Tansley review

Proanthocyanidins – a final frontier in flavonoid research?

Author for correspondence:
Richard Dixon
Tel: +1 580 224 6601
Fax: +1 580 224 6692
Email: radixon@noble.org

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Richard A. Dixon, De-Yu Xie and Shashi B. Sharma

Plant Biology Division, Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, Oklahoma 73401, USA

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Summary

Proanthocyanidins are oligomeric and polymeric end products of the flavonoid biosynthetic pathway. They are present in the fruits, bark, leaves and seeds of many plants, where they provide protection against predation. At the same time they give flavor and astringency to beverages such as wine, fruit juices and teas, and are increasingly recognized as having beneficial effects on human health. The presence of proanthocyanidins is also a major quality factor for forage crops. The past 2 years have seen important breakthroughs in our understanding of the biosynthesis of the building blocks of proanthocyanidins, the flavan-3-ols (+)-catechin and (–)-epicatechin. However, virtually nothing is known about the ways in which these units are assembled into the corresponding oligomers *in vivo*. Molecular genetic approaches are leading to an understanding of the regulatory genes that control proanthocyanidin biosynthesis, and this information, together with increased knowledge of the enzymes specific for the pathway, will facilitate the genetic engineering of plants for introduction of value-added nutraceutical and forage quality traits.

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I. Introduction

Proanthocyanidins, also known as condensed tannins, are flavonoid polymers that have a long history of use as tanning agents for animal skins, and are determinants of flavor and astringency in teas, wines and fruit juices. The chemistry of proanthocyanidins has been studied for many decades. Their name reflects the fact that, on acid hydrolysis, the extension units

are converted to colored anthocyanidins, and this forms the basis of the classical assay for these compounds (Porter, 1989).

This review concentrates on recent advances in our appreciation of the broad biological activities of proanthocyanidins as they affect plant, animal and human health, and therefore become targets for genetic modification to improve yield, forage traits and the 'healthiness' of the human diet. Such engineering will require detailed understanding of the biochemistry and molecular genetics of proanthocyanidin biosynthesis. Although there have been important recent advances in these areas, a number of critical questions still remain to be addressed. Several excellent reviews on the subject of proanthocyanidins have appeared over the past 25 yr (Haslam, 1977; Foo & Porter, 1980; Stafford, 1988; Porter, 1989; Stafford, 1990; Scalbert, 1991; Schofield *et al.*, 2001). The older reviews should not be passed over, as they document critical and original thinking on a difficult biosynthetic problem before the application of modern molecular tools (Haslam, 1977), and introduce concepts such as metabolic channeling (Stafford, 1983) that were several years ahead of their time. More recent reviews provide excellent summaries of the agricultural benefits (Aerts *et al.*, 1999), chemistry (Ferreira & Slade, 2002; Ferreira *et al.*, 2003), and biochemistry (Marles *et al.*, 2003) of proanthocyanidins.

II. Sources and structures of proanthocyanidins

Proanthocyanidin structures vary depending upon the nature (stereochemistry and hydroxylation pattern) of the flavan-3-ol starter and extension units, the position and stereochemistry of the linkage to the 'lower' unit, the degree of polymerization, and the presence or absence of modifications such as esterification of the 3-hydroxyl group. These different features are illustrated in the structures shown in Fig. 1.

The building blocks of most proanthocyanidins are the flavan-3-ols (+)-catechin and (–)-epicatechin (Fig. 1). Note the 2,3-*cis* stereochemistry of (–)-epicatechin and the 2,3-*trans*-stereochemistry of (+)-catechin. These stereochemical differences are of major importance in proanthocyanidin biosynthesis, as all chiral intermediates in the flavonoid pathway up to and including leucoanthocyanidin are apparently of the 2,3-*trans* stereochemistry, raising important questions about the origin of the 2,3-*cis* stereochemistry of (–)-epicatechin, the commonest extension unit in proanthocyanidins (Foo & Porter, 1980). The B-ring hydroxylation pattern of the catechin/epicatechin pair is determined by the presence or absence of the enzymes flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylase, cytochrome P_{450} monooxygenases that act early in the pathway after formation of the flavanone naringenin (Menting *et al.*, 1994; Kaltenbach *et al.*, 1999; Schoenbohm *et al.*, 2000) (Fig. 2). Lack of these activities results in a B-ring hydroxylated only at the 4′-position, yielding the (–)-epiafzelechin/(+)-afzelechin pair (Fig. 1) or, in the case of the corresponding anthocyanidin, pelargonidin. The presence of both F3′H and F3′5′H would lead to formation of the (–)-epigallocatechin/(+)-gallocatechin pair or, in the case of the corresponding anthocyanidin, delphinidin. Homo-oligomeric proanthocyanidins with two (3′,4′) B-ring hydroxyl groups are termed procyanidins, whereas mixed oligomers with at least one unit containing only a 4′-hydroxyl, or the 3′,4′5′-tri-hydroxy pattern, are termed propelargonidins or prodelphinidins, respectively.

Generally, the linkage between successive monomeric units in proanthocyanidins is between the 4-position of the 'upper' unit and the 8-position of the 'lower' unit (Fig. 1). The stereochemistry of the linkage at C_4 may be either α or β, although it is not clear how this is determined. Alternative linkages can occur between C_4 of the 'upper' unit and C_6 of the 'lower' unit (as in the mopane proanthocyanidins) (Ferreira *et al.*, 2003), or in the case of the A-type proanthocyanidins, between both C_2 and C_4 of the upper unit and the oxygen at C_7 and positions 6 or 8, respectively, of the lower unit (see proanthocyanidins A1 and A2 and the cranberry and peanut proanthocyanidins in Fig. 1).

Proanthocyanidins B1–B4 differ only in the arrangement of (+)-catechin and (–)-epicatechin starter and extension units, as shown in Fig. 1. Their formation must be under strict enzymatic control because the different types of dimers are characteristic of specific plant species. For example, procyanidin B1 is found in grape, sorghum and cranberry; B2 in apple, cocoa bean and cherry; B3 in strawberry, hops and willow catkins; and B4 in raspberry and blackberry (Haslam, 1977).

