Tobacco methyl salicylate esterase mediates nonhost resistance

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**A B S T R A C T**

Nonhost resistance is a type of broad-spectrum resistance exhibited by a given plant species to most strains of a pathogen which are generally pathogenic to other plant species. In this study, we have examined the role of tobacco SABP2 (Salicylic acid-Binding Protein 2) in nonhost resistance. SABP2, a methyl salicylate esterase is a critical component of SA-signaling pathway in tobacco plants. The transgenic tobacco SABP2-silenced lines treated with tetraFA, a known inhibitor of esterase activity of SABP2 exhibited enhanced susceptibility to nonhost pathogen, Pseudomonas syringae pv. phaseolicola compared to the control plants. The increased accumulation of SABP2 transcripts upon Psp infection supports the involvement of SABP2 in nonhost resistance. The tetra-FA treated plants also showed delayed expression of pathogenesis related-1 gene upon Psp inoculations. The expression of nonhost marker genes CDM1 and HIN1 was also monitored in tobacco plants infected with host-pathogen P.s. pv. tabaci and P.s. pv. phaseolicola. Overall, results presented in this manuscript suggest that SABP2 has a role in nonhost resistance in tobacco plants.

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1. Introduction

SABP2 catalyzes the conversion of methyl salicylic acid (MeSA) to SA which is a key component in the signal transduction pathway(s), leading to the activation of defense responses in plants following pathogen attack [1,2]. SABP2 displays high affinity for SA and play a crucial role in the activation of systemic acquired resistance (SAR) to plant pathogens [1]. SABP2 is known to mediate SA-mediated SAR signaling in tobacco, potato, Arabidopsis and other plants.

Nonhost resistance (NHR), shown by an entire plant species to a specific parasite or pathogen, is the most common and durable form of plant resistance to disease-causing organisms [3]. A potential plant pathogen has to overcome many barriers to become a successful virulent pathogen. Studies using SA defective NahG transgenic Arabidopsis plants suggested a role for SA in NHR resistance [4]. Further investigation using T-DNA insertion mutants in SA-signaling/biosynthetic pathways (sid2, pad4, eds5, eds1, and npr1) questioned the role of SA in NHR in Arabidopsis [5]. It was suggested that the loss of NHR in Arabidopsis NahG plants was not due to loss of SA but due to the accumulation of catechol, an SA degradation product [5]. In recent years, there is renewed interest in studying NHR and a number of recent studies have indicated the involvement of various stress signaling pathways [6–10].

The plants in their natural habitat due to an easy source of nutrition are being continuously attacked by a variety of microbial pathogens. This is being further complicated by changes in global climate. With the changes in climatic conditions, the pathogens are increasingly finding conditions more suitable for their growth and reproduction. In response to continuous pathogen attacks, complex immune systems have evolved to tackle these pathogens and overcome disease. The innate immune system in plants is divided into two main branches, host resistance and nonhost resistance depending on the adaptability and host range of the pathogen. All plants are not susceptible to all pathogens and all pathogens cannot infect and cause disease in all plants. The adaptability of a pathogen to overcome all the pre-formed chemical and physical barriers and its ability to cause a disease renders the plant “host” to that particular pathogen and the pathogen is known as a “host-pathogen”. The resistance exerted by the plant towards host-pathogen is termed “host resistance”. This form of resistance is “specific” as the host possesses the cognate R proteins to the microbial avirulent (Avr) proteins. Therefore, this type of resistance is always associated with gene-for-gene resistance. It likely involves the SA-mediated signaling followed by the expression of pathogenesis-related (PR) and other defense genes leading to disease resistance [11]. Either the absence of...
microbial *avr* gene or the host *R* gene leads to the slow activation of defenses and results in the development of disease [12].

Most plant species are resistant to most pathogens, a phenomenon termed as nonhost resistance. The pathogen that cannot evade or suppress the constitutive and inducible mechanisms and cannot cause a disease in the plant is termed as a “nonhost pathogen.” NHR is a broad-based, durable form of resistance and results from the poor adaptability of the pathogen to the physiology and growth habit of the plant. It also results from the plant’s recognition of the invading pathogen or its components by plant surveillance systems and activation of the defense responses leading to a hypersensitive response (HR) related cell death. NHR is durable because pathogens do not acquire new hosts very frequently. This feature leads to the stability of NHR. Both constitutive and inducible defense mechanisms constitute NHR [13–15].

