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Short communication

Salicylic acid and cysteine contribute to arbutin-induced alleviation of angular leaf spot disease development in cucumber

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Abbreviations:
ARB, arbutin
ARB+PSL, plants treated with arbutin and infected
CS, cysteine synthase
CSH, cysteine
dai, days after inoculation
p-CSH, protein-bound cysteine
PAL, phenylalanine ammonia lyase
PSL, *Pseudomonas syringae* pv *lachrymans*
SA, salicylic acid
SAC, salicylic acid conjugates
SAT, serine acetyltransferase

ABSTRACT

Arbutin induced suppression of angular leaf spot disease in cucumber resulting from lower populations of *Pseudomonas syringae* pv *lachrymans* in the infected tissues. This study provides insight into mechanisms that may potentially account for this effect. In the absence of the pathogen, exogenous arbutin-induced expression of *PR1*, the marker of salicylic acid signaling, increased the content of salicylic acid and modulated the cysteine pool. This suggested that arbutin promoted cucumber plants to a "primed" state. When challenged with the pathogen, the arbutin-treated plants showed strongly reduced infection symptoms 7 days after inoculation. At this time point, they were characterized by higher contents of free and protein-bound cysteine due to higher cysteine biosynthetic capacity related to increased activities of serine acetyltransferase and cysteine synthase when compared with plants infected without arbutin treatment. Moreover, in the arbutin-treated and infected plants the contents of free salicylic acid and its conjugates were also increased, partly owing to its biosynthesis via the phenylpropanoid pathway. We suggest that arbutin-induced abrogation of angular leaf spot disease in cucumber could be mediated by salicylic acid and cysteine-based signaling.

Introduction

The plant hormone salicylic acid (SA, 2-hydroxy benzoic acid) is mainly recognized as a key signaling compound mediating defense against biotrophic and hemibiotrophic pathogens, both locally and systemically (Kumar, 2014). SA is also known to mediate some positive acclimation responses to abiotic stress (Mateo et al., 2006; Gémes et al., 2011). This hormone can regulate sugar metabolism and affect photosynthetic performance in plants (Janda et al., 2014). Moreover, it plays diverse roles in plant growth and development (Rivas-San Vicente and Plasencia, 2011). SA is derived from the shikimic acid pathway linking the metabolism of sugars and SA biosynthesis. Initially assumed to be produced in the cytoplasm by the phenylalanine ammonia lyase (PAL) pathway, SA is now considered to be synthesized preferentially in chloroplasts via the isochorismate route. In *Arabidopsis*, however, the SA signal for local resistance to *Botrytis cinerea* is generated via the PAL pathway (Dempsey et al., 2011). Once synthesized, SA is converted into conjugates (SAC), mainly SA *O*-β-glucoside, salicyloyl glucose ester, methyl salicylate and methyl salicylate *O*-β-glucoside (Vlot et al., 2009).

SA-dependent responses rely on redox signals. It has been shown that SA cooperates with reactive oxygen species and redox sensitive cellular components to induce a hypersensitive response

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Table 1
Primers used for RT-PCR.

Name	Primer's sequences
Reference gene – 18S	Ambion 18S primer pair universal kit
Investigated gene – PR1	Right: GTAAGGTCCGCCAGAGTTCALeft: CCTCAAGACTTGGTCGGTGT

and systemic acquired resistance during the plant–pathogen interaction, as well as to regulate photosynthesis and redox homeostasis in plant cells during acclimation to light (Mateo et al., 2006; Mühlenbock et al., 2008; Vlot et al., 2009; Kuźniak et al., 2013). The antioxidant enzymes catalase and cytosolic ascorbate peroxidase have been identified as SA-binding proteins and SA regulates the redox-dependent monomerization of NPR1 (Non-expressor of Pathogenesis Related genes 1) which leads to its nuclear translocation and defense gene activation (Kumar, 2014).

The redox-regulated signaling pathways in plant responses to changing environment are based on reversible posttranslational modifications such as thiol-disulfide switches and S-thiolation (Zaffagnini et al., 2012; Zagorchev et al., 2013). As glutathione constitutes the major low molecular weight thiol compound in plants, it plays a central role in redox-based regulations of the plant stress response. Recently, however, cysteine (CSH) has emerged as a critically important molecule for redox signaling underpinning stress tolerance (Álvarez et al., 2012; Couturier et al., 2013; Kuźniak et al., 2014). Cysteine synthesis is catalyzed by the cysteine synthase complex formed by two enzymes, serine acetyltransferase (SAT) and cysteine synthase [O-acetylserine (thiol)-lyase] (CS) (Wirtz and Hell, 2007).

