Journal of Plant Physiology 221 (2018) 66–73

Contents lists available at ScienceDirect

Journal of Plant Physiology

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

Salicylaldehyde synthase activity from *Venturia inaequalis* elicitor-treated cell culture of apple

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ARTICLE INFO

Keywords:
Apple
Cell culture
Malus domestica
Salicylic acid
Salicylaldehyde synthase

ABSTRACT

Salicylic acid (SA) is known to trigger a number of plant defense responses upon pathogen attack. It is well known that apple (*Malus domestica*) plants respond to pathogen invasion by synthesizing SA, but its biosynthesis is not well understood. In this study, we report salicylaldehyde synthase (SAS) activity from *Venturia inaequalis* elicitor (VIE)-treated cell suspension cultures of apple (*Malus domestica* 'Florina'). SAS catalyzes non-oxidative C_2-side chain cleavage of *2*-coumaric acid to form salicylaldehyde (SALD) in the presence of a reducing agent such as cysteine. The side chain cleavage mechanism was found to be very similar to that of salicylaldehyde synthase activity from tobacco and 4-hydroxybenzaldehyde synthase activity from *Vanilla planifolia* and *Daucus carota*. A basal SAS activity was observed in the non-elicited cell cultures, and a 7-fold increase in SAS activity was observed upon elicitation. In parallel to SAS activity, the level of total SA accumulation increased by 5.6-fold after elicitation compared to the untreated control cells. Elicitor treatment further resulted in an 8.7-fold increase in the activity of the phenylalanine ammonia-lyase (PAL) enzyme that preceded the peak of SAS activity and total SA accumulation, suggesting the involvement of the phenylpropanoid pathway in SA metabolism. The preferred substrate for SAS was *2*-coumaric acid (*Km* = 0.35 mM), with cysteine being the preferred reducing agent. In addition, a 1.8-fold enhancement in the SA content and 0.7-fold enhancement in the SALD content was observed when elicited cell cultures were fed with 2-coumaric acid. These observations suggest the involvement of SAS in SALD biosynthesis.

1. Introduction

Apple is the main deciduous fruit crop growing in the temperate world zone and is known for its attractive colorful texture and high nutritional value. Apple belongs to the subtribe Malinae of the Rosaceae family, which also contains a number of other valuable fruit crops (Potter et al., 2007). Importantly, several commercially grown apple cultivars are afflicted by many diseases, leading to the dramatic losses of both the trees and the fruits. Fire blight disease caused by the bacterium *Erwinia amylovora* (Thomson, 2000) and apple-scab disease caused by the fungus *Venturia inaequalis* (Jha et al., 2009; Machardy., 1996) are the two most devastating apple diseases worldwide. Over the centuries, apple plants have been mostly reproduced by the clonal propagation of single trees, thus bringing genetic uniformity that seriously affects the disease resistance potential (Gessler and Patocchi, 2007). Considering the economic importance of apple, the development of new disease tolerant cultivars is urgently required. During biotic or abiotic stress, apple plants are known to produce enhanced levels of salicylic acid (SA) (Wang et al., 2016). It has been recently reported that upon SA application, apple plants showed enhanced resistance towards *Glomerella* leaf spot disease. However, the biosynthesis of SA in apple is not well understood (Zhang et al., 2016).

SA, a C_6-C_1 phenolic metabolite, is considered to be one of the most important endogenous signaling molecules involved in triggering numerous plant defense responses, either locally or systemically (Yalpani et al., 1993; Chong et al., 2001). SA-induced local resistance is indicated by the hypersensitive reaction (HR) while in the case of systemic resistance, the signal is transported over long distances where it induces the expression of pathogenesis-related proteins (PR proteins) to trigger systemic acquired resistance (SAR) (Vasyukova and Ozeretskovskaya, 2007). Both HR and SAR are associated with the biosynthesis of SA at the site of infection and in systemic parts of the plant (Gaffney et al., 1993). The critical role of SA as a signaling molecule in the regulation of pathogenesis-induced HR and SAR is well

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https://doi.org/10.1016/j.jplph.2017.12.002
Received 11 July 2017; Received in revised form 20 November 2017; Accepted 2 December 2017
Available online 06 December 2017
0176-1617/

established (Vlot et al., 2009). However, apart from pathogen-induced SA production, a constitutive level of SA is also detected in many plant species. The constitutive SA level in different plant species is variable. Some plants such as *Arabidopsis thaliana* have a lower level of endogenous SA, while plants such as *Oryza sativa* and *Vicia faba* have a higher level of endogenous SA (Yu et al., 1997).

