Cloning, Prokaryotic Expression, and Subcellular Localization of the PtCHI Gene from *Pueraria thomsonii* (Postprint)

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Abstract

To investigate the molecular mechanism differences of the key enzyme gene PtCHI in isoflavonoid metabolism among Pueraria varieties, and to preliminarily elucidate the causes for inter-varietal differences in isoflavonoid content, this study employed wild Pueraria and the starch-rich Pueraria cultivar 'Guige No. 1' as experimental materials. Following ethanol extraction, the contents of puerarin and total flavonoids in both wild and starch-rich Pueraria were determined via high-performance liquid chromatography. Based on the previously reported CHI gene from wild Pueraria, the PtCHI gene from starch-rich Pueraria was isolated through homologous cloning, expressed in vitro, and its subcellular localization was investigated in Arabidopsis thaliana protoplasts. The results demonstrated: (1) The puerarin content in wild Pueraria was significantly higher than that in starch-rich Pueraria, while the total flavonoid content in wild Pueraria was also elevated compared to starch-rich Pueraria, albeit not reaching statistical significance; (2) The PtCHI gene from starch-rich Pueraria was successfully isolated, spanning 742 bp and containing a complete 672 bp open reading frame encoding 223 amino acids, exhibiting 99% homology with the CHI gene from wild Pueraria; (3) The expression profile of the CHI gene in starch-rich Pueraria followed the order stem > root > leaf, whereas in wild Pueraria it was root > stem > leaf; moreover, except in leaves, the expression level of the CHI gene in wild Pueraria was significantly higher than that in starch-rich Pueraria; (4) The encoded protein was predicted to be a stable hydrophilic protein of 27.8 kDa, with -helices predominating in its secondary and tertiary structures, possessing 25 phosphorylation sites, showing close phylogenetic relationships with wild Pueraria, soybean, and Glycyrrhiza uralensis, and exhibiting high potential for interaction with F3H2, F3H, 4CL4, DFR2, and CHS; (5) A single 27.8 kDa PtCHI protein was successfully induced and purified in vitro; (6) Subcellular localization analysis using Arabidopsis thaliana protoplasts further revealed

that PtCHI is predominantly localized in chloroplasts. This study establishes a foundation for further dissecting the differences in flavonoid content between starch-rich and wild Pueraria, as well as for functional validation of PtCHI and mechanistic investigations of isoflavonoid metabolic pathways.

Full Text

Cloning, Prokaryotic Expression and Subcellular Localization of the PtCHI Gene from *Pueraria thomsonii*

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Abstract

This study investigated the molecular mechanisms underlying differences in the isoflavone metabolic enzyme gene PtCHI between *Pueraria* varieties to elucidate the causes of inter-varietal variation in isoflavone content. Using wild *Pueraria montana* and the cultivated *Pueraria thomsonii* variety "Guige No. 1" as materials, we extracted puerarin and total flavonoids with ethanol and quantified them by high-performance liquid chromatography. Based on the previously reported CHI gene from *P. montana*, we isolated the PtCHI gene from *P. thomsonii* through homologous cloning, expressed the protein *in vitro*, and examined its subcellular localization in *Arabidopsis* protoplasts. The results revealed: (1) *P. montana* contained significantly higher puerarin content than *P. thomsonii*, and its total flavonoid content was also elevated, though not significantly; (2) The PtCHI gene was successfully isolated from *P. thomsonii*, comprising 742 bp with a complete 672 bp open reading frame encoding 223 amino acids, sharing 99% homology with the *P. montana* CHI gene; (3) CHI expression patterns differed between species, with stem > root > leaf in *P. thomsonii* and root > stem > leaf in *P. montana*, and expression levels in *P. montana* were significantly higher than in *P. thomsonii* in all tissues except leaves; (4) Bioinformatic analysis predicted PtCHI to be a stable hydrophilic protein of 27.8 kD, with -helices dominating its secondary and tertiary structures, containing 25 phosphorylation sites, showing close phylogenetic relationships with *P. montana*, soybean, and *Glycyrrhiza uralensis*, and likely interacting with F3H2, F3H, 4CL4, DFR2, and CHS; (5) A single 27.8 kD PtCHI protein was successfully induced and purified *in vitro*; (6) Subcellular localization in *Arabidopsis* protoplasts demonstrated that PtCHI primarily localizes to chloroplasts. These findings provide a foundation for further investigating flavonoid content differences between *P. montana* and *P. thomsonii*, as well as for functional verification of PtCHI and

mechanistic studies of isoflavone metabolism.

