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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

performed in an essentially similar manner as described in Section 2.3.

2.5. Bimolecular fluorescence complementation and subcellular localization studies

Multi-cassette pDOE vectors [The Arabidopsis Information Resource (https://www.arabidopsis.org/index.jsp)], which were designed to reduce non-specific signal from self-assembly of fragments of fluorescent proteins [31], were used. For bimolecular fluorescence complementation (BiFC) assays, the binary vector pDOE-05 was used to express proteins of interest, which were, respectively, fused in-frame with the N-terminal (residues 1–210) and C-terminal (residues 210–238) fragments (termed NmVen210 and CVen210, respectively) of the mVenus protein [termed mVen, a spectral variant of green fluorescent protein (GFP)]. To study the subcellular localization of GmIFS1, GmCHS1, GmCHI1A, and GmCHI2, the binary vector pDOE-13 was used to express their chimeras with mTurquoise2 [mTq2 (an additional spectral variant of GFP)] or mVen. Construction of the plasmids for BiFC and subcellular localization studies are described in Supplemental Information S3.

Agrobacterium tumefaciens GV3101 (pMP90) cells that harbored one of the pDOE derivatives (see Supplemental Information S3) were incubated at 28 °C for 1 day with 20 μM acetosyringone. The cells were collected by centrifugation, washed with infiltration buffer [10 mM MES-KOH (pH 5.7) containing 10 mM $MgCl_2$ and 200 μM acetosyringone], and resuspended in the same buffer to an OD_{600} of 0.05–0.5. Agroinfiltration was performed using wild-type tobacco *Nicotiana benthamiana* plants grown at 25 °C for 4–5 weeks under long-day conditions (16 h light and 8 h dark). After infiltration, the plants were incubated at 25 °C under long-day conditions for 2 days.

2.6. Confocal microscopy

Tobacco leaf pieces were directly mounted on glass slides with a drop of water and then covered with glass covers. Fluorescence in the cells of tobacco leaves was observed using a laser scanning confocal microscope (Leica TCS-SP8; Mannheim, Germany) with a white light laser and a HyD detector. For detection of mTq2 fluorescence, the excitation wavelength was 458 nm and signals of 460–500 nm were recorded using a HyD detector. For detection of mVen fluorescence, the excitation wavelength was 514 nm and signals of 520–560 nm were recorded using a HyD detector. Simultaneously, a transmission image was recorded using a photomultiplier tube-type detector. Each image was collected using a 'between lines' sequential scanning mode.

3. Results

3.1. Identification of binary protein–protein interactions by yeast assay systems

GmIFS1 is a membrane-bound P450 protein [16–18], which contains an N-terminal sequence that anchors to the ER membrane [3,32]. Thus, a SU system [29] was first used to screen interactions of GmIFS1 with cytoplasmic enzymes (i.e., GmCHSs and GmCHIs; see Section 3.2, also see Ref. [33] for GmCHIs) and other putative cytoplasmic enzymes (i.e., GmCHR1, GmHID1, GmUGT1, and GmMaT1) involved in isoflavonoid biosynthesis. In these assays, SUC2-GmIFS1-Cub-LexA-VP16 was designed to ensure the membrane insertion of the N-terminal peptide of this P450. Moreover, interactions of homologs of GmCHS and GmCHI (i.e., GmCHS1 and GmCHS7 for CHS and GmCHI1A, GmCHI1B2, and GmCHI2 for CHI) with GmIFS1 were examined to assess whether there are any homolog-dependent differences in their interactions.

On the basis of the results of the yeast-growth and β-galactosidase assays in the SU system (Fig. 1), protein–protein interactions with GmIFS1 were unambiguously observed with GmCHS1, GmCHI1A, GmCHI1B2, and GmCHI2. Interaction of GmIFS1 with GmCHS7 was weaker than that with GmCHS1 under the same conditions (Fig. 1), where the expression levels of NubG-GmCHS7 and NubG-GmCHS1 in the yeast cells were comparable (Supplemental Fig. S3). GmCHS1-NubG also interacted with SUC2-GmIFS1-Cub-LexA-VP16 (Supplemental Fig. S4). No appreciable yeast growth nor β-galactosidase activity was detected when interactions between GmIFS1 and other cytoplasmic enzymes were assayed (Fig. 1). We also examined all possible combinations of binary interactions between the putative cytoplasmic enzymes (see above) by means of yeast two-hybrid assays. No appreciable yeast growth was detected with any combinations of proteins under the assay conditions (Supplemental Fig. S5).