As part of the immune responses, plants have developed active signaling pathways against these pathogens to signal the defense responses. Important among phytohormones mediating defense responses is salicylic acid (SA) [11]. Plants infected with pathogens and exhibiting resistance response showed a manifold increase in the levels of SA and increased resistance [16]. SA plays a very important role in conferring disease resistance in infected tissues (local resistance, LR) and in distal uninfected tissues (systemic acquired resistance, SAR) [17,18]. Methyl salicylic acid (MeSA) is considered as the mobile signal for SAR development from the infected tissues [19]. Increase in the MeSA levels in infected tissues is correlated with the increase in the SAR [19]. MeSA which is an inactive form of SA synthesized by salicylic acid methyltransferase (SAMT), both locally and distally, is converted back to SA by salicylic acid-binding protein 2 (SABP2) [19–21]. It is a soluble protein with esterase activity present in very low abundance (10 fmol/mg). It exhibits high affinity for SA (Kₐ = 90 nM) and has a molecular weight of 29 kDa [1]. Previously conducted studies have shown that silencing of SABP2 compromises LR as well as SAR upon pathogen infections [1].

Recently, SA, a key signaling molecule, is presumed to play a role in NHR. *Arabidopsis* is a nonhost for cowpea rust fungus (*Uromyces vignae*) and hence restricts the growth of this fungus. *Arabidopsis* mutant sid2, which is defective in ICS1 (Isocitrstrate synthase 1), an important enzyme in the biosynthesis of SA, supports the growth of *Uromyces vignae* indicating that the SA pathway is required for NHR [22]. Mutation in *Arabidopsis* *EDSI* (enhanced disease susceptibility 1) in *Ws-0* ecotype, an important activator of SA signaling, resulted in the enhancement of sporulation by *Hyaloperonospora arabidopsidis* (downy mildew), which is a nonhost pathogen in *Arabidopsis* (*Ws-0* ecotype) when compared to the wild-type plants [23]. Previous experiments showed that SA accumulated in *Pseudomonas syringae* pv. *phaseolicola* challenged wild-type tobacco plants indicating a relationship between NHR and SA [5].

SABP2 converts MeSA into SA that is responsible for downstream signaling may also have a role to play in the NHR. In this study, the role of SABP2 in NHR is being investigated. For this study, transgenic tobacco lines [1,2] silenced in SABP2 expression were used [1]. As a control, C3 lines with empty vector was used. These transgenic plants were infected with tobacco nonhost pathogen *Pseudomonas syringae* pv. *phaseolicola* NPS5121 (*Psp*) to study NHR. These results were then compared to the effect of host pathogens *Pseudomonas syringae* pv. *tabaci* (*Pst*) on C3 and 1-2 plants. Changes in the expression levels of previously reported nonhost resistant genes such as *Cell Death Marker 1* (*CDM1*) and *Harpin Induced 1* (*HIN1*), defense-related gene like *Pathogenesis-Related 1* (*PR1*) and a critical gene in SA signaling, SABP2 was monitored and studied.

### 2. Materials and methods

#### 2.1. Reagents, plant materials, pathogen inoculations

Most reagents were obtained from Sigma-Aldrich and Fisher Scientific. 2,2,2,2′-tetra FA was obtained from Rieke Metals, Inc (Lincoln, NE). Oligonucleotide primers were synthesized through Fisher Scientific. Reagents for RT-PCR were obtained from Promega and Invitrogen.

Two transgenic lines of tobacco (*Nicotiana tabacum* cv. Xanthi nc (NN)) were used in this study. Transgenic line C3 contained empty silencing vector (pHANNIBAL) and line 1-2 in which SABP2 expression is silenced by RNA interference [1]. Seeds of these tobacco lines were sown in soil containing peat moss (Fafard F-15, Agawam, MA) and allowed to grow in a plant growth chamber (PGW 36, Conviron, Canada) set at 16-h day cycle maintained at 22 °C. Fully grown 6 to 8 weeks old plants were used for the experiments.

