Molecular and biochemical studies of SABP2 in defense signaling pathway induced by Acibenzolar-S-Methyl in plants**.** Talk presented at *Southern Section of American Society of Plant Biologists meeting*.

Awards:

- Member of Sigma Xi - Research Honor Society, Phytochemical Society of North America (PSNA), American Society of Plant Biologist (ASPB) and ETSU Graduate & Professional Student Association (GPSA),
- Awarded the W. H. Fraley and N. M. Fraley Memorial Scholarship for Graduate Research, 2009.
- Second prizewinner in poster competition at Appalachian Research Forum, ETSU, 2009.
- Second prizewinner in talk Competition at Appalachian Research Forum, ETSU, 2010.