Rarely, units other than flavan-3-ols are found in proanthocyanidins, one example being flavans, such as in the *Cassia petersiana* butiniflavan–epicatechin (Coetzee *et al.*, 1999) (Fig. 1). Descriptions of pigmented proanthocyanidins containing covalently bound anthocyanin residues should be interpreted with caution, and may reflect artifactual adducts formed during extraction (Kennedy *et al.*, 2001; Ferreira & Slade, 2002). Quite commonly, the 3-hydroxyl group of the flavan-3-ol units is esterified, often with gallic acid, as in the grape seed proanthocyanidin shown in Fig. 1. Indeed, free (–)-epigallocatechin gallate is a major phenolic compound in tea (Forrest & Bendall, 1969), and gallic acid-substituted epicatechin, epigallocatechin and epiafzelechin units also occur in tea proanthocyanidins (Lakenbrink *et al.*, 1999).

Proanthocyanidin levels have been estimated from a multitude of plant sources since the pioneering work of the Robinsons, Bate-Smith and Haslam (reviewed by Haslam, 1977). However, much of this work has used simple colorimetric methods for assessment of proanthocyanidin concentrations. When applied carefully and combined with a good extraction protocol, use of the vanillin–HCl, butanol–HCl and dimethylaminocinnamaldehyde reagents can provide reproducible estimates of proanthocyanidin content (Porter, 1989; Li *et al.*, 1996; Dalzell & Kerven, 1998; Schofield *et al.*, 2001). Recently, more sensitive and specific methods utilizing HPLC and mass spectrometry have been developed for the fractionation and identification of proanthocyanidins, with accurate

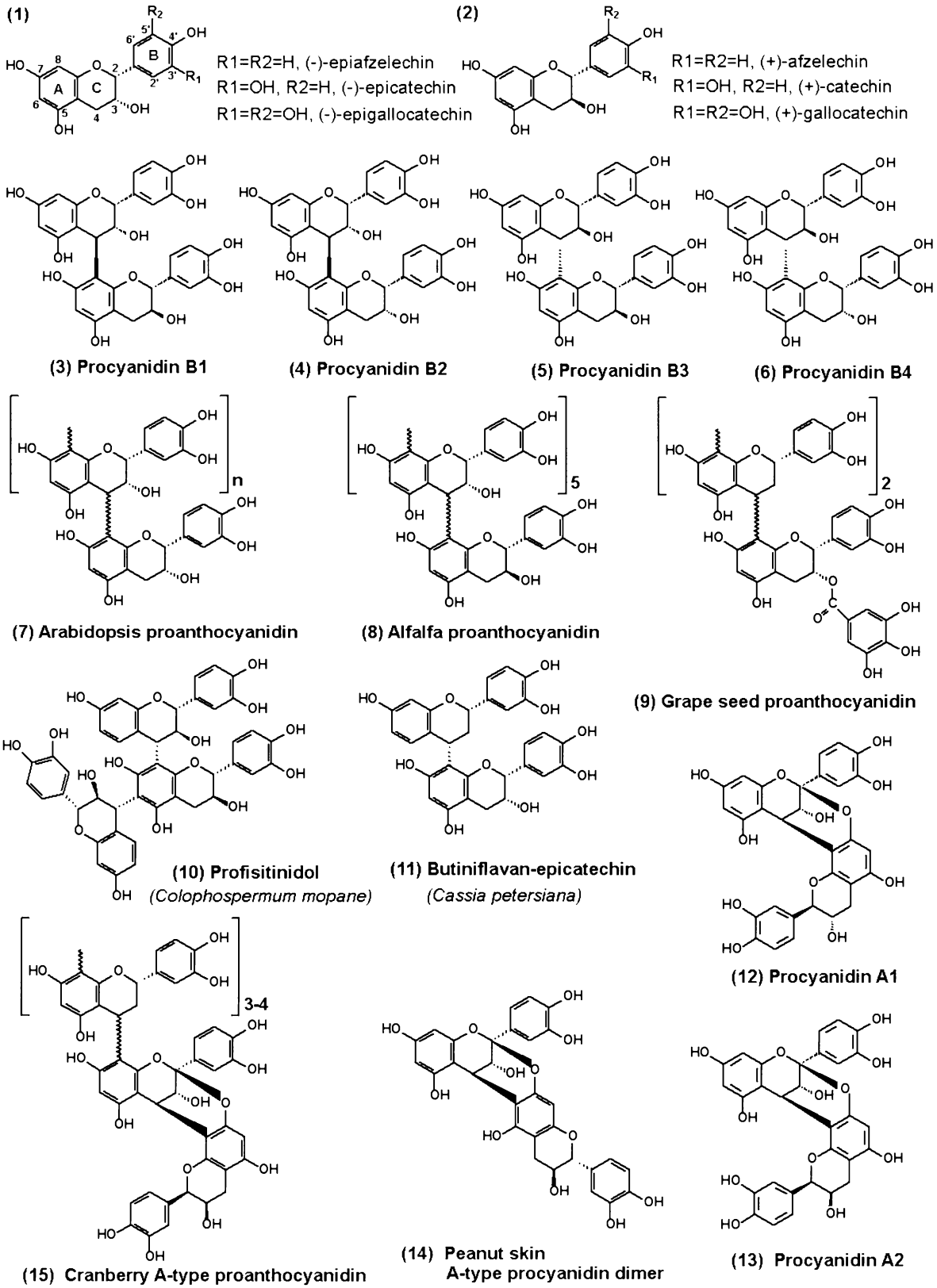

Fig. 1 Structures of the flavan-3-ol building blocks of proanthocyanidins (1, 2); simple B-type proanthocyanidins (3–6); complex proanthocyanidins based on the B-type structure (7–10); a flavan-containing proanthocyanidin (11); simple A-type proanthocyanidins (12, 13); and complex proanthocyanidins based on the A-type structure (14, 15).