Tobacco host-pathogen, *Pseudomonas syringae* pv. *tabacii* (*Pst*) which caused wildfire disease on tobacco and nonhost pathogen *Pseudomonas syringae* pv. *phaseolicola* NPS3121 (*Psp*) which causes halo blight disease on beans were used. Both the *Pst* and *Psp* were cultured on King’s B (KB) medium at 28 °C. For *Psp* the media contained 25 μg/ml rifampicin. The bacterial cultures were suspended in 10 mM MgCl₂ to obtain a final concentration of 10⁵ for *Pst* and 10⁶ colony-forming units (CFU)/ml for *Psp* (calculated as 0.2 OD₅₆₀ = 10⁵ CFU/ml). Bacterial suspensions were then infiltrated using a needleless syringe, into the intercellular spaces of the leaves of both C3 and 1-2 plants [24].

For tetraFA treatments, leaves selected for pathogen infiltration were spray treated with 1 mM tetraFA in 10 mM Hepes, pH 7.0 solution 48 h prior to bacterial infiltrations [25]. Treated leaves were later infiltrated with bacterial suspension as described earlier.

#### 2.2. Determination of growth of bacteria in plants

To determine the growth of *Psp* in both C3 and 1-2 plants, two leaf discs from the inoculated area were punched out using a cork borer at various times post inoculations. Samples were homogenized in 1 ml of 0.1 M sucrose solution (filter sterilized) using Fast Prep-24 (MP Bio). Serial dilutions (10⁻¹ to 10⁻⁵) of each sample was prepared in 0.1 M sucrose and 20 μl of diluted sample was spotted on a KB media plate in duplicate. Bacterial colonies were allowed to grow at 28 °C (~36–48 h) and were counted to determine the colony forming units (CFU). The experiment was repeated at least three times.

#### 2.3. Isolation of total RNA and RT-PCR analysis

Samples from the inoculated leaves were collected at 1.5, 3, 6, 9, 12, 24, 48, 72 h-post-inoculation (hpi) and used for RNA isolation. Total leaf RNA was isolated using Tri-Reagent (Sigma) following manufacturer’s instructions. First-strand complementary DNA (cDNA) was synthesized using 1 μg of total RNA. RT-PCR analysis was performed by using 1 μl of cDNA in a 10 μl PCR reaction mixture. The PCR amplifications of *CDM1* (Fwd-5′-CTCGACGTTTTCCAAAGCA3′ and Rev-5′-TTATTCG GCCAATGTTGC3′), *HIN1* (Fwd-5′-GACCGATCGCCGAAT- CCAAT3′ and Rev-5′-GCTACAACTGAGTTGCTGG3′), *SABP2* (Fwd-5′-TGGCCCAAGATTCTGGGC3′ and Rev-5′-AGATGACTGTTGATATATG3′) and *PR1* (Fwd-5′-GATGCC ATAACACAGTTCG3′ and Rev-5′-TTTACAGATGTTCTTCTCAG3′) an annealing temperature of 55 °C for 35, 33 and 30 cycles respectively. Samples were analyzed by agarose gel electrophoresis.
Results no significant difference in the growth of Psp in SABP2-silenced [1,2] plants when compared to control (C3) plants (Fig. 1A).

Similarly, both these transgenic lines, C3 and 1–2 were also inoculated with the host-pathogen Pst (10^6 CFU/ml). Although both C3 and 1–2 tobacco lines showed enhanced growth (almost a log higher) of Pst compared to Psp but no significant difference was observed (Fig. 1B). Both the transgenic lines C3 and 1–2 showed sustained growth of Pst until 7th day (last time point tested in this experiment). This experiment was repeated more than three times with similar results.

3.2. Differential expression of CDM1 and HIN1 gene in Psp infected SABP2-silenced [1,2] plants compared to the Pst-infected plants

To study the expression of nonhost marker genes, CDM1 and HIN1 both the SABP2-silenced [1,2] and control (C3) plants were inoculated with Psp and Pst. Samples were collected at various time points, total RNA isolated and first strand cDNA synthesized as described in the methods section.