3.2. In planta analysis of protein–protein interactions and subcellular localization

Some of the protein–protein interactions that were unambiguously identified in the yeast systems (i.e., binary interactions of GmIFS1 with GmCHS1, GmCHI1A, and GmCHI2, see above) were further examined *in planta* by means of BiFC. When GmIFS1-NmVen210 was co-expressed with GmCHS1-CVen210 in tobacco cells, yellow fluorescence signals with a sharp network-like pattern were clearly observed in the transformed cells (Fig. 2A–D; see Supplemental Fig. S6 for their negative controls), indicating that GmIFS1 and GmCHS1 interacted *in planta*. Co-expression of GmIFS1-NmVen210 with GmCHI1A-CVen210 or GmCHI2-CVen210 yielded similar patterns of fluorescence signals in the cells (Fig. 2E–L). Thus, GmIFS1 and these GmCHI proteins also interacted with each other *in planta*.

The patterns of intracellular fluorescence signals due to these protein–protein interactions were then compared with those of intracellular localization patterns of GmIFS1, GmCHS1, GmCHI1A and GmCHI2, which were, respectively, expressed as chimeric proteins with a spectral variant of GFP in tobacco cells (Fig. 3). The cytoplasmic space and nucleus of the cells were also visualized by expression of one of the free GFP derivatives (see Fig. 3B, E, I and M). The fluorescence signals of the chimeric GmIFS1-mTq2 showed a sharp network-like pattern in the cells of tobacco leaves (Fig. 3A), which were very similar to those observed with the interaction assays (Fig. 2B, F and J). By contrast, the fluorescence signals of mVen showed a cloudy distribution pattern in the cytoplasm and nucleus (Fig. 3B) and were distinct from the sharp pattern observed with GmIFS1-mTq2 (see the *insets* of Fig. 3A and B for details). The fluorescence signals of GmCHS1-mVen, GmCHI1A-mVen, and GmCHI2-mVen also showed cloudy distribution patterns in the cytoplasm and nucleus (Fig. 3F, J and N, respectively), as in the case of free mVen (Fig. 3B) and mTq2 (Fig. 3E, I and M), and were different from those of GmIFS1-mTq2 and those observed when the interactions of these cytoplasmic proteins with GmIFS1 were assayed by BiFC (Fig. 2).

4. Discussion

In this study, we identified binary interactions between GmIFS1 and GmCHS1 and between GmIFS1 and GmCHIs (GmCHI1A, GmCHI1B2, and GmCHI2) by means of protein–protein interaction assay systems using yeast cells. These interactions were further confirmed *in planta* by BiFC, providing the first evidence for binary associations of flavonoid biosynthetic enzymes with a P450 protein.