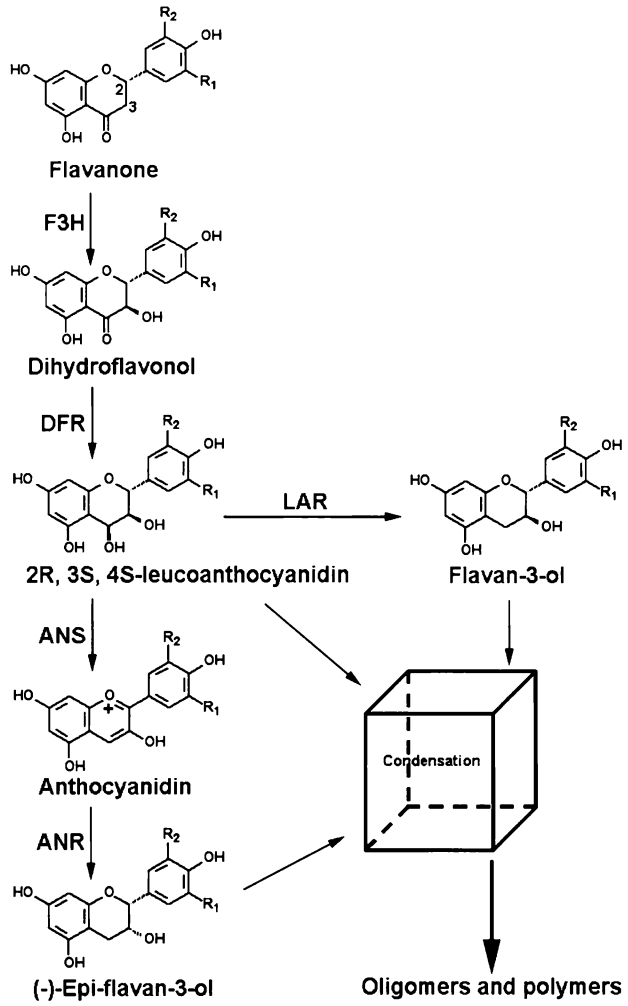

Fig. 2 Biosynthetic pathway for formation of proanthocyanidins. The enzymes are: F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol reductase; LAR, leucoanthocyanidin reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase. Condensation reactions are shown as a black box.

determination of both the amount and degree of polymerization of the different-sized oligomeric fractions (Cheynier *et al.*, 1999; Gu *et al.*, 2002). Armed with this technology, the detailed proanthocyanidin profiles and compositions were recently determined for over 40 common food sources (Gu *et al.*, 2004). The foods with the highest levels of total proanthocyanidins were, in decreasing order, ground cinnamon, sorghum (sumac bran), dry grape seed, unsweetened baking chocolate, raw pinto beans, sorghum (high-tannin whole grain), choke berries, red kidney beans, hazelnuts and pecan nuts (Gu *et al.*, 2004).

There is considerable interest in the proanthocyanidins found in grape skins because of their importance for the flavor and astringency of wine. Because of inclusion of the skins during fermentation, the total proanthocyanidin content of red wines, averaging around 175 mg l^{-1} (Sanchez *et al.*, 2003), is approximately 20-fold higher than that of white wines, from which the skins are removed. The changes occurring in proanthocyanidins during development of the grape are complex, involving increases in the degree of polymerization, in the proportion of (–)-epigallocatechin extension units, and in polymer-associated anthocyanins (Kennedy *et al.*, 2001). The proanthocyanidin compositional changes that occur during storage of the wine itself are even more complex, also involving changes in chain length and formation of anthocyanin adducts (Vidal *et al.*, 2002). Grape seed extract is being studied intensively as a source of proanthocyanidins in view of its health-promoting potential in a number of model cell and animal systems (Bagchi *et al.*, 2000) (see below).

III. Functions of proanthocyanidins in the plant

Proanthocyanidins and monomeric flavan-3-ols and their derivatives are present in the fruits, bark, leaves and seeds of many plants, where their major function is to provide protection against microbial pathogens, insect pests and larger herbivores. The deposition of proanthocyanidins in the endothelial layer of the seed coat in many species would appear to be a classic example of a pre-formed protective barrier. Coffee varieties susceptible to the fungal pathogen *Hemileia vastatrix* contain lower levels of proanthocyanidins, and the proanthocyanidin fraction from pulp or leaves inhibits fungal uredospore germination *in vitro* (Gonzalez de Colmenares *et al.*, 1998). Consistent with a role of proanthocyanidins as a first line of defense against insect predation, several reports have documented the presence of proanthocyanidins in glandular trichomes (Kelsey *et al.*, 1984; Li *et al.*, 1996; Aziz *et al.*, 2004).

Esterified flavan-3-ols such as (–)-epigallocatechin gallate accumulate to very high levels in the leaves of the tea plant, along with oligomeric proanthocyanidins. Most interest in the tea polyphenols is now concentrated on their potential health beneficial effects (Ahmad *et al.*, 2000), although high levels of monomeric flavan-3-ols such as (–)-epicatechin have been linked with resistance of plants to fungal attack (Ardi *et al.*, 1998). The unusual epimer (–)-catechin has recently been shown to be a powerful allelochemical, responsible for the adaptive advantage of the invasive species spotted knapweed (*Centaurea maculosa*) in the western USA (Bais *et al.*, 2003). (–)-Catechin triggers active oxygen production and a calcium-signaling cascade that leads to death of the roots of susceptible species (Bais *et al.*, 2003).

Proanthocyanidins bind metals through complexation involving their *o*-diphenol groups. This property is often viewed as imparting negative traits as regards bioavailability of essential mineral micronutrients, especially iron and zinc (House, 1999). Iron depletion causes severe limitation to bacterial growth, and it has been suggested that the ability to bind iron represents one mechanism for the antibacterial activity of proanthocyanidins (Scalbert, 1991). The metal-binding

properties of proanthocyanidins might, however, be of benefit to the plant, as in the case of the legume *Lotus pedunculatus*, which is tolerant to aluminum and may complex the metal in tannin-rich vacuoles in the root (Stoutjesdijk *et al.*, 2001).

