Dual activity of anthocyanidin reductase supports the dominant plant proanthocyanidin extension unit pathway

Ji Hyung Jun, Nan Lu, Maite Docampo-Palacios, Xiaqiang Wang, Richard A. Dixon*

Proanthocyanidins (PAs) are plant natural products important for agriculture and human health. They are polymers of flavan-3-ol subunits, commonly (−)-epicatechin and/or (+)-catechin, but the source of the in planta extension unit that comprises the bulk of the polymer remains unclear, as does how PA composition is determined in different plant species. Anthocyanidin reductase (ANR) can generate 2,3-cis-epicatechin as a PA starter unit from cyanidin, which itself arises from 2,3-trans-leucocyanidin, but ANR proteins from different species produce mixtures of flavan-3-ols with different stereochemistries in vitro. Genetic and biochemical analyses here show that ANR has dual activity and is involved not only in the production of (−)-epicatechin starter units but also in the formation of 2,3-cis-leucocyanidin to serve as (−)-epicatechin extension units. Differences in the product specificities of ANRs account for the presence/absence of PA polymerization and the compositions of PAs across plant species.

INTRODUCTION

Proanthocyanidins (PAs, polymers of flavan-3-ol units) are a major class of polyphenolic compounds that can accumulate to high levels in leaves, fruits, seeds, and bark in most plant families (1–3) and that have beneficial effects for preventing development of conditions such as Alzheimer’s and cardiovascular diseases (4, 5). In addition, the non-specific protein binding properties of PAs affect forage quality (6, 7) and taste and storage life of beverages (8, 9). PAs also protect plants against herbivory (10) and may contribute to carbon capture and soil nitrogen recycling (11). The lack of full understanding of PA biosynthesis limits our ability to manipulate PA structures in plants to enhance nutritional value, organoleptic properties, forage quality, herbivore resistance, and environmental sustainability.

PAs are composed of both 2,3-trans flavan-3-ols, e.g., (+)-gallo catechin, and 2,3-cis flavan-3-ols, e.g., (−)-epi(gallo)catechin. PA biosynthesis has been elucidated genetically through studies of transparent testa (tt) mutants lacking seed pigments in thale cress (Arabidopsis thaliana) (12) and shares early steps with those of anthocyanin biosynthesis. Anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR) lead to production of the two flavan-3-ols (−)-epicatechin and (−)-catechin, respectively (Fig. 1A) (13, 14). However, ANR from different species can generate more than one flavan-3-ol stereoisomer (15–17), and LAR also generates flavan-3-ol from epicatechin-cysteine (epi-cys), a potential PA extension unit (18). Two closely related dioxygenases, leucoanthocyanidin dioxygenase (MtLDOX) and anthocyanidin synthase (MtANS), function in parallel routes to provide starter and extension units, respectively, in the model legume Medicago truncatula (Fig. 1A) (19).

The biochemical control of product stereochemistry in PA biosynthesis remains unclear. Although the chemical patterns of PAs found in plants suggest simple thermodynamic control mediated by nonenzymatic conjugation of leucocyanidin and flavan-3-ol (20), the mechanisms of PA polymerization and precursor supply have proven difficult to confirm in planta. Flavan-3,4-diols (leucocyanidins) readily dissociate to carbocation or quinone methide intermediates that can react with nucleophilic centers such as C-6 or C-8 on flavan-3-ols (21–24). Retention of the 2,3-trans stereochemistry of (−)-dihydroquercetin (DHQ) in leucocyanidin formed by dihydroflavanol reductase (DFR) suggests that, if leucocyanidin is an in planta PA extension unit, then another reaction, such as the activity of ANR with cyanidin (Fig. 1A) (19), is necessary to generate the 2,3-cis-stereochemistry in (−)-epicatechin, the predominant extension unit across many plant species (Fig. 1A) (25–27). Although this role of ANR is supported genetically (13, 18), the production of more than one flavan-3-ol stereoisomer by different ANRs (15–17) and the possession of active ANRs in plants that do not accumulate substantial levels of PAs (28, 29) remain paradoxical.

Here, we show that 2,3-cis-3,4-cis-leucocyanidin produced from 2R-flav-3-en-3,4-diol via a second activity of ANR can act as the source of epicatechin extension units in planta. The dual activity of ANR explains the compositional variation and presence or absence of PA accumulation in different plants.

RESULTS

PA levels and composition in seeds of different plant species

To provide a basis for understanding potential relationships between ANR specificity and PA composition across species, we first subjected soluble PA extracts from the seeds of the model plants M. truncatula and A. thaliana and the crop plants soybean (Glycine max), barley (Hordeum vulgare), maize (Zea mays), and wheat (Triticum aestivum) to phloroglucinolysis coupled with accurate mass liquid chromatography–mass spectrometry (LC/MS) to confirm identity of PA starter and extension units. High-performance LC (HPLC) with ultraviolet (UV) detection was used to distinguish epicatechin and catechin extension units. A. thaliana and M. truncatula seeds contained the highest levels of soluble PAs (Fig. 1B). PAs from M. truncatula, A. thaliana, and soybean were mainly composed of epicatechin, whereas PAs from barley contained mostly catechin starter and extension units (Fig. 1C). Little to no PA accumulation was detected in maize and wheat seed samples (Fig. 1B).
Product formation from different ANR enzymes in vitro

ANR proteins cluster phylogenetically according to plant family, with separation of legume and monocot ANRs (fig. S1). To determine whether there is a relationship between the in vitro ANR activity of a particular species and its corresponding PA content or composition as determined above, we cloned ANR open reading frames from A. thaliana, M. truncatula, soybean, the high-PA-containing legume Desmodium uncinatum, tea (Camellia sinensis), grapevine (Vitis vinifera), barley, wheat, and maize and expressed their recombinant proteins in Escherichia coli (fig. S2). LDOX from M. truncatula converts (+)-catechin to flav-2-en-3-ol, which is subsequently oxidized to cyanidin (19). We therefore examined the ANR-mediated formation of epicatechin in vitro by two routes: directly from cyanidin or via coupled reactions with LDOX and (+)-catechin as substrate. In coupled reactions of LDOX with recombinant AtANR, MtANR, or GmANR, conversion of (+)-catechin to epicatechin was efficient and stereospecific to generate the (−)-(2,3-cis) isomer (Fig. 2, A and B). Similarly, VvANR, CsANR2, and DuANR produced only (−)-epicatechin in such coupled reactions but produced a mixture of flavan-3-ol stereoisomers directly from cyanidin (fig. S3). Recombinant ANR proteins from maize, wheat, and barley generated varying mixtures of (+)/(−)-epicatechin or catechin directly from cyanidin,
with (+)-epicatechin as the most common product (fig. S3, A and B). These enzymes produced (+)-epicatechin, at low efficiency, in coupled reactions from catechin with LDOX (Fig. 2, A and B).

The flavylum ion of cyanidin undergoes conversion to different forms as a function of pH (30). We therefore compared the products of MtANR, AtANR, and ZmANR with cyanidin at pH 5.0 and 7.0 (fig. S4). For MtANR and AtANR, (+)-epicatechin remained the major product, with increased production of (−)-epicatechin at lower pH, whereas (+)-epicatechin increased with ZmANR at pH 5.0 (fig. S4, A and B). pH had little effect on (−)-epicatechin production from (+)-catechin by MtANR and AtANR in coupled reactions with LDOX, but the (+)-epicatechin formed by ZmANR at pH 7.0 was mostly replaced by (−)-catechin at pH 5.0 (fig. S4, C and D).

