The flexibility of proanthocyanidin biosynthesis in plants

Nan Lu,1 Ji Hyung Jun,1,† Chenggang Liu1 and Richard A. Dixon1,*

1 BioDiscovery Institute and Department of Biological Sciences, University of North Texas, Denton TX 76203, USA

*Author for correspondence: Richard.Dixon@unt.edu
†Present address: Children’s Research Institute and the Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, Texas 75390, USA

Dear Editor,

Proanthocyanidins (PAs) are natural oligomers or polymers of flavan 3-ols that accumulate in plants. Increasing evidence suggests benefits of PAs in agriculture, environmental sustainability, and human health (Mueller-Harvey et al., 2019; Rauf et al., 2019; Roldan et al., 2022). Our understanding of PA biosynthesis was until recently based on the model dicot species Arabidopsis (Arabidopsis thaliana), utilizing the large pool of T-DNA insertional mutants. Through forward genetic screening, a set of A. thaliana transparent testa (tt) mutants with impaired PA deposition in the seed coat has been identified and a PA biosynthesis pathway proposed based on characterized enzymes (Lepiniec et al., 2006). Subsequently, further progress has been made studying PA biosynthesis in the model legume Medicago truncatula, and other crops such as grapevine (Vitis vinifera). As expected, many genes involved in PA biosynthesis in A. thaliana are functionally conserved across species. However, it is now becoming apparent that the PA-specific branch of flavonoid biosynthesis is unusually flexible, and that different species have adopted different solutions to the problem of “managing” the trafficking and chemical condensation of precursors that act as PA starter and extension units.

Conserved proteins involved in PA biosynthesis include the transcription factors TRANSPARENT TESTA 2 (AtTT2, with ortholog MYEOLOBLASTOSIS FAMILY 14 (MtMYB14) in M. truncatula), AtMYB5/MtMYB5, AtTT8/MtTT8, TRANSPARENT TESTA GLABROUS 1 (AtTTG1, with ortholog WD40-DOMAIN PROTEIN (MtWD40-1) in M. truncatula), and AtMYB2/MtMYB2; the transporter AtTT12, with ortholog MULTIDRUG AND TOXIC COMPOUND EXTRUSION FAMILY 1 (MtMATE1) in M. truncatula; and anthocyanidin reductase (ANR; Figure 1, A and B). ANR catalyzes the reaction that generates flavan 3-ols (e.g. epicatechin), the building blocks of PAs (Xie et al., 2003). Surprisingly, however, it now appears that the biosynthetic routes to epicatechin starter and extension units are different (Jun et al., 2018, 2021), and an additional function of ANR in generating the epicatechin-based PA extension unit 2,3-cis-leucocyanidin was recently demonstrated (Jun et al., 2021; Figure 1, A and B). The carbocation intermediates derived from leucocyanidins are highly reactive, and the reaction to generate PA dimers from 2,3-cis-leucocyanidin and epicatechin occurs spontaneously in vitro. PA polymerization may be modulated by the chemical sequestration or physical separation of stable starter and reactive extension units until they reach their site for nonenzymatic polymerization (the vacuole or vesicles in route to the vacuole), thereby avoiding accumulation in the cytosol of potentially self-toxic monomers. The need to physically protect or separate PA starter and/or extension units may account for the difficulty in reconciling the functions of nonconserved genes implicated in PA biosynthesis between species.

Several PA-related gene functions and mutant phenotypes differ between A. thaliana, M. truncatula, and other species. For example, M. truncatula leucoanthocyanidin dioxygenase (Iodox; Jun et al., 2018) and leucoanthocyanidin reductase (lar; Liu et al., 2016) mutants show strongly reduced PA levels.
but wild-type anthocyanin levels, but there are no apparent LDOX or LAR homologs in *A. thaliana* (Figure 1, A and B). The *A. thaliana* tt19 mutant, caused by loss of function of a glutathione S-transferase (AtGSTF12, TT19), shows reduced soluble PAs and increased insoluble PAs in young seeds (Kitamura et al., 2004, 2010), strongly resembling the lar mutant in *M. truncatula* (Liu et al., 2016). However, tt19 also exhibits a strong loss-of-anthocyanin phenotype, indicating that although its exact function remains unclear, TT19 participates in both PA and anthocyanin pathways in *A. thaliana*. The involvement of TT19 and its homologous genes in PA and anthocyanin biosynthesis in other species has been extensively studied. However, with only few exceptions (e.g. Wang et al., 2012), TT19 homologs from multiple species, including *M. truncatula* (see Wang et al., 2022 and references therein), are associated with anthocyanin biosynthesis but fail to complement the reduced PA phenotype of the tt19 mutant background. Additionally, an anthocyanin-related GST, BRONZE 2 (BZ2), was identified in maize (*Zea mays*), a species that does not appear to produce PAs. Why
TT19-like genes are required for PA polymerization in some but not all species remains unresolved.

