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Tobacco methyl salicylate esterase mediates nonhost resistance☆

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ABSTRACT

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.

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

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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 system 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 multifold 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 (K_d = 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 (Isochorismate 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 EDS1* (*enhanced disease susceptibility 1*) in Ws-0 ecotype, an important activator of SA signaling, resulted in the enhancement of sporulation by *Hyaloperonospora arabidopsis* (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* NPS3121 (*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. *tabaci* (*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 $MgCl_2$ to obtain a final concentration of 10^5 for *Pst* and 10^6 colony-forming units (CFU)/ml for *Psp* (calculated as 0.2 OD_{600} = 10^8 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′CTCGACGTTTTTCAAGCACA3′ and Rev-5′TTAATTCCGCCAGTGGTG AC3′), *HIN1* (Fwd-5′GAGCCATGCCGGAATCCAAT3′ and Rev-5′GCTACCAATCAAGATGGCATCTGG3′), *SABP2* (Fwd-5′TGGCCCAAAGTTCTTGGC3′ and Rev-5′AGAGATCAGTTGTATTTATG3′) and *PR1* (Fwd-5′GATGCCC ATAACACAGCTCG3′ and Rev-5′TTTACAGATCCAAGTTCTTCAGA3′) an annealing temperature of 55 °C for 35, 33 and 30 cycles respectively. Samples were analyzed by agarose gel electrophoresis.

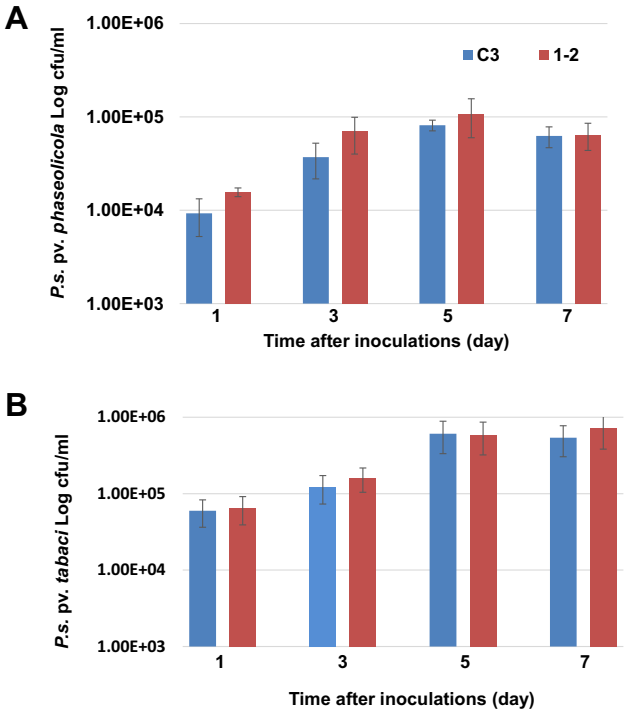

Fig. 1. Effect of SABP2-silencing on the growth of host and non-host bacterial plant pathogens. A. SABP2-silenced [1,2] and control plants were syringe infiltrated with *P.s. pv. phaseolicola* and its growth monitored over seven (1, 3, 5 and 7) days after inoculations. The graph shows the average bacterial count (CFU/ml) at each time point. B. SABP2-silenced [1,2] and control plants (C3) were syringe infiltrated with *P.s. 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.

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. 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* (10^5 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).