Flav-2-en-3-ol is the preferred substrate for ANR for formation of PA starter units

The LAR/LDOX pathway that generates flav-2-en-3-ol is involved in the formation of PA starter units, but not extension units, in M. truncatula (Fig. 1A) (19). Flav-2-en-3-ol can theoretically be reduced directly to epicatechin. To determine whether flav-2-en-3-ol or cyanidin is the preferred substrate for ANR in coupled reactions with LDOX and (+)-catechin, we performed time-course experiments. On incubating (+)-catechin with MtLDOX and 2-oxoglutarate at pH 7.0, flav-2-en-3-ol was detectable at 2 and 5 min and then gradually decreased, with a corresponding increase in cyanidin (fig. S5, A and B). At each time point, recombinant MtANR was added to the reaction mixtures, and the products were analyzed after 30 min. Epicatechin generation was most efficient at initial time points up to 10 min. Smaller peaks of epicatechin and traces of catechin were detected when ANR was added after 1 and 2 hours of initial incubation, and cyanidin was still present in these mixtures (fig. S5, C and D). These results suggest that flav-2-en-3-ol is the preferred substrate for ANR to stereospecifically produce (−)-epicatechin.

ANRs from species with epicatechin extension units produce 2,3-cis-leucocyanidin from flav-en-3,4-diol in vitro

2,3-trans-Leucocyanidin (2R,3S,4S) is assumed to be the origin of catechin extension units in wild-type plants with catechin-rich PAs and is incorporated as a catechin extension unit in PAs in M. truncatula ans and ans lar mutants (19). This suggests that 2,3-cis-leucocyanidin (2R,3R,4R) could correspondingly serve as an epicatechin extension unit.
unit, although this compound has yet to be identified in plants. ANS can convert 2,3-trans-leucocyanidin to a flav-3-en-3,4-diol (or flav-2-en-3,4-diol) with double bonds between the 3 and 4 (or 2 and 3) positions of the C-ring (31, 32). This compound could theoretically be reduced to 2,3-cis-leucocyanidin. We therefore tested whether MtANR can reduce a flaven-3,4-diol to introduce cis configuration at the 2,3 positions. (+)-2R,3S-DHQ was used as initial substrate for coupled reactions with recombinant MtDFR1, MtANS, and MtANR according to the scheme in Fig. 2E, monitored by ultraperformance LC/MS (UPLC/MS). Recombinant DFR1 alone converted DHQ to a compound matching the retention time (RT, 4.4 min) of an HPLC analysis, this compound had the same RT as chemically synthesized 2,3-trans-leucocyanidin (fig. S6A) but was not seen in coupled reactions with DFR1 and ANS (Fig. 2C and fig. S6A). However, in reactions containing MtANR, a compound with the molecular mass of leucocyanidin [mass/charge ratio \((m/z) 305.0667\)] was seen at a RT of 3.8 min (Fig. 2C). To confirm that this compound was 2,3-cis-leucocyanidin, the products at RT of 3.8 and 4.4 min were subjected to tandem MS (MS/MS) analysis along with chemically synthesized 2,3-trans-leucocyanidin. The \([M-H]^-\) ion with \(m/z 305.0667\) produced the same fragment ions for both compounds, suggesting that the products generated by DFR1 and DFR1 + ANS + ANR have the same formula and structure (Fig. 3C).

To determine their stereochemistries, the two compounds were subjected to nuclear magnetic resonance (NMR) spectrometry (Fig. 3, D to G, and table S1). 2,3-trans-Leucocyanidin was a mixture of 3,4-cis (79%) and 3,4-trans (11%) as previously reported (table S1) (34), and the compound generated by the coupled DFR1 + ANS + ANR reactions showed \(^1H\) - and \(^13C\)-NMR spectra characteristic of three consecutive methane-proton systems (Fig. 3, D to G, and table S2). The one-dimensional (1D) proton NMR spectrum and chemical shifts (table S2) were consistent with an isomer of leucocyanidin as previously reported (32). H-2 and H-3 were used to determine the \(J\) coupling constants between H-2, H-3, and H-4, because H-4 was obscured by the residual water peak (Fig. 3G). This measurement was possible by measuring \(\gamma_{2,3}\) from the splitting of the H-2 signal because H-3 was coupled to both H-2 and H-4. Therefore, the other splitting of H-3 was due to coupling with H-4. Small coupling constants between H-2 and H-3 (1.2 Hz) and between H-3 and H-4 (2.9 Hz) confirmed that there is cis-stereochemistry between the

---

**Fig. 3. Identification of 2,3-cis-3,4-cis-leucocyanidin.** (A) Phenyl reversed-phase HPLC profiles at 280 nm (32) for purification of product of coupled reaction of DFR1, ANS and MtANR with dihydroquercetin as substrate (purification 1) or with DFR1 only and dihydroquercetin as substrate (purification 2). (B) C18 reversed-phase HPLC profiles at 280 nm of the purified compounds [shaded in (A)] as standards along with products of the single and coupled enzyme reactions. (C) MS/MS spectra of chemically synthesized 2,3-trans-3,4-cis-leucocyanidin (2R,3S,4S), putative 2,3-cis-leucocyanidin and 2,3-trans-leucocyanidin purified in (A). All samples were analyzed with an accurate mass Sciex TripleTOF 6600 mass spectrometer (detected mass values for each compound underlined). (D) 1D proton NMR spectrum of putative 2,3-cis-leucocyanidin. (E) 2D COSY NMR spectrum of putative 2,3-cis-leucocyanidin. (F) 2D HSQC NMR spectra of putative 2,3-cis-leucocyanidin. (G) Two regions of the homonuclear 2D/NMR spectrum of the putative 2,3-cis-leucocyanidin for determination of \(\gamma\) coupling constants. NMR spectra were acquired at 600 MHz with 20 \(\mu\)g of 2,3-cis-3,4-cis-leucocyanidin, purified from the DFR + ANS + ANR reaction with dihydroquercetin as initial substrate, concentrated in 5 \(\mu\)l of water and diluted in 280 \(\mu\)l of D2O. cis-L, putative 2,3-cis-leucocyanidin; trans-L, 2,3-trans-3,4-cis-leucocyanidin; 2,3-trans-3,4-trans-L, 2,3-trans-3,4-trans-leucocyanidin; Sd, chemically synthesized 2,3-trans-leucocyanidin standard.
protons of vicinal diols in a ring, identifying the compound as 2,3-cis,3,4-cis-leucocyanidin (2R,3R,4R-leucocyanidin).