Keywords: *Pueraria thomsonii*, PtCHI, cloning, prokaryotic expression, subcellular localization

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Introduction

Pueraria thomsonii, the dried root of the leguminous vine *Pueraria thomsonii* Benth., is widely distributed throughout China and characterized by woody stems with thick tuberous roots. Its primary bioactive components include isoflavonoids such as puerarin, daidzein, genistein, and isoliquiritigenin, as well as saponins like sophoradiol, sophoradiol, and soyasapogenol. Additionally, *P. thomsonii* contains abundant nutrients including vitamin C, protein, reducing sugars, starch, and dietary fiber. As both a medicinal and edible crop with high starch content, it is commonly processed into various commercial products such as kudzu powder, chips, cakes, and wine. Previous research has demonstrated its pharmacological effects, including antipyretic, antiviral, hypoglycemic, lipid-lowering, blood pressure-reducing, hepatoprotective, anti-tumor, and kidney function-improving properties.

Chalcone isomerase (CHI) represents a critical enzyme in the isoflavonoid metabolic pathway, with its enzymatic activity playing a significant role in flavonoid accumulation and consequently influencing plant stress resistance and flower coloration. The CHI superfamily is classified into three main types: Type I, Type II, and Type III. Most plant CHI genes belong to Type I, while Type II is generally restricted to legumes and evolved from Type I; Type III comprises CHI-like proteins predominantly found in fungi and bacteria. The CHI gene has been successfully isolated and functionally characterized in numerous plant species. For instance, Ling (2016) isolated five FhCHI proteins from *Freesia hybrida in vitro*, demonstrating that FhCHI2 and FhCHI5 exhibited catalytic activity in converting naringenin chalcone to naringenin, whereas FhCHI3 and FhCHI4 were inactive. Heterologous expression in *Arabidopsis* revealed that FhCHI1, FhCHI2, and FhCHI5 could restore anthocyanin and flavonol metabolism in CHI mutants, revert shoot apex pigmentation to purple, and restore seed coat color to brown, while FhCHI3 and FhCHI4 failed to rescue the mutant phenotype. Guo (2011) transformed the *Ipomoea batatas* IbCHI gene into *Arabidopsis* mutants, showing that IbCHI restored seed coat color from light yellow to wild-type dark brown and enhanced resistance to high light stress, as evidenced by slower reduction in chlorophyll fluorescence parameters (Fv/Fm and Yield) compared to mutants. Under drought conditions, transgenic plants expressing IbCHI showed less wilting than mutant controls. He (2011) reported that overexpression of soybean GmCHI4A and GmCHI4B in hairy roots enhanced salt tolerance under 150 $mmol \cdot L^{1}$ NaCl treatment, with transgenic lines showing significantly higher expression of GmSOD1 and GmSOS1 and elevated isoflavone content compared to controls.

Subcellular localization studies have revealed diverse patterns for CHI proteins, with variations even among homologous genes across species. Guo (2019) demonstrated that the safflower CtCHI1 localized to the nucleus, while Li (2019) found that the *Salvia miltiorrhiza* SmCHI localized not only to the Golgi apparatus, plasma membrane, and nucleus in tobacco epidermal cells, but also to the endoplasmic reticulum, peroxisomes, and plastids.

Building upon our previous findings of significant differences in puerarin and total flavonoid content between *P. montana* and *P. thomsonii*, and based on established knowledge that chalcone isomerase serves as a key enzyme in flavonoid biosynthesis, we referenced the work of Yoshiya Terai et al. (1996) who cloned a 756 bp CHI gene from *P. montana* containing a 675 bp ORF encoding 225 amino acids with a protein size of 23,803 Da. The present study employed homologous cloning, IPTG-induced *in vitro* protein expression, and *Arabidopsis* protoplast transformation to address three key questions: (1) Do CHI genes differ between *P. thomsonii* and *P. montana*? (2) How does CHI expression vary between these species? (3) Where does the CHI protein localize within cells? These findings will inform discussions on puerarin and total flavonoid content differences between wild and cultivated *Pueraria* species and establish a foundation for elucidating the mechanisms by which PtCHI regulates flavonoid accumulation in *P. thomsonii*.

Materials and Methods

1.1 Plant Materials The experimental material consisted of the edible *Pueraria thomsonii* Benth. variety "Guige No. 1" bred by our research group, cultivated in the botanical garden of the College of Agriculture, Guangxi University.