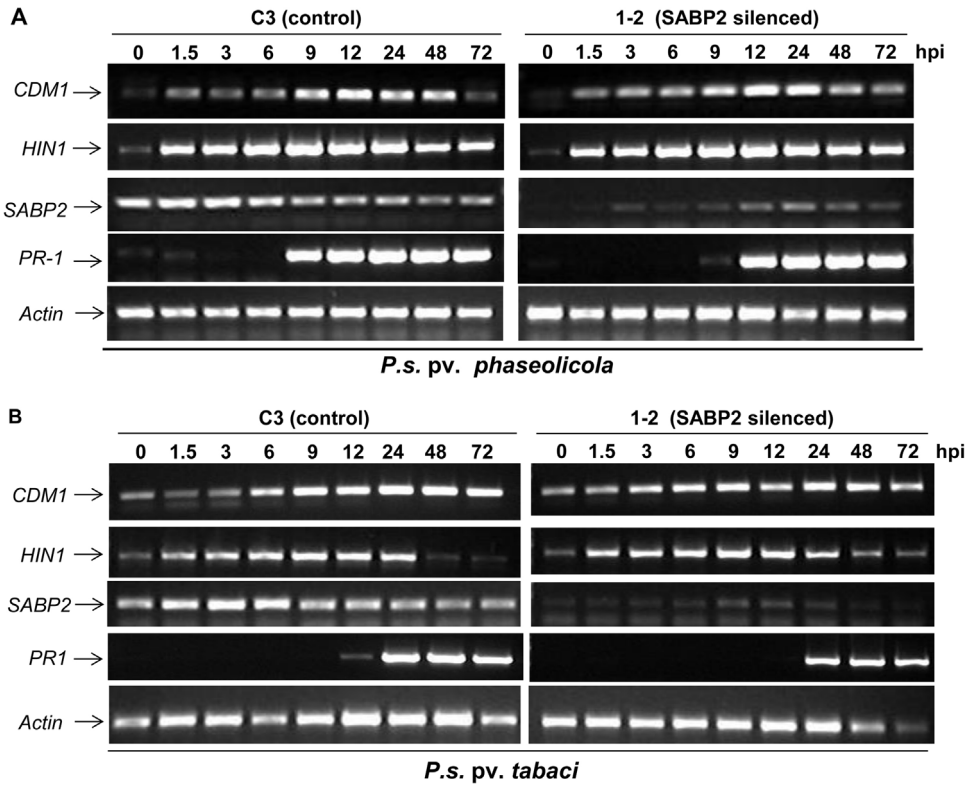

Fig. 2. RT-PCR expression analysis of defense genes in SABP2-silenced tobacco plants infected with nonhost *P.s. phaseolicola* and host *P.s. tabaci pathogens*. A. Agarose gel showing expression of programmed cell death-related genes, *CDM1*, *HIN1* in C3 and 1-2 plants infected with *P.s. phaseolicola.* 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 loading control. B. *Expression of CDM1, HIN1, SABP2 and PR1 in C# and 1-2 plants infected with P.s. tabaci.* The experiment was repeated three times. The result presented here accurate representations of all experiments.

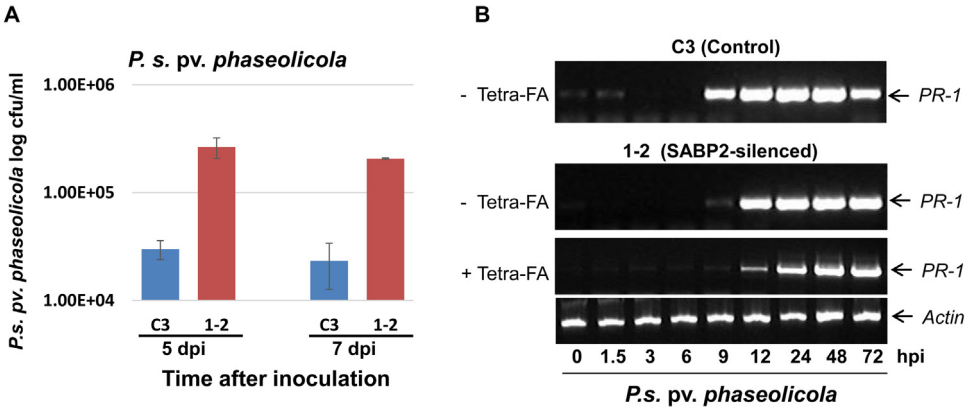

Fig. 3. SABP2-silenced plants treated with tetraFA show enhanced susceptibility to nonhost pathogen *P.s. pv. phaseolicola.* A. The graph shows the average bacterial number (CFU/ml) of *Psp* in control, C3 and tetra-FA treated SABP2-silenced, 1-2 plants. Plants were treated with tetra FA 48 h prior to inoculation with the pathogen. B. Agarose gel showing expression of *PR1* gene in C3 and 1-2 plants infected with *P.s. 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 loading control.