Recombinant ANRs from multiple species were then compared in coupled reactions with MtDFR1 and MtANS, with DHQ as initial substrate (Fig. 2C). A similar peak with m/z 305.0667 was detected at RT of 3.8 min for A. thaliana and soybean, and 2,3-cis-leucocyanidin production confirmed with ANRs from these species, as well as grapevine, tea, and D. uncinatum, by HPLC analysis (Fig. 2C and fig. S6A). However, recombinant ANR proteins from maize, wheat, and barley produced the same profile as that from a mixture of DFR1 + ANS, indicating that these proteins do not catalyze in vitro conversion of flavan-3,4-diol to 2,3-cis-leucocyanidin (Fig. 2C and fig. S6A); quercetin was detected as the major product along with cyanidin, accompanied by a color change in the reaction (fig. S6, B and C).

To attempt to explain these differences in ANR activities, multiple sequence alignments were performed (fig. S7), and a homology model for ANRs was generated from the structures of grape VvANR [Protein Data Bank (PDB) ID: 2RH8] and DFR (PDB ID: 3BXX) (fig. S8). MtANR has a similar structure to VvANR and DFR, with a large N-terminal domain adopting a Rossman fold and a small C-terminal domain forming a cleft for NADPH and substrate binding (fig. S8). Hydrophobic (Val91, Ala132, Ala133, Ile137, Leu197, Met213, Ala227, and Ile233) and polar (Thr165 and Ser209) residues in the putative substrate binding pocket position the B-ring of flav2-en (or 3-en)-3-ol and flav-3-en-3,4-diol close to the nicotinamide ring of NADPH and Tyr188, forming Van der Waals interactions. In the substrate binding pocket of HvANR (with 50.6% amino acid–sequence identity to MtANR), Phe195 and Ile225 correspond to Leu197 and Ala227 in MtANR, and in ZmANR (with 48.9% amino acid–sequence identity to MtANR), Val133, Tyr134, Ile202, Val222, and Lys228 correspond to Ala132, Thr136, Leu197, Ala227, and Ile233 in MtANR (figs. S7 and S8). The presence of these larger side chains may provide steric hindrance to interfere with substrate binding in HvANR and ZmANR, preventing activity with flav-2-en (or 3-en)-3-ol and flav-3-en-3,4-diol.

2,3-cis-Leucocyanidin can act as a PA extension unit in vitro

To determine whether both isomers of leucocyanidin generated enzymatically can undergo nucleophilic addition to form PA dimers, 200 μM (−)-epicatechin was added to single and coupled enzyme reactions (DHQ with MtDFR1 or MtDFR1 + MtANS + ANRs from different species) after 2 hours of incubation (Fig. 2D). Product profiles were compared to extracted ion chromatograms (EICs) of procyanidins B1 [(−)-epicatechin-(4β→8)-(+)catechin], B2 [(−)-epicatechin-(4β→8)-(−)-catechin], B3 [(+)-catechin-(4α→8)-(−)-catechin], and B4 [(+)-catechin-(4α→8)-(−)-epicatechin] (m/z 577,1348). B4 was detected in incubations with DFR1 alone, and B2 was detected in incubations with DFR1 + ANS + ANR from M. truncatula, A. thaliana, or soybean (Fig. 2D). Trace amounts of B4 were observed in coupled reactions including wheat, maize, or barley ANRs (Fig. 2D). When (±)-epicatechin-2,3,4-13C3 was incubated with purified cis-leucocyanidin, extracted ions of dimers with m/z 580.1452 and trimers with m/z 688.2086 were detected (figs. S9 and S10), indicating that labeled epicatechin was only incorporated as a starter unit and the extension units originated from 2,3-trans-leucocyanidin for B4 or 2,3-cis-leucocyanidin for B2 (Fig. 2D).

Epi-cys and catechin-cysteine are present in plants and may serve as a pool of extension units (18). Incubation of 200 μM cysteine with 2,3-cis- or 2,3-trans-leucocyanidins resulted in production of catechin-cysteine or epi-cys (figs. S11, A and B, S12A, and S13), indicating that cysteine adducts of flavan-3-ols can be generated directly and nonenzymatically from leucocyanidin. Dimer formation in coupled enzyme reactions occurred only upon adding (−)-epicatechin (compare Fig. 2D with fig. S11C) and was reduced by adding cysteine (fig. S11D) in a dose-dependent manner (fig. S12).

2,3-cis-Leucocyanidin is present as a functional PA extension unit in plants

To address whether the 2,3-cis-leucocyanidin pathway to PA extension units operates in plants, crude PA extracts from A. thaliana mutants with loss of function of PA pathway genes were analyzed by LC/MS and the profiles compared to EICs of purified trans- or cis-leucocyanidin produced by coupled enzymatic reactions (Fig. 4A). Extracts from wild-type Columbia (Col-0) plants contained 2,3-cis-leucocyanidin, which was absent in extracts from the tt3-1 mutant. The tds4-4 mutant showed a peak corresponding to 2,3-cis-leucocyanidin, which was absent in the tds4-4 tt3-1 double mutant (Fig. 4, A and B), confirming that it is produced via DFR and that DFR is epistatic to ANS. In the ban-5 mutant, both cis- and trans-leucocyanidin disappeared, but only the cis-leucocyanidin peak was lost in the tds4-4 ban-5 double mutant (Fig. 4A), indicating that production of 2R,3R,4R-leucocyanidin requires both ANS and ANR and that 2,3-trans-leucocyanidin, the product of DFR, is consumed by ANS as in the ban-5 mutant (Fig. 4, A and B). These data, along with detection of virtually no PAs in the above mutants (fig. S14, A and B), confirm the involvement of 2,3-cis-leucocyanidin in the otherwise classical pathway to epicatechin-derived PAs. Accumulation of cyanidin-3-O-glucoside, derived from flav-2-en-3,4-diol as a product of ANS and subsequent modification by glycosylation (35), was observed as expected in the ban-5 mutant but not in the tds4-4 ban-5 double mutant (fig. S14, C and D), consistent with the seed color phenotype of ban-5 (Fig. 4C) (36).

Next, we analyzed seeds of other species for leucocyanidin accumulation. M. truncatula presents an analytical challenge because it produces traces of epigallocatechin and gallocatechin with the same chemical formula as leucocyanidin, and gallocatechin chromato- graphed with cis-leucocyanidin. To overcome this problem, we made use of the observation that the carbocation derived from leucocyanidin can be captured by methanol to generate the flavan-3-ol-4-O-methyl ether (37). The purified leucocyanidin was therefore incubated with acidic methanol and subjected to LC/MS analysis interrogating EICs at m/z 319.0823 for epicatechin- or catechin-4-O-methyl ether derived from 2,3-cis- or 2,3-trans-leucocyanidin, respectively (Fig. 4D and fig. S15), using reference standards generated from procyanidins B2 and B3 (Fig. 4D) (37). Generation of epicatechin- and catechin-4-O-methyl ether in extracts of A. thaliana wild type and tds4-4 mutant plants correlated with 2,3-cis- and 2,3-trans-leucocyanidin accumulation, respectively (Fig. 4, A and D, and fig. S15). Treatment of crude PA extracts from M. truncatula wild type (R108) and soybean with acidic MeOH generated epicatechin-4-O-methyl ether, while catechin-4-O-methyl ether was detected in corresponding extracts from barley seeds (Fig. 4D and fig. S15). Only catechin-4-O-methyl ether was detected in a parallel experiment with extracts from the M. truncatula ans-1 ldox-1 double mutant, consistent with its accumulation of catechin-based PAs (Fig. 4D) (19). The specific 4-O-methyl adduct was consistent with the PA composition reported in other M. truncatula mutants (Fig. 4D) (18, 19). No flavan-3-ol-4-O-methyl ether was detected in extracts from maize and wheat in parallel experiments (Fig. 4D).
To verify dual activity of ANR to generate epicatechin extension units in vivo, genetic complementation of the *A. thaliana* ban-5 mutant was performed (fig. S16). Analysis of more than 20 transgenic plants for each construct showed that the lack of both monomer and dimer accumulation in ban-5 was fully rescued by expression of ANR genes from *A. thaliana*, *M. truncatula*, soybean, and *D. uncinatum*, partially rescued (epicatechin only) by maize ANR but not rescued by barley ANR (fig. S16).