In various peanut genotypes, levels of constitutive procyanidins in the leaf-bud petioles appeared to be inversely proportional to the fecundity of aphids feeding on the plants (Grayer *et al.*, 1992). There is also evidence that inducible proanthocyanidin accumulation may represent a defense mechanism of some seeds against piercing–sucking insects (Ceballos *et al.*, 2002), and of leaves against mammalian and insect herbivory. However, the importance of constitutively formed tannins in herbivore choice has been questioned (Briggs, 1990). In a recent study in Kenya, although correlations were observed between tannin levels in leaves of *Acacia* and the extent of mammalian herbivory, the leaves in the upper canopy, which were not naturally subjected to herbivory, had the highest tannin levels (Ward & Young, 2002).

Not all effects of proanthocyanidins benefit the plant at the expense of microorganisms and insects. For example, proanthocyanidins in the bark of *Pinus densiflora* act as oviposition stimulants for the cerambycid beetle *Monochamus alternatus* (Sato *et al.*, 1999).

Bacillus thuringiensis (Bt) endotoxin genes have recently been introduced into crop plants for control of insect herbivores. Cotton proanthocyanidins, although functioning as feeding deterrents, may paradoxically provide the insect with some level of protection against the effects of the endotoxin, and this effect probably occurs post-ingestion (Navon *et al.*, 1993). Clearly, transgenic crop protection strategies need to take into account the possibility of interactions with the plant's natural defensive arsenal.

Levels of plant secondary metabolites are often affected by leaf carbon/nitrogen ratios, and this has been confirmed in the case of proanthocyanidins, where increased levels have been associated with high ambient CO_2 concentrations and low soil fertility, suggesting a potential positive effect on proanthocyanidin production as a result of greenhouse gas emissions. This response was shown to be genotype-dependent in trembling aspen (*Populus tremuloides*) (Mansfield *et al.*, 1999). Sulfur pollution from a steam power station in Japan was shown to be responsible for reduced levels of both proanthocyanidins and hydrolyzable tannins in Japanese Cedar trees, with a resulting increase in insect predation and damage. Clearly, atmospheric changes resulting from human activity can have profound and varying ecological impacts through effects on proanthocyanidin biosynthesis (Katoh *et al.*, 1989).

IV. Proanthocyanidins and plant quality traits

The protective effects from herbivory resulting from the astringency of proanthocyanidins have important implications in agriculture. Thus high concentrations of proanthocyanidins (6–12% of dry matter) can decrease the palatability of forages and have a negative impact on nutritive value, including forage digestibility, by binding bacterial enzymes or forming complexes with cell-wall polysaccharides (Reed, 1995; Aerts *et al.*, 1999; Smulikowska *et al.*, 2001). Paradoxically, however, the presence of tannins can also be a positive trait in some forage species. This is because forage from species with very high leaf protein content, such as the legume alfalfa (Marshall *et al.*, 1980), is fermented too rapidly in the rumen. This results in loss of by-pass protein (protein nitrogen available to the animal after exiting the rumen) with resulting increased nitrogen excretion in the urine, and generation of excessive amounts of the greenhouse gas methane, with the potential for development of pasture bloat, a dangerous condition that can be lethal to the animal (Table 1).

Table 1 Potential benefits of transgenic forage crops with low (2–4% d. wt) levels of proanthocyanidins

Change	Process
Reduced	Rumen fermentation leading to reduction in incidence of pasture bloat
Reduced	Methane gas emissions from ruminants
Reduced	Protein degradation during ensiling
Improved	Absorption of essential amino acids leading to increased meat, milk and wool production
Reduced	Excretion of soluble nitrogen in urine
Reduced	Mineralization of carbon and nitrogen in soil

In laboratory studies, treatment of feed proteins with modest amounts of proanthocyanidins (around 2–4% of dry matter) reduced both proteolysis during ensiling and rumen fermentation. In studies performed with sheep in New Zealand (Douglas *et al.*, 1999), increasing dietary proanthocyanidin from trace amounts to 4% of dry matter increased by-pass protein, and a diet containing only 2% proanthocyanidin strongly increased absorption of essential amino acids by the small intestine by up to 60%. In addition, low concentrations of proanthocyanidins can help counter intestinal parasites in lambs and confer bloat safety (Aerts *et al.*, 1999), presumably by interacting with both leaf protein and microbial enzymes such that the rate of protein degradation in the rumen is reduced. These properties of proanthocyanidins are the main driving force behind efforts to genetically introduce the proanthocyanidin pathway into forage crops (Reed, 1995; Aerts *et al.*, 1999).

Proanthocyanidins can also have a negative impact on nutritional quality through their ability to complex metal ions, thus reducing the bioavailability of essential micronutrients such as iron and zinc; this may be a serious problem in developing countries where the majority of the population relies on diets rich in beans for protein nitrogen. Beans and cereals also contain high levels of phytic acid (*myo*-inositol hexaphosphate), a compound with strong metal-chelating ability. After removing phytic acid with phytase, treatment of water slurries from high proanthocyanidin cereal flours with

polyphenol oxidase resulted in increased iron bioavailability, presumably because of oxidation of the iron-chelating proanthocyanidins (Matuschek *et al.*, 2001). However, in a study using genetic selection for enhanced iron bioavailability in bean seeds, there was, surprisingly, no correlation between iron availability and levels of phytic acid or proanthocyanidins, suggesting that additional factors are critical for micronutrient availability (Welch *et al.*, 2000). Genetic approaches, such as quantitative trait locus (QTL) analysis, may be helpful for characterizing these factors; such an approach has already been taken to analyze calcium, iron, zinc and proanthocyanidin levels in beans (Guzmán-Maldonado *et al.*, 2003). In this study, four QTLs, together accounting for 42% of the variance in proanthocyanidin levels, were identified (Guzmán-Maldonado *et al.*, 2003).

The dietary effects of proanthocyanidins are clearly target species-dependent. Bean (*Vicia faba*) seeds, which contain high levels of proanthocyanidins, are often used to supplement pig diets. While somewhat reducing non-nitrogen matter digestibility, inclusion of bean seeds (containing nearly 600 mg proanthocyanidin kg^{-1}) in pig diets did not appear to reduce weight gain or to have a negative impact on protein nitrogen availability (Flis *et al.*, 1999).

