Phytochemistry xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Benzoylsalicylic acid isolated from seed coats of *Givotia rottleriformis* induces systemic acquired resistance in tobacco and Arabidopsis

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ARTICLE INFO

Article history:
Received 6 October 2015
Received in revised form 2 March 2016
Accepted 7 March 2016
Available online xxxx

Keywords:
Givotia rottleriformis (Euphorbiaceae)
Benzoylsalicylic acid
Salicyclic acid
Acetylsalicylic acid
Non-expressor of pathogenesis-related gene-1
Pathogenesis-related genes
Systemic acquired resistance
Hypersensitive response

ABSTRACT

Systemic acquired resistance (SAR), a whole plant defense response to a broad spectrum of pathogens, is characterized by a coordinated expression of a large number of defense genes. Plants synthesize a variety of secondary metabolites to protect themselves from the invading microbial pathogens. Several studies have shown that salicylic acid (SA) is a key endogenous component of local and systemic disease resistance in plants. Although SA is a critical signal for SAR, accumulation of endogenous SA levels alone is insufficient to establish SAR. Here, we have identified a new acyl derivative of SA, the benzoylsalicylic acid (BzSA) also known as 2-(benzoyloxy) benzoic acid from the seed coats of *Givotia rottleriformis* and investigated its role in inducing SAR in tobacco and Arabidopsis. Interestingly, exogenous BzSA treatment induced the expression of *NPR1* (Non-expressor of pathogenesis-related gene-1) and pathogenesis related (*PR*) genes. BzSA enhanced the expression of hypersensitivity related (*HSR*), mitogen activated protein kinase (*MAPK*) and *WRKY* genes in tobacco. Moreover, Arabidopsis *NahG* plants that were treated with BzSA showed enhanced resistance to tobacco mosaic virus (TMV) as evidenced by reduced leaf necrosis and TMV-coat protein levels in systemic leaves. We, therefore, conclude that BzSA, hitherto unknown natural plant product, is a new SAR inducer in plants.

1. Introduction

Plants respond to pathogens by inducing both local and systemic responses thus preventing pathogen multiplication and spread to systemic parts of the plant. The development of disease resistance response against pathogen infection depends on 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 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 (NB-LRR) 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). HR, a localized resistance reaction is characterized by a rapid cell death at the site of infection that prevents further spread of pathogen to systemic parts (Morel and Dangl, 1997; Wright et al., 2000). Concomitantly, a secondary systemic response spreads to the tissues distant from the initial site of infection and develop resistance to subsequent infections known as SAR (Durrant and Dong, 2004). Molecularly, HR and SAR are characterized by the coordinate expression of a large number of *PR* genes (Ryals et al., 1996; Ward et al., 1991).

SAR is a mechanism of induced defense that confers long-lasting protection against a broad spectrum of pathogens. SA-induced SAR is a well characterized response to pathogen infection in both tobacco and Arabidopsis and is associated with accumulation of PR proteins (Gaffney et al., 1993; Delaney et al., 1994). Several chemical inducers have been identified that induced SAR either dependent or independent of SA accumulation. Probenazole (PBZ) and its metabolite 1,2-benzirothiazol-3 (2H)-one 1,1-dioxide (BIT)

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http://dx.doi.org/10.1016/j.phytochem.2016.03.002
0031-9422/

induced SAR in Arabidopsis through SA accumulation (Yoshioka et al., 2001). Similarly, 2-acetonyl-3-hydroxyoindole (AHO), isolated from the extracts of *Strobilanthes cusia* exhibited enhanced resistance to TMV by stimulating the SAR-signaling pathway upstream of SA (Li et al., 2008). In contrast, chemicals such as N-cyanomethyl-2-chloroisonicotinamide (NCI), 2,6-dichloroisonicotinic acid (INA), benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) induced SAR by triggering the signaling pathway downstream of SA accumulation (Nakashita et al., 2002; Vernooij et al., 1995; Gorlach et al., 1996). Recent studies have implicated additional small metabolites in the SAR-associated rapid activation of defenses in response to subsequent exposure to the pathogens (Shah et al., 2014).

The biosynthesis of SA has been well studied in plants, which takes place through two pathways using the primary metabolite chorismate; the phenylalanine ammonia lyase (PAL) pathway and the isochorismate (IC) pathway (Dempsey and Klessig, 2012). The PAL pathway occurs *via* trans-cinnamic acid (CA) to benzoic acid (BA) and subsequent conversion to SA through a series of enzymatic reactions (Coquoz et al., 1998; Meuwly et al., 1995; Ribnicky et al., 1998; Yalpani et al., 1993). The IC pathway takes place in chloroplasts in which the chorismate is converted to SA *via* isochorismate, with isochorismate synthase 1 (ICS) being the critical enzyme (Wildermuth et al., 2001). Recently, two transcription factors, NTM1-LIKE-9 (NTL9) and CCA1 HIKING EXPEDITION (CHE), were reported as activators of ICS during specific immune responses (Zheng et al., 2015). Although relative contributions of PAL versus IC branches toward SA biosynthesis vary between different plant species, at least in Arabidopsis majority of the pathogen-induced SA appears to be derived from the ICS branch (Gao et al., 2015). Much research has been carried out to understand the mechanisms by which the SA signal is perceived in plants due to its diverse functions (Yan and Dong, 2014). Several genetic screens for mutants defective in SA responses revealed that *NPR1* (*Nonexpressor of PR gene-1*) is a key regulator of the SA signalling pathway (Cao et al., 1994, 1997; Delaney et al., 1995; Ryals et al., 1997; Shah et al., 1997). The NPR1 protein is an oligomeric protein and its monomerization and nuclear translocation is required for defense gene expression (Mou et al., 2003; Tada et al., 2008).