1.2 Quantification of Puerarin and Total Flavonoids Puerarin extraction was performed by weighing 0.1 g of kudzu powder into a flask containing 50 mL of 30% ethanol, sealing with plastic wrap, and sonicating at 250 W for 30 minutes. After cooling to room temperature, the weight was restored with 30% ethanol, the solution was filtered, and 5 mL of filtrate was diluted to 25 mL with 30% ethanol to obtain a 20 $g \cdot mL^{1}$ puerarin extract. HPLC analysis employed a mobile phase of methanol:0.1% acetic acid (30:70) at a flow rate of 1.0 $mL \cdot min^{1}$ with detection at 250 nm. Total flavonoid extraction and quantification followed the method of Li and Wu (2008).

1.3 Cloning and Expression Analysis of *P. thomsonii* PtCHI Total RNA was extracted from "Guige No. 1" and reverse-transcribed to cDNA. PCR amplification was performed using primers PtCHI-F and PtCHI-R (Table 1). The purified product was ligated into the pMD-19T vector and transformed into DH5 competent cells. Positive clones were sequenced and aligned using DNA MAN. Expression analysis in *P. montana* and *P. thomsonii* was conducted using primers PtCHI-DLF and PtCHI-DLR.

1.4 Bioinformatic Analysis of PtCHI The open reading frame was identified using NCBI ORF Finder. Physicochemical properties were predicted with ProtParam. Secondary and tertiary structures were predicted using SOPMA and SWISS-MODEL, respectively. Subcellular localization was predicted with WoLF PSORT. Phosphorylation sites were identified using NetPhos 3.1 Server. Protein-protein interactions were analyzed using STRING with the soybean protein database. Phylogenetic analysis was performed using MEGA 7.0.

1.5 Prokaryotic Expression and Western Blot Verification The PtCHI ORF was amplified using primers PtCHI-YHF and PtCHI-YHR (Table 1), purified, and ligated into the linearized pET-28a vector to generate the pET28a-PtCHI construct. Following IPTG induction, SDS-PAGE analysis confirmed soluble protein expression. The recombinant protein was purified using Ni-NTA affinity chromatography and verified by Western blot using anti-His tag mouse monoclonal antibody as primary antibody and goat anti-mouse IgG (H+L) as secondary antibody.

1.6 Subcellular Localization The PtCHI ORF was amplified using primers PtCHI-YXBF and PtCHI-YXBR (Table 1), ligated into the linearized pSAT6-EYFP-N1 vector to generate pSAT6-EYFP-N1-PtCHI. *Arabidopsis* protoplasts were isolated from 25-day-old seedlings by enzymatic digestion (2.5% Cellulase R10 and 0.3% Macerozyme R10) of finely sliced leaves for 3-4 hours, followed by filtration and washing. Transformation was performed using 20% PEG4000. After incubation, images were captured using confocal microscopy.

Table 1. Specific primers for expression vector construction

Primer name	Primer sequence
PtCHI-F	GATCGAAACCCTTAATTTCA
PtCHI-R	ATCACTTTCCCTCAACTCAG
PtCHI-DLF	CAGCAGCAGTAGCAACCATC
PtCHI-DLR	CGAGTGATGACACCGCTTTA
PtCHI-YHF	cagcaaatgggtcgcggatccATGGCGGCAGCAGCAGCA
PtCHI-YHR	gtggtggtggtggtgctcgagGACTATAATGCCGTGGCTCAATAC
PtCHI-YXBF	ggtccggactcagatctcgagCATGGCGGCAGCAGCAGCA
PtCHI-YXBR	gctcaccatcaggatcccgggGACTATAATGCCGTGGCTCAATAC

Results

2.1 Quantification of Puerarin and Total Flavonoids As shown in Figure 1, *P. montana* contained significantly higher puerarin content than *P. thomsonii* (Guige No. 1), approximately 3.5-fold greater. Although total flavonoid content was also elevated in *P. montana*, the difference was not statistically significant.

Figure 1. Content of puerarin and total flavonoids
** indicates significant difference at the 0.01 level.

2.2 Cloning and Expression Analysis of PtCHI Using primers designed from the reported *P. montana* CHI sequence and leaf cDNA from *P. thomsonii* as template, we amplified the PtCHI gene fragment (Figure 2). The cDNA sequence was 742 bp in length, containing a complete 672 bp ORF spanning from the start codon ATG at position 55 to the stop codon TGA at position 726, encoding 223 amino acids (Figure 3). BLAST analysis revealed 99% homology with the *P. montana* CHI protein. Expression analysis showed tissue-specific patterns: in *P. thomsonii*, expression was highest in stem, followed by root and leaf (stem > root > leaf), whereas *P. montana* showed root > stem > leaf expression. Notably, CHI expression in *P. montana* was significantly higher than in *P. thomsonii* in all tissues except leaves (Figure 4).