PCR amplification was conducted to analyze the expression of nonhost marker CDM1 gene. In the Psp infected C3 plants, CDM1 expression peaked between 9 and 48 hpi and reduced at 72 hpi (Fig. 2A). In the SABP2-silenced [1,2] plants the expression of CDM1 showed similar pattern except that it peaked between 9 and 24 hpi (Fig. 2A).

The expression of CDM1 in host-pathogen, Pst-infected plants showed slightly different results when compared to Psp infected plants. Following inoculation with Pst, sustained increased expression of CDM1 was observed between 6 and 72 hpi in control (C3) and SABP2-silenced [1,2] plants (Fig. 2B). These results show that expression of CDM1 was not significantly affected in SABP2 silenced [1,2] plants when compared to control (C3) plants infected with Pst.

In Psp infected SABP2-silenced [1,2] and control (C3) plants, there was no significant difference in the timing and the pattern of HIN1 expression between the two plant types. Enhanced expression was observed starting 1.5 hpi and reduced at around 48 and 72 hpi in both control (C3) and SABP2-silenced [1,2] plants (Fig. 2A). In the case of Pst-infected plants, the expression of HIN1 peaked between 9 and 12 hpi whereas it’s expression reduced almost to basal levels by 48 hpi in both C3 and SABP2-silenced [1,2] plants (Fig. 2B).

The expression of SABP2 was also monitored upon infection by both host and nonhost pathogens. The C3 plants showed sustained expression of SABP2 transcripts upon infection by both Psp and Pst (Fig. 2A, and B). In the SABP2-silenced [1,2] plants, the expression of SABP2 in both the Psp and Pst-infected plants was much weaker compared to the C3 plants. The expression level of SABP2 transcripts started to increase at 3 hpi with a peak at 24 hpi in Psp infected SABP2-silenced [1,2] plants (Fig. 2A). In Pst-infected SABP2-silenced [1,2] lines, the expression of SABP2 was highest between 9 and 12 hpi (Fig. 2B). Clearly, the levels of expression of SABP2 in 1–2 plants was lower compared to C3 plants in both Psp and Pst-infected plants. Significantly, there was increased expression of SABP2 in SABP2-silenced [1,2] lines upon both Psp and Pst inoculations.

Next, we tested the expression of defense gene PR-1 in both plant types upon infection by Psp and Pst. Expression of PR1 was delayed by 12 h in Pst-infected SABP2-silenced [1,2] plants whereas it was delayed by 3 h upon Psp infection when compared to PR1 expression in control (C3) plants (Fig. 2A and B). The expression pattern of PR1 is similar in both SABP2-silenced [1,2] and control (C3) plants when infected by Pst. PR1 expression in Psp infected control (C3) plants showed up at 9 hpi whereas it occurred much later at 12 hpi in Pst-infected control (C3) plants (Fig. 2A and B).

2.4. In-silico analysis of SIP423

SIP423, an SABP2-interacting protein was identified in a yeast-two hybrid screen using SABP2 as a bait and total tobacco cDNA library as prey. The partial sequence of SIP423 was used to search NCBI database for similar proteins. SIP423 showed high homology to glycolate oxidase (GOX) like enzymes for various plants. Both the NCBI and Solanaceae database were searched to obtain full-length nucleotide sequence of SIP423. The full-length nucleotide sequence was translated into an amino acid sequence which was further used to search for similar proteins in NCBI database by BLAST analysis [26]. Multiple sequence alignment using Clustal Omega was performed to determine the similarities between the translated amino acid sequence of SIP423 and other similar proteins [27]. The 3-D structure of SIP423 was predicted by using I-TASSER [28].

3. Results

3.1. Growth of nonhost pathogen Psp and host-pathogen Pst in SABP2-silenced [1,2] plants

To determine if SABP2 has any role in supporting/resisting the growth of Psp, a nonhost pathogen, the 1–2 (SABP2-silenced) and C3 (control) transgenic lines were inoculated with Psp (10^6 CFU/ml). Inoculum concentration of 10^6 CFU/ml for Psp was used because it was the highest concentration at which the plant did not show cell death/necrosis. The 1–2 transgenic lines did not show any significant difference in the growth of Psp compared to the C3 lines. At 7 days post infection, both the C3 and 1–2 lines showed decreased the growth of Psp. This is most likely due to overall tissue necrosis.