The proanthocyanidins found in barley seed coat are of commercial importance because of their potentially negative effects on the shelf life of beer, caused by formation of a colloidal haze involving complexation with protein (von Wettstein *et al.*, 1985). Barley proanthocyanidins consist primarily of (+)-catechin residues. Several mutant lines have been developed in which proanthocyanidin formation is blocked; these have been evaluated both as breeding material for improved varieties (Horsley *et al.*, 1991; Garvin *et al.*, 1998), and as tools for understanding the structural and regulatory genes involved in proanthocyanidin biosynthesis (Jende-Strid, 1991; Meldgaard, 1992).

V. Flavanols, proanthocyanidins and human health

A large body of literature has emerged over the past several years ascribing potential health beneficial effects to dietary monomeric flavan-3-ol derivatives and to various crude and purified proanthocyanidin fractions from fruits, leaves and bark (Table 2). A significant percentage of this work has been aimed at validating the already documented heath effects of green tea (Dufresne & Farnworth, 2001), grapes (wines, juices and grape seed extracts) (Bagchi *et al.*, 2000), and cranberry juice (Foo *et al.*, 2000).

Nonpolymerized flavan-3-ols and their derivatives have significant pharmacological activities, and a number of medicinal plants contain high levels of these compounds. For example, on the basis of an assay measuring *in vitro* inhibition of cyclooxygenase-2-catalyzed prostaglandin biosynthesis, (+)-catechin, (+)-gallocatechin, and 4′-*O*-methyl-*ent*-gallocatechin were isolated from *Atuna racemosa*, *Syzygium corynocarpum*, *Syzygium malaccense* and *Vantanea peruviana*, species traditionally used to treat inflammatory conditions (Noreen *et al.*, 1998). Rhamnose conjugates of these flavanols were also present in the extracts but were inactive in the *in vitro* assay. Green tea is a rich source of catechin derivatives, particularly (–)-epigallocatechin gallate, and this compound and the related (–)-epicatechin have been shown to protect mammalian cells from free radical-mediated oxidative stress and to cause apoptosis of cancer cells (Zhan *et al.*, 1997; Ahmad

Table 2 Some potential health-protective effects of proanthocyanidins and flavan-3-ol monomers

Effect	Source	Chemical constituent	Reference
Anticancer	Green tea	EGCG	Ahmad *et al.* (2000)
Anticancer	Cranberry	C, EC, GC2, EGC2, oligomers	Kandil *et al.* (2002)
Antioxidant	Grape seed	GSPE	Bagchi *et al.* (2000)
Antioxidant	Chocolate	EC	Serafini *et al.* (2003)
Inhibition of bacterial adhesion to urinary tract	Cranberry	ProA	Foo *et al.* (2000)
Immunomodulatory	*Ecdysanthera utilis*	ProA	Lin *et al.* (2002)
Promotion of cardiac recovery after ischemia	Grape seed	Crude proanthocyanidin	Pataki *et al.* (2002)
Inhibition of prostaglandin biosynthesis	*Syzygium* spp.	C, GC, ProA	Noreen *et al.* (1998)
Protection from renal failure	Grape	ProBP1	Avramovic *et al.* (1999)
Analgesic, anti-inflammatory	*Polypodium feei*	shellegueain A	Subarnas & Wagner (2000)
Prevention of atherosclerosis	Grape seed	GSPE	Vinson *et al.* (2002)
Antimutagenic	*Hamamelis virginiana* bark	proanthocyanidins	Dauer *et al.* (2003)
Reduction of skin irritation	*H. virginiana* bark	proanthocyanidins	Deters *et al.* (2001)
Inhibition of cholesterol synthesis	Green tea	EGCG	Abe *et al.* (2000)
Protection from hemorrhage	Peanut skin	A-type proanthocyanidins	Lou *et al.* (1999)

EGCG, epigallocatechin gallate; C, catechin; EC, epicatechin; GC2, gallocatechin dimer; EGC2, epigallocatechin dimer; GSPE, grape seed proanthocyanidin-rich extract; ProA, A-type proanthocyanidin; Pro3–5, proanthocyanidin trimers-pentamers; ProG3-5, gallates of proanthocyanidin trimers to pentamers.

et al., 2000; Spencer *et al.*, 2001). The differential apoptotic effect of epigallocatechin gallate on cancer as compared with noncancer cells was proposed to be the result of differential dose–response effects on inhibition of NF-κB expression, with cancer cells being the more sensitive (Ahmad *et al.*, 2000). Chocolate contains (–)-epicatechin, and a recent article received much interest in the popular press when it was suggested that consumption of dark chocolate (but not milk chocolate) was potentially cardioprotective because of increases in blood plasma antioxidant potential associated with raised plasma epicatechin levels (Serafini *et al.*, 2003). Consumption of milk at the same time as dark chocolate negated the effect as milk protein can bind polyphenols and reduce their bioavailability (Serafini *et al.*, 2003).

The benefits associated with the antioxidant activity of grape seed proanthocyanidin extract (GSPE) have been evaluated in a number of animal and cell model systems (Table 2; Bagchi *et al.*, 2000). For example, feeding rats with GSPE from red grape seeds for 3 wk resulted in improved cardiac recovery during reperfusion after ischemia, and this was associated with significant reduction in free radical levels as determined by electron spin resonance spectroscopy (Pataki *et al.*, 2002). Protective effects of GSPE on chick cardiomyocytes appear to be the result of direct scavenging of reactive oxygen species, and do not require protein kinase C or nitric oxide synthase activities (Shao *et al.*, 2003). It has been proposed that the *in vivo* antioxidant potential of GSPE is greater than that of vitamins C, E and β-carotene (Bagchi *et al.*, 2003). Clinical trials with human subjects have suggested that consumption of GSPE can significantly reduce oxidized low-density lipoprotein, a marker for cardiovascular disease (Vinson *et al.*, 2002), and reduce plasma lipid hydroperoxide levels during the postprandial phase (Natella *et al.*, 2002), one possible reason for the benefits of drinking red wine with a meal. The beneficial effects of GSPE on cardiovascular markers in hypercholesterolemic humans are increased by coingestion of niacin-bound chromium (NBC); GSPE alone, and in combination with NBC, strongly reduces atherosclerosis in hamster aorta (Vinson *et al.*, 2002). Extensive studies have been performed documenting the safety of administering standardized GSPEs to laboratory animals over long time periods (Ray *et al.*, 2001).