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.

```
AT_GOX1 -MEITNVTEYDAIAKQKLPKMVYDYYASGAEDQWTLQENRNAFARILFRPRILIDVSKID 59
At_GOX2 -MEITNVTEYDAIAKAKLPKMVYDYYASGAEDQWTLQENRNAFARILFRPRILIDVNKID 59
At_GOX3 -MEITNVTEYDAIAKAKLPKMVYDYYASGAEDQWTLQENRNAFARILFRPRILIDVNKID 59
So_GOX  -MEITNVNEYEAIAKQKLPKMVYDYYASGAEDQWTLAENRNAFSRILFRPRILIDVTNID 59
SIP423  MEEVTNVMEYEAIAKKKLPKMVFDYYASGAEDQWTLAENRNAFSRILFRPRILIDVSKID 60
N.b_GOX MEEVTNVMEYEAIAKKKLPKMVFDYYASGAEDQWTLAENRNAFSRILFRPRILIDVSKID 60
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At_GOX2 MATTVLGFKISMPIMVAPTAFQKMAHPDGEYATARAASAAGTIMTLSSWATSSVEEVAST 119
At_GOX3 MATTVLGFKISMPIMVAPTAFQKMAHPDGEYATARAASAAGTIMTLSSWATSSVEEVAST 119
So_GOX  MTTTILGFKISMPIMIAPTAMQKMAHPEGEYATARAASAAGTIMTLSSWATSSVEEVAST 119
SIP423  MSTTVLGFKISMPIMIAPTAMQKMAHPEGEYATARAASAAGTIMTLSSWATSSVEEVAST 120
N.b_GOX MSTTVLGFKISMPIMIAPTAMQKMAHPEGEYATARAASAAGTIMTLSSWATSSVEEVAST 120
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N._GOX  GPGIRFFQLYVYKDRNVVAQLVRRAERAGFKAIALTVDTPRLGRREADIKNRFVLPPFLT 180
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SIP423  LKNFEGLDLGKMDQA------SDSGLASYVAGQIDRTLSWKDVQWLQTITSLPILVKGVL 234
N._GOX  LKNFEGLDLGKMDQA------SDSGLASYVAGQIDRTLSWKDVQWLQTITSLPILVKGVL 234
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SIP423  TAEDARLAVQAGAAGIIVSNHGARQLDYVPSTIMALEEVVKAAQGRIPVFLDGGVRRGTD 294
N.b_GOX TAEDARLAVQAGAAGIIVSNHGARQLDYVPSTIMALEEVVKAAQGRIPVFLDGGVRRGTD 294
        *.****:*:* ******************:** ********:***:**************
AT_GOX1 VFKALALGASGIFIGRPVVFSLAAEGEAGVRKVLQMLRDEFELTMALSGCRSLKEISRNH 353
At_GOX2 VFKALALGASGIFIGRPVVFALAAEGEAGVKKVLQMLRDEFELTMALSGCRSLSEITRNH 359
At_GOX3 VFKALALGASGIFIGRPVVFALAAEGEAGVKKVLQMLRDEFELTMALSGCRSLSEITRNH 359
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N.b_GOX VFKALALGASGIFIGRPVVFSLAAEGEAGIKKVLQMLRDEFELTMALSGCRSLNEITRNH 354
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At_GOX2 IVTEWDTPRHLPRL--- 373
At_GOX3 IVTEWDTPRHLPRL--- 373
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SIP423  IVTEWDAPRAALPAPRL 371
N._GOX  IVTEWDAPRAALPAPRL 371
        *.::** *
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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).

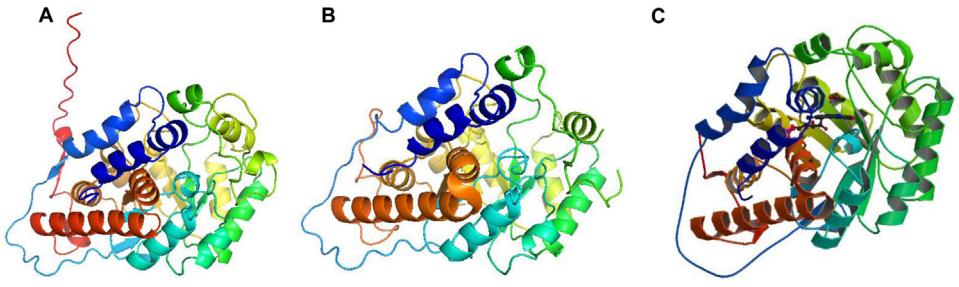

Fig. 5. Predicted structure of SIP423. A & B. SBIP-423 shows high structural similarity with Spinach GOX. C. Human glycolate oxidase in complex with glycolate. The SIP423 structure was predicted using I-TASSER.

3.4. SIP423 an SABP2-interacting protein is putative glycolate 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 glycolate oxidase. Its shows strong homology to glycolate oxidase from *Nicotiana benthamiana* and Arabidopsis GOX1, GOX 2 and GOX 3 (Fig. 4). The predicted structure of SIP423 showed similarity to the spinach glycolate oxidase (Fig. 5). Its structure was also compared to human glycolate 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 phytoalexins, 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 (0 h) 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 *hsr203J* [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 spermine, a polyamine which induces the expression of *HIN1* by activating the MAPK pathway [44,45]. Strengthening these reports, in this study, 1-2 plants with the lower expression level of *SABP2* showed the 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 326 bp 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 NHR.

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 72 hpi whereas the expression started as early as 1.5 hpi and a similar level of expression 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 *NgCDM1* 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 *NgCDM1* 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 *CDM1* 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 *SABP2* upon pathogen infection was examined in both C3 as well as 1-2 plants. SABP2, 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 *SABP2* is activated upon nonhost pathogen infection. Surprisingly 1-2 plants which are silenced in SABP2 also showed increased *SABP2* expression but at very low levels compared to C3 plants. Levels of *SABP2* 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 *SABP2* transcripts that produced in response to pathogen infection. Although, increased level of *SABP2* 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 SABP2 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 *SABP2 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 *SABP2*. The timing of *SABP2* 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 SABP2 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* (*PR3*) 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 SABP2 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 SABP2 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 SABP2 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 SABP2 plays an important role in providing resistance against nonhost pathogens.

It is highly likely that other proteins that may be interacting with SABP2 which regulate or controls its downstream signaling activity. In a yeast-two hybrid screen using SABP2 as a bait, SIP423 (SABP2-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* pv *syringae* strain B728A [6]. It is highly possible that SIP-423 might be playing an effective role in mediating NHR by interacting with SABP2. This need to be studied in future.

To summarize, we have demonstrated a clear role for SABP2 in NHR. We further hypothesize that SABP2 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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