Figure 2. PCR amplification of PtCHI gene
M: DL2000 DNA Marker; 1: PCR product of PtCHI gene.

Figure 3. cDNA sequence and deduced amino acid sequence of PtCHI gene

Figure 4. Expression of CHI genes in different tissues
Different lowercase letters indicate significant differences ($P < 0.05$).

2.2.1 Physicochemical Properties and Subcellular Localization Prediction Physicochemical analysis predicted PtCHI to be a stable hydrophilic protein of 27.8 kD with a theoretical pI of 5.34 and an instability index of 32.25. The aliphatic index was 95.40, with negatively charged residues comprising 12.05% and positively charged residues 9.82% of total amino acids, suggesting an overall negative charge. Subcellular localization prediction indicated highest probability (64.28%) for cytoplasmic localization, followed by chloroplast (14.28%), with nuclear, mitochondrial, and Golgi localization each predicted at 7.14%.

2.2.2 Secondary and Tertiary Structure Prediction SOPMA analysis revealed that the secondary structure consisted predominantly of -helices (48.66%), followed by random coils (24.11%) and extended strands (17.41%), with -turns being least abundant (9.82%) (Figure 5). SWISS-MODEL tertiary structure prediction confirmed these findings, showing a structure dominated by -helices, consistent with the secondary structure analysis (Figure 6).

Figure 5. Secondary structure prediction of PtCHI protein

Figure 6. Tertiary structure prediction of PtCHI protein

2.2.3 Phosphorylation Site Prediction NetPhos 3.1 Server identified 25 potential phosphorylation sites, with the highest probability scores at serine-107 (0.969) and proline-169 (0.964), followed by serine-29 (0.901) and serine-203

(0.902). Additional high-probability sites included serine-26 (0.894), threonine-44 (0.863), serine-101 (0.844), and serine-112 (0.827) (Figure 7).

Figure 7. Phosphorylation site prediction of PtCHI protein

2.2.4 Phylogenetic Analysis MEGA 7.0 phylogenetic analysis of PtCHI amino acid sequences from 11 species revealed two major clusters. PtCHI from Guige No. 1 grouped with *P. montana*, soybean (*Glycine max*), *Glycyrrhiza uralensis*, *Mucuna pruriens*, and *Spatholobus suberectus*, while *Phaseolus vulgaris*, *Vigna radiata*, and *V. unguiculata* formed a separate cluster, and *Abrus precatorius* and *Arabidopsis thaliana* constituted a distinct group. This indicates close evolutionary relationships between *P. thomsonii* PtCHI and CHI proteins from *P. montana*, soybean, and *G. uralensis* (Figure 8).

Figure 8. Phylogenetic analysis of PtCHI amino acid sequence

2.2.5 Protein Interaction Prediction STRING analysis using the soybean protein database predicted strong interactions between CHI and flavanone 3-hydroxylases F3H2 and F3H (score 0.994 each), which are iron/ascorbate-dependent oxidoreductases involved in anthocyanin and proanthocyanidin accumulation. Additional high-confidence interactions included 4-coumarate-CoA ligase 4CL4 (score 0.980), involved in lignin synthesis, and dihydroflavonol reductase DFR2 (score 0.974), associated with anthocyanin accumulation. Interactions with flavonoid 3' -hydroxylase SF3' H1 (score 0.956) and chalcone synthase CHS8 (score 0.952) were also predicted (Figure 9).

Figure 9. PtCHI protein interaction analysis

2.3.1 Prokaryotic Expression Vector Construction PCR amplification using specific primers (Table 1) yielded a ~669 bp product matching the target size (Figure 10A). The product was ligated into linearized pET-28a, transformed into DH5 , and verified by restriction digestion with XhoI and SpeI, which released bands at approximately 5,300 bp and 669 bp (Figure 10B). Sequencing-confirmed plasmids were transformed into BL21 competent cells, with PCR verification confirming successful insertion (Figure 10C), demonstrating successful construction of the pET28a-PtCHI expression strain.

Figure 10. Construction of prokaryotic expression vector pET28a-PtCHI
A: PCR amplification of PtCHI (1-5: amplification products). B: 1: Double digestion of pET28a-PtCHI; 2: Plasmid extraction. C: PCR verification of pET28a-PtCHI in BL21 (1-3: amplification products). M: DL2000 Marker.

