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Research article

Tissue-specific and intracellular localization of indican synthase from *Polygonum tinctorium*

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ABSTRACT

The plant *Polygonum tinctorium* produces the secondary metabolite indican (indoxyl-β-D-glucoside), a precursor of the blue dye indigo. *P. tinctorium* synthesizes indican through the actions of the UDP-glucosyltransferase (UGT), indican synthase. Herein, we partially purified an indican synthase from the leaves and subsequently performed peptide mass fingerprinting analysis. Consequently, we identified a fragment that was homologous to a UDP-glucosyltransferase 72B (UGT72B) family member. We named it *PtIgs* (*P. tinctorium* indoxyl-β-D-glucoside synthase) and obtained the full-length cDNA using rapid amplification of the cDNA ends. The primary structure of *Pt*IGS, which *PtIgs* encoded, showed high identity with indican synthases (*It*UGT1 and *It*UGT2) from *Indigofera tinctoria* (Inoue et al., 2017). Moreover, in expression analyses of *P. tinctorium*, *Pt*IGS mRNA was virtually found only in the leaves, was most highly expressed in the 1st leaves, and decreased with leaf age. Because *Pt*IGS expression tended to reflect indican contents and synthesis activities, we concluded that *Pt*IGS functions as an indican synthase in plant cells.

To examine intracellular localization of *Pt*IGS, crude leaf extracts were separated into cytosol and microsome fractions, and found *Pt*IGS in the cytosol and in microsome fractions. Furthermore, microsomal *Pt*IGS was soluble in the presence of detergents and urea and was strongly associated with membranes. Finally, we confirmed endoplasmic reticulum (ER) membrane localization of *Pt*IGS using ultracentrifugation with a sucrose density gradient. These data suggest that *Pt*IGS interacts with some kind of proteins on ER membranes to certainly carry out a delivery of substrate.

1. Introduction

Cultivated plants such as *Polygonum tinctorium*, *Indigofera tinctoria*, *Baphicacanthus cusia*, and *Isatis tinctoria* have been used to produce blue indigo dye since ancient times. Cells of these plants contain the secondary metabolites indican (indoxyl-β-D-glucoside) and/or isatan B (1H-Indol-3-yl β-D-ribo-3-hexosulopyranoside) as indigo precursors (Maier et al., 1990). In previous studies, we elucidated mechanisms of indigo production in *P. tinctorium* (Minami et al.; 1996, 1997, 2000) and showed that indican is stored in vacuoles and is degraded to indoxyl and β-D-glucose following the release of β-D-glucosidase from chloroplasts. Subsequently, indoxyl is oxidized by air to form the dimer indigo but only under conditions of cell lysis due to physical wounds, disease, or attack by insects or fungi. Hence, indican may contribute to defenses against infectious organisms. In agreement, young leaves contain larger amounts of indican than more mature leaves and are more important to plant growth and survival (Minami et al., 2000).

Indican synthase is a UDP-glucosyl transferase that uses UDP-glucose and indoxyl as substrates. UDP-glucosyltransferases play roles in various aspects of secondary metabolism in plants (Tiwari et al., 2016; Bowles et al., 2005; Gachon et al., 2005). Moreover, in the indican synthesis pathway of *P. tinctorium*, the indican synthase substrate indoxyl may be produced from indole. However, the related enzymes, including cytochrome P450s (CYPs), have not yet been identified in *P. tinctorium*. Multiple studies show roles of CYPs in various aspects of secondary metabolism and demonstrate that these proteins are

Abbreviations: indican, indoxyl-β-D-glucoside; indigo, 2-(1,3-dihydro-3-oxo-2H-indazol-2-yliden)-1,2-dihydro-3H-indol-3-on; UGT, UDP-glucosyltransferase; RACE, rapid amplification of cDNA ends; IGS, indoxyl-β-D-glucoside synthase; BiP, binding protein; G6PDH, glucose-6-phosphate dehydrogenase