**Fig. 4. Leucocyanidin isomers in PA pathway mutants and different plant species.** (A) EIC of leucocyanidin (m/z 305.0667 ± 10 ppm) from crude extracts of *A. thaliana* tt3-1, tds4-4, ban-5, tt3-1 ban-5, tds4-4 ban-5, and tt3-1 tds4-4 seeds extracted in 80% MeOH. (B) Enzymatic reactions for leucocyanidin biosynthesis. (C) Seed coat phenotypes of wild-type Col, tt3-1, tds4-4, ban-5, tt3-1 ban-5, tds4-4 ban-5, and tt3-1 tds4-4 mutant *A. thaliana* seeds. Photo credit: Ji Hyung Jun, University of North Texas. (D) EIC of flavan-3-ol-methyl ether (m/z 319.0823 ± 10 ppm) from crude extracts of *A. thaliana*, soybean, barley, maize, wheat, *M. truncatula* wild type (R108), and *M. truncatula* ans-1, idox-1, ans-1 idox-1, anr-1, ans-1 anr-1 and lar-1 mutant seeds extracted with 80% MeOH/0.2% HCl. Epicatechin-4-O-methyl-ether and catechin-4-O-methyl-ether derived from 2,3-cis-leucocyanidin (2,3-cis-leu), 2,3-trans-leucocyanidin (2,3-trans-leu), procyanidin B2, and procyanidin B3 were run as standards. B3, procyanidin B3; cis-L, 2,3-cis,3,4-cis-leucocyanidin standard; O-methyl-C, catechin-4-O-methyl-ether; O-methyl-EC, epicatechin-4-O-methyl-ether; trans-L, 2,3-trans,3,4-cis-leucocyanidin standard.

**DISCUSSION**

2,3-trans-Leucocyanidin has been established as the candidate for thermodynamically driven conjugation of catechin extension units, making 2,3-cis-leucocyanidin a logical precursor of the dominant epicatechin extension units of PAs (25–27). Unexpectedly, to the
best of our knowledge, there have been no reports of the detection of leucocyanidins in PA-accumulating plant tissues. Here, we show that relatively recent neofunctionalization of ANR proteins is associated with both PA accumulation and compositional variation across plant species. Flav-2-en-3-ol is a more efficient substrate for ANR than cyanidin, a bona fide substrate in vitro (13), and functions as an intermediate in the biosynthesis of epicatechin starter units. Epicatechin produced from flav-2-en-3-ol is stereochemically pure (−)-epicatechin, whereas that produced from cyanidin is usually a mixture of isomers. The previously unrealized activity of ANR to produce the extension unit 2,3-cis-3,4-cis-leucocyanidin from 2R-flav-3-en-3,4-diol was demonstrated here by genetic, biochemical, and chemical approaches. The dual activity of ANR to produce both starter and extension unit precursors explains why anr mutants lose most of their PA accumulation (18). Because 2R-flav-3-en-3,4-diol and flav-2-en-3-ol (or flav-3-en-ol) only differ in the hydroxyl group at C-4, a less stringent substrate binding site could allow for the observed dual activity of ANRs, as supported by molecular modeling. The active site pocket residues in ANRs from barley and maize do not appear to allow this relaxed specificity.

The ability of ANR to use both 3-ol and 3,4-diol explains PA composition across species, as shown for epicatechin- and catechin-based PAs in the model in Fig. 5. In D. uncinatum, the absence of LDOX expression in leaves results in PAs derived from catechin starter units, whereas the PAs in the seed are wholly composed of epicatechin (19). Such compositional changes can be achieved in

---

**Fig. 5. Dual roles of ANR in the biosynthesis of epicatechin starter and extension units.** Model explaining how PAs are mostly composed of epicatechin subunits in plants such as M. truncatula (red shadow) or catechin subunits in H. vulgare (blue shadow). In M. truncatula, the separate contribution of ANS and LDOX in parallel pathways provides starter and extension units via dual ANR activity. To generate the direct precursor of extension units, flav-3-en-3,4-diol is reduced to 2,3-cis-3,4-cis-leucocyanidin by ANR. Water elimination from C-4 and subsequent protonation at C-3 may result in the carbocation that can react with nucleophilic centers such as C-8 on flavan-3-ols as proposed (48). Nonenzymatic conjugation of leucocyanidin and cysteine leads to flavan-3-ol-cysteine, representing a potential regulatory mechanism to control the level of carbocation and PA polymerization (18). In barley seeds, possession of an ANR enzyme lacking ability to produce 2,3-cis-3,4-cis-leucocyanidin and (−)-epicatechin is consistent with accumulation of catechin-based PAs via conjugation of 2,3-trans-3,4-cis-leucocyanidin and (+)-catechin.
different tissues of the same plant through possession of bi-functional ANR to produce both 2,3-cis-leucocyanidin extension units and (−)-epicatechin starter units. In A. thaliana, a yet to be defined micro-environment could favor the nonenzymatic production of flav-2-en-3-ol from cyanidin, generating (−)-epicatechin starter units, whereas the coupled DFR + ANS + AtANR reaction generates 2,3-cis-leucocyanidin extension units.

The ability of ANR to change the stereochemistry of the hydroxyl group at C-4 from 4S to 4R suggests a role for ANR in determining the differences of stereochemistry at C-4 between the linkages found in procyanidin B1 [(−)-epicatechin-(4β → 8)-(−)-catechin] and B2 [(−)-epicatechin-(4β → 8)-(−)-catechin] or B3 [(+)-catechin-(4α → 8)-(−)-catechin) and B4 [(+)-catechin-(4α → 8)-(−)-epicatechin)].

Nonenzymatic conjugation of leucocyanidin and cysteine leads to the formation of flavan-3-ol-cysteine as another potential source of extension units (18). It is not clear whether this reaction occurs in vivo. However, reduced efficiency of dimer formation with increased cysteine concentration suggests a possible regulatory mechanism to control the level of available carboxylation and PA polymerization, consistent with our previous model implicating LAR in conversion of extension units (epi-cys) to starter units (epicatechin) (18) and the genetic link between polymorphisms in LAR and PA chain length in grapevine (38). Given that PA assembly in planta now appears to be nonenzymatic, contrary to the assembly of other major phenolic plant polymers such as lignin, it is now important to address the molecular mechanisms that protect the plant from the reactive carboxylic intermediates that are intimately associated with PA assembly (39).

