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 oxidation 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’-hydroxylase (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, anthocyanin reductase; ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; F3’H, flavonoid 3’-hydroxylase; LAR, leucoanthocyanidin reductase.

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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 (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

**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; F3'H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3’5’H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, UDP-glucose-flavonoid 3-O-glycosyltransferase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase.

**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/W1ca1 (InWDR1 mutant), and (G) Q0306 (brown mutant). (H) Seed pigmentation and trichome formation in NS/W1ca1 (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.
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 phyto melanins 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 IpHLH2 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 IpHLH2 are also involved in activating both proanthocyanidin and phyto melanin 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 IpHLH2 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.

Table 1. Summary of proanthocyanidin mutant screening.

<table>
<thead>
<tr>
<th>Seed color</th>
<th>Line</th>
<th>PAs*</th>
</tr>
</thead>
</table>
| 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, Q0597, Q0626, Q0635, Q0639, Q0640, Q0644, Q0645, Q0652, Q0661, Q0663, Q0664, Q0666, Q0667, Q0668, Q0669, Q0670, Q0671, Q0672, Q0673, Q0676, Q0677, Q0679, Q0703, Q0726, Q0751, Q0771, Q0783, Q0789, Q0821, Q0829, Q0830, Q0837, Q0840, Q0889, Q0895, Q0933, Q0943, Q0961, Q0962, Q0963, Q0964, Q0965, Q1055, Q1057, Q1058, Q1065, Q1067, Q1071, Q1072, Q1075, Q1083, Q1094, Q1095, Q1096, Q1097, Q1098, Q1099, Q1122, Q1124, Q1243, Q1245, Q1246, Q1247, Q1248, Q1249, Q1250, Q1251, Q1252, Q1255, Q1256, Q1257, Q1258, Q1259, Q1261, Q1263, Q1267, Q1268, Q1270, Q1271, Q1272, Q1274, Q1275, Q1276, Q1277, Q1278, Q1279, Q1280, Q1281, Q1282, Q1283, Q1284, Q1285, Q1286, Q1287, Q1288, 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, Q1321, Q1322 | +
| Light-brown         | Q0306, Q0314, Q0332, Q0338, Q0341, Q0350, Q0356, Q0359, Q0545, Q0607, Q0674, Q0790, Q0810, Q1041, Q1264, Q1296, Q1307, Q1311 | –
| Ivory               | Q0466, Q0660, Q1059          | –

*Proanthocyanidins detected using chemical staining.

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 IpHLH2 mutants of I. nil (NS/W1ca1) 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; Taki & 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; lida 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.
(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 GCC ATT GAC GAC GTC CT-3′, 5′-CAG ATG GAA ATA GCA GCC GA-3′); F3HB-F2/R1 (5′-GGC ATT GAC GAC GTC CT-3′, 5′-CTC CAC ATC CCT CAT CCT TG-3′); and F3HC-F2/R1 (5′-CAT CGA CGG CGG GGT 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 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 IpβHLH2 mutants (Park and Hoshino 2012; Park et al. 2007), we did not observe staining in these lines (Figure 3B, C). Our data suggested that...
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.](image)

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Gene ID</th>
<th>RNA expression</th>
<th>RNA-seq</th>
<th>Mutation</th>
<th>Necessity**</th>
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<td>NCBI</td>
<td>Petal</td>
<td>Seed coat</td>
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<td><strong>CHS-D</strong></td>
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<td>−</td>
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<td><strong>F3H-C</strong></td>
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</table>

*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).*
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 (2S)- and (2R)-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
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/W1ca1) 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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