Fig. 1. Effect of SABP2-silencing on the growth of host and non-host bacterial plant pathogens. A. SABP2-silenced [1,2] and control plants were syringe infiltrated with Ps. pv. phaseolicola and its growth monitored over seven (1, 3, 5 and 7) days after inoculations. The graph shows the average bacterial count (CFU/ml) at each time point. B. SABP2-silenced [1,2] and control plants (C3) were syringe infiltrated with Ps. pv. tabaci and its growth monitored over seven days after inoculations. The graph shows the average bacterial number post inoculation. Error bars represent the standard deviation among 20 replicate samples.
3.3. PR1 expression and growth of Psp upon infection of tetra-FA treated SABP2-silenced [1,2] plants

Since the increase in SABP2 transcript levels upon pathogen (Psp and Pst) infection was observed in SABP2-silenced 1-2 plants, these were treated with 2,2,2,2′-tetra-fluoroacetophenone (tetraFA), a strong inhibitor of esterase activity of SABP2 [25]. The growth of Psp and the expression of defense marker gene PR1 was monitored in Psp inoculated, tetraFA untreated or treated (48 h prior to Psp inoculations) C3 and 1-2 plants. The growth of Psp in tetraFA treated 1-2 plants was significantly affected in SABP2-silenced [1,2] plants when compared to control (C3) plants (Fig. 3A). The tetraFA treated SABP2-silenced lines showed significant (almost a log difference) increase in growth of Psp compared to control (C3) plants. The expression of PR1 gene in tetraFA treated 1-2 plants was compromised both in timing and magnitude of expression. The PR1 transcript level started to increase gradually after 12 hpi in the tetraFA treated 1-2 plants (Fig. 3). This was significantly different compared to 1-2 plants that were not treated with tetraFA in which the expression of PR1 peaked at 12 hpi.

Fig. 2. RT-PCR expression analysis of defense genes in SABP2-silenced tobacco plants infected with nonhost P. phaseolicola and host P. tabaci pathogens. A. Agarose gel showing expression of programmed cell death-related genes, CDM1, HIN1 in C3 and 1-2 plants infected with P. phaseolicola. Leaf samples were collected at various time points (as indicated) following inoculation with pathogens. Apart from the non-host resistance genes, expression of SABP2 and PR1 was also monitored. Actin was used as loading control. B. Expression of CDM1, HIN1, SABP2 and PR1 in C# and 1-2 plants infected with P. tabaci. The experiment was repeated three times. The result presented here accurate representations of all experiments.

Fig. 3. SABP2-silenced plants treated with tetraFA show enhanced susceptibility to nonhost pathogen P. pv. phaseolicola. A. The graph shows the average bacterial number (CFU/ml) of Psp in control, C3 and tetra-FA treated SABP2-silenced, 1-2 plants. Plants were treated with tetra FA 48 h prior to inoculation with the pathogen. B. Agarose gel showing expression of PR1 gene in C3 and 1-2 plants infected with P. phaseolicola. Plants were pretreated with tetraFA as described above. Leaf samples were collected at various time points (as indicated) following inoculation with pathogens. Actin was used as loading control.
Fig. 4. Multiple sequence alignment of SIP423 with similar proteins. Translated amino acid sequence of SBIP-423 used to search for similar proteins in NCBI database. Multiple sequence alignment was performed using CLUSTAL O(1.2.2). Protein sequences used in the alignments are SIP423 (XM_016622629), Nb-GOX; Nicotiana benthamiana GOX (HQ110098), So-GOX, Spinacia oleracea (P05414.1), At-GOX1 (At3g14420), At-GOX2 (AT3G14415.2) and At-GOX3 (At4g18360).

Fig. 5. Predicted structure of SIP423. A & B. SBIP-423 shows high structural similarity with Spinach GOX. C. Human glycolate oxidase in complex with glycolate. The SIP423 structure was predicted using I-TASSER.
3.4. SIP423 an SABP2-interacting protein is putative glycate oxidase

SIP423 was identified as an SABP2-interacting protein in a yeast two-hybrid screen using tobacco SABP2 as a bait. SIP423 was partially characterized by in-silico analysis and was found to be a putative glycate oxidase. Its shows strong homology to glycate oxidase from Nicotiana benthamiana and Arabidopsis GOX1, GOX 2 and GOX 3 ([Fig. 4]). The predicted structure of SIP423 showed similarity to the spinach glycate oxidase ([Fig. 5]). Its structure was also compared to human glycate oxidase ([Fig. 5]).