**MATERIALS AND METHODS**

**Chemicals**

(−)-Epicatechin, (−)-catechin, (+)-catechin, and 2R,3R-DHQ were purchased from Sigma-Aldrich. (±)-Epicatechin-2,3,4-13C3 was purchased from Cambridge Isotope Laboratories. (+)-Epicatechin was purchased from Nacalai Tesque. Procyanidin B1, B2, and B3 were purchased from AdooQ BioScience. Procyanidin B4 was synthesized by in vitro conjugation of 4β-(S-cysteinyl)-catechin and epicatechin that were added to a 50-μl reaction volume containing 50 mM potassium phosphate buffer (pH 8.0). Reactions were carried out for 1 hour at room temperature and terminated by extraction with 200 μl of ethyl acetate. Ethyl acetate extracts were dried under vacuum, dissolved in 50 μl of water and analyzed by UPLC/MS. 4β-(S-Cysteinyl)-catechin and 4β-(S-cysteinyl)-epicatechin were synthesized using procyanidin B3 and B2 and purified as described (18).

2,3-trans-3,4-cis-Leucocyanidin was prepared according to (40). 2,3-trans-3,4-cis-Leucocyanidin and 2,3-cis-3,4-cis-leucocyanidin from enzymatic reactions were purified by reversed-phase HPLC using a μBondapak phenyl column (10 μm, 3.9 mm by 300 mm; Waters) under isocratic conditions with H2O under a flow rate of 1 ml/min at room temperature; the fractions were cut and collected into a flask on the basis of monitoring absorbance at 280 nm. The collected aqueous samples were subsequently concentrated under vacuum. Both purified compounds were used as standards in subsequent experiments. To generate flavan-3-O-methyl ether from procyanidins, 20 μg of procyanidin B2 or B3 was dissolved in 80% methanol/0.2% HCl and incubated for 2 hours (37). To generate flavan-3-O-methyl ether from leucocyanidins, purified 2,3-cis- or 2,3-trans-leucocyanidins concentrated in 10 μl of water were diluted to 100 μl in 80% methanol/0.2% HCl (v/v) and incubated for 2 hours.

**Plant materials**

*A. thaliana* Columbia (Col-0) was used as the wild-type plant. The *tds4-4* (ANS, SALK_073183), *ban-5* (BAN, SALK_040250), and *tr3-1* (DFR, CS84) mutants were obtained from the Arabidopsis Biological Resource Center. *M. truncatula* ecotype R108 was used as the wild-type plant. Seeds of the *M. truncatula* *ans-1* (NF20424), Idox-1 (NF11718), *anr-1* (NF9161), *lar-1* (NF9870), and *wd40-1* (NF2745) mutants were obtained from the Noble Research Institute, Ardmore, OK. *M. truncatula* seeds were scarified with concentrated sulfuric acid for 10 min and then washed with water five times to remove sulfuric acid. *M. truncatula* and *A. thaliana* seeds were sterilized with 30% bleach for 10 min and then rinsed five times with sterile water. Sterilized seeds were vernalized at 4°C for 4 days on B5 medium. Vernalized seeds were germinated on B5 medium for 10 days before transfer to soil in pots. The plants were grown in a growth chamber set at 16-hour day/8-hour night cycle, 22°C. Seeds of *G. max* (PI547438), *H. vulgare* (cv Robust), *T. aestivum* (cv Chinese Spring), and *Z. mays* (cv Suntava) were germinated on B5 medium as above and transferred to soil. The plants were grown in a greenhouse set at 16-hour day/8-hour night cycle, 24°C.

**Determination of PA content**

PAs were determined essentially as described previously (19). About 50 mg samples were ground into powder in liquid nitrogen and used for extraction with 1.5 ml of PA extraction solvent (70% aceton with 0.5% acetic acid) by sonicating in a water bath for 30 min at room temperature. The resulting slurry was centrifuged at 5000g for 5 min, and supernatants were collected. The pellets were reextracted twice, and supernatants were pooled. Equal volumes of chloroform were added to the pooled supernatants, the mixtures were vortexed for 30 s and centrifuged at 5000g for 5 min, and the supernatants were further extracted twice with chloroform and twice with hexane. The resulting aqueous phase (soluble PA fraction) was lyophilized and redissolved in 50% methanol. PAs in the soluble fraction were quantified by the dimethylaninocinnamaldehyde (DMACA) method (41). Soluble PA fraction (5 μl) was mixed with 100 μl of 0.2% DMACA in methanol/HCl 1:1 ratio, and the absorbance at 640 nm was measured after 5 min. Epicatechin was used as standard. All measurements were the average of three biological replicates with three technical replicates for each, and error bars show SDs.

**Phloroglucinolysis**

About 20 μg of soluble PAs were mixed with 150 μl of a slurry of Sephadex LH20 resin prepared with MeOH (dry powder, 4 ml/g) in an Eppendorf tube, incubated for 10 min with rotation, and the resin was then washed twice with 1 ml of 50% MeOH and eluted three times with 200 μl of 50% aceton. Collected aceton fractions were dried in a speed vacuum centrifuge, dissolved in 50 μl of phloroglucinol solution (5% phloroglucinol and 1% ascorbic acid (w/v) in 1 N HCl in MeOH), and incubated for 20 min at 50°C. The reaction was stopped by adding 50 μl of 0.2 M sodium acetate solution. The reaction mixture was centrifuged for 5 min at 13,000 rpm at 4°C, filtered with ZipTip protein concentrator with C18 resin (Sigma-Aldrich), and analyzed by HPLC (42).

**Preparation of crude polyphenol extracts from plant tissues**

To extract polyphenols from plant materials, 50 mg of fresh samples were ground in liquid nitrogen, vortexed for 30 s, and then sonicated for 15 min at 4°C in 500 μl of 80% methanol. After centrifugation for 5 min at 16,000g, the supernatant was centrifuged for 15 min at 16,000g, filtered with Ziptip with C18 resin (Sigma-Aldrich), and analyzed by
UPLC/MS as described below. To generate flavan-3-O-methyl ether from leucocyanidin, 80% methanol was replaced with 80% methanol/0.2% HCl following the above protocol as described (37).

Expression of recombinant ANR proteins and assay of ANR activity
The full-length ANR complementary DNAs (cDNAs) from A. thaliana (Col-0), M. truncatula (R108), C. sinensis (TRI2043), G. max (cv Clark), Z. mays (cv Hi-II), T. aestivum (cv Chinese Spring), and DFR1 and ANS from M. truncatula (R108) were cloned into pMal-c5x vector [New England Biolabs (NEB)] at the multiple-cloning site. HvANR, BAJ96327, and VvANR, NP_001267885 open reading frames were synthesized (Gene Universal) and cloned into pMal-c5x vector. Protein expression and purification was as described previously (19). The expression constructs were transformed into E. coli strain Express Competent E. coli (NEB). Transformed bacteria were grown in LB medium supplemented with 0.2% glucose to optical density at 0.5, and isopropyl-β-D-thiogalactopyranoside was added at 0.3 mM to induce protein expression. Bacteria were harvested after a 20-hour induction. ANR proteins were purified with amylose resin (NEB, E8021) following the manufacturer’s protocol. Bacteria were lysed by sonication at 4°C in extraction buffer [20 mM tris (pH 7.0), 200 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride]. The bacterial lysates were centrifuged at 12,000g for 15 min at 4°C. The supernatants were loaded on amylose resin that was washed with wash buffer (extraction buffer). Last, proteins were eluted by elution buffer [20 mM tris (pH 7.0), 200 mM NaCl, 1 mM DTT, 10 mM maltose]. Purified proteins were concentrated with an Amicon Ultra-4 Centrifugal Filter (Millipore), and aliquots were stored at −80°C.

