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Original Paper

Anthocyanin mutants of Japanese and common morning glories exhibit normal proanthocyanidin accumulation in seed coats

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Abstract Anthocyanin and proanthocyanidin biosynthesis pathways are believed to overlap. This study examined proanthocyanidin accumulation in seed coats of morning glories (*Ipomoea nil* and *I. purpurea*) carrying mutations in *CHS-D*, *CHI*, and *ANS* genes encoding chalcone synthase, chalcone isomerase, and anthocyanidin synthase, respectively. Chemical staining revealed that mutants accumulate proanthocyanidin normally. Thus, the tested genes are not essential to proanthocyanidin biosynthesis, but are essential to anthocyanin biosynthesis in flowers and stems. Based on the results and the *I. nil* draft genome sequence, the genes involved in proanthocyanidin biosynthesis, including a new copy of the flavanone 3-hydroxylase gene could be predicted. Moreover, the genome has no homologs for known enzymes involved in producing flavan-3-ols, the starter and extension units of proanthocyanidin. These results suggested that *I. nil* produces flavan-3-ols through an undiscovered biosynthesis pathway. To characterize proanthocyanidin pigmentation further, we conducted mutant screening using a large *I. nil* population. We discovered that the *brown* mutant lines (exhibiting brown seeds and normal anthocyanin pigmentation) do not accumulate proanthocyanidin in their seed coats. Thus, the *brown* mutation should be useful for further investigations into the various mechanisms controlling anthocyanin and proanthocyanidin pathways.

Key words: Anthocyanin, *Ipomoea nil*, *Ipomoea purpurea*, proanthocyanidin, seed coat.

Introduction

Proanthocyanidins (or condensed tannins) are flavan-3-ol polymers and a class of flavonoid pigments. Distributed in a wide range of plant species, proanthocyanidins play an important role in defense against UV radiation, microbial pathogens, insect pests, and herbivore predation (Barbehenn and Peter Constabel 2011; Dixon et al. 2005). Their oxidization in seed coats confers the typical brown color indicating seed maturation (Marles et al. 2003; Tanner et al. 2003). Proanthocyanidins also have economic importance as the source of astringency in fruit, wines, and teas, while also being powerful antioxidants with potential protective effects against cancers, reactive oxygen species, and cholesterol accumulation (Dixon et al. 2005). Thus, considerable effort has been expended on increasing proanthocyanidin quantity in fruits and legumes (Dixon et al. 2013).

Proanthocyanidins and anthocyanins are thought to be more recently evolved flavonoids (Koes et al. 1994). The early steps of their biosynthesis pathway involve the same enzymes (Figure 1). In *Arabidopsis*, both anthocyanin and proanthocyanidin are absent if genes encoding any one of the following enzymes are defective: chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydoroxylase (F3′H), dihydroflavonol 4-reductase (DFR), and anthocyanidin synthase (ANS) (Feinbaum and Ausubel 1988; Koornneef 1990; Shirley et al. 1992). However, it is unclear if these genes mediate both anthocyanin and proanthocyanidin production in other plants.

Abbreviations: ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydoroxylase; LAR, leucoanthocyanidin reductase.

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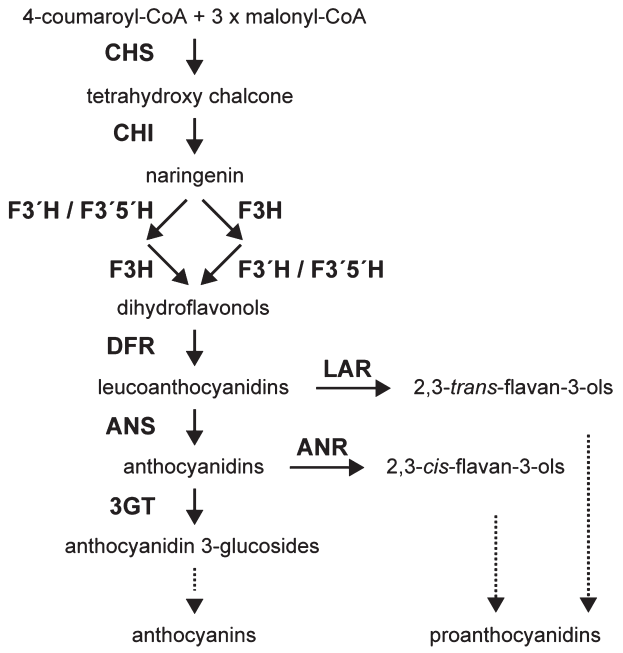

Figure 1. Schematic diagram representing the flavonoid pathway of anthocyanin and proanthocyanidin biosynthesis in plants. Enzymes are shown in boldface. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydoroxylase; F3′5′H, flavonoid 3′,5′-hydoroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, UDP-glucose: flavonoid 3-*O*-glycosyltransferase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase.

Leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) are two enzymes involved in the late steps of proanthocyanidin biosynthesis (Devic et al. 1999; Tanner et al. 2003; Xie et al. 2003). Respectively, LAR and ANR catalyze 2,3-*trans*-flavan-3-ol formation from leucoanthocyanidins and 2,3-*cis*-flavan-3-ol from anthocyanidins (Figure 1) (Winkel-Shirley 2006). In addition, 2,3-*trans*-flavan-3-ols (catechins) and 2,3-*cis*-flavan-3-ols (epicatechins) are the starter and extension units of proanthocyanidin. *Arabidopsis* produces proanthocyanidin based on 2,3-*cis*-flavan-3-ols, whereas many other plants base their proanthocyanidin production on both flavan-3-ol types (Abrahams et al. 2003; Harborne and Williams 2000; Routaboul et al. 2006; Tanner et al. 2003). No *LAR* genes have been found in *Arabidopsis* (Lepiniec et al. 2006).

The expression of proanthocyanidin and anthocyanin biosynthesis genes is controlled by transcriptional activators containing the R2R3-MYB domain, basic helix-loop-helix (bHLH) domain, and WD40 repeats (WDR). These activators interact to regulate anthocyanin/proanthocyanidin pigmentation and other epidermal traits, including root hair and trichome formation, as well as seed coat mucilage production (Hichri et al. 2011; Koes et al. 2005; Lepiniec et al. 2006; Ramsay and Glover 2005).

Morning glory species, the Japanese morning glory

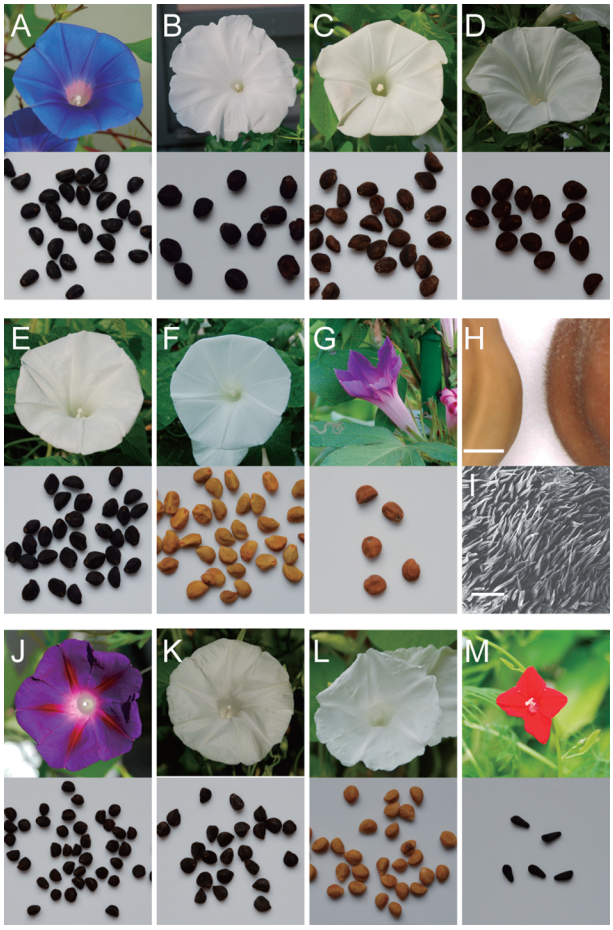

Figure 2. Flower and seed phenotypes of *Ipomoea* species. (A–G) *Ipomoea nil* lines. (A) TKS (wild-type), (B) AK19 (*CHS-D* mutant), (C) AK40 (*CHI* mutant), (D) AK10 (*DFR-B* mutant), (E) AK23 (*ANS* mutant), (F) NS/W1*ca1* (*InWDR1* mutant), and (G) Q0306 (*brown* mutant). (H) Seed pigmentation and trichome formation in NS/W1*ca1* (left) and Q0306 (right). The photograph was taken using a digital microscope (VHX-100, Keyence, Osaka, Japan), and the scale bar represents 1 mm. (I) SEM image of seed trichome in Q0306; the scale bar represents 200 μm. (J–L) Phenotypes of *I. purpurea* lines. (J) FP39 (wild-type), (K) PR640 (*CHS-D* mutant), and (L) YJH/DR-4 (*bHLH2* mutant). (M) Wild-type *I. quamoclit* line, Q0055.