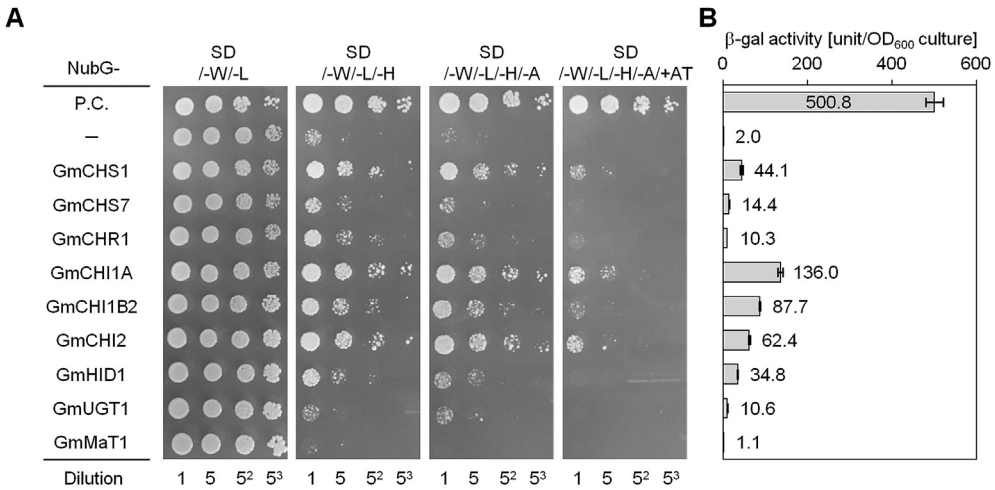

Fig. 1. Interaction of isoflavonoid biosynthetic enzymes with GmIFS1 as assayed by means of the SU system. (A) Yeast cells co-expressing SUC2-GmIFS1-Cub-LexA-VP16 with NubG-fused enzymes (NubG-GmCHS1, etc.). As a negative control (–), yeast cells co-expressing SUC2-GmIFS1-Cub-LexA-VP16 with NubG are shown. For other negative controls, see Supplemental Fig. S2 (B) β-Galactosidase activities of the yeast transformant cells as assayed in the SU system. See Section 2.3 for details.

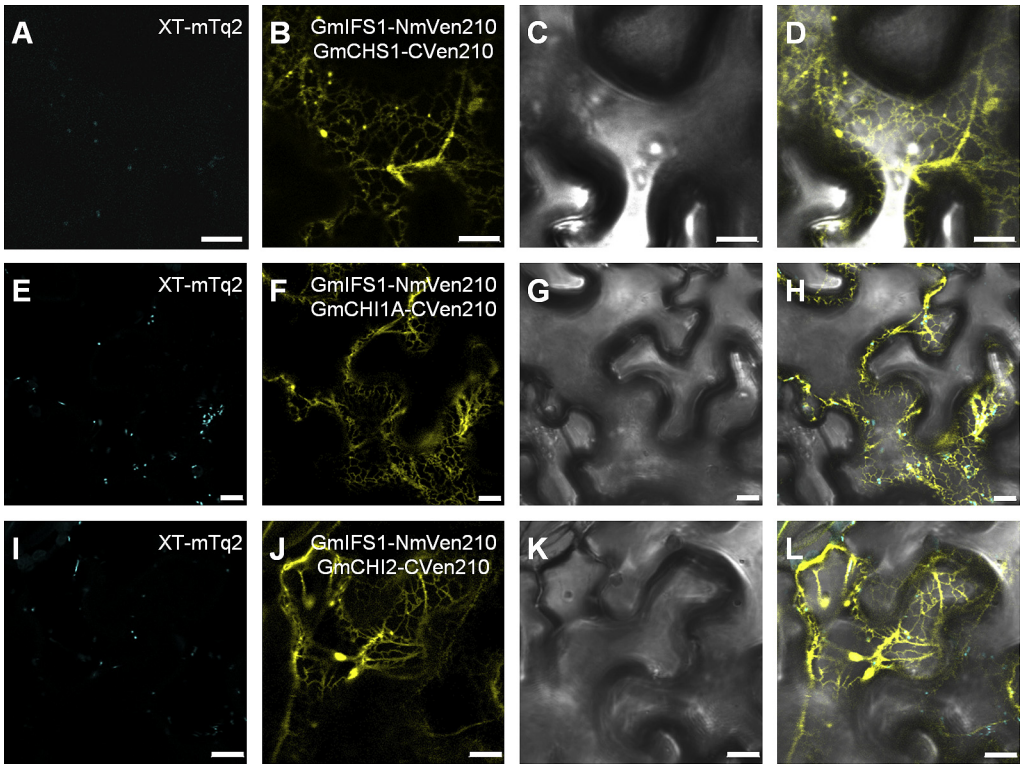

Fig. 2. Detection of binary interactions of GmIFS1 with GmCHS1, GmCHI1A, and GmCHI2 by BiFC in tobacco leaf cells. GmIFS1-NmVen210 and XT-mTq2 (a Golgi-localized marker [31]) were co-expressed with GmCHS1-CVen210 (A–D), GmCHI1A-CVen210 (E–H), and GmCHI2-CVen210 (I–L) in tobacco leaf cells. Intracellular fluorescence within a single focal plane was observed by means of confocal laser fluorescent microscopy. (A, E and I) Identification of the transformed cells on the basis of fluorescence of XT-mTq2. (B, F and J) Fluorescence images generated from BiFC between the NmVen210 and CVen210 portions of the chimeric proteins expressed in the transformed cells shown in A, E and I, respectively. (C, G and K) Transmitted-light images of the cells shown in A, E and I, respectively. (D) The images of A, B and C merged. (H) The images of E, F and G merged. (L) The images of panels I, J and K merged. Scale bars = 10 μm.

