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Benzoylsalicylic acid derivatives as defense activators in tobacco and Arabidopsis

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ABSTRACT

Systemic acquired resistance (SAR) is a long lasting inducible whole plant immunity often induced by either pathogens or chemical elicitors. Salicylic acid (SA) is a known SAR signal against a broad spectrum of pathogens in plants. In a recent study, we have reported that benzoylsalicylic acid (BzSA) is a SAR inducer in tobacco and *Arabidopsis* plants. Here, we have synthesized BzSA derivatives using SA and benzoyl chlorides of various moieties as substrates. The chemical structures of BzSA derivatives were elucidated using Infrared spectroscopy (IR), Nuclear magnetic spectroscopy (NMR) and High-resolution mass spectrometer (HRMS) analysis. The bioefficacy of BzSA derivatives in inducing defense response against tobacco mosaic virus (TMV) was investigated in tobacco and SA abolished transgenic *NahG Arabidopsis* plants. Interestingly, pre-treatment of local leaves of tobacco with BzSA derivatives enhanced the expression of SAR genes such as *NPR1* [*Non-expressor of pathogenesis-related* (*PR*) *genes 1*], *PR* and other defense marker genes (*HSR203*, *SIPK*, *WIPK*) in systemic leaves. Pre-treatment of BzSA derivatives reduced the spread of TMV infection to uninfected areas by restricting lesion number and diameter both in local and systemic leaves of tobacco in a dose-dependent manner. Furthermore, pre-treatment of BzSA derivatives in local leaves of SA deficient *Arabidopsis NahG* plants induced SAR through *AtPR1* and *AtPR5* gene expression and reduced leaf necrosis and curling symptoms in systemic leaves as compared to BzSA. These results suggest that BzSA derivatives are potent *SAR* inducers against TMV in tobacco and *Arabidopsis*.

1. Introduction

Plants have evolved both innate and acquired immunity to counter microbial pathogens by activating a variety of inducible defense mechanisms (van Loon et al., 2006). Plants develop disease resistance by two branched innate immune system. The first branch recognizes pathogen (microbe)-associated molecular patterns (PAMPs/MAMPs) through plant pattern-recognition receptors (PRR) resulting in PAMP/MAMP-triggered immunity (PTI) that can halt further colonization (Boller and Felix, 2009; Jones and Dangl, 2006). The second branch innate immune system responds to pathogen effectors either directly or through their effects on the host cellular targets. Recognition of pathogen effectors by nucleotide-binding leucine rich repeat (NBLRR) proteins encoded by disease resistance (R) genes, activate effector-triggered immunity (ETI) that accelerates and amplifies PTI response leading to induction of HR (hypersensitive response) and SAR (systemic acquired resistance) in the host (Jones and Dangl, 2006; Schwessinger and Zipfel, 2008; Zipfel, 2009). The plant genome encodes hundreds of resistance (R) proteins that allow the plants to recognize specific pathogen-derived molecules known as avirulence (avr) factors. The R-avr recognition between plants and pathogens often triggers a localized reaction at the site of infection known as hypersensitive response (HR) (Dangl and Jones, 2001; Durrant and Dong, 2004). It was demonstrated that during HR several events such as programmed cell death, production of reactive oxygen species (ROS) and synthesis of anti-microbial compounds can occur at the site of infection (Dangl and Jones, 2001; Hammond-Kosack

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and Jones, 1996). The signaling compounds generated at the primary site of infection elicit increased resistance to secondary infection in uninfected parts of the plants and this phenomenon is known as systemic acquired resistance (SAR) (Delaney et al., 1994; Ross, 1961; Ryals et al., 1994; Ward et al., 1991). It is largely considered that salicylic acid (SA) is a major contributor to the development of SAR. The development of SAR is mainly associated with the coordinate expression of a large number of defense genes in systemic parts of the plants (Ryals et al., 1995).