Despite its important role in triggering plant defense responses, the biosynthesis of SA is not well understood (Yang et al., 2015) at the level of the enzymes or the genes. Two main pathways of SA biosynthesis in plants have been hypothesized: the chorismate pathway and the phenylpropanoid pathway. The chorismate pathway of SA biosynthesis is well studied in *A. thaliana* (Wildermuth et al., 2001) and *Nicotiana tabbacum* (Catinot et al., 2008). Arabidopsis contains two isochorismate synthase (ICS) genes: *ICS1* and *ICS2* that catalyze the isomerization of chorismate to isochorismate (Widhalm and Dudareva, 2015). Isochorismate serves as the intermediate for SA biosynthesis. The *Arabidopsis* chorismate mutase (*CM1*) gene is known to possess isochorismate pyruvate lyase (IPL) activity that converts isochorismate to SA (Strawn et al., 2007). Newly synthesized SA is then exported from the plastids using the enhanced disease susceptibility 5 (EDS5) transporter (Serrano et al., 2013).

In the phenylpropanoid pathway, phenylalanine is first converted to *trans*-cinnamic acid in a reaction catalyzed by the phenylalanine ammonia-lyase (PAL) enzyme. From the *trans*-cinnamic acid, two routes have been reported for SA biosynthesis in different plant species. In the first route, *trans*-cinnamic acid undergoes direct C_2-chain cleavage to yield benzoic acid. The mechanism of the C_2-side chain shortening is either *β*-oxidative or non-*β*-oxidative as previously reported. In the *β*-oxidative pathway, *trans*-cinnamic acid is first converted to cinnamoyl-CoA (Gaid et al., 2012; Klempien et al., 2012), followed by the formation of benzoic acid (Widhalm and Dudareva, 2015; Yalpani et al., 1993). Benzoic acid is then hydroxylated at the C_2 position to yield SA. A soluble CYP 450 monooxygenase, benzoate-2-hydroxylase, catalyzing C2-hydroxylation has been reported in tobacco (León et al., 1993). In tobacco, cucumber and rice, the SA biosynthesis was proposed to proceed exclusively via free benzoic acid (Meuwly et al., 1995; Silverman et al., 1995).

However, it was demonstrated that when radiolabeled benzoic acid was fed to tobacco mosaic virus (TMV)-inoculated tobacco plants, the level of induced SA accumulation was higher than the level of substrate supplied, suggesting the involvement of other SA biosynthetic routes apart from benzoic acid-mediated biosynthesis (Yalpani et al., 1993). In the non-*β*-oxidative pathway, it was hypothesized that *trans*-cinnamic acid is converted to benzoic acid through the intermediate formation of benzaldehyde, following a mechanism similar to that of 4-hydroxybezoic acid formation in potato tubers (French et al., 1976) and *Daucus carota* (Sircar and Mitra, 2008) and 4-hydroxybenzaldehyde synthesis in the cell cultures of *Vanilla planifolia* (Podstolski et al., 2002). However, it was later demonstrated that application of benzaldehyde to tobacco plants caused many-fold enhancements in the SA level, and upon the application of radio-labeled benzaldehyde, the radioactivity moved to SA via benzoic acid (Ribnicky et al., 1998). In contrast, the application of radiolabeled *trans*-cinnamic acid showed no radioactivity incorporated into benzaldehyde which raised questions about the involvement of benzaldehyde in SA biosynthesis (Jarvis et al., 2000; Malinowski et al., 2007). In the second route, *trans*-cinnamic acid is first hydroxylated to *2*-coumaric acid which then undergoes oxidative or non-oxidative C_2-side chain shortening. In the oxidative mechanism, 2-coumaroyl-CoA undergoes oxidative C_2-side chain cleavage to form SA as reported in *Gaulteria procumbens* (El-Basyouni et al., 1964) and *Agrobacterium tumefaciens*-infected tomato seedlings (Chadha and Brown, 1974). In the non-oxidative route, *2*-coumaric acid was proposed to be converted to SA through the intermediate formation of salicylaldehyde. Such conversion of *2*-coumaric acid to salicylaldehyde was reported in *N. tabacum* involving salicylaldehyde synthase activity (Malinowski et al., 2007). The next enzyme involved in the conversion of salicylaldehyde to SA is still elusive. Until now, information on salicylaldehyde synthase activity has been unavailable.

