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Benzaldehyde dehydrogenase-driven phytoalexin biosynthesis in elicitor-treated *Pyrus pyrifolia* cell cultures

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ABSTRACT

Pyrus pyrifolia (Asian pear) cell cultures respond to yeast extract (YE) treatment by accumulating benzoate-derived biphenyl phytoalexins, namely, noraucuparin and aucuparin. Biphenyl phytoalexins are defense-marker metabolites of the sub-tribe Malinae of the family Rosaceae. The substrates for biphenyl biosynthesis are benzoyl-CoA and malonyl-CoA, which combine in the presence of biphenyl synthase (BIS) to produce 3,5-dihydroxybiphneyl. In the non-β-oxidative pathway, benzoyl-CoA is directly derived from benzoic acid in a reaction catalyzed by benzoate-CoA ligase (BZL). Although the core β-oxidative pathway of benzoic acid biosynthesis is well-understood, the complete cascade of enzymes and genes involved in the non-β-oxidative pathway at the molecular level is poorly understood. In this study, we report the detection of benzaldehyde dehydrogenase (BD) activity in YE-treated cell cultures of *P. pyrifolia*. BD catalyzes the conversion of benzaldehyde to benzoic acid. BD and BIS activities were coordinately induced by elicitor treatment, suggesting their involvement in biphenyl metabolism. Changes in phenylalanine ammonia-lyase (PAL) activity preceded the increases in BD and BIS activities. Benzaldehyde was the preferred substrate for BD (K_m = 52.0 µM), with NAD^+ being the preferred co-factor (K_m = 64 µM). Our observations indicate the contribution of BD towards biphenyl phytoalexin biosynthesis in the Asian pear.

1. Introduction

The genus *Pyrus* involves important fruit plants of the rosaceous subtribe Malinae. This genus is represented by 24 species, up to three artificial hybrids and six natural interspecific hybrids (Fotirić Akšić et al., 2015). *Pyrus communis* L. is commonly known as the European pear, whereas *Pyrus pyrifolia* (Burm.) Nak is popularly known as the Asian pear. *P. pyrifolia* is primarily cultivated in China, India and Japan (Hancock, 2008). The most important pear diseases are pear scab (caused by *Venturia pirina*) and fire-blight (caused by *Erwinia amylovora*). Another important pear pest is *Cacopsylla pyri*, which feeds on pear leaves and fruits. Considering the economic importance of pears, developing new disease- and pest-tolerant pear cultivars is one of the most challenging tasks for pear breeders. Upon pathogen infection, resistant pear cultivars produce the Malinae marker phytoalexins, biphenyls and dibenzofurans (Chizzali et al., 2016). These compounds are Malinae inducible defense metabolites, primarily produced and accumulated in the woody part and more rarely in the leaves (Chizzali et al., 2012; Kokubun and Harborne, 1994). It has been recently reported that upon *E. amylovora* infection, *P. communis* accumulates the biphenyl and dibenzofuran class of phytoalexins (Chizzali et al., 2016). The formation of this biphenyl scaffold is known to be catalyzed by a type III polyketide synthase, biphenyl synthase (BIS) (Liu et al., 2007). The BIS enzyme catalyzes the condensation of one molecule of benzoyl-CoA with three molecules of malonyl-CoA to produce one molecule of 3,5-dihydroxybiphenyl, the precursor for other substituted biphenyl phytoalexins (Gaid et al., 2009). The starter substrate benzoyl-CoA is either derived from cinnamoyl-CoA (in β-oxidative pathway) or from free benzoic acid (non-β -oxidative pathway) in a reaction catalyzed by benzoate:CoA ligase (BZL), which promotes thio-esterification of benzoic acid by coenzyme A. BZL activity has been detected in *Clarkia breweri* and *Hypericum androsaemum* (Abd El-Mawla and Beerhues, 2002; Beuerle and Pichersky, 2002).