Arbutin (hydroquinone *O*-β-D glucoside, ARB) is a phenolic compound of plant origin but its physiological role in plants remains largely unknown. ARB protects membranes and acts as an antioxidant (Ioku et al., 1992; Oliver et al., 2001). It has been suggested that, due to these properties, ARB accumulated in high concentrations in resurrection plants could contribute to their extreme resistance to environmental stress (Moore et al., 2007). Moreover, ARB as well as hydroquinone and benzoquinone originating from ARB have been shown to be involved in resistance to fire blight (Powell and Hildebrand, 1970) and in different types of allelopatic interactions (Manners and Galitz, 1985).

To gain further insight into the role of ARB in plant resistance to biotic stress, we focused on the potential of exogenous ARB to promote defense against *Pseudomonas syringae* pv *lachrymans* (PSL) in cucumber. To the best of our knowledge, the ability of ARB to elicit plant defense responses against pathogens has not been investigated. Since we previously showed that SA and protein cysteinylation mediated the benzothiadiazole-induced resistance to PSL in cucumber (Kuźniak et al., 2014), in this study the influence of exogenous ARB on PSL infection development as well as the involvement of SA and cysteine in plant defense responses were analyzed.

Materials and methods

Plant material and experimental design

Cucumber (*Cucumis sativus* L. cv. Polan) seedlings were grown hydroponically in modified Hoagland's medium as described earlier (Kuźniak et al., 2014) at 174 μmol photons m^{-2} s^{-1} (photosynthetically active radiation range 400–700 nm). Four-week-old plants were sprayed with 2.0 mM water solution of arbutin (ARB; *Sigma-Aldrich*) to the run-off point. The concentration of ARB was selected on the basis of preliminary screening experiments (data not shown). Control plants were sprayed with distilled water. Seven days after treatment abaxial surfaces of the third leaves of plants were infiltrated with distilled water (mock inoculation) or with *Pseudomonas syringae* pv *lachrymans* (PSL) suspension using a 1 ml plastic syringae (Kuźniak et al., 2014). The inoculation level was 10^7 cfu/ml. The experimental variants were as follows: (1) Control; (2) ARB-treated plants (ARB); (3) PSL-inoculated plants (PSL) and (4) ARB-treated and PSL-inoculated plants (ARB + PSL). Angular leaf spot disease development and bacterial population size in the inoculated leaves were recorded 7 days after inoculation (dai). For biochemical analyses, the inoculated leaves were taken 0, 2 and 7 dai.

Disease development and bacterial population size

The leaf area affected by disease symptoms was quantified by an algorithm run in MATLAB (Gocławski et al., 2012). The population size of *P. syringae* pv *lachrymans* in the inoculated leaves was enumerated (cfu/g FW) by the plate count method as described by Libik-Konieczny et al. (2011). For homogenization, five leaf discs (1 cm diameter) from the areas adjacent to necrotic spots were taken from each leaf. The results obtained for PSL were treated as 100%, and the data for ARB + PSL were expressed as percentage of PSL.

PR1 expression analysis

RNA was isolated from the plant material 7 dai according to standard procedure with Aurum™ Total RNA Mini Kit (*Bio-Rad*). cDNA was produced on the basis of achieved RNA after reverse transcription with the use of *T* Script cDNA. Real Time PCR was carried out with the use of Sso Fast Eva Greek Supermix (*Bio-Rad*) using the following program: (1) initial denaturation: 95 °C, 3 min; (2) denaturation with amplification: 95 °C, 15 s; (3) annealing: 55 °C, 20 s; (4) DNA extension: 72 °C, 25 s; (5) fluorescence reading: 79 °C, 1 s.

Primers for *PR*1 gene (Table 1) were designed using Protein3 program on the basis of *C. sativus PR1*1 gene sequence found in the NCBI (National Center of Biotechnology) data base. Optimal pair of primers was chosen on the basis of their: 1/length (18–22 nucleotydes), 2/melting temperature (T_m in the range between 52–58 °C, primers from the pair did not differ in melting temperature more than 5 °C), 3/percentage of GC content in the range 40–60% and even distribution of GC residues within the primers. Results were analyzed according to $2^{\Delta tc}$ method, where delta cycle threshold of cDNA from the control plants was defined as 100% transcript presence. Three biological replicates and two technical replicates were analyzed.

Quantification of SA and cysteine

SA was extracted as described by Molina et al. (2002). SA and SAC as well as CSH and p-CSH were quantified by HPLC (Kuźniak et al., 2014).

PAL activity

PAL activity was assayed as described by Skłodowska et al. (2010). One unit of PAL was defined as the amount of enzyme catalyzing the formation of 1 μmol of trans-cinnamic acid (ε = 9000 M^{-1} cm^{-1}) per minute per mg protein.