The contribution of SA to SAR was investigated in *NahG* transgenic plants harbouring a bacterial gene encoding salicylate hydroxylase, which converts SA to catechol and thus these plants are defective in their ability to induce SAR againt TMV (Gaffney et al., 1993). In these plants, exogenously applied SA was unable to induce SAR leading to enhanced susceptibility to pathogens suggesting that SA is essential for the establishment of SAR in healthy systemic tissue (Delaney et al., 1994; Friedrich et al., 1995; Vernooij et al., 1994). The SAR inducing activity of INA was studied in *NahG* plants and was found that INA stimulates SAR response without accumulation of SA whereas AHO stimulates SA-mediated defense response (Li et al., 2008; Vernooij et al., 1995).

The key event in the development of plant disease resistance is the timely recognition of the invading pathogens and the rapid activation of signal transduction pathways (Yang et al., 1997). Extensive research has showed that several plant mitogen-activated protein kinase (*MAPK*) cascades are involved in plant defense signalling after recognition of pathogens or pathogen-derived elicitors (Fiil et al., 2009; Meng and Zhang, 2013; Rasmussen et al., 2012; Tena et al., 2001). Two tobacco *MAPKs*, *SIPK* and *WIPK* have been shown to be expressed upon wounding and pathogen infections (Seo et al., 1995). The protein levels of MIPK3, MIPK4 and MIPK6 were reduced in Arabidopsis *NahG* plants showing that these MAPK proteins were modulated by SA level (Zhou et al., 2009). Moreover, MAPK3 and MAPK6 have been shown to lead to SA biosynthesis and the expression of *PR1* and *PR5* genes (Bartels et al., 2009).

The expression of tobacco *WRKY* genes were rapidly enhanced by TMV, SA and its biologically active analogues that are capable of inducing PR proteins and enhanced resistance (Chen and Chen, 2000). The role of *WRKY* genes in plant defense was demonstrated and their expression was associated with *SIPK* and *WIPK* (Kim and Zhang, 2004). Recent studies showed that *NPR1* also controls expression of transcription factors, such as *WRKY* transcription factors, which are required for SA-mediated transcriptional reprogramming (Pajerowska-Mukhtar et al., 2012).

The development of disease resistance is mainly associated with rapid cell death at the site of infection by synthesizing endogenous SA around the necrotic lesions, leading to the induction of several defense genes (Morel and Dangl, 1997). The hypersensitive-related (*hsr*, *hsr203*, *hsr201*, *hsr515* and *HIN1*) genes are activated preferentially during HR, and therefore used as a HR marker genes (Czernic et al., 1996; Gopalan et al., 1996; Pontier et al., 1999). The gene *HSR201* is a BAHD family of acyl-transferase induced by pathogen in tobacco (D'Auria et al., 2002). The gene sequence of *HSR201* of tobacco was almost similar to the sequence of *BEBT* gene that encodes an enzyme benzyl alcohol benzoyl transferase (BEBT) capable of synthesizing benzoyl benzoate in flowers and damaged leaves of *Clericka breweri* (D'Auria et al., 2002).

Although SAR induced by different chemicals has been studied in the last two decades resulting in the elucidation of many crucial aspects of defense mechanisms in plants SAR (Dempsey and Klessig, 2012; Gao et al., 2015; Shah et al., 2014), identification of new metabolites with SAR inducing activity might provide better insights into the precise mechanisms of SAR. In the present investigation, we identified BzSA, SA and its precursors BA, BD and CA in seed coats of *Givotia rottleriformis* Griff., a soft-wood tree species belonging to Euphorbiaceae family. Exogenous treatment of BzSA induced SAR gene expression more efficiently than known SAR inducers such as SA and ASA in tobacco and Arabidopsis *NahG* plants.

2. Results and discussion

The rationale of the present investigation was based on the HR lesions of a unknown pathogenic infection observed on the leaves of *G. rottleriformis* (Supplementary Fig. S1). We observed that the development of necrotic HR lesions were restricted even after several days of infection in the leaves of *G. rottleriformis* plants growing in the field. This important observation guided us towards SAR research. Based on these field observations, we hypothesized that there may be some metabolic compound synthesized to develop resistance in this plant. We extracted total metabolic compounds from different parts of the plants such as leaf, bark and seed coats and assessed the potentiality of these extracts in SAR induction in tobacco. We have selected a resistant tobacco variety because tobacco is a suitable model plant to study SAR, and thus treated with these extracts on local leaves and challenged with TMV in systemic leaves. Surprisingly, seed coat extract was found to be more bioactive than leaf and bark extracts as evidenced by reduction in the development of TMV-induced necrotic HR lesions in terms of number and diameter in systemic leaves of tobacco (Supplementary Table S1). The active seed coat extract was used for isolation of bioactive metabolic compounds.

2.1. Identification of benzoylsalicylic acid in seed coats of G. rottleriformis

We extracted the total seed coat compounds in methanol (MeOH) and the methanolic crude seed coat extract was fractionated by open silica column chromatography (see Experimental procedure, Section 4.3.1) and the eluted fractions (1–7) were tested

Please cite this article in press as: Kamatham, S., et al. Benzoylsalicylic acid isolated from seed coats of *Givotia rottleriformis* induces systemic acquired resistance in tobacco and Arabidopsis. Phytochemistry (2016), http://dx.doi.org/10.1016/j.phytochem.2016.03.002