In this paper, we report VIE-induced accumulation of salicylic acid in apple (*Malus domestica* cv. Florina) cell cultures along with the detection and biochemical characterization of salicylaldehyde synthase (SAS) activity catalyzing the conversion of *2*-coumaric acid to salicylaldehyde. The biochemical properties of SAS were studied using partially purified protein. Changes in the SAS activity were correlated with phenylalanine ammonia-lyase (PAL) activity and total SA accumulation.

2. Materials and methods

2.1. Plant material and chemicals

Apple cultivar 'Florina' (*Malus domestica* cv. Florina) was obtained from the Central Institute of Temperate Horticulture (CITH), Srinagar, India. Plants were kept under temperate conditions in a greenhouse (temperature 20–22 °C and relative humidity of 65–70%). The reagents used in the sample preparation were analytical grade, and all solvents used for the High-performance liquid chromatography (HPLC) analyses were HPLC grade. All authentic standards were obtained from Sigma-Aldrich Chemical Co. Ltd. (India).

2.2. Induction and maintenance of cell suspension cultures

Apple cell suspension cultures were derived from the young leaves (8–10 days old) of greenhouse grown plants as described previously (Sarkate et al., 2017). Cultures were grown at 26 °C in the dark under continuous shaking at 120 rpm. The cells were harvested by vacuum filtration on the 7th day.

2.3. Elicitor preparation and treatment

Venturia inaequalis elicitor was used in this study. The strain of *V. inaequalis* (MTCC No.: 1109) was purchased from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. *V. inaequalis* elicitor (VIE) was prepared from fungal cell extracts as described by (Zhang et al., 2016). Briefly, 10 g ground fungal mycelium was added to 1 L acidified water with a final pH of 2. The water extract was then boiled for 1 h, cooled to room temperature and filter sterilized. After filtration, the pH of the fungal extract solution was adjusted to 5.0, and the final volume adjusted to 1 L by adding distilled water. This solution was used as the VIE. For elicitation, 2.5 mL VIE (equivalent to 70 mg fungal polysaccharide) was added to the seven-day-old cell suspension culture (50 mL). After the onset of elicitation, the cell cultures were harvested at defined time intervals: 0, 6, 9, 12, 24 and 48 h post elicitation (hpe). In the control treatment, a similar volume of sterile distilled water was added in place of the VIE. At least three replicates were used for each treatment.

2.4. Extraction and quantification of salicylic acid

Frozen cells (approximately 2 g) were used to extract and detect the total (free and conjugated) salicylic acid (SA) precisely as described previously (Fragnire et al., 2011). SA was detected by HPLC using a YMC-Triart (Kyoto, Japan) C_{18} analytical column (reverse phase, 250 × 4.6 mm, 5 µm, 12 nm). A binary gradient solvent system consisting of 1 mM TFA in water (A) and methanol (B) with a flow rate of 1.0 mL/min for 80 min was used to eluate the SA and other phenolics. The two mobile phases were used in the gradient mode under the following concentration times (%/min) of B: 0–10% B in 27 min, 10–40% B over a 28 min period, 40% B for 5 min, 40–44% B for 2 min, 44% B for 8 min, from 44 to 10% B in 3 min and 7 min at 10% B to re-establish the initial conditions before the injection of another sample. SA was identified by comparing the retention time and UV-spectra with authentic