It should be noted that GmCHS1 and GmIFS1 were observed to interact regardless of which terminus, N- or C-terminus, of GmCHS1 a protein tag (NubG or CVen210) was added to (see Results). This was also the case for the interaction of GmCHI with GmIFS1—for example, NubG-GmCHI1A interacted with SUC2-GmIFS1-Cub-LexA-VP16 in the SU assay (Fig. 1) and GmCHI1A-CVen210 interacted with GmIFS1-NmVen210 in the BiFC assay (Fig. 2E–H). These results were consistent with the fact that N- and C-termini of these enzymes could be spatially in close proximity to each other in their stereo structures, as revealed by the crystallographic studies of CHS [34] and CHI of *Medicago sativa* (alfalfa) [35].

The results of fluorescence imaging analyses using GmIFS1-mTq2 showed a sharp network-like fluorescence pattern of this protein chimera in the transformed cells. This pattern is very

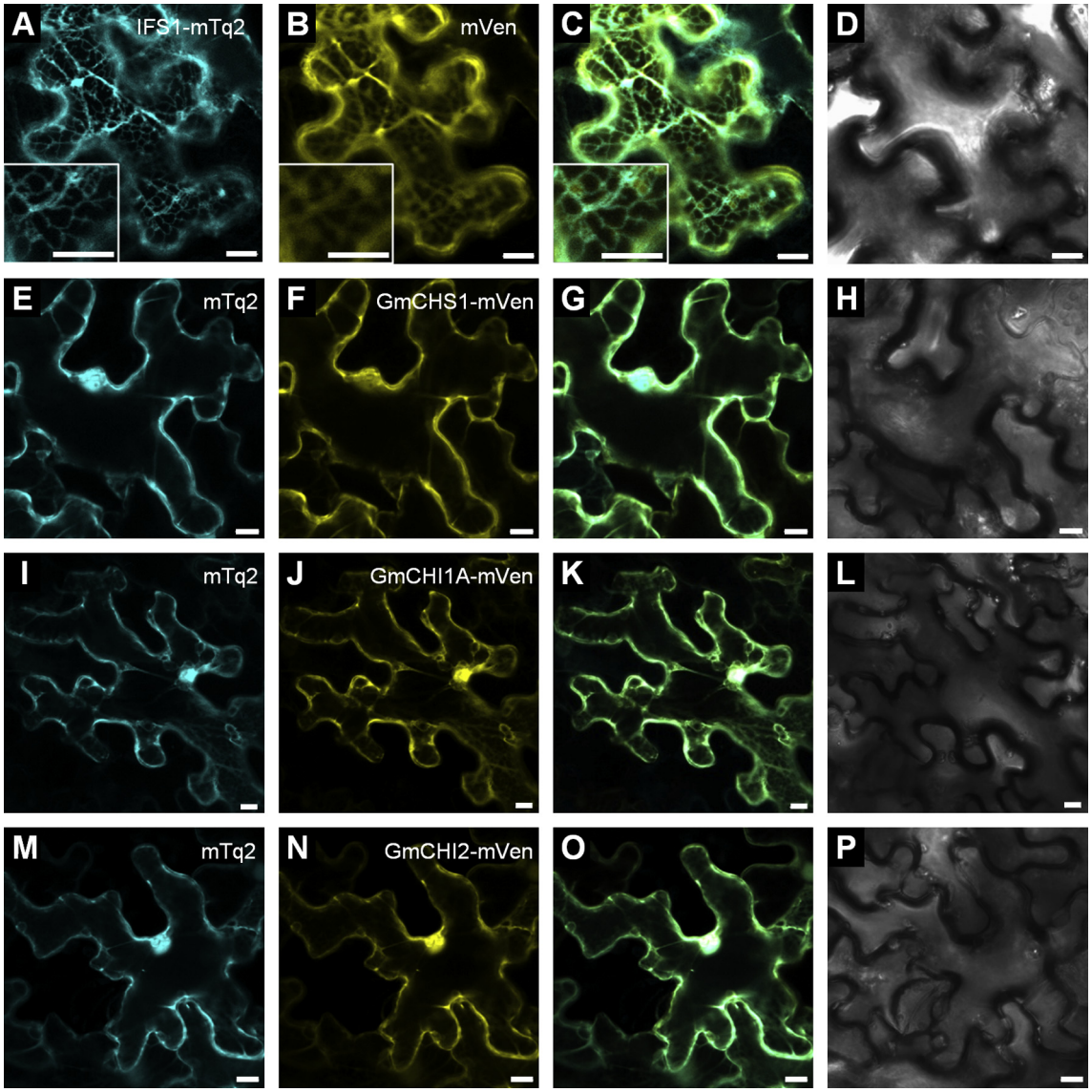

Fig. 3. Subcellular localization of GmIFS1, GmCHS1, GmCHI1A, and GmCHI2 as studied by transient expression of their fluorescent-protein chimeras in tobacco leaf cells. Intracellular fluorescence within a single focal plane in the tobacco leaf cells was observed by means of confocal laser fluorescent microscopy. (A–D) Co-expression of GmIFS1-mTq2 and mVen. (E–H) Co-expression of mTq2 and GmCHS1-mVen. (I–L) Co-expression of mTq2 and GmCHI1A-mVen. (M–P) Co-expression of mTq2 and GmCHI2-mVen. (A, E, I and M) Detection of fluorescence from GmIFS1-mTq2 (A) and free mTq2 (E, I and M). (B, F, J and N) Detection of fluorescence from free mVen (B) and protein chimeras with mVen (F, J and N). (C) The images of A and B merged. (G) The images of E and F merged. (K) The images of I and J merged. (O) The images of M and N merged. (D, H, L and P) Transmitted-light images of the cells shown in A–C, E–G, I–K and M–O, respectively. In the *insets* to A–C, the identical area of the transformed cell was magnified to show the difference between the fluorescence distribution patterns of GmIFS1-mTq2 (A) and mVen (B). Scale bars = 10 μm.

similar to those observed previously by other research groups and is consistent with the fact that GmIFS1 is a P450 localized on the ER [3,32], which shows an intricate tubular network throughout the cytoplasm of tobacco cells [36,37]. By contrast, intracellular distribution patterns of fluorescence signals in the cells expressing GmCHS1-mVen, GmCHI1A-mVen, and GmCHI2-mVen (Fig. 3F, J and N) were similar to those observed with cells expressing free mTq2 (Fig. 3E, I and M) and were consistent with cytoplasmic and nuclear localization of these enzymes. Most importantly, the interaction of these cytoplasmic enzymes with GmIFS1 yielded sharp network-like fluorescence patterns in the BiFC analyses (Fig. 2B, F and J), which was very similar to the fluorescence pattern of GmIFS1-mTq2 (Fig. 3A). All of these results strongly suggested that, *in planta*, interaction of these cytoplasmic enzymes with GmIFS1 take place on the ER, where GmIFS1 is located.