(*Ipomoea nil*) and the common morning glory (*I. purpurea*) are commercially important horticultural plants (Figure 2A–L). Wild type *I. nil* and *I. purpurea* have blue and purple flowers, respectively (Figure 2A, J), through the production of anthocyanins. Several mutations resulting in flower-color alterations have been isolated in these species (Chopra et al. 2006; Iida et al. 2004), allowing for characterization of the structural and regulatory genes for anthocyanin biosynthesis (Koes et al. 2005; Petroni and Tonelli 2011). Structural genes, *CHS-D*, *CHI*, *DFR-B*, and *ANS* in *I. nil*, as well as *CHS-D* in *I. purpurea*, are essential for flower and stem anthocyanin production (Figure 2B–F, K) (Chopra et al. 2006; Habu et al. 1998; Hoshino et al. 2009; Iida et al. 2004; Inagaki et al. 1994), but their exact roles in proanthocyanidin production are unclear. *Ipomoea*

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seed coats accumulate proanthocyanidins, but seeds of mutants for the above genes are dark brown and indistinguishable from wild-type seeds (Park and Hoshino 2012; Park et al. 2007), likely because phytomelanins comprise the dark-brown pigment along with proanthocyanidins. Thus, proanthocyanidin accumulation cannot be characterized using seed appearance alone. In a recent study, we used chemical staining to show that the *DFR-B* gene is not essential for proanthocyanidin synthesis in *I. nil* (Park and Hoshino 2012). Previous work has also demonstrated that *InMYB1* and *InWDR1* in *I. nil* and *IpMYB1* and *IpbHLH2* in *I. purpurea* all activate anthocyanin pigmentation in flowers (Chang et al. 2005; Morita et al. 2006; Park and Hoshino 2012; Park et al. 2007). Furthermore, *InWDR1* and *IpbHLH2* are also involved in activating both proanthocyanidin and phytomelanin pigmentation in seed coats, as evidenced by the lack of brown seed pigmentation among mutants (Figure 2F, L). However, *InWDR1* does not regulate *CHS-D*, *CHI*, or *ANS*, whereas *IpbHLH2* activates *DFR-B* and *ANS* expression, but not *CHS-D* expression in seed coats (Park and Hoshino 2012; Park et al. 2007).

From these observations, we hypothesized that *CHS-D*, *CHI*, and *ANS* are not involved in proanthocyanidin production, similar to our findings for *DFR-B*. To test this hypothesis, we measured proanthocyanidin accumulation in *CHS-D*, *CHI*, and *ANS* loss-of-function morning glory mutants. We then predicted genes involved in proanthocyanidin biosynthesis using the published *I. nil* draft genome sequence (Hoshino et al. 2016). Finally, we screened a large collection of *I. nil* lines and related *Ipomoea* species to isolate novel mutants exhibiting altered proanthocyanidin pigmentation in seed coats.

Materials and methods

Plant materials

Wild-type *I. nil* (Tokyo-kokei standard, TKS; Kawasaki and Nitasaka 2004), *I. purpurea* (YO/FP-39, FP39; Habu et al. 1998), and *I. quamoclit* (Q0055; Sakata Nursery, Yokohama, Japan) all have dark brown seeds, but produce blue, purple, and red flowers, respectively. Negative controls for the proanthocyanidin assay were recessive *InWDR1* and *IpbHLH2* mutants of *I. nil* (NS/W1*ca1*) and *I. purpurea* (YJH/WR-4); both produce ivory seeds that do not accumulate proanthocyanidins (Morita et al. 2006; Park et al. 2007). The study also used lines AK19 (Ginsekai; Takii & Co., Ltd., Kyoto Japan), AK40, AK10, and AK23 (r3) that were, respectively, *I. nil CHS-D*, *CHI*, *DFR-B*, and *ANS* mutants (Chopra et al. 2006; Habu et al. 1998; Hoshino et al. 2001, 2009; Iida et al. 2004; Inagaki et al. 1994). The *I. purpurea CHS-D* mutant (PR640) was also included. All mutant lines produced white flowers and green stems. Drs. Keiichi Shimizu, Norio Saito, and Caitilin Corberly provided AK10, AK23, and PR640, respectively. Line AK40 was from our own collection. Finally, proanthocyanidin mutants were screened using 205 lines (Table 1) from the National BioResource Project (NBRP) morning glory (http://www.shigen.nig.ac.jp/asagao/index.jsp).

Proanthocyanidin analysis

Seed proanthocyanidins were quantified using vanillin and DMACA staining, as described previously (Abrahams et al. 2002; Debeaujon et al. 2000; Park et al. 2007). Immature seeds were soaked in either 5% vanillin solution (MeOH : HCl = 2 : 1, v/v) or 0.15% DMACA (MeOH : HCl = 3 : 1, v/v) solutions for 10 min at room temperature. Proanthocyanidin presence resulted in pink-reddish (vanillin) or bluish (DMACA) staining.

Sequence and expression analysis

To identify proanthocyanidin biosynthesis genes, *Arabidopsis* proteins were searched against the *I. nil* draft genome sequence

Table 1. Summary of proanthocyanidin mutant screening.