2.3.2 Recombinant Protein Induction The pET28a-PtCHI strain was cultured with 0.5 mM IPTG at 16 °C and 30 °C. SDS-PAGE revealed a specific protein band at ~30 kD corresponding to the target protein, absent in uninduced controls, confirming successful expression. Notably, higher temperature

induction yielded less soluble protein in the supernatant, indicating that lower temperatures promote soluble expression (Figure 11).

Figure 11. SDS-PAGE analysis of PtCHI prokaryotic expression
A: Induction at 16 °C, 100 rpm, 0.5 mM IPTG. B: Induction at 30 °C, 100 rpm, 0.5 mM IPTG. M: Protein marker; 1: Total uninduced protein; 2: Total IPTG-induced protein; 3: IPTG-induced supernatant; 4: IPTG-induced pellet.

2.3.3 Protein Purification and Quantification Ni-NTA affinity chromatography exploited the binding of nickel ions to histidine imidazole rings for purification. Increasing imidazole concentration in the mobile phase enabled elution of target protein. Figure 12 shows effective purification: contaminating proteins were removed at low imidazole concentrations (20-100 mM), while target protein eluted above 120 mM. At 160 mM imidazole, a single prominent band indicated high purity. Buffer exchange yielded a final PtCHI concentration of 6.39 $g \cdot L^{1}$.

Figure 12. Purification of PtCHI recombinant protein by Ni-affinity chromatography
M: Protein marker; 1: Eluate; 2: 20 mM imidazole; 3: 40 mM imidazole; 4: 80 mM imidazole; 5: 100 mM imidazole; 6: 120 mM imidazole; 7: 160 mM imidazole; 8: 200 mM imidazole.

2.3.4 Western Blot Verification The pET28a vector' s His-tag enabled Western blot detection using anti-His antibodies. Exposure revealed a target band at ~27.8 kD on PVDF membrane (Figure 13). Specific bands were detected across different protein loadings (10-50 ng), confirming the purified protein as PtCHI.

Figure 13. Western blot analysis of PtCHI recombinant protein
M: Protein marker; 1: 10 ng loading; 2: 20 ng loading; 3: 30 ng loading; 4: 50 ng loading.

2.4.1 Subcellular Localization Vector Construction PCR with primers PtCHI-YXBF/R yielded a single 669 bp band (Figure 14A). After purification, the product was ligated into linearized pSAT6-EYFP-N1 and transformed into DH5 . Restriction digestion with XhoI and XmaI verified the construct, releasing bands at ~4,600 bp and 669 bp (Figure 14B). Sequence-confirmed plasmids were prepared for *Arabidopsis* transient expression.

Figure 14. Construction of subcellular localization recombinant plasmid
A: PCR amplification of PtCHI. B: Double digestion of pSAT6-EYFP-N1-PtCHI.

2.4.2 Subcellular Localization Analysis PEG-CaCl -mediated transformation of pSAT6-EYFP-N1-PtCHI into *Arabidopsis* protoplasts revealed green flu-

orescence from the EYFP channel localized around chloroplasts after 16 hours (Figure 15A), overlapping with chloroplast autofluorescence (Figure 15B). This demonstrates that PtCHI localizes specifically to chloroplasts.

Figure 15. Subcellular localization of PtCHI
A: YFP channel green fluorescence; B: Chloroplast autofluorescence; C: Merged image.

Discussion

3.1 Relationship Between CHI Expression and Isoflavonoid Accumulation Isoflavonoids serve as the primary bioactive components in *Pueraria*, with their content representing a key criterion for medicinal quality evaluation. Previous studies have documented substantial differences in total flavonoid content between wild and cultivated *Pueraria*, with *P. montana* containing significantly higher isoflavonoid levels. Wang et al. (2009) reported that *P. montana* from the Funiu Mountain region exhibited three-fold higher total flavonoid content than *P. thomsonii*, with puerarin content 2.5 times greater. Meng et al. (2020) found that 100 g of *P. montana* powder contained 5.08 g total isoflavones, significantly exceeding the 0.463 g in *P. thomsonii*, with correspondingly higher puerarin and daidzin content. Zhang et al. (2017) similarly observed significantly higher cellulose, soluble sugars, total flavonoids, and puerarin in Guangxi *P. montana* compared to *P. thomsonii*.