4. Discussion

SA, synthesized by catalytic conversion of MeSA to SA by SABP2, likely plays role in downstream signaling resulting in the expression of SA-related defense genes [2]. The signal for an increase in the synthesis of SA comes from the plant resistant (R) proteins on recognition of pathogen-encoded avirulent (Avr) proteins. This results in activation of gene-for-gene resistance involving strengthening of basal defenses such as cell-wall depositions of callose and lignins, transcriptional activation of PR genes resulting in the production and accumulation of lytic enzymes such as chitinases, glucanases, and proteases, production of anti-microbial proteins like defensins, antimicrobial secondary metabolites like phthalexins, reactive oxygen species and nitric oxide and activation of mitogen-activated protein kinases (MAPK) signaling cascades. This ultimately results in the hypersensitive response (HR) leading to programmed cell death [29–34]. Therefore, activation of SA-mediated defense signaling, which involves SABP2, is often related to gene-for-gene resistance [35]. The main objective of this research was to determine if SABP2 has a role in the defense signaling pathway against nonhost pathogens.

To determine the effect of SABP2 on the growth of the nonhost pathogen, its growth in control (C3) and SABP2 silenced [1,2] tobacco plants was studied ([Fig. 1]). There was no significant difference in growth of Psp growth observed in 1-2 plants compared to C3 plants ([Fig. 1A]). At times a two-fold difference was observed in 1-2 plants compared to the C3 plants (data not shown). To determine if SABP2-silenced plants continued to express very low levels of SABP2 (due to RNAi-mediated silencing) or if there was any significant change in its expression upon infection by nonhost pathogen, P. s. pv. phaseolicola, the expression of native SABP2 was analyzed by RT-PCR. Results showed low levels of SABP2 transcripts in the uninfected C3 plants and it gradually increased on pathogen inoculation over time. As expected there was no detectable expression of SABP2 in 1-2 plants in uninfected (0h) plants but upon inoculation with pathogens, there was a significant increase in the level of SABP2 transcripts ([Fig. 2A and B]). This was a surprising observation but it did explain the likely reason for the insignificant difference in growth of pathogens in 1-2 lines compared to C3 plants during pathogen growth experiments. These results suggest that SABP2 silencing becomes less effective in transgenic 1-2 plants when infected by pathogens and the growth experiment results could have been different in the complete absence of SABP2 in 1-2 plants even after pathogen infection.

Upon pathogen infection, there is an accumulation of SA at the site of infection and it is linked with the expression of the defense-related genes and HR-assisted cell death [36,37]. So, attempts were made to examine the expression of defense genes such as HIN1 and CDM1 in relation to SABP2-mediated SA signaling. Harpins induce the expression of HR-related cell death genes such as HIN1 [38] and hs203 [39]. HIN1 expression in C3 and 1-2 plants was monitored post infection with Psp, and Pst. The results revealed no significant difference in its expression in the absence of SABP2 when compared to control (C3) plants ([Fig. 2]). This suggests that SABP2 and SA synthesized via SABP2 have no role in HIN1 expression upon pathogen infection. Our results were consistent with the previous experiments where tobacco leaves sprayed with SA did not show any increase in the expression of HIN1 [38]. In tobacco plants infected with Ralstonia solanacearum, which is a nonhost pathogen on tobacco and a close relative of Pseudomonas, it was also found that HIN1 gene was activated independently of the SA signaling pathway [40]. Harpin’s bind to the plasma membrane triggering the pH shift and rapid increase in the cytosolic calcium levels which initiates the oxidative burst [41–43]. These events activate the MAPKs such as SIPK (SA-induced protein kinase) and WIPK (wound-induced protein kinase) which signal downstream leading to the expression of HIN1 [42,43]. Another possibility, according to previous reports, could be that the biotic stress caused by the harpins may result in the accumulation of a perimene, a polyanime which induces the expression of HIN1 by activating the MAPK pathway [44,45]. Strengthening these reports, in this study, 1-2 plants with lower expression level of SABP2 showed similar expression levels of HIN1 as in control C3 plants that exhibit a stronger expression of SABP2 ([Fig. 2]). This suggests that HIN1 gene expression may be due to a separate signaling pathway induced by the harpins from the pathogens, therefore HIN1 gene expression is independent of SABP2. On the other hand, according to the previous reports, harpin presence enhances the accumulation of SA which is required for harpin induced HR cell death [43,46]. Previous reports demonstrated the involvement of EDS1 and NDR1 in the harpin induced resistance [46] which are usually considered to work upstream of SA and help in SA accumulation [36]. Altogether, it could be concluded that the expression of HIN1 by harpin is not induced by SA, but the signals upstream of SA are involved in the expression of HIN1 without the signals being passed through the SA pathway [38].