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localized on ER membranes (Chapple, 1998; Ralston and Yu, 2006). Furthermore, indole may be synthesized by the tryptophan synthase α-subunit or its homolog indole synthase. Tryptophan and its precursor indole are generally synthesized in chloroplasts. However, Jin et al. (2016) recently reported that indole synthase is present in *P. tinctorium* cytosolic fractions and may contribute to indican synthesis. Although the final product (indican) of the indican synthesis pathway accumulates in vacuoles, the precursors indoxyl and indole are probably produced in the cytosol or chloroplasts. In addition, indican synthase is highly active under alkaline conditions, whereas it is relatively inactive under the acidic conditions of vacuoles, suggesting the presence of an as yet uncharacterized indican transport pathway into vacuoles.

In this study, we investigated the timing and localization of indican synthesis in plant cells by analyzing indican synthase in cells and tissues. In a previous study, we isolated indican synthase from *P. tinctorium* leaves but were unable to determine the N-terminal sequence, produce antibodies, or perform other investigations because the protein quantities were considerably low (Minami et al., 2000). Furthermore, cDNA cloning techniques were not available at that time. However, more recently, we successfully cloned indican synthase cDNA from *Indigofera tinctoria* using transcriptome data (Inoue et al., 2017), and in the present study, we used the same techniques to clone indican synthase cDNA from *P. tinctorium*.

To determine where indican is synthesized and its mechanism of transportation into vacuoles, we investigated intracellular localization of indican synthase. Specifically, we identified *P. tinctorium* indican synthase following partial purification using peptide mass fingerprinting (PMF) analyses and cDNA cloning. Subsequently, we expressed the recombinant protein in *Escherichia coli* (*E. coli*) to prepare its specific antibody and determined tissue and intracellular localization using immunoblotting and sucrose density gradients.

2. Materials and methods

2.1. Plant materials

P. tinctorium was grown in a greenhouse at 24 °C. Calluses were grown on Murashige and Skoog medium containing 2 mg.mL^{-1} 1-naphthylacetic acid and 0.5 mg.mL^{-1} 6-benzyladenine, and experiments were performed using tissues from leaves, stems, roots, and calluses.

2.2. UGT assays (indican synthesis reactions)

Indican synthesis was measured according to previously described methods (Minami et al., 2000). Briefly, reactions were performed under anaerobic conditions in mixtures containing 2 mM indoxyl, 2 mM UDP-glucose, 0.1 M 2-(N-cyclohexylamino)ethanesulfonic acid (CHES)-NaOH buffer (pH 10.0), 0.1 M dithiothreitol, and 10 mM ascorbic acid. Reactions were started by adding appropriate concentrations of enzyme solution and were incubated at 37 °C for 10 min. Reactions were then terminated by adding trichloroacetic acid solution and were then neutralized using NaOH. After centrifugation at 18, 000 × g for 5 min, synthesized indican in supernatants from reaction mixtures was quantified as described in section 2.3 and enzyme activities were calculated as units corresponding to the synthesis of 1 μmol of indican per minute.

2.3. Indican quantification

Indican concentrations were determined as described in our previous report (Minami et al., 2000). Briefly, fresh tissues (0.1 g) were added to 0.5 mL aliquots of methanol–chloroform solution and were ground using a pestle. Indican in the aqueous layers was then applied to a YMC-Triart C_{18} column (150 × 4.6 mm, YMC, Co. Ltd., Kyoto, Japan) on high-performance liquid chromatography system. After elution, indican fluorescence was detected at 400 nm (excitation at 290 nm) using a fluorescence detector (L-7480, Hitachi, Tokyo, Japan).