Other studies have attempted to define the chemical components in GSPE responsible for specific protective effects. Wines and grape juices can cause endothelium-dependent relaxation of blood vessels *in vitro* by increasing nitric oxide production (Fitzpatrick *et al.*, 2000), and this forms the basis of an activity-directed fractionation using a rat aorta preparation bioassay. Using this assay, the most active fractions obtained by reversed phase HPLC were proanthocyanidin trimers, tetramers, pentamers, higher polymers and their gallate derivatives (Fitzpatrick *et al.*, 2000). A specific proanthocyanidin component of GSPE, proanthocyanidin-BP1 (Fig. 1), is protective in a rat model for oxidative renal injury (Avramovic *et al.*, 1999). In red wines, oxygen radical absorbance capacity can be correlated with the presence of malvidin compounds and proanthocyanidins. In white wines, a significant correlation was found between the trimeric proanthocyanidin fraction and peroxyl radical scavenging activity (Sanchez *et al.*, 2003).

Extracts from witch hazel (*Hamamelis virginiana*) are widely used in the therapy of skin diseases and in cosmetic formulas, as well as in treating gastrointestinal disorders. They contain a range of polysaccharides, hydrolysable tannins, proanthocyanidins, and flavan-3-ol derivatives (Hartisch & Kolodziej, 1996; Deters *et al.*, 2001), among which are dimeric proanthocyanidins with galloylated epigallocatechin and epicatechin, as well as an unusual 4-hydroxybenzoate-esterified catechin starter unit (Hartisch & Kolodziej, 1996). In a study of the antimutagenic activity of compounds found in the bark of witch hazel, the proanthocyanidin fraction was found to be the most effective in protecting against benzo(a)pyrene-induced DNA damage (Dauer *et al.*, 2003), and the proanthocyanidins likewise were the effective fraction in relation to stimulation of keratinocyte proliferation and *in vivo* protection of skin from irritation (Deters *et al.*, 2001). Grape proanthocyanidins are likewise effective as sun damage-preventing agents when applied topically to humans (Bagchi *et al.*, 2000).

Juice of the American cranberry (*Vaccinium macrocarpon*) is well known for its ability to protect the urinary tract from adherence of urinopathogenic bacteria; drinking cranberry juice is a recommended treatment for various urinary tract infections and prostatitis. Cranberry juice contains high concentrations of anthocyanins, flavonol glycosides, phenolic acids and proanthocyanidins. Using a bioassay to detect binding of uropathogenic P-fimbriated *Escherichia coli* cells to surfaces containing the urinary tract α-Gal(1→4)β-Gal receptor motifs, the active fraction(s) were shown to contain proanthocyanidins consisting primarily of epicatechin tetramers and pentamers, with at least one A-type linkage (Fig. 1; Foo *et al.*, 2000). A more complex picture was obtained when fractionating cranberry extracts on the basis of antioxidant activity, and refinement of the bioassay to detect only compounds that inhibited phorbol ester-mediated ornithine decarboxylase activity (an assessment of potential antitumor activity) still resulted in a complex mixture containing (–)-epicatechin, (+)-catechin, dimers of gallocatechin and epigallocatechin, and a series of proanthocyanidin oligomers (Kandil *et al.*, 2002).

Several A-type proanthocyanidins were recently isolated from the shrub *Ecdysanthera utilis*, used in Taiwanese folk medicine as an analgesic, antiphlogistic and spasmolytic agent. The extracts have strong anti-inflammatory activity, and the major immunomodulatory compound was shown to be procyanidin A1 (Fig. 1; Lin *et al.*, 2002). This compound appears to act by blocking interferon-γ and interleukin-2 production (Lin *et al.*, 2002). Similarly, the proanthocyanidin

shellegueain A from the roots of the fern *Polypodium feei* has been shown to have nearly comparable anti-inflammatory activity to aspirin, and has been proposed to function through inhibition of prostaglandin biosynthesis (Subarnas & Wagner, 2000).

Little is known about the structural features that affect the bioavailability and metabolism of proanthocyanidins within the body. Similarly to their monomeric units, some oligomeric proanthocyanidins are absorbed and bioavailable in the human body, as procyanidin B1 (Fig. 1) can be detected in human plasma as early as 2 h after ingestion of GSPE (Sano *et al.*, 2003). Incubating proanthocyanidins *in vitro* with human colonic microflora can result in complete degradation to hydroxylated derivatives of phenylacetic, phenylpropionic and phenylvaleric acids (Deprez *et al.*, 2000), compounds that had previously been shown to arise from degradation of flavonoid monomers.

VI. Biosynthesis of proanthocyanidins

1. Formation of (–)-epicatechin and (+)-catechin monomers

Proanthocyanidins are products of the flavonoid pathway leading to the anthocyanin flower pigments, a pathway that has been investigated intensively at the biochemical and genetic levels (Holton & Cornish, 1995). For many years the biosynthesis of proanthocyanidins had been assumed to branch from the anthocyanin pathway at the level of leucoanthocyanidin (Fig. 2). Several reviews excellently detail our current understanding of the biosynthesis of anthocyanins and the other flavonoid classes derived from earlier intermediates in the anthocyanin pathway (Shirley, 1996; Winkel-Shirley, 2001a, 2001b; Saito & Yamazaki, 2002); the early reactions are summarized in Fig. 2, but not discussed further here. Understanding the proanthocyanidin branch of the pathway has been difficult because the potential substrates are not commercially available, have multiple potential stereochemistries, and are unstable and readily oxidized in biological extracts. Biochemical studies laid the foundations for our understanding of the pathway, but only with the application of genetic and molecular genetic approaches has it been possible critically to evaluate this understanding and to move beyond *in vitro* assay data.