Enzyme reactions (100 μl) included 20 mM potassium phosphate buffer (pH 7.0) or 20 mM MES (pH 5), 1 mM 2-oxoglutarate, 0.4 mM ammonium iron(II) sulfate, 4 mM sodium ascorbate, 100 μM NaCl, 10 mM maltose, 5 mM DTT, and 50 μg of recombinant proteins. The reactions were carried out for 2 hours (unless indicated otherwise) at 30°C and terminated by transferring to −80°C. The reaction mixture was centrifuged for 5 min at 13,000 rpm at 4°C, filtered with ZipTip with C18 resin (Sigma-Aldrich), and analyzed by HPLC.

Conjugation of leucocyanidin, flavan-3-ol, and cysteine
The enzyme reaction of MtDRF1 + MtANS+ANR, MtDRF1 + MtANS, or DFR1 alone was carried out for 2 hours as above, and then 200 μM (−)-epicatechin and/or 0.2 μM to 2 mM l-cysteine were added and incubated for 5 hours under the same conditions. A mixture of the purified 2,3-cis-leucocyanidin or 2,3-trans-leucocyanidin and 200 μM (−)-epicatechin, 200 μM (+)-epicatechin, (+)-epicatechin-2,3,4,1’C₄, or 200 μM l-cysteine was incubated in 50 μl of 20 mM potassium phosphate buffer (pH 7.0) for 5 hours at 25°C.

RNA isolation, reverse transcription PCR, and quantitative reverse transcription PCR
These procedures were as described previously (19). RNA was isolated from developing siliques of wild-type and ANR overexpressing A. thaliana plants, using a Qiagen RNeasy kit (Qiagen) according to the manufacturer’s instructions. RNA was treated with deoxynucleobase I (Invitrogen) to remove trace amounts of DNA contamination. One microgram of total RNA was used for reverse transcription with SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). Quantitative polymerase chain reaction (PCR) was performed using the ABI QuantStudio 6 Flex Real-Time PCR System, using primers listed in table S3.

HPLC and UPLC/MS analysis
Reversed-phase HPLC analysis was carried out as previously described (19) on an Agilent HP1100 system equipped with a 250 mm by 4.6 mm, 5-μm, C18 column (Varian Metasil 5 Basic) and diode array detector, using the following gradient: solvent A (1% phosphoric acid) and B (acetonitrile) at a flow rate of 1 ml/min: 0 to 5 min, 5% B; 5 to 10 min, 5 to 10% B; 10 to 25 min, 10 to 17% B; 25 to 30 min, 17 to 23% B; 30 to 65 min, 23 to 50% B; 65 to 79 min, 50 to 100% B; and 79 to 80 min, 100 to 5% B. Data were collected at 280 and 530 nm for PA intermediates and anthocyanidins, respectively. Identiﬁcations were based on chromatographic behavior, and UV spectra were compared with those of authentic standards. For the analysis of products from chlorogluconolysis, solvent A (1% acetic acid) and B (methanol) were used (42).

LC/MS analysis of the metabolites was carried out as previously described (43) using an Exion UPLC system coupled with a high-resolution TripleTOF6600+ mass spectrometer from AB Sciex. The compounds were separated using a C18 Acquity UPLC HSS T3 (100 mm by 2.1 mm, 1.8 μm) column from Waters. The temperatures of the column compartment and the autosampler were kept at 42° and 15°C, respectively. The analytes were eluted using a gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B) under a flow rate of 0.4 ml/min. The following gradient was applied: 0 to 1.0 min, 5.0% B; 1.0 to 2.0 min, 5.0 to 10.0% B; 2.0 to 7.0 min, 10.0 to 28.2% B; 7.0 to 11.0 min, 28.2 to 70.0% B; 11.0 to 11.1 min, 70.0 to 95.0% B; 11.1 to 13.0, 95% B; 13.0 to 13.1 min, 95.0 to 5.0% B; and 13.1 to 15.0 min, 5.0% B. The mass spectrometer was set to scan metabolites from m/z 250 to 1000 atomic mass unit (amu) in negative mode with an ion spray voltage of 4000 V. The accumulation time was 100 ms. Declustering potential and collision energy were 50 and 10 V, respectively. MS/MS spectra were acquired from m/z 30 to 1000 amu with an accumulation time of 25 ms. Declustering potential, collision energy, and collision energy spread were set to 50, 25, and 10 V, respectively. The curtain gas (nitrogen), nebulizing, and heating gas were ﬁxed at 30, 60, and 60 psi, respectively. The temperature of the source was 500°C. An Atmospheric Pressure Chemical Ionization (APCI) negative calibration solution was delivered by a calibrant delivery system every 10 samples to correct for any mass drift that may occur during the run. MS spectra were acquired using Analyst TF 1.8.1 software. The analysis of the data was performed using Scix OS software.

The products of ANR reactions were further distinguished using normal-phase HPLC analysis on an analytical chiral column (Chiral Technologies, catalog no. 80325) with a guard cartridge (Chiral Technologies, catalog no. 80311) on the same HPLC device. For chiral chromatographic analysis, reaction mixtures were puriﬁed with ethyl acetate, dried under vacuum, and dissolved in hexane: ethanol (80:20 v/v). Separation was with the following gradient: solvent A (hexanes with 0.5% acetic acid) and B (ethanol with 0.5% acetic acid) at a flow rate of 1 ml/min: 0 to 20 min, 20% B; 20 to 23 min, 20 to 50% B; 23 to 38 min, 50% B; and 38 to 40 min, 50 to 20% B. UV absorption data were collected at 280 nm. Identiﬁcations were based on comparison of chromatographic behavior and UV spectra with authentic standards.
Plasmid constructions for generating ANR-overexpressing plants

To generate binary vectors for ANR overexpression, full-length ANR cDNAs from *A. thaliana*, *M. truncatula*, *G. max*, *D. uncinate*, and *Z. mays* (Hi-II), and *H. vulgare* were first cloned into pENTR-D vector and then cloned into pB7GW2D binary vector containing the CaMV 35S promoter by Gateway™ LR recombination reaction to generate ANR-pB7GW2D binary vector. For each construct, the binary vector in the *Agrobacterium tumefaciens* strain AGL1 was introduced into plants by the floral dip method (44). Primer sequences are listed in table S3.

Phylogenetic analysis

Multiple protein sequence alignments were performed using the ClustalW alignment. *M. truncatula* DFR1 (KEH40173) was selected as outgroup. The phylogenetic tree was constructed using the Jukes-Cantor genetic distance model and neighbor-joining tree builder ClustalW alignment.