In plants, benzoic acid is derived from cinnamic acid by C_2 shortening of the propyl side chain. Multiple biosynthetic pathways have been reported for this chain shortening reaction, proceeding either *via* a

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CoA-dependent and β-oxidative mechanism involving cinnamoyl-CoA as an intermediate or *via* a CoA-independent and non-β-oxidative route involving benzaldehyde as an intermediate (Sircar and Mitra, 2008; Gaid et al., 2012). The CoA-dependent and β-oxidative mechanism of C_2 side chain shortening was detected in *Cucumis sativus*, *Nicotiana attenuata, Arabidopsis thaliana, and Petunia hybrida* (Ribnicky et al., 1998; Jarvis et al., 2000; Bussell et al., 2014; Widhalm and Dudareva, 2015). Recently, all of the genes and enzymes involved in the core β-oxidative pathway of benzoic acid biosynthesis were identified and functionally characterized. In the CoA-dependent and β-oxidative route, cinnamic acid is first activated into cinnamoyl-CoA in a reaction catalyzed by cinnamate-CoA-ligase (CNL) (Colquhoun et al., 2012; Gaid et al., 2012; Klempien et al., 2012). Cinnamoyl-CoA is later converted into 3-oxo-3-phenylpropanoyl-CoA through intermediate formation of 3-hydroxy-3-phenylpropanoyl-CoA in a reaction catalyzed by a bi-functional enzyme, cinnamic acid-CoA hydratase/dehydrogenase (Ph-CHD) (Bussell et al., 2014; Qualley et al., 2012). Finally, 3-oxo-3-phenylpropanoyl-CoA is converted to benzoyl-CoA by 3-ketoacyl thiolase (PhKAT) (Van Moerkercke et al., 2009; Widhalm and Dudareva, 2015). Furthermore, the CoA-independent and non-β-oxidative route of C_2 side chain shortening was detected for benzoic acid formation in cell cultures of *Sorbus aucuparia* (Gaid et al., 2009), as well as 4-hydroxybenzoic acid formation in *Solanum tuberosum* (French et al., 1976) and *Daucus carota* (Schnitzler et al., 1992; Sircar and Mitra, 2008). A benzaldehyde dehydrogenase-encoding cDNA was cloned from *Antirrhinum majus* (Long et al., 2009) and shown to contribute to methyl benzoate biosynthesis. A third route of C_2 side chain shortening involving CoA-dependent and non-β-oxidative C_2 cleavage was reported in certain plant species via direct conversion of cinnamoyl-CoA into benzaldehyde. A *CHY* gene encoding 3-hydroxyisobutyryl-CoA hydrolase, which converts cinnamoyl-CoA into benzaldehyde, was reported in *A. thaliana* (Ibdah and Pichersky, 2009).Similar C_2 side chain shortening was observed in the hairy root cultures of *Datura stramonium*, which express a bacterial gene encoding hydroxycinnamoyl-CoA hydratase/lyase (HCHL) (Mitra et al., 2002), the cell cultures of *H. androsaemum* (Abd El-Mawla and Beerhues, 2002), and *Vanilla planifolia* pods (Gallage et al., 2014).

In the present work, we report elicitor-induced accumulation of benzoate-derived biphenyl phytoalexins in *P. pyrifolia* cell cultures, as well as the detection and characterization of benzaldehyde dehydrogenase (BD; EC 1.2.1.28) in *P. pyrifolia.* The biochemical properties of BD, which catalyzes the conversion of benzaldehyde to benzoic acid (Fig. 1), were studied in desalted cell-free extracts. BD activity was correlated with phenylalanine ammonia-lyase (PAL) and BIS activities. The detection of BD activity in pear cell cultures provides important insight into the benzoic acid metabolism.