Seed color	Line	PAs*
Black or dark-brown	Q0114, Q0160, Q0188, Q0191, Q0205, Q0240, Q0243, Q0254, Q0255, Q0270, Q0273, Q0303, Q0304, Q0312, Q0313, Q0316, Q0321, Q0325, Q0330, Q0333, Q0335, Q0336, Q0337, Q0339, Q0342, Q0343, Q0344, Q0346, Q0347, Q0349, Q0352, Q0353, Q0355, Q0357, Q0370, Q0371, Q0373, Q0374, Q0375, Q0410, Q0415, Q0426, Q0438, Q0441, Q0442, Q0448, Q0449, Q0459, Q0464, Q0465, Q0466, Q0467, Q0468, Q0470, Q0471, Q0515, Q0525, Q0537, Q0538, Q0539, Q0575, Q0584, Q0626, Q0635, Q0640, Q0644, Q0645, Q0652, Q0661, Q0663, Q0664, Q0666, Q0667, Q0668, Q0669, Q0670, Q0671, Q0672, Q0673, Q0677, Q0679, Q0703, Q0726, Q0751, Q0771, Q0783, Q0789, Q0821, Q0829, Q0830, Q0837, Q0840, Q0889, Q0893, Q0895, Q0933, Q0943, Q0961, Q0962, Q0963, Q0964, Q0965, Q1055, Q1057, Q1058, Q1065, Q1071, Q1072, Q1075, Q1083, Q1094, Q1095, Q1096, Q1097, Q1098, Q1099, Q1202, Q1214, Q1243, Q1245, Q1246, Q1247, Q1248, Q1249, Q1250, Q1251, Q1252, Q1255, Q1256, Q1257, Q1258, Q1259, Q1261, Q1263, Q1267, Q1268, Q1270, Q1271, Q1272, Q1273, Q1274, Q1275, Q1276, Q1277, Q1278, Q1279, Q1280, Q1281, Q1282, Q1283, Q1284, Q1285, Q1286, Q1287, Q1288, Q1289, Q1290, Q1291, Q1292, Q1293, Q1294, Q1295, Q1297, Q1298, Q1299, Q1300, Q1301, Q1302, Q1303, Q1304, Q1305, Q1306, Q1308, Q1309, Q1310, Q1312, Q1313, Q1314, Q1315, Q1316, Q1317, Q1319, Q1320, Q1322	+
Light-brown	Q0306, Q0314, Q0332, Q0338, Q0341, Q0350, Q0356, Q0359, Q0545, Q0607, Q0674, Q0790, Q0810, Q1041, Q1264, Q1296, Q1307, Q1311	−
Ivory	Q0646, Q0660, Q1059	−

*Proanthocyanidins detected using chemical staining.

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(Hoshino et al. 2016) using BLASTP. Protein databases from NCBI and NBRP (morning glory, http://viewer.shigen.info/asagao/) were used. Distribution of *LAR* and *ANR* were tested using the KEGG database that includes protein sequences predicted from the whole genome sequences of multiple plant species (Kanehisa et al. 2016).

The wild-type *I. nil* (TKS) was used for reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from immature seed coats at 4, 8, 12, 16, 20, 24, and 28 days after pollination (DAP) was extracted using a Get pureRNA Kit (Dojindo Molecular Technologies, Kumamoto, Japan). RNA extraction from flower buds was performed as described previously (Park et al. 2007). First-strand cDNA was synthesized using the SuperScript III reverse transcriptase kit (Thermo Fisher Scientific, Waltham, MA, USA). The internal control was *glyceraldehyde 3-phosphate dehydrogenase 2* (*GAPDH2*) (Park and Hoshino 2012). Amplification of *F3H-A, F3H-B, and F3H-C* cDNA was performed with the following primer sets, respectively: F3HA-F2/R1 (5′-AAG GGC ATT GAC GAC GTC CT-3′, 5′-CAG ATG GAA ATA GCA GCC GA-3′); F3HB-F2/R1 (5′-GGC ATT GAC GAC GAC GTC CA-3′, 5′-CTC CAC ATC CCT CAT CCT TG-3′); and F3HC-F2/R1 (5′-CAT CGA CGA CGG CGG CGT TA-3′, 5′-GAA ATT ATG GAG CGG GCT AG-3′). Thermocycling conditions were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation (98°C for 10 s), annealing (60°C for 15 s), and extension (68°C for 1 min).

Scanning electron microscopy

Mature seeds were glued to a stage, frozen in liquid nitrogen for 1 min, and examined using a scanning electron microscope (XL30, Philips, Amsterdam, Netherlands) at 10 kV.

Results

Proanthocyanidin accumulation in I. nil seed coats over time

Proanthocyanidins were previously observed in immature *I. nil* seeds at 24 DAP (Park and Hoshino 2012), but the pigment's temporal accumulation during seed development has not been characterized. Therefore, we assayed proanthocyanidin accumulation every 4 day from 12 to 32 DAP (Figure 3A). Proanthocyanidin accumulation was undetectable at 12 DAP but increased from 16 to 24 DAP, peaking at 24–28 DAP, and then decreasing at 32 DAP, when seeds turned black (Figure 3A). As seeds mature, proanthocyanidin content likely decreases through forming insoluble, oxidized complexes with other phenolics and cell wall polysaccharides, thus causing seed color to darken (Marles et al. 2003).

