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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.