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Identification of protein–protein interactions of isoflavonoid biosynthetic enzymes with 2-hydroxyisoflavanone synthase in soybean (*Glycine max* (L.) Merr.)

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ABSTRACT

Metabolic enzymes, including those involved in flavonoid biosynthesis, are proposed to form weakly bound, ordered protein complexes, called "metabolons". Some hypothetical models of flavonoid biosynthetic metabolons have been proposed, in which metabolic enzymes are believed to anchor to the cytoplasmic surface of the endoplasmic reticulum (ER) via ER-bound cytochrome P450 isozymes (P450s). However, no convincing evidence for the interaction of flavonoid biosynthetic enzymes with P450s has been reported previously. Here, we analyzed binary protein–protein interactions of 2-hydroxyisoflavanone synthase 1 (GmIFS1), a P450 (CYP93C), with cytoplasmic enzymes involved in isoflavone biosynthesis in soybean. We identified binary interactions between GmIFS1 and chalcone synthase 1 (GmCHS1) and between GmIFS1 and chalcone isomerases (GmCHIs) by using a split-ubiquitin membrane yeast two-hybrid system. These binary interactions were confirmed *in planta* by means of bimolecular fluorescence complementation (BiFC) using tobacco leaf cells. In these BiFC analyses, fluorescence signals that arose from the interaction of these cytoplasmic enzymes with GmIFS1 generated sharp, network-like intracellular patterns, which was very similar to the ER-localized fluorescence patterns of GmIFS1 labeled with a fluorescent protein. These observations provide strong evidence that, *in planta*, interaction of GmCHS1 and GmCHIs with GmIFS1 takes place on ER on which GmIFS1 is located, and also provide important clues to understand how enzymes and proteins form metabolons to establish efficient metabolic flux of (iso)flavonoid biosynthesis.

1. Introduction

Enzymes involved in sequential metabolic pathways have been proposed to form weakly bound, ordered protein complexes, called "metabolons" [1]. In many known cases, metabolons are bound to cellular structural elements. In plant specialized metabolism, some metabolons are anchored to specific domains of the cytoplasmic surface of the endoplasmic reticulum (ER) via ER-bound cytochrome P450 isozymes (termed P450s) [2,3]. Dynamic formation and dissociation of metabolons potentially provide metabolic regulatory mechanisms [2,4–6]. Thus, elucidation of the structural organization of metabolons provides a basis for a better understanding of the regulation of metabolic pathways and its application to metabolic engineering.

Isoflavones are a class of plant flavonoids with a 3-phenylchromone structure and are distributed almost exclusively

Abbreviations: BiFC, bimolecular fluorescence complementation; GmCHS, chalcone synthase of *Glycine max*; GmCHR, chalcone reductase of *G. max*; GmCHI, chalcone isomerase of *G. max*; GmHID, 2-hydroxyisoflavanone dehydratase of *G. max*; GmIFS, 2-hydroxyisoflavanone synthase of *G. max*; GmUGT, UDP-glucose:isoflavone 7-*O*-glucosyltransferase of *G. max*; GmMaT, malonyl-CoA:isoflavone 7-*O*-glucoside 6″-*O*-malonyltransferase of *G. max*; P450, cytochrome P450; mTq2, monomeric Turquoise2; mVen, monomeric Venus.

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in legumes, including soybean [*Glycine max* (L.) Merr.]. In soybean, 7-*O*-(6″-*O*-malonyl)-glucosides of daidzein and genistein are major forms of isoflavones that mainly accumulate in the roots and leaves of seedlings and the seeds [7,8]. These flavonoids are involved in symbiotic and defense mechanisms of soybean [9] and are of nutritional and biomedical significance in human health [10].

In the proposed pathway of isoflavone biosynthesis in soybean (Supplemental Fig. S1), the first important step is catalyzed by chalcone synthase (CHS or GmCHS) to produce 4,2′,4′,6′-tetrahydroxychalcone (THC) [11]. Moreover, 4,2′,4′-trihydroxychalcone, a deoxy form of THC, can also be produced via the coupled catalytic actions of CHS and chalcone reductase (CHR or GmCHR) [12,13]. Chalcones thus produced subsequently undergo stereo-specific isomerization catalyzed by chalcone isomerase (CHI or GmCHI) to produce (*2S*)-flavanones [14,15]. (*2S*)-Flavanones then undergo 2-hydroxylation catalyzed by 2-hydroxyisoflavanone synthase (GmIFS), a microsomal cytochrome P450 enzyme (CYP93C), to produce 2-hydroxyisoflavanones [16–18]. 2-Hydroxyisoflavanone dehydratase (GmHID) then catalyzes 2,3-dehydration of 2-hydroxyisoflavanones to produce daidzein and genistein [19,20]. These isoflavone aglycones further undergo 7-*O*-glucosylation and subsequent 6″-*O*-malonylation, which are respectively catalyzed by UDP-glucose:isoflavone 7-*O*-glucosyltransferase (GmUGT) [8,20,21] and malonyl-CoA:isoflavone 7-*O*-glucoside 6″-*O*-malonyltransferase (GmMaT) [22]. The resulting isoflavone conjugates finally accumulate in vacuoles [23]. The soybean genome encodes a number of paralogs of these biosynthetic enzymes [24,25]. Paralogs of each biosynthetic enzyme may show differential substrate specificities and differential expression patterns, and hence perform differential physiological roles in the plant [8,14].