Proanthocyanidin accumulation in anthocyanin mutants

We examined proanthocyanidin production in seed coats

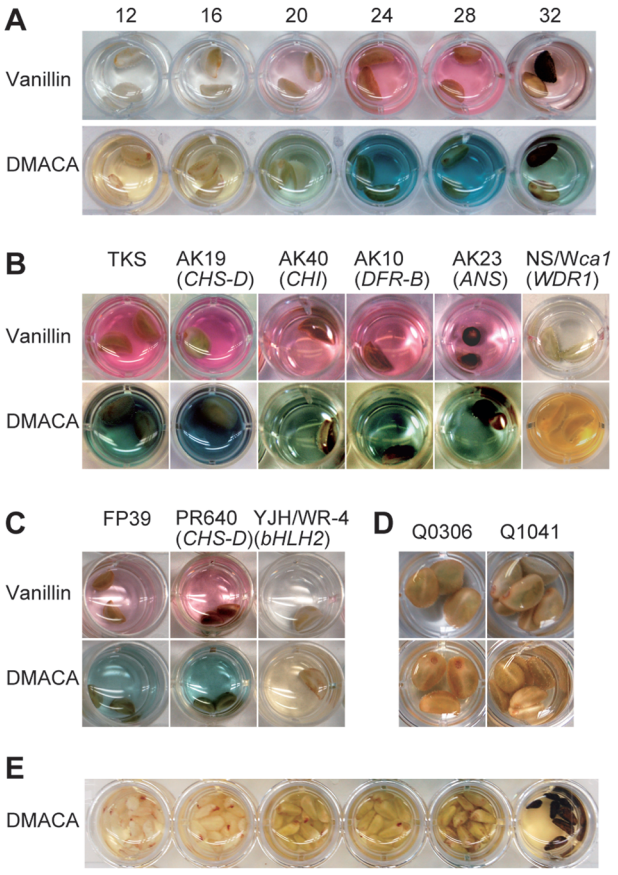

Figure 3. Proanthocyanidin accumulation in *Ipomoea* seeds. (A) Temporal accumulation of proanthocyanidins in wild-type *I. nil* (TKS). Numerals on photos indicate days after pollination (DAP); seeds were soaked in a vanillin or DMACA solution for staining. (B) Proanthocyanidin accumulation in immature (28 DAP) seeds of anthocyanin-deficient *I. nil* mutants. The lines used were TKS (wild-type), AK19 (*CHS-D* mutant), AK40 (*CHI* mutant), AK10 (*DFR-B* mutant), AK23 (*ANS* mutant), NS/W1*ca1* (*InWDR1* mutant). Flower and seed phenotypes of these lines are shown in Figure 2. (C) Proanthocyanidin accumulation in seed coat of the *CHS-D* mutant *I. purpurea* line (PR640) at 28 DAP. Lines FP39 (wild-type) and YJH/WR-4 (*bHLH2* mutant) were used as positive and negative controls, respectively. (D) Proanthocyanidin mutant screening revealed that *brown* mutant lines Q0306 and Q1041 were proanthocyanidin mutants. (E) Immature seeds at various developmental stages of wild-type *I. quamoclit* (Q0055) remained unstained after being soaked in a DMACA solution.

of *CHS-D*, *CHI*, and *ANS* mutants. Proanthocyanidin accumulation was highest at 28 DAP in wild-type plants (Figure 3A). When immature mutant seeds of *I. nil* and *I. purpurea* were treated with vanillin-HCl and DMACA-HCl solutions at 28 DAP, all exhibited reddish and bluish staining (Figure 3B, C), indicating proanthocyanidin accumulation. Overall, staining results were indistinguishable across mutants and the wild type. Corroborating previous studies showing a lack of proanthocyanidin accumulation in the seed coats of *InWDR1* and *IpbHLH2* mutants (Park and Hoshino 2012; Park et al. 2007), we did not observe staining in these lines (Figure 3B, C). Our data suggested that

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proanthocyanidins accumulate normally in seed coats of *CHS-D*, *CHI*, and *ANS* mutants. Therefore, despite being essential for anthocyanin production in flower petals, these three genes (along with *DFR-B*) in *I. nil*, as well as *CHS-D* in *I. purpurea*, are not required for seed-coat proanthocyanidin accumulation.