Harpin encoding gene from Pst lacks 326bp in the central region thus making it defective in activating the defense responses. Recombinant harpins which resemble harpins from Pst failed to elicit HR in tobacco [47]. Therefore, HIN1 expression induced by Pst in our studies may be due to some other effector molecules but may not be by harpins. Studies conducted on Pst flagellin showed that HIN1 was expressed in tobacco leaves when infiltrated with polymerized flagella from Pst [48]. Expression levels of HIN1 upon nonhost pathogen infection are higher compared to host pathogens, Pst ([Fig. 2B]). Hence, the expression profile of HIN1 can be a marker to distinguish between host and NRH.

CDM1 expression was monitored in C3 and 1-2 plants inoculated with Psp and Pst. Strong expression of CDM1 was observed from 9 to 48 hpi in C3 plants infected with nonhost pathogen, Psp whereas the expression pattern slightly differed in the case of host-pathogen, Pst. Strong CDM1 expression in Pst-infected C3 plants was observed at 9 hpi and lasted until 24 hpi ([Fig. 2B]). The pattern of CDM1 expression in 1-2 plants infected with Psp or Pst revealed no significant difference when compared to the C3 plants. CDM1 was previously reported to be associated with the HR related cell death during incompatible interaction between avirulent pathogen and tobacco plants [49]. The expression of CDM1 against the nonhost pathogens was similar whereas its expression differed against the host-pathogen, Pst. The results presented in this manuscript suggests that the expression of CDM1 is either SABP2-independent or it is not expressed through the SA-mediated signaling pathway. Previous studies conducted to see if the expression is due to the signaling molecules like SA or JA, failed as there was no change in expression of NtCDM1 in response to SA and MeJA treatments of tobacco leaves [49]. HIN1 transcripts accumulated much earlier than CDM1 which shows that there is a possibility that induction of these two HR-related genes
is driven by two separate signaling pathways. Supporting evidence for this could be the involvement of biotic stress in the expression of these two genes. Previous studies reported that biotic stress caused by oxidative burst, upon pathogen attack, has no role in NgCDMI induction whereas HIN1 expression could be triggered by biotic stress which suggests that the signaling pathways leading to the expression of these two genes must be different [49]. In this study, it was observed that mock treatment of the plants also induced the expression of CDMI and HIN1 although the pattern of expression was different when compared to the pathogen infected plants (data not shown). This suggests that stress created by infiltration may have affected the gene expression.

The expression level of SABPB upon pathogen infection was examined in both C3 as well as 1–2 plants. SABPB, which is a critical protein in the SA-mediated signaling pathway, showed a gradual increase in expression in nonhost pathogen Psp infected C3 plants. This suggests that SABPB is activated upon nonhost pathogen infection. Surprisingly 1–2 plants which are silenced in SABPB also showed increased SABPB expression but at very low levels compared to C3 plants. Levels of SABPB expression in 1–2 plants at 24 hpi was comparable to basal gene expression (0 hpi) in C3 plants (Fig. 2A, B). This suggests that the RNAi gene silencing machinery is incapable of silencing the increased SABPB transcripts that produced in response to pathogen infection. Although, increased level of SABPB transcripts started accumulating as early as 1.5 hpi but its peaked at 24 hpi in both Psp and Pst-infected C3 and 1–2 plants and then started reducing by 48 and 72 hpi (Fig. 2A and B). This raises a doubt if SABPB expression is transient or temporal or may be its transcription is inhibited by certain inhibitors after reaching certain levels, in order to control the levels of SA in the cell as high levels of SA can be toxic to the cell organelles and their function. The expression pattern of SABPB gene against the nonhost pathogens is almost similar to its expression against host pathogens in C3 plants.