NMR spectroscopy

NMR spectra were obtained with the sample dissolved in 280 μL of D₂O (99.96%, Cambridge Isotope Laboratories). 1D proton NMR spectra were obtained with the sample dissolved in 280 μL NMR spectroscopy method with 1000 bootstrap replicates. Jukes-Cantor genetic distance model and neighbor-joining tree builder ClustalW alignment.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/20/eaabg4682/DC1

REFERENCES AND NOTES

1. R. L. Prior, L. Gu, Occurrence and biological significance of proanthocyanidins in the American diet. *Phytochemistry* 66, 2264–2280 (2005).
2. J. R. Donaldson, M. T. Stevens, H. R. Barnhill, R. L. Lindroth, Age-related shifts in leaf chemistry of clonal aspen (*Populus tremuloides*). *J. Chem. Ecol.* 32, 1415–1429 (2006).
3. D. A. Balentine, S. A. Wiseman, L. C. Bouwens, The chemistry of tea flavonoids. *Crit. Rev. Food Sci. Nutr.* 37, 693–704 (1997).
4. P. Cos, T. De Broyne, N. Hermans, I. Apes, D. Vanden Berge, A. J. Vlietinck, Proanthocyanidins in health care: Current and new trends. *Curr. Med. Chem.* 11, 1345–1359 (2004).
5. D. Bagchi, M. Bagchi, S. J. Stohs, D. K. Das, S. D. Ray, C. A. Kuszynski, S. S. Joshi, H. G. Pruess, Free radicals and grape seed proanthocyanidin extract: Importance in human health and disease prevention. *Toxicology* 148, 187–197 (2000).
6. G. L. Lees, Condensed tannins in some forage legumes: Their role in the prevention of ruminant pasture bald. *Basic Life Sci.* 59, 915–934 (1992).
7. B. R. Min, W. E. Pinchak, J. D. Fulford, R. Puchala, Effect of feed additives on in vitro and in vivo rumen characteristics and frothy bloat dynamics in steers grazing wheat pasture. *Anim. Feed Sci. Tech.* 123–126, 615–629 (2005).
8. E. M. Jurgens, A. B. Marin, J. A. Kennedy, Analysis of the oxidative degradation of proanthocyanidins under basic conditions. *J. Agric. Food Chem.* 52, 2292–2296 (2004).
9. W. Ma, A. Guo, Y. Zhang, H. Wang, Y. Liu, H. Li, A review on astringency and bitterness perception of tannins in wine. *Trends Food Sci. Tech.* 40, 6–19 (2014).
10. C. Ullah, S. B. Unskicser, M. Reichelt, J. Gershenzon, A. Hammerbacher, Accumulation of catechin and proanthocyanidins in black poplar stems after infection by *Pectostomaphora populii*: Hormonal regulation, biosynthesis and antifungal activity. *Front. Plant Sci.* 10, 1441 (2019).
11. B. Adamczyk, J. Heimansola, J. Simon, Mechanisms of carbon sequestration in highly organic ecosystems—Importance of chemical ecology. *ChemistryOpen* 9, 464–469 (2020).
12. L. Lepiniec, I. Debeaujon, J.-M. Routaboul, A. Baudry, L. Pourcel, N. Nesi, M. Caboche, Genetics and biochemistry of seed flavonoids. *Annu. Rev. Plant Biol.* 57, 405–430 (2006).
13. D. Y. Xie, S. B. Sharma, N. L. Paiva, D. Ferreira, R. A. Dixon, Role of anthocyanidin reductase, encoded by *BANYULS* in *Arabidopsis thaliana*. *Science* 299, 396–399 (2003).
14. G. J. Tanner, K. T. Francki, S. Abrahams, J. M. Watson, P. J. Larkin, A. R. Ashton, Proanthocyanidin biosynthesis in plants. Purification of legume leucoanthocyanidin reductase and molecular cloning of its cDNA. *J. Biol. Chem.* 278, 31647–31656 (2003).
15. Y. Pang, I. B. Abeyesinghe, J. He, X. He, D. Huhman, K. M. Mewan, L. W. Sumner, J. Yun, R. A. Dixon, Functional characterization of proanthocyanidin pathway enzymes from tea and their application for metabolic engineering. *Plant Physiol.* 161, 1103–1116 (2013).
16. L. Zhao, X.-L. Jiang, Y.-M. Qian, P.-Q. Wang, D.-Y. Xie, L.-P. Gao, T. Xiu, Metabolic characterization of the anthocyanidin reductase pathway involved in the biosynthesis of flavan-3-ols in elite Shuchazao tea (*Camellia sinensis*) cultivar in the field. *Molecules* 22, 2241 (2017).
17. M. Gargouri, C. Manigand, C. Maugue, T. Granier, B. Langlois d’Estaintot, O. Cala, I. Planet, K. Bathany, J. Chaudriere, B. Gallois, Structure and epimerase activity of anthocyanidin reductase from *Vitis vinifera*. *Acta Crystallogr.* D 65, 989–1000 (2009).
et al. 2021; 18.

...jung, J. H.; Kim, R. M.; Abbott, M. Cotton, A. Levy, P. Marchetto, K. Ochoa, S. M. Jackson, B. Gillam, W. Chen, L. Yan, J. Higginbotham, M. Cardenas, J. Waligorski, E. Applebaum, L. Phelps, J. Falcone, K. Kanchi, T. Thane, A. Scimone, T. Thane, J. Ken, T. Wang, J. Ruppert, N. Shah, K. Rotter, J. Hodges, E. Ingenthoorn, M. Cordes, S. Kolberg, J. Sgro, B. Delgado, K. Mead, A. Chinwalla, S. Leonard, K. Crous, K. Collura, D. Kudrna, J. Currie, R. He, A. Angelova, S. Rajasekhar, T. Mueller, R. Loneli, G. Scarca, A. Ko, J. Delaney, M. Wissotski, G. Lopez, D. Campos, M. Braidotti, E. Ashley, W. Golser, H. R. Kim, S. Lee, J. Lim, Z. Dujmic, W. Kim, J. Talag, A. Zuccolo, C. Fan, A. Sebastian, M. Kramer, L. Spiegelt, L. Nascimento, T. Zutavern, B. Miller, C. Ambroseia, S. Muller, W. Spooner, A. Narechania, L. Ren, S. Wei, S. Kumari, B. Faga, M. J. Levy, L. I. Mahan, P. Van Buren, M. W. Vaughn, K. Ying, C.-T. Yeh, S. J. Emmich, Y. Jia, A. Kalyanaraman, A.-P. Hsia, W. B. Barbazuk, R. S. Baucom, T. P. Bruttell, N. C. Carpita, C. Chaparro, J.-M. Chia, J.-M. Deragon, J. C. Estill, Y. Fu, J. A. Jedeloh, Y. Han, L. Lee, P. Li, D. R. Lish, S. Liu, L. Z. Liu, D. H. Nagel, M. C. McCann, P. S. Miguel, A. M. Myers, D. Nettleton, J. Nguyen, B. W. Penning, L. Ponnala, K. L. Schneider, D. C. Schwartz, A. Sharma, C. Soderlund, N. C. Springer, Q. Sun, H. Wang, M. Waterman, R. Westerman, T. K. Wolfgruber, J. Yang, Y. Yu, L. Zhang, S. Zhou, Q. Zhu, J. L. Bennetzen, R. K. Dawe, J. Jiang, J. Jiang, G. G. Presting, S. R. Wessler, S. Aluru, R. A. Martienssen, S. W. Clifton, W. R. McCombie, R. A. Wing, K. R. Wilson, The B73 maize genome: complexity, diversity, and dynamics. Science 326, 1112–1115 (2009).