Identification of additional copies of proanthocyanidin biosynthesis genes from the I. nil draft genome

Although flavonoid biosynthesis genes have been extensively characterized in *I. nil*, their genomic copy numbers remain unknown. We therefore employed BLASTP searches with *Arabidopsis* flavonoid biosynthesis enzyme sequences against *I. nil* protein databases to confirm copy number. The results are summarized in Table 2. No additional gene copies were found, except for *F3H*. Previous studies have shown that *F3H-A* is likely responsible for anthocyanin pigmentation in flowers because its expression is activated by *InWDR1* and *InMYB1* (Morita et al. 2006). The *I. nil* genome carries two additional *F3H* genes: *F3H-B* and *F3H-C* (Table 2). *F3H-B* seems to be a pseudogene because its deduced protein sequence is shorter at the 3′ end than either F3H-A (36 amino acids) or F3H-C (30 amino acids). Publicly available RNA-seq data (Hoshino et al. 2016) and RT-PCR analysis indicated that all three *F3H* are transcribed in both flower and seed coats (Table 2, Figure 4). As no *F3H* mutants have been isolated, we do not know the exact roles of these three *F3H* genes in anthocyanin and proanthocyanidin biosynthesis.

Among the flavonoid biosynthesis genes given in Table 2, *CHS-D*, *CHS-E*, *F3′H*, *ANS*, and *3GT* genes have a single intron, while *CHI* carries two introns. *F3H* and *DFR* genes contain three and five introns, respectively. Two of three *F3H* (*F3H-A* and *F3H-B*) and three *DFR* genes were duplicated in tandem on chromosomes 2 and 5, respectively. The publicly available RNA-seq data (Hoshino et al. 2016) indicated that all the genes are transcribed in leaves and stems in addition to flowers. The data also indicated that all genes, except for *F3H-B* and *3GT*, are expressed in roots. Only *CHI*, *F3H-B*, *F3H-C*, and *F3′H* transcripts were found in the RNA-seq data from embryos.

Moreover, BLASTP searches indicated that *I. nil* does not possess genes related to flavan-3-ol production, given that the best-match sequences using *Medicago truncatula* LAR and *Arabidopsis* ANR as queries were a putative isoflavone reductase (XP_019174833) and *DFR-B*, respectively. To test whether *LAR* and *ANR* absence is unique to *I. nil*, we evaluated putative flavonoid biosynthesis pathways using the KEGG database. We found that *LAR* and *ANR* are absent from the genomes of *Cucumis sativus* (cucumber), *Cucumis melo* (muskmelon), *Momordica charantia* (bitter melon), *Cucurbita maxima* (winter squash), *Capsicum annuum* (red pepper), *Sesamum indicum* (sesame), and *Dendrobium officinale*.

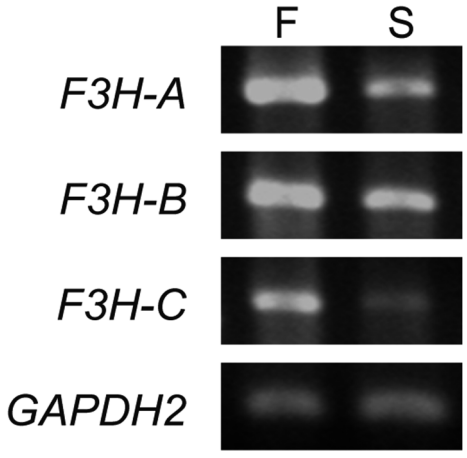

Figure 4. RT-PCR analysis for the *F3H* genes in *Ipomoea nil*. "F" and "S" indicate flower petals and seed coats, respectively.

Table 2. Flavonoid biosynthesis genes found in the *Ipomoea nil* genome.

Gene	Accession No.	Gene ID		RNA expression		RNA-seq		Mutation	Necessity**	
		Original*	NCBI	Petal	Seed coat	Flower	Seed coat		Petal	Seed coat
CHS-D	AB001818	INIL12g08537	109176547	+	−	+	−	transposon	essential	unnecessary
CHS-E	AB001819	INIL14g35461	109161594	+	+	+	+		essential***	essential
CHI		INIL11g18443	109186979	+	+	+	+	transposon	essential	not essential
F3H-A	D83041	INIL02g39928	109167724	+	+	+	+		(redundant)	(redundant)
F3H-B		INIL02g39929	109167725	+	+	+	+	pseudo gene	unnecessary	unnecessary
F3H-C		INIL05g09512	109177773	+	+	+	+		(redundant)	(redundant)
F3′H	AB113261	INIL04g34064	109160581	+	+	+	+	substitution	not essential	not essential
DFR-A		INIL05g09558	109177611	+	+	+	−		unnecessary	redundant
DFR-B	AB006792	INIL05g09559	109177619	+	+	+	−	transposon	essential	not essential
DFR-C		INIL05g09560	109177620	+	−	+	−		unnecessary	unnecessary
ANS	AB073920	INIL13g40254	109168138	+	−	+	−	transposon	essential	unnecessary
3GT	LC019108	INIL10g12321	109182028	+	+	+	+	transposon	essential	(unnecessary)

*The original gene ID is from a previous publication (Hoshino et al. 2016). **Predictions without mutant analyses are presented in parentheses. ****CHS-E* expression is responsible for flower tube pigmentation in some *I. nil* lines (Hoshino et al. 2009).