Salicylic acid (SA) plays a critical role in SAR induction by acting as an endogenous signal for the induction of SAR genes (Delaney et al., 1994; Dempsey and Klessig, 2012). It was also reported that the translocating, SAR-inducing signal is not SA (Vernooij et al., 1994). Reciprocal grafts demonstrated that the signal requires the presence of SA in tissues distant from the infection site to induce systemic resistance (Vernooij et al., 1994). The intermediates of SA biosynthesis and the molecular events in plants were detailed by several researchers (Dempsey et al., 2011; Lee et al., 1995; Ribnicky et al., 1998; Silverman et al., 1995; Wildermuth et al., 2001). During pathogen infection, the endogenously accumulated SA is rapidly metabolized to its conjugates such as SA-*O*-β-D-glucoside and methylsalicylate (MeSA) and subsequent studies have demonstrated that MeSA is a critical mobile signal for SAR in plants (Enyedi et al., 1992; Lee et al., 1995; Park et al., 2007). Recently, it is also reported that plants regulate SA levels by converting it to 2, 3-dihydroxybenzoic acid (2, 3-DHBA) to prevent SA over-accumulation (Zhang et al., 2013). The requirement of SA in SAR development was demonstrated in transgenic *NahG* plants that harbor a bacterial gene encoding salicylate hydroxylase, which converts SA into inactive catechol (Gaffney et al., 1993). In our previous study, we reported benzoylsalicylic acid (BzSA) as a new derivative of SA that induces SAR more potently than SA and its derivative acetyl salicylic acid (ASA) (Kamatham et al., 2016). Induction of SAR was blocked when SA methyltransferase that converts SA to MeSA was silenced in primary infected leaves, and therefore it was concluded that MeSA is a SAR signal in tobacco (Park et al., 2007).

Recently, several metabolites have been identified as SAR inducing signals that work either dependent or independent of SA accumulation (Chaturvedi et al., 2012; Dempsey and Klessig, 2012; Gao et al., 2015; Shah et al., 2014). Dehydroabietinal (DA), an abietane diterpenoid, purified from vascular sap of *Arabidopsis thaliana* leaves induced SAR through the accumulation of SA (Chaturvedi et al., 2012). The non-protein amino acid pipecolic acid (Pip) regulates SAR and basal immunity to bacterial pathogen infection and biosynthesis of Pip in systemic tissues contributing to SAR establishment (Ding et al., 2016; Hartmann et al., 2017). Azelaic acid (AzA) a putative SAR signal, mobilizes *Arabidopsis* immunity in a concentration-dependent manner (Jung et al., 2009; Wittek et al., 2014). The other SAR inducers such as Benzo (1, 2, 3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) (Gorlach et al., 1996; Lawton et al., 1996) and 2, 6-dichloroisonicotinic acid (INA) (Metraux et al., 1990; Uknes et al., 1992) induced SAR independent of SA accumulation. In contrast, probenazole (PBZ) and its active metabolite 1, 2-benzisothiazol-3 (2H)-one 1,1-dioxide (BIT) induced SAR *via* SA accumulation (Yoshioka et al., 2001). During SAR, SA activates *NPR1* gene a transcriptional coregulator that activates SA-dependent defense genes (Wu et al., 2012). The expression of *PR* genes leads to the enhancement of SAR in plants (Durrant and Dong, 2004; Ryals et al., 1996). Loss of function studies involving mutations in *NPR1* gene, showed compromised SAR and therefore unable to develop resistance to pathogen infection (Kinkema et al., 2000; Mou et al., 2003; Spoel et al., 2009). Previously, it has been reported that SA and INA derivatives are biologically active and some of them induced *PR-1* expression in tobacco plants (Conrath et al., 1995). In addition, benzothiadiazole, N-cyanomethyl-2-chloroisonicotinamide (NCI), 3-chloro-1-methyl-1H-pyrazole-5-carboxylic acid (CMPA), 3-acetonyl-3-hydroxyoxindole (AHO), glycerol-3-phosphate (G3P) induced SAR *via PR* gene expression (Chanda et al., 2011; Gorlach et al., 1996; Lawton et al., 1996; Li et al., 2008; Nakashita et al., 2002; Yasuda et al., 2003a, 2003b).