In *M. truncatula*, LAR has been proposed to modulate the degree of PA polymerization by balancing epicatechin (starter unit) and epicatechin–cysteine (extension unit) levels (Liu et al., 2016). It was hypothesized that TT19 might modulate PA polymerization in *A. thaliana* by transiently binding to either starter or extension units until they mix (Dixon and Sarnala, 2020). To test this hypothesis, we analyzed PA profiles in Arabidopsis *tt19-8* and Col-0 (wild-type) young seeds using liquid chromatography–mass spectrometry (Figure 2). In the soluble PA fraction, the levels of free PA starter units (epicatechin) were much lower in *tt19-8* than in Col-0, but levels of the free potential extension unit 4β-(S-cysteinyl)-epicatechin were similar or even higher. These data indicate that TT19 is necessary for maintenance of PA starter units, but likely not the more reactive extension units.

**URIDINE DIPHOSPHATE GLYCOSYL TRANSFERASE 72L1** (UGT72L1) is a glucosyltransferase from *M. truncatula* that forms epicatechin-3′-O-glucoside (Pang et al., 2008), the substrate for the vacuolar transporter MtMATE1 (the homolog of TT12; Zhao and Dixon, 2009; Figure 1B). However, no UGT responsible for epicatechin hexoside formation in *A. thaliana* has been unequivocally identified. UGTs are involved in detoxification processes, and it is therefore reasonable to suppose that UGT72L1 facilitates transfer of PA starter units to vacuoles. However, to date, there are no genetic data implicating UGTs in PA biosynthesis. Furthermore, it is possible that epicatechin is not the in vivo substrate for the formation of epicatechin-3′-O-glucoside; by analogy, it was recently suggested that 5,7,3′,4′-tetrahydroxy-flav-2-en-3-ol 3′-O-glucoside (2F3G), rather than cyanidin itself, is the immediate precursor of cyanidin 3-O-glucoside (Yoshida et al., 2020), and flav-en-ol intermediates have also been implicated in epicatechin biosynthesis (Jun et al., 2018, 2021).

Currently, the preferred strategy for engineering PAs in plants is through overexpression of transcription factors (TFs), which has proven effective in some species (Roldan et al., 2022). However, ectopic TF expression can affect off-target genes that are not directly linked to flavonoid biosynthesis, potentially leading to pleiotropic effects on plant development (Liu et al., 2014; James et al., 2017; Lu et al., 2021). Furthermore, several crop species, such as maize, lack an endogenous traditional PA pathway. Therefore, an alternative strategy is to use synthetic biology to build the PA pathway in desired tissues, which requires identification of the minimal gene set for PA production in a particular species. This is made more difficult by the apparent lack of enzymatic control of the final polymerization step, species differences in PA precursor trafficking and sequestration, and the lack of a clear understanding of the cell biology of these processes.

![Figure 2](image-url) Analysis of PA starter and extension units in wild-type Col-0 and *tt19-8* seeds. Detection of epicatechin (epi; starter unit, left) and 4β-(S-cysteinyl)-epicatechin levels (epi-cys; extension unit, right) by accurate mass liquid chromatography–mass spectrometry according to Lu et al. (2021) and Jun et al. (2021). Top panels show authentic standards. Inset shows epicatechin scan in *tt19-8* at increased resolution (×10^3). SD, standards; cps, counts per second.
In conclusion, current data suggest that different species have evolved different routes for PA biosynthesis. In the models in Figure 1, plants may utilize either a GST or LAR to modulate the polymerization process. Strategies for engineering PAs and tailoring PA compositions in forage and row crops will benefit from a better understanding of the ways in which different plant species handle the trafficking and polymerization of reactive PA pathway intermediates.

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