The expression of defense gene PR1 serves as a marker indicating the activation SA signaling pathway. PR1 expression was observed in both pathogen-inoculated C3 and 1–2 plants. In 1–2 plants the PR1 expression was delayed by ~3 h in both Psp and Pst-infected plants (Fig. 2A and B). If the delayed response of PR1 in 1–2 plants had any impact on the growth of Psp, during pathogen growth experiments, is unknown and will be an interesting study. Surprisingly PR1 was also expressed in 1–2 plants which had diminished levels of SABPB. The timing of SABPB expression and PR1 expression does not overlap which suggest that PR1 expression may have induced through other alternative signaling pathways which are normally repressed by SA thereby resulting in the different pattern of PR1 expression in 1–2 plants. The possibilities could be due to the activation of several defense pathways against pathogen infection. The SABPB that was expressed in 1–2 plants may have been sufficient for the expression of PR1 or may be activated by MAPK cascade induced by harpins from the pathogens. It was observed from the previous studies that cultured tobacco cells accumulated the transcripts of PR genes such as PR1, PR2, and chitinase (PR5) when treated with harpins from Psp [42]. In addition to this, JA, another phytohormone that also takes part in the defense signal transduction acting antagonistically to the SA, also mediate expression of PR genes (PR1b, PR5, and PR6 genes) [50]. In normal conditions upon pathogen attack SA represses the JA signaling of some defense responses. So, under low SABPB levels in 1–2 plants, JA might get activated to result in the expression of defense genes. Spermine also induced the expression of PR1 in SA-independent manner when the tobacco leaves were exogenously applied [45]. Altogether, the expression of PR1 in 1–2 plant treated with pathogens can be attributed to the signaling by SABPB or MAPK or JA or Spermine. However, the exact mechanism through which these molecules activate the PR1 is not known.

The experiments conducted using tetraFA, which is an inhibitor of SABPB activity, resulted in further delay in PR1 expression in tetraFA treated, Psp infected 1–2 plants than in untreated, Psp infected 1–2 plants (Fig. 3B). Also, the level of expression was also decreased when compared to untreated, Psp infected 1–2 plants (Fig. 3B). The pathogen growth assay showed that the growth of Psp in tetraFA treated 1–2 plants was significantly affected compared to the control (C3) plants (Fig. 3A). This clearly demonstrates that SABPB plays an important role in providing resistance against nonhost pathogens.

It is highly likely that other proteins that may be interacting with SABPB which regulate or controls its downstream signaling activity. In a yeast-two hybrid screen using SABPB as a bait, SIP423 (SABPB-interacting protein 423) was identified as an interacting protein (Kumar et al. unpublished). In-silico analysis showed that SIP-423 is a putative glycolate oxidase (GOX). It shows high sequence similarity with known GOX from N. benthamiana and Arabidopsis (GOX1, 2 and 3) (Fig. 4). SIP-423 shows high structural similarity with spinach and human glycolate oxidases (Fig. 5). Interestingly Nicotiana benthamiana plants silenced in GOX expression showed an increase in the growth of nonhost pathogens P. syringae pv tomato strain T1, P. syringae pv glycinea, and X. campestris pv vesicatoria [6].

NHR was also compromised in Arabidopsis GOX mutants against nonhost pathogens P. syringae pv tabaci and P. syringae strain B728A [6]. It is highly possible that SIP–423 might be playing an effective role in mediating NHR by interacting with SABPB. This need to be studied in future.

To summarize, we have demonstrated a clear role for SABPB2 in NHR. We further hypothesize that SABPB2 likely modulates tobacco NHR through its interactions with SIP423, a glycolate oxidase-like protein. Further investigation is required to explore this hypothesis.

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