The SU assay results suggested that GmCHS1 could bind to GmIFS1 more strongly than GmCHS7 (see Fig. 1), implying that strength of protein–protein interactions of enzymes may vary among paralogous members. The observed differential strength of interactions of these CHS paralogs with GmIFS1 might be potentially related to their proposed differences in regulation and physiological roles in the soybean plant (see Ref. [38] for example).

No other appreciable binary interactions were detected between any two of GmIFS1 and other cytoplasmic enzymes examined in this study (Fig. 1 and Supplemental Fig. S2). It would be noteworthy, in this regard, that the channeling of products/substrates between CHS and CHR enzymes was previously proposed [27] to account for the specificity of the CHS/CHR-catalyzed reactions, providing the possibility that these two enzymes might interact with each other. However, previous three-dimensional structural characterizations

of CHS and CHR [13,34] strongly suggest that direct contact of the active sites of these two enzymes was highly unlikely, and the present observation that binary interaction between CHS and CHR was absent (or very weak, if at all) was consistent with the proposal from the structural studies. The possibility still remains, however, that the active sites of CHS and CHR could be in close proximity when these two enzymes are organized in a metabolon [27].

In conclusion, we established binary interactions of GmIFS1 with GmCHS1 and GmCHIs, which take place on the ER *in planta*. To understand how enzymes and proteins form dynamic metabolons to establish efficient metabolic flux of (iso)flavonoid biosynthesis, however, some other important points remain to be studied. For example, the involvement of an enzyme in the metabolon may require pre-existing binary (or ternary, or higher) complexes of other biosynthetic enzymes. Moreover, binding of an "adaptor" protein(s) with no enzymatic activity might be needed to establish the formation of a functional metabolon. These issues should be addressed in future studies.

Disclosures

The authors have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.12.038.

Transparency document

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