...A. V. Zimin, D. Puiu, R. Hall, S. Kingan, B. J. Clavijo, S. L. Salzberg, The first near-complete assembly of the hexaploid wheat bread wheat genome, Triticum aestivum. GigaScience 6, 1–7 (2017).

...D. Y. Xie, S. B. Sharma, R. A. Dixon, Anthocyanidin reductases from Medicago truncatula and Arabidopsis thaliana. Arch. Biochem. Biophys. 422, 91–102 (2004).

...R. W. Welford, J. T. Turnbull, D. T. Clarke, A. G. Prescott, C. J. Schofield, Evidence for oxidation at C3 of the flavonoid C-ring during anthocyanin biosynthesis. Chem. Commun., 1828–1829 (2001).

...J. J. Turnbull, M. J. Nagle, J. F. Seibel, R. W. D. Welford, G. H. Grant, C. J. Schofield, The C4 stereocchemistry of leucocyanidin substrates for anthocyanidin synthesis affects product selectivity. Bioorg. Med. Chem. Lett. 13, 3853–3857 (2003).

...S. Martens, T. Teeri, G. Forkmann, Heterologous expression of dihydroflavonol 4-reductases from various plants. FEBS Lett. 531, 453–458 (2002).

...J. R. Zhang, J. Tolchard, K. Bathany, B. Langlois d’Estaintot, J. Chaudriere, Production of 3,4-cis- and 3,4-trans-leucocyanidin and their distinct MS/MS fragmentation patterns. J. Agric. Food Chem. 66, 351–358 (2018).

...Y. Lee, H. R. Yoon, Y. S. Paik, J. R. Liu, W.-i. Chung, G. Choi, Reciprocal regulation of Arabidopsis UGT78D2 and BANYULS is critical for regulation of the metabolic flux of anthocyanidins to condensed tannins in developing seed coats. J. Plant Biol. 48, 356–370 (2005).

...S. Albert, M. Deleryn, M. Devic, BANYULS, a novel negative regulator of flavonoid biosynthesis in the Arabidopsis seed coat. Plant J. 11, 289–299 (1997).

...P. Wang, Y. Liu, L. Zhang, W. Wang, H. Hou, Y. Zhao, X. Jiang, Y. Hu, T. Yang, D.-Y. Xie, L. Gao, T. Xia, Functional demonstration of plant flavonoid carboxylates proposed to be involved in the biosynthesis of proanthocyanidins. Plant J. 101, 18–36 (2020).

...Y. F. Huang, A. Doligez, A. Fournier-Level, L. Le Cunff, Y. Bertrand, A. Canaguier, C. Morel, V. Miralles, F. Veran, J.-M. Souquet, V. Cheynier, N. Terrier, P. This, Dissecting genetic architecture of grape proanthocyanidin composition through quantitative trait locus mapping. BMC Plant Biol. 12, 30 (2012).

...R. A. Dixon, S. Sarnala, Proanthocyanidin biosynthesis—A matter of protection. Plant Physiol. 184, 579–591 (2020).

...G. J. Tanner, K. N. Kristiansen, Synthesis of 3,4-cis-[3H]leucocyanidin and enzymatic reduction to catechin. Anal. Biochem. 209, 274–277 (1993).

...Y. Pang, G. J. Peel, S. B. Sharma, Y. H. Tang, R. A. Dixon, A transcript profiling approach reveals an epicuticle-specific glucosyltransferase expressed in the seed coat of Medicago truncatula. Proc. Natl. Acad. Sci. U.S.A. 105, 14210–14215 (2008).

...J. A. Kennedy, G. P. Jones, Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. J. Agric. Food Chem. 49, 1740–1746 (2001).

...N. Lu, X. Rao, Y. Li, J. H. Jun, R. A. Dixon, Dissecting the transcriptional regulatory machinery for proanthocyanidin and anthocyanin biosynthesis in soybean (Glycine max). Plant Biotech. J. 1, 1–14 (2021).

...S. J. Clough, A. F. Bent, Floral dimp: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16, 735–743 (1998).

...A. Fiser, A. Sali, Modeller: Generation and refinement of homology-based protein structure models. Methods Enzymol. 374, 461–491 (2003).

...N. Trabelsi, P. Petit, C. Manigand, B. Langlois d’Estaintot, T. Granier, J. Chaudière, B. Gallois, Structural evidence for the inhibition of grape dihydroflavonol 4-reductase by flavonols. Acta Crystallogr. D 64, 883–891 (2008).

...G. M. Morris, R. Huew, L. Lindstrom, M. F. Sanner, R. K. Belew, D. S. Goodsell, A. J. Olson, AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J. Comput. Chem. 30, 2785–2791 (2009).

...P. Emshley, M. Cowtan, Coot: Model-building tools for molecular graphics. Acta Crystallogr. D 60, 2126–2132 (2004).

...J. Pfeiffer, C. Kühnel, J. Brandt, D. Duy, P. A. Punyasiri, G. Forkmann, T. C. Fischer, Biosynthesis of flavonol 3,5-diols by leucocyanidin 4-reductases and anthocyanidin reductases in leaves of grape (Vitis vinifera L.), apple (Malus x domestica Borkh.) and other crops. Plant Physiol. Biochem. 44, 323–334 (2006).

Acknowledgments: We thank S. Temple for critical reading of the manuscript, the Biocatalytic Analysis Facility of the University North Texas, V. Shulaev for assistance with MS, and P. Azadi (Complex Carbonate Research Center, University of Georgia) for assistance with NMR analysis. Funding: This work was supported by Forage Genetics International Inc.; the University of North Texas; and the Chemical Sciences, Geosciences, and Biosciences Division, Office of Basic Energy Sciences, U.S. Department of Energy (grant DE-SC0015662 to the Complex Carbonate Research Center). The M. truncatula plants used in this research project, which are jointly owned by the Centre National de la Recherche Scientifique and the Noble Research Institute, Ardmore, OK, USA, were created through research funded, in part, by the National Science Foundation (grant no. 703285). Author contributions: R.A.D. and J.H.J. conceived and designed the study. J.H.J., N.L., M.D.-P., and X.W. acquired data. R.A.D., J.H.J., N.L., M.D.-P., and X.W. analyzed and interpreted data. The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Submitted 7 January 2021 Accepted 25 March 2021 Published 14 May 2021

Citation: J. H. Jun, N. Lu, M. Docampo-Palacios, X. Wang, R. A. Dixon, Dual activity of anthocyanidin reductase supports the dominant plant proanthocyanidin extension unit pathway. Sci. Adv. 7, eabg4682 (2021).
Dual activity of anthocyanidin reductase supports the dominant plant proanthocyanidin extension unit pathway
Ji Hyung Jun, Nan Lu, Maite Docampo-Palacios, Xiaoqiang Wang and Richard A. Dixon

Sci Adv 7 (20), eabg4682
DOI: 10.1126/sciadv.eabg4682