The *in vitro* biosynthesis of the 2,3-*trans*-(+)-catechin series was established in the early 1980s by Stafford and Lester, who showed that crude extracts from cell or tissue cultures of *Ginko biloba* and Douglas fir (*Pseudotsuga menziesii*) could convert dihydroflavonol [(+)-dihydromyrecitin or (+)-dihydroquercetin] to the corresponding flavan-3,4-diol and catechin derivative [(+)-gallocatechin or (+)-catechin, respectively] (Stafford & Lester, 1984, 1985). Similar conversion of dihydroquercetin to catechin was observed in extracts from developing grains of barley (Kristiansen, 1986). Note that the dihydroflavonol intermediates of the anthocyanin pathway already possess the 2,3-*trans* stereochemistry, which is established by the chalcone isomerase reaction (Fig. 2). The *in vitro* formation of catechins was presumed to occur via the consecutive action of a dihydroflavonol reductase (DFR), to yield leucoanthocyanidin, and a leucoanthocyanidin reductase (LAR). *DFR* genes were cloned from several plant species during the late 1980s and 1990s (Beld *et al.*, 1989; Charrier *et al.*, 1995; Tanaka *et al.*, 1995), and LAR activity was detected in extracts from leaves of tannin-rich legumes (Tanner & Kristiansen, 1993; Skadhauge *et al.*, 1997). However, it was only in 2003 that the cloning of a gene encoding an enzyme capable of converting leucoanthocyanidin to (+)-catechin was reported (Tanner *et al.*, 2003); this LAR, from the forage legume *Desmodium uncinatum*, is encoded by a member of the isoflavone reductase-like group of the plant reductase–epimerase–dehydrogenase (RED) supergene family. Notably, *Arabidopsis thaliana*, which produces a seed-coat proanthocyanidin consisting entirely of 2,3-*cis*-(–)-epicatechin (Abrahams *et al.*, 2003), does not appear to possess an ortholog of the *Desmodium LAR* gene (Tanner *et al.*, 2003).

Most models of proanthocyanidin biosynthesis have asserted that the extension units arise by condensation of an electrophile, derived from leucoanthocyanidin, with the nucleophilic 8 or 6 position of the starter unit. This process is discussed in more detail below. However, this model fails to account for the fact that, as formed by the classical pathway, the stereochemistry of leucoanthocyanidin is 2,3-*trans*, whereas in most cases the major extension units are 2,3-*cis*. One possible solution to this stereochemical paradox was discovered through a genetics-based approach. Mutations in the *BANYULS* (*BAN*, named after the color of a French red wine) gene in *A. thaliana* result in a transparent testa (*tt*) associated with a lack of proanthocyanidins and precocious accumulation of anthocyanins in the seed coat (Devic *et al.*, 1999) (Fig. 3a). On the basis of this phenotype, and the amino acid sequence similarity of BAN to DFR, it was suggested that *BAN* encodes LAR (Devic *et al.*, 1999) and therefore converts flavan-3,4-diols to the corresponding 2,3-*trans*-flavan-3-ols such as (+)-catechin. However, expression of recombinant BAN proteins from *Arabidopsis* or *Medicago truncatula* in *E. coli* failed to give *in vitro* LAR activity. Instead, the *BAN* genes from both *Arabidopsis* and *M. truncatula* were shown to encode a new enzyme, anthocyanidin reductase (ANR), that converts cyanidin to 2,3-*cis*-(–)-epicatechin (Xie *et al.*, 2003). Therefore anthocyanins are not, as previously believed, only end products of flavonoid metabolism. Although *BAN* expression in *M. truncatula* is primarily limited to young seed coats, transgenic expression of *Medicago* BAN in tobacco leads to accumulation of condensed tannins throughout the pigmented portions of the petals, with concomitant reduction in anthocyanin levels (Xie *et al.*, 2003; Fig. 3b). The final stages in biosynthesis of (+)-catechin and (–)-epicatechin are therefore as illustrated in Fig. 2. The alteration in stereochemistry at C_2–C_3 is brought about through the intermediacy of

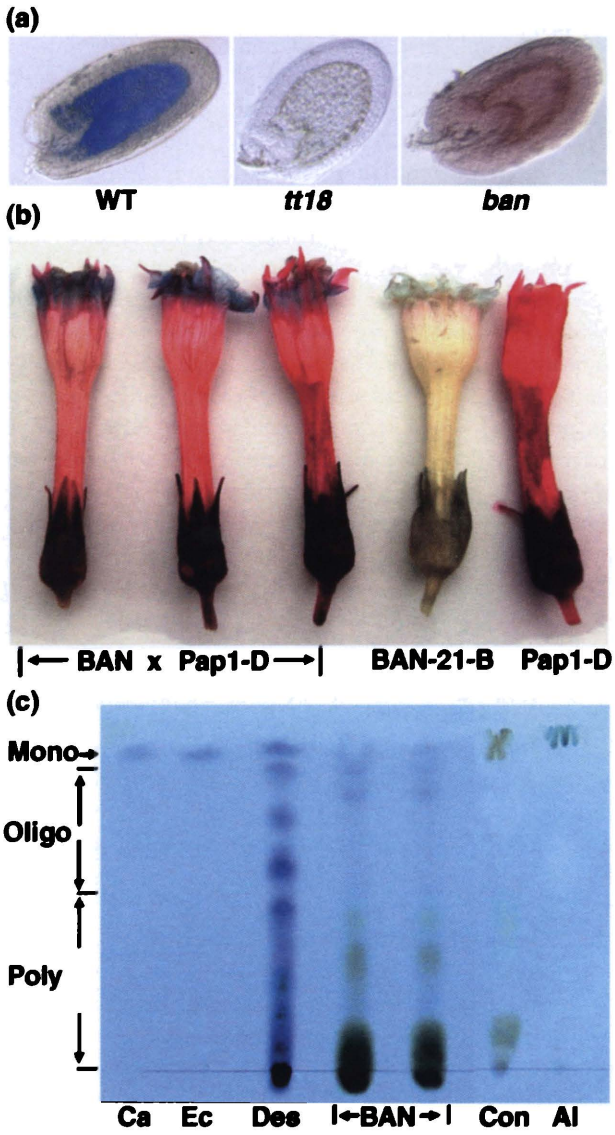

Fig. 3 Phenotypes of plants modified in proanthocyanidin biosynthesis. (a) Seeds of wild-type (WT), *tt18* (ANS mutant) and *ban* (ANR mutant) *Arabidopsis* stained with DMACA reagent. Note accumulation of proanthocyanidins in WT, anthocyanins in *ban*, and neither in *tt18*. (b) Tobacco flowers stained with DMACA reagent. The three flowers on the left are progeny of a cross between a plant expressing *Medicago truncatula* BAN and a plant expressing *Arabidopsis* PAP1. The two plants on the right are the respective parents. (c) Thin-layer chromatogram showing separation of oligomeric and polymeric proanthocyanidins from extracts of leaves of *Desmodium uncinatum* (Des); BAN-expressing tobacco flowers (BAN); nontransgenic tobacco flowers (Con); and leaves of alfalfa (Al). Catechin (Ca) and epicatechin (Ec) standards are also shown.