2. Materials and Methods

2.1. Plant material and chemicals

Pear (*P. pyrifolia*) plants were obtained from the Central Institute of Temperate Horticulture (CITH), Srinagar, India. Pear plants were maintained in a green house at 25 °C. Analytical-grade solvents were used in sample preparation, and all solvents used for HPLC analyses were HPLC grade. All authentic standards were procured from Sigma-Aldrich Chemical Co. Ltd. (India). Cell culture medium and plant growth regulators were purchased from Himedia (India). Aucuparin and noraucuparin were synthesized as per previously described (Hüttner et al., 2010).

2.2. Callus induction and establishment of cell suspension cultures

Primary callus cultures were derived from young leaves. Five top young leaves (2 months old) were collected and surface sterilized (Thakur et al., 2008). After surface sterilization, leaf segments were cut into small sections and put on basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 2.2 µM 6-benzylaminopurine (BAP), 30 g/l sucrose and 7 g/l agar in dark conditions at 26 °C for callus induction. Calli were propagated by regular sub-culturing at 4-week intervals. Medium without plant growth regulators served as the control. Friable soft calli were selected for the initiation of cell suspension culture. Cell suspension was initiated in the dark by shaking 3 g of calli at 160 rpm in 50 ml of the liquid MS medium (in 250 ml Erlenmeyer flasks) containing 2,4-dichlorophenoxyacetic acid (2,4-D; 1.0 µM) and indole acetic acid (IAA; 1.0 µM). Subculturing was done at 3-week intervals.

2.3. Elicitor treatment

Eight-day-old cell cultures from the linear growth phase were treated with YE at a final concentration of 3 g/l. The YE stock solution was prepared by dissolving 1.5 g of YE in 10 ml of distilled water followed by filter sterilization. Flasks were kept at 26 °C in dark in an orbital shaker at 160 rpm. After the onset of elicitation, cell cultures were harvested at defined post-elicitation time points: 0, 12, 24, 48 and 72 hours post elicitation (hpe) for phytoalexin analyses. In the control treatment, a similar volume of sterile distilled water was added in lieu of the YE. Three replicates were used for each treatment, and the experiment was repeated two times.

2.4. Precursor feeding experiments

To ascertain whether benzaldehyde-derived benzoic acid is the precursor for biphenyl phytoalexins, a feeding experiment was performed. Benzaldehyde was dissolved in DMSO (50% v/v with water), filter sterilized and subsequently fed to the cell culture (on 8th day of subculture) in a final concentration of 1 mM at the same time as the YE elicitor. Culture treated with an equal amount of only DMSO served as control. After 24 h, the cells were harvested, extracted and analyzed for phytoalexin content by HPLC.

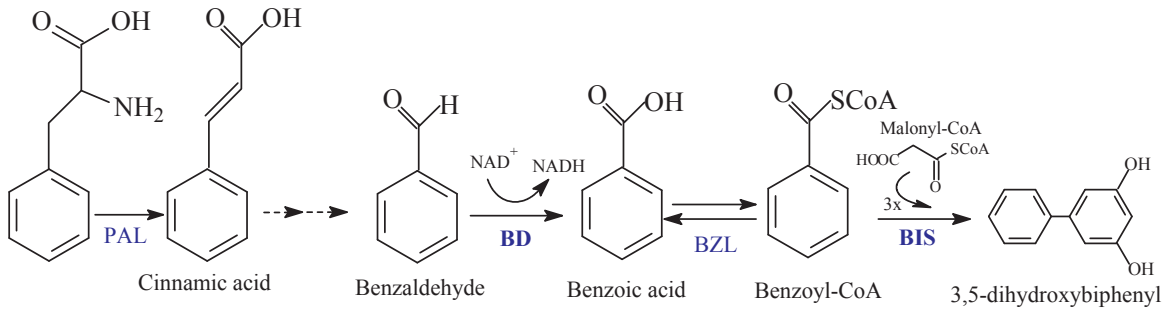

Fig. 1. Benzaldehyde dehydrogenase (BD)-dependent biphenyl phytoalexin biosynthesis in elicitor-treated cell cultures of *P. pyrifolia.* BD catalyzes the conversion of benzaldehyde to benzoic acid.