As the second rate-limiting enzyme in flavonoid biosynthesis, chalcone isomerase critically influences isoflavonoid accumulation, pigment synthesis, and stress resistance. Overexpression studies in *Glycyrrhiza uralensis* demonstrated that transgenic hairy roots accumulated 1.394 g·100 g ^{1} total flavonoids, significantly higher than wild-type (0.842 g·100 g ^{1}), with combined 2% PEG8000 and 0.1% yeast extract treatment further elevating flavonoid content to 2.838 g·100 g ^{1} while upregulating CHI transcription. In *Talinum paniculatum*, overexpression of soybean GmCHI increased total flavonoid content 4.8-7.4 fold compared to wild-type (VU et al., 2018). Our findings align with these reports, showing both higher puerarin and total flavonoid content in *P. montana* and significantly elevated CHI expression in roots and stems compared to *P. thomsonii*. These results strongly suggest a close relationship between CHI expression and flavonoid accumulation in *Pueraria*, providing valuable insights for future quality improvement of cultivated *P. thomsonii*.

3.2 Structural Differences in CHI Genes and Isoflavonoid Accumulation Functional divergence often arises from structural variations within gene families. In sugarcane, ScJAZ family members ScJAZ22, ScJAZ24, and ScJAZ30 exhibited distinct gene structures, amino acid numbers, and molecular weights, displaying differential expression patterns during *Xanthomonas albilineans* infection of resistant variety ROC22, with ScJAZ22 downregulated

while ScJAZ24 and ScJAZ30 were upregulated. Similarly, Dong et al. (2021) identified 197 bHLH genes in pear with variable gene structures, coding sequences, and exon numbers, showing differential expression under cold and heat treatments, where PbrbHLH7, PbrbHLH8, PbrbHLH128, PbrbHLH160, PbrbHLH161, and PbrbHLH195 were upregulated while other members remained unchanged. These examples demonstrate that structural differences within gene families confer functional diversity.

The PtCHI gene cloned from *P. thomsonii* contained a 672 bp ORF encoding 223 amino acids, differing by 3 bp and one amino acid from the *P. montana* PmCHI (675 bp, 225 amino acids) reported by Yoshiya Terai et al. (1996). Sequence alignment revealed a phenylalanine (Ala) deletion at position 5 in *P. thomsonii*, though this site is neither a phosphorylation site nor a conserved domain. The observed differences in ORF length, amino acid number, and expression levels between the two species parallel findings in sugarcane and pear. Whether these structural variations confer functional differences that contribute to flavonoid content variation requires further investigation.

3.3 Subcellular Localization, Protein Interactions, and Isoflavonoid Accumulation Phylogenetic analysis revealed close relationships between *P. thomsonii* PtCHI and CHI proteins from *P. montana*, soybean, and *G. uralensis*. CHS-CHI interactions have been documented in rice, *Arabidopsis*, *Selaginella moellendorffii*, and *Physcomitrella patens* (Ban et al., 2018). Fujino et al. (2018) reported that *Antirrhinum majus* AmCHS and AmCHI localize to the endoplasmic reticulum and nucleus, where CHI interacts with CHS, FNSII, F3H, and DFR to enhance -galactosidase activity. Ban et al. (2018) demonstrated that hop CHIL2 and CHS_H1 both localize to the cytoplasm and interact to increase xanthohumol and quercetin chalcone production 1.4-fold and 1.5-fold, respectively, compared to individual expression. Waki et al. (2016) showed that soybean GmIFS interacts with GmCHS1, GmCHS7, GmCHI1A, GmCHI1B2, and GmCHI2, with stronger GmIFS-GmCHI interactions and higher -galactosidase activity than GmIFS-GmCHS. Dastmalchi et al. (2016) observed cytoplasmic interactions between soybean GmIFS1/GmIFS2 and GmCHR14, GmCHI2, GmCHS7, and GmCHS8, with strongest fluorescence for GmIFS2-GmCHR14 and weakest for GmIFS2-GmCHS7/GmCHS8.

Our study localized PtCHI exclusively to chloroplasts in the cytoplasm, differing from previous reports, likely due to species-specific variations. Predicted interactions with CHS, F3H, and DFR2 align with previous studies linking these proteins to anthocyanin, pigment, and lignin accumulation. The absence of detected interaction with PtIFS raises the question of whether substrate competition among enzymes results in minimal PtIFS-CHI interaction, potentially limiting isoflavonoid biosynthesis in *P. thomsonii*. This warrants further investigation.

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Note: Figure translations are in progress. See original paper for figures.