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Screening of I. nil proanthocyanidin mutants

We screened *I. nil* proanthocyanidin mutants to further examine the genetic mechanisms underlying proanthocyanidin synthesis. Most mutant lines exhibited morphological alterations in flower and leaf shape or pigmentation. Of the 205 screened lines, 184 had black or dark-brown seeds, 18 had light-brown seeds, and 3 had ivory-colored seeds (Table 1, Figure 2). Classical genetic studies showed that *brown* and *ca-white* (*ca*) mutations confer light-brown and ivory-colored seeds, respectively (Hagiwara 1931, 1937; Miyake and Imai 1920; Miyazawa 1923). The gene *Ca* (encoding InWDR1) regulates both flower and seed-coat pigmentation (Morita et al. 2006). Immature seeds from all 205 lines were subjected to DMACA-HCl assays. Every line with black or dark-brown seeds exhibited staining characteristic of proanthocyanidin accumulation. Lines Q0114, Q0353, and Q0830 were *F3′H* mutants (Morita et al. 2005), suggesting that *F3′H* is not essential for proanthocyanidin biosynthesis.

In contrast, the 18 light-brown and ivory lines did not exhibit vanillin-HCl and DMACA-HCl staining (Table 1, Figure 3D), indicating that proanthocyanidin accumulation did not occur in their seed coats.

Dark-brown seed pigmentation in I. quamoclit without proanthocyanidin accumulation

We also investigated proanthocyanidin accumulation in the cypress vine (*I. quamoclit*), a species related to *I. nil*. Although mature *I. quamoclit* seeds were dark brown (Figure 2M), blue staining after DMACA-HCl treatment was not observed in seeds at any developmental stage (Figure 3E). This outcome strongly suggests that proanthocyanidins are not the source of dark-brown pigmentation in *I. quamoclit* seeds.

Discussion

In this study, we demonstrated that *CHS-D*, *CHI*, and *ANS* in *I. nil*, as well as *CHS-D* in *I. purpurea*, are not essential for proanthocyanidin accumulation in seed coats (Figure 3B, C). These genes, however, are essential to anthocyanin production in flowers and stems (Table 2) (Chopra et al. 2006; Habu et al. 1998; Hoshino et al. 2009; Iida et al. 2004; Inagaki et al. 1994). Our previous study similarly showed that *DFR-B* in *I. nil* is not essential for proanthocyanidin accumulation in seed coats, but essential for anthocyanin pigmentation (Park and Hoshino 2012). Together, the data suggest that different genes are involved in the early steps of anthocyanin and proanthocyanidin biosynthesis. In contrast, *Arabidopsis CHS*, *CHI*, *F3H*, *F3′H*, *DFR*, and *ANS* are essential for both anthocyanin and proanthocyanidin biosynthesis; their mutants do not accumulate proanthocyanidin in seed coats and thus present a transparent test of phenotype (Abrahams et al. 2003; Shikazono et al. 2003; Shirley et al. 1992, 1995; Wisman et al. 1998).

Both *I. nil* and *I. purpurea* have at least two active *CHS* genes (*CHS-D* and *CHS-E*), but only the latter is expressed in seed coats (Johzuka-Hisatomi et al. 1999; Park and Hoshino 2012; Park et al. 2007). This finding coincides with our conclusion that *CHS-D* is unnecessary for proanthocyanidin accumulation. Instead, *CHS-E* is likely to be responsible for proanthocyanidin biosynthesis in *I. nil* and *I. purpurea*.

We had previously shown that among the three active *DFR* genes in *I. nil, DFR-A* and *DFR-B* transcripts accumulate in the seed coats (Park and Hoshino 2012). Because *DFR-B* mutants nevertheless accumulate proanthocyanidin in seed coats (Figure 3B) (Park and Hoshino 2012), we conclude that *DFR-A* and *DFR-B* are functionally redundant, at least in terms of proanthocyanidin production.