The formation of metabolons in flavonoid biosynthesis was first proposed in 1974 [26]. Several lines of evidence for the occurrence of membrane-bound flavonoid biosynthetic metabolons have been reported in several plant species (see Ref. [2] for a review). In *Arabidopsis thaliana*, direct binary interactions among cytoplasmic enzymes involved in flavonoid biosynthesis have been intensively studied by means of molecular biological, immunological, and physicochemical methods [27,28]. Thus, models for the flavonoid biosynthetic metabolons (including those for isoflavone biosynthesis) have been proposed [3]. In these models, a linear array of biosynthetic enzymes is associated with the cytoplasmic surface of the ER, and some of these enzymes are anchored via P450 protein(s). However, to date, an entire picture of flavonoid biosynthetic metabolons remains elusive, and no direct evidence that flavonoid biosynthetic enzymes interact with P450 proteins has been reported. Here we present the first evidence for protein–protein interactions of flavonoid biosynthetic enzymes with a P450 in soybean.

2. Materials and methods

2.1. cDNAs of soybean isoflavonoid biosynthetic enzymes

Full-length cDNAs of soybean isoflavonoid biosynthetic enzymes with the following DDBJ/ENA/Genbank accession numbers (shown in parentheses) were obtained: *GmCHS1* (X54644), *GmCHS7* (M98871), *GmCHR1* (X55730), *GmCHI1A* (AY595413), *GmCHI1B2* (AY595419), *GmCHI2* (AY595415), *GmIFS1* (AF195818), *GmHID1* (AB154415), *GmUGT1* (AB292164), and *GmMaT1* (AB291058). The entire open reading frames coding for these enzymes were obtained as described in Supplemental Information S1.

2.2. Construction of plasmids for split-ubiquitin system

The split-ubiquitin membrane yeast two-hybrid system (termed here the SU system) [29] was used to identify protein–protein interactions of GmIFS1 with other isoflavonoid biosynthetic enzymes using the DUALmembrane Kit 3 (Dualsystems Biotech, Zurich, Switzerland). The full-length *GmIFS1* (without a translation termination codon) was obtained by digestion of the pGEM-T-Easy-based construct (see Supplemental Information S1) with the restriction enzyme *Sfi*I, then gel-purified and subcloned into the *Sfi*I sites of the pBT3-SUC vector included in the kit. By using this plasmid for protein expression, the SUC2 peptide and the Cub-LexA-VP16 protein were added to the N- and C-terminus, respectively, of the expressed protein (GmIFS1), where SUC2 refers to the 19-residue signal peptide of *Saccharomyces cerevisiae* invertase and Cub-LexA-VP16 refers to the chimeric protein of the C-terminal half of ubiquitin (Cub) and a transcription factor cassette (LexA-VP16). For construction of a plasmid that expressed a protein (GmCHS1, GmCHS7, GmCHR1, GmCHI1A, GmCHI1B2, GmCHI2, GmHID1, GmUGT1, or GmMaT1) fused with the N-terminal half of a mutated ubiquitin (termed NubG [29]) at their N-terminus (designated, for example, NubG-GmCHS1), the *Sfi*I-digested fragment of each cDNA was subcloned into the pPR3-N vector included in the kit. To construct a plasmid that expresses GmCHS1 fused with NubG at its C-terminus (i.e., GmCHS1-NubG), the *GmCHS1*-encoding fragment was amplified by PCR using gene-specific primers (see Supplemental Table S1), digested with *Sfi*I, and subcloned into the pPR3-C vector included in the kit.

2.3. Protein–protein interaction assays using SU system

S. cerevisiae strain NMY51 (*MATα his3Δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)4-HIS3 ura3::(lexAop)8-lacZ ade2::(lexAop)8-ADE2 GAL4*), which was included in the DUALmembrane Kit 3 (see above), was transformed with one of the following pairs of plasmids (i.e., derivatives of pBT3-SUC and pPR3-N; derivatives of pBT3-SUC and pPR3-C; or, as a positive control, pOst1-NubI and derivatives of pBT3-SUC) by means of the polyethylene glycol–lithium acetate method in accordance with the manufacturer's guidelines. A single colony formed on a synthetic dropout (SD) agar medium lacking tryptophan and leucine (SD/−W/−L) was subsequently transferred to a liquid SD/−W/−L medium and the cells were further grown overnight at 30 °C with shaking. The cells were collected by centrifugation and suspended in sterile water to an optical turbidity at 600 nm (OD_{600}) of 1.0. Five microliters each of 5-fold serial dilutions of this cell suspension were placed on agar plates of SD/−W/−L, SD/-W/-L/-H (SD lacking tryptophan, leucine and histidine), SD/−W/−L/−H/−A (SD lacking tryptophan, leucine, histidine, and adenine), and SD/−W/−L/−H/−A/+AT [SD/−W/−L/−H/−A supplemented with 1 mM 3-aminotriazole (Sigma, St Louis, MO, USA), a competitive metabolic inhibitor of histidine biosynthesis] and the cells were grown at 30 °C for 2–4 days. The occurrence of interactions between the test proteins was also examined by assaying β-galactosidase activity of the transformant cells in accordance with the manufacturer's guidelines. Under the present assay conditions, proteins to be tested were considered to be interacted with each other when the assay results met the following two criteria: (i) the yeast growth on SD/−W/−L/−H/−A was observed with 25-fold or higher dilution of the cell suspension; and (ii) the yeast cells displayed β-galactosidase activity at a level of 10 U or higher per OD_{600} culture.

2.4. Yeast two-hybrid assays

Yeast two-hybrid assays [30] were performed using the Matchmaker Gold Yeast Two-Hybrid System (Clontech, Mountain View, CA, USA) as described in Supplemental Information S2. The yeast-growth assays for protein–protein interactions were