In the present study, we have chemically synthesized and characterized BzSA derivatives. Pre-treatment of BzSA derivatives (**1**–**14**) activated *NPR1* dependent SAR gene expression in systemic leaves of tobacco plants. Similarly, pre-treatment of SA- deficient *NahG* transgenic *Arabidopsis* plants with BzSA derivatives induced *PR* gene expression. Furthermore, pre-treatment of local leaves of tobacco plants with BzSA derivatives (**1**–**14**) prevented the spread of tobacco mosaic virus (TMV) infection to uninfected systemic leaves, thus reducing TMV lesion number and diameter more potently than known SAR inducers such as SA, ASA, and BzSA. Likewise, pre-treatment of these derivatives reduced leaf necrosis and curling in case of *Arabidopsis NahG* plants.

2. Results and discussion

2.1. Chemical synthesis of BzSA derivatives

In the previous study, we have purified BzSA from the seed coats of *Givotia rottleriformis* and reported as an efficient SAR inducer as compared to SA and ASA (Kamatham et al., 2016). In the present study, we have synthesized BzSA and its derivatives (**1**–**14**) with different chemical modifications (Fig. 1a and b) according to previously described method (Cheong et al., 2008). The chemical synthesis of BzSA derivatives was performed in two steps, in the first step benzoic acid with different moieties were converted into corresponding benzoyl chlorides. The commercially available various substituted precursors such as (3, 4-dimethoxy), (4-methoxy), (3, 4, 5 − tri methoxy), 4-nicotinyloxy, (4-fluoro), (6-chloronicotinolyoxy), (4-chloro), (4-bromo), (4-ido), (3-chloro), (4-trifluoro), (4-nitro), (3-bromo), (thiophenyl-2 carbonyloxy) benzoyl chlorides were purchased from Avera chemicals, India. In the second step, the acid chloride was conjugated to SA and the resultant BzSA derivatives were named according to their moieties as 2-(4-methoxy) BzSA [**1**], 2-(4-iodo) BzSA [**2**], 2- (3-chloro) BzSA [**3**], 2-(3, 4-dimethoxy) BzSA [**4**], 2- (3, 4, 5-trimethoxy) BzSA [**5**], 2-(6-chloro nicotinoyloxy) BzSA [**6**], 2-(4-nitro) BzSA [**7**], 2-(4-chloro) BzSA [**8**], 2-(4-fluoro) BzSA [**9**], 2-(4-bromo) BzSA [**10**], 2-(4-nicotinoyloxy) BzSA [**11**], 2-(thiophenyl-2-carbonyloxy) BzSA [**12**], 2-(4-trifluoromethyl) BzSA [**13**] and 2-(3-bromo) BzSA [**14**]. The unreacted substrates were removed by open silica column chromatography and the purity of BzSA derivatives (**1**–**14**) was verified using thin layer chromatography (TLC). The chemical structural analysis of purified BzSA derivatives (**1**–**14**) was performed using IR, ^{1}H and ^{13}C NMR analysis and the molecular mass of each derivative was determined by HRMS (Supplementary Figs. S1–S30). Recently, we have reported that purified BzSA from the seed coats of *Givotia rottleriformis* induced SAR in tobacco and *Arabidopsis* (Kamatham et al., 2016). None of the BzSA derivatives (**1**–**14**) are reported as SAR inducers in plants so far.

2.2. BzSA derivatives induced SAR genes expression in tobacco plants

The establishment of SAR is mainly associated with the accumulation of a large number of defense genes such as *NPR1*, *PR*, *HR* and *MAPK* genes upon pathogen infection or by exogenous application of chemical elicitors (Chaturvedi et al., 2012; Dempsey and Klessig, 2012; Kinkema et al., 2000; Mou et al., 2003; Spoel et al., 2009).