2.4. Partial purification of indican synthase from leaves

Fresh young leaves (approximately 54 g) were homogenized in ten (w/v) volumes of buffer A containing 50 mM potassium phosphate (pH 7.0), 5 mM β-mercaptoethanol, 2 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mg.mL^{-1} Pefabloc SC (Sigma-Aldrich, Missouri, USA) in a chilled homogenizer (model AM-11, Nihon Seiki, Niigata, Japan). Crude extracts were filtered through a layer of gauze and were then centrifuged at 49,000 × *g* for 30 min. Supernatants were then dialyzed against buffer B containing 20 mM potassium phosphate buffer (pH 7.5), 2 mM β-mercaptoethanol, and 1 mM EDTA at 4 °C overnight. After centrifugation at 49,000 × *g* for 20 min, polyethylene glycol (PEG)-6000 was added to supernatants at a final concentration of 30% (w/v), and suspensions were again centrifuged at 18,000 × *g* for 1 h. Subsequently, 10 mL aliquots of DEAE-Toyopearl 650M resin (Tosoh Bioscience, Tokyo, Japan) were equilibrated with buffer B and were added to supernatants. Enzymes were absorbed to the resin by gently stirring the mixture at 4 °C overnight. After collecting by centrifugation at 2000 × *g* for 5 min, the resin was again suspended in buffer B and was packed into a glass column (1.7 × 4.4 cm). The enzyme was then eluted from the column with buffer B containing 50 mM KCl and was then dialyzed against buffer C containing 20 mM potassium phosphate buffer (pH 7.8), 2 mM β-mercaptoethanol, and 1 mM EDTA at 4 °C overnight. The enzyme was then applied to a Source 15Q (GE Healthcare Life Science, Pennsylvania, USA) column (0.4 × 8.0 cm) equilibrated with buffer C, was eluted with buffer B containing 50 mM KCl, and was finally concentrated by ultrafiltration using an Amicon Ultra centrifugal filter (NMWL 30,000; Merck Millipore Corporation, Dermstadt, Germany). The resulting enzyme solutions were separated using a HiLoad 16/60 Superdex 200 pg column (GE Healthcare Life Science) equilibrated with buffer C containing 150 mM KCl. Protease inhibitor cocktail (Nakarai tesque, Kyoto, Japan) was added to all enzyme fractions.

2.5. PMF analysis

Enzyme fractions from Superdex 200 column chromatography were concentrated to approximately 1 unit using an Amicon Ultra centrifugal filter (NMWL 30,000) and were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% polyacrylamide gel. Molecular weights of proteins were estimated using the molecular marker WIDE-VIEW III (Wako Pure Chemical Industries Ltd., Osaka, Japan), and polyacrylamide gel was stained with Coomassie brilliant blue prior to excising the desired bands. Gel pieces were then washed with distilled water and 0.1 M NH_4HCO_3, immersed in acetonitrile for 5 min, and dried in a desiccator. Proteins in gel pieces were then digested with 4.5 μg. mL^{-1} trypsin in 40 mM NH_4HCO_3 containing 10% (v/v) acetonitrile at 37 °C overnight. Subsequently, 1 μL samples were mixed with 1 μL aliquots of matrix solution containing 0.2 mg. mL^{-1} α-cyano-4-hydroxycinnamic acid, 90% (v/v) acetonitrile, and 0.1% (v/v) trifluoroacetic acid, and were loaded onto an anchor tip (MTP AnchorChip 384 T F, Bruker Daltonics, Massachusetts, USA). Mass analyses of samples were performed using matrix-assisted laser desorption ionization-time of flight mass spectrometry with an Autoflex Speed instrument (Bruker Daltonics). Proteins from samples were then analyzed using the Mascot server on the basis of the resulting mass spectrometric and previously reported transcriptomic data (GenBank, accession SRX692542; Sarangi et al., 2015; Minami et al., 2015).

2.6. cDNA cloning of PtIGS

To generate full-length *Pt*IGS cDNA (*PtIgs*), 5′- and 3′- rapid amplification of cDNA ends (RACE) were performed using SMARTer RACE 5′/3′ kits according to the manufacturer's instructions (Takara Bio Inc.,