We found a single gene copy of *CHI*, *F3′H*, and *ANS* in *I. nil* (Table 2). This outcome appears to be inconsistent with the observation of normal proanthocyanidin accumulation in *CHI* mutants. The most plausible explanation is that CHI activity is not essential for proanthocyanidin synthesis in the *I. nil* seed coats. Indeed, chalcones can spontaneously isomerize into flavanones without CHI activity (Davies and Schwinn 2005). The reaction is nonstereospecific, resulting in (2*S*)- and (2*R*)-flavanone; CHI activity simply guarantees generation of the former. Additionally, *F3′H* is also non-essential for proanthocyanidin production because its mutants (Q0114, Q0353, and Q0830; Morita et al. 2005) accumulate proanthocyanidins (Table 1). This result suggests that both flavan-3-ols with and without the 3′-hydroxyl group are proanthocyanidin precursors in *I. nil*. Our current study examining publicly available RNA-seq data (Table 2) further confirmed that *ANS* is not expressed in *I. nil* seed coats (Park and Hoshino 2012). Because ANS activity is essential for 2,3-*cis*-flavan-3-ol but not 2,3-*trans*-flavan-3-ol production (Park and Hoshino 2012), we suggest that *I. nil* uses the latter as starter and extension units of proanthocyanidins. This hypothesis is further supported by the observation that *ANS* mutants accumulate proanthocyanidin normally. Taken together, existing data allow us to predict the roles of proanthocyanidin-production genes in *I. nil* seed coats (Table 2). Specifically, *CHS-E*, *CHI*, and the two *F3H* (*F3H-A, F3H-C*) and *DFR* (*DFR-A* and *DFR-B*) genes are involved in the biosynthesis pathway, but *CHI* and *DFR-B* are not essential.

Previous work has suggested that LAR converts leucoanthocyanidins to 2,3-*trans*-flavan-3-ols in the proanthocyanidin pathway (Figure 1) (Xie and Dixon 2005). Unexpectedly, our BLASTP search revealed that the *I. nil* genome does not contain *LAR* or *ANR* genes. Moreover, the absence of *LAR* or *ANR* in some plant

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species, especially plants belonging Cucurbitaceae, was supported by the KEGG database survey. Among Cucurbitaceae plants, proanthocyanidins were found in the seeds and whole fruits of cucumber and bitter melon, respectively (Tan et al. 2014; Zhu et al. 2016). Thus, some plants may produce proanthocyanidins via undiscovered biosynthesis pathways without LAR and ANR involvement. Further analysis of *I. nil* proanthocyanidins will benefit the identification of such pathways.

Our screening of *I. nil* lines revealed that *brown* and *InWDR1* (*ca*) mutant lines did not accumulate proanthocyanidin in their seed coats. Previously, we used another *InWDR1* mutant line (NS/W1*ca1*) to show that *InWDR1* activates proanthocyanidin pigmentation in seeds (Park and Hoshino 2012), and our current findings confirm those results. Although *InWDR1* mutation removes anthocyanin, resulting in white flowers and green stems (Hagiwara 1931, 1937; Miyake and Imai 1920; Miyazawa 1923), the effects of the *brown* mutation on anthocyanin pigmentation have not been reported until now. Here we demonstrate that *brown* mutants show normal anthocyanin accumulation. This study is the first to describe mutations that result in alterations to proanthocyanidin but not anthocyanin pigmentation among *Ipomoea* species. Based on our results, we propose that *brown* mutants are a useful material for detailed examinations of differential mechanisms underlying anthocyanin and proanthocyanidin production. We observed that *brown* mutants have slightly darker seeds than *InWDR1* mutants (Figure 2F–H). The latter accumulate phytomelanins at approximately 20% of wild-type levels. Therefore, pigmentation differences between *brown* and *InWDR1* mutants may be attributable to differing phytomelanin content in seed coats. More research is necessary to determine whether the *brown* gene controls phytomelanin in addition to proanthocyanidin. Notably, *brown* mutants produce normal seed trichomes (Figure 2H–I). *InWDR1* and *IpbHLH2* control seed trichome formation in *I. nil* and *I. purpurea*, respectively, besides anthocyanin biosynthesis in flowers and proanthocyanidin and phytomelanin pigmentation in seeds (Morita et al. 2006; Park and Hoshino 2012; Park et al. 2007). These observations suggest that the *Brown* gene product is not a component of the transcriptional activator complex including InWDR1 and InbHLH2, which is the product of *IpbHLH2* ortholog in *I. nil*.

Finally, our screening revealed that despite having dark-brown seed coats, *I. quamoclit* does not accumulate proanthocyanidins (Figure 3E). We suggest that the brown pigmentation of *I. quamoclit* seed coats may comprise phytomelanins. Additionally, previous studies have shown that *ANS* is expressed in *I. purpurea* but not *I. nil* seed coats, indicating that their respective flavan-3-ols units are 2,3-*trans*-flavan-3-ols and 2,3-*cis*-flavan-3-ols (Park and Hoshino 2012; Park et al. 2007). Together, the data suggest that *Ipomoea* species exhibit considerable diversity in the compositions of their seed coat pigments.

In conclusion, our anthocyanin mutant analyses suggested that *I. nil* uses a different set of genes for the early steps in anthocyanin and proanthocyanidin biosynthesis pathways, although *Arabidopsis* uses the same set of genes for these steps. This allows normal proanthocyanidin accumulation in seed coats of anthocyanin mutants of *I. nil*. The lack of *LAR* and *ANR* genes in *I. nil* implied the possibility that some plant species produce proanthocyanidins via unelucidated biosynthesis pathways. The *brown* mutants and *I. quamoclit* are useful materials for further elucidation of anthocyanin and proanthocyanidin production.

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