an achiral intermediate (the anthocyanidin), rather than through the action of an epimerase, as proposed earlier (Stafford, 1983). According to the new model, anthocyanidin synthase (ANS, also known as leucoanthocyanidin dioxygenase) now becomes an enzyme of the proanthocyanidin pathway, and mutations in ANS do indeed result in a deficiency in proanthocyanidin accumulation (Abrahams *et al.*, 2003; Xie *et al.*, 2003; Fig. 3a).

The biochemical properties of ANR from *M. truncatula* (MtANR) and *Arabidopsis* (AtANR) show interesting differences (Xie *et al.*, 2004b). MtANR uses both NADPH and NADH as reductant with a slight preference for NADPH over NADH. In contrast, AtANR uses only NADPH and exhibits positive cooperativity for this cosubstrate. MtANR shows preference for potential anthocyanidin substrates in the order cyanidin > pelargonidin > delphinidin, with typical Michaelis–Menten kinetics for each substrate (Xie *et al.*, 2004b). The products, as determined by mass spectrometry, NMR and circular dichroism spectrometry, are (–)-epicatechin (–)-epiafzelechin, and (–)-epigallocatechin, respectively. In contrast, AtANR exhibits the reverse preference, and substrate inhibition is observed at high concentrations of cyanidin and pelargonidin. (+)-Catechin and (±)-dihydroquercetin inhibit AtANR but not MtANR, whereas quercetin inhibits both enzymes (Xie *et al.*, 2004b).

To date, other than the case of the *BANYULS* mutation in *Arabidopsis*, no genetic validation of the roles of either ANR or LAR in proanthocyanidin biosynthesis has been reported. However, biochemical correlations link these enzymes to proanthocyanidin biosynthesis *in vivo* in several species. In the case of ANR, very high activities have been observed in extracts from *Desmodium* and *Lotus corniculatus* leaves, grape skins and developing barley seeds, all sources rich in proanthocyanidins (unpublished results). In contrast, the forage legume alfalfa contains no proanthocyanidins in the leaves (Skadhauge *et al.*, 1997), and no ANR or LAR activity is detectable. Likewise, LAR activity has been determined in barley, *Desmodium* leaves and the seeds of several legumes (Kristiansen, 1986; Skadhauge *et al.*, 1997; Tanner *et al.*, 2003). In a survey of developing tissues of five different legume species there was an absolute correlation between the presence of extractable LAR activity and the accumulation of proanthocyanidins (Skadhauge *et al.*, 1997). However, the temporal and quantitative correlations were less clear. In the tannin-rich forage legume sanfoin (*Onobrychis viciifolia*), proanthocyanidin levels increase on a per leaf basis throughout development, but remain relatively constant on a per weight basis (Joseph *et al.*, 1998). The relative levels of chalcone synthase protein likewise remain relatively constant throughout development, but the activities of DFR and LAR are highest in the youngest leaves, and decline dramatically at the later stages of development (Joseph *et al.*, 1998). It should be noted that, in most experiments to date measuring LAR activity, the chemical identity of the product was not confirmed beyond cochromatography of labeled product with a (+)-catechin standard.

A special comment needs to be made concerning the involvement of DFR in proanthocyanidin biosynthesis. Early

biochemical studies demonstrated the two-step conversion of dihydroflavonol to 2,3-*trans*-flavan-3-ol in crude extracts incubated with NADPH (Stafford & Lester, 1984, 1985), and a subsequent study provided evidence that formation of (+)-gallocatechin from (+)-dihydromyrecetin occurred through a high molecular-weight enzyme complex in extracts from *O. viciifolia* (Singh *et al.*, 1997). Is it possible that a specific isoform of DFR, capable of forming a complex with LAR, is specifically involved in proanthocyanidin biosynthesis? In *M. truncatula*, in which proanthocyanidin synthesis occurs predominantly in the seed coat, two *DFR* genes are present with basically overlapping tissue specificities but different relative substrate preferences (Xie *et al.*, 2004a). However, in many other species, including *Arabidopsis*, DFR is encoded by a single gene. In trembling aspen, insect herbivory, wounding or treatment with methyl jasmonate all result in strong induction of the single *DFR* gene and, at least in the case of wounding, local and systemic accumulation of proanthocyanidin (Peters & Constabel, 2004). If a second *DFR*-like gene product were involved in a parallel complex (with ANS) leading to anthocyanidin, this could also be common to proanthocyanidin biosynthesis via ANR, unless there were also specific forms of ANS that were differentially involved in anthocyanin and proanthocyanidin synthesis.

The discovery of ANR raises the question of the control point between anthocyanin and proanthocyanidin biosynthesis, previously believed to be at the level of leucoanthocyanidin oxidation (to form anthocyanidin) or reduction (to form catechin). The revised pathway to proanthocyanidins (Fig. 2) suggests that control, at least in the case of the 2,3-*cis*- units, might reside in competition between anthocyanidin glucosyltransferase and ANR for substrate, as ANR does not reduce glycosylated anthocyanidins. However, if LAR is essential for the formation of 2,3-*trans*- units, control of this branch would still be at the leucoanthocyanidin level. Such dual position control is less attractive than that proposed in earlier models, now seen to be inconsistent with the biochemistry and genetics of proanthocyanidin formation, in which nonchiral, enzyme-bound common intermediates are converted, in parallel, to monomers with either *cis*- or *trans*- stereochemistry depending on the direction of hydride transfer (Haslam, 1977). Such models have not, however, been completely dismissed from discussion (Tanner *et al.*, 2003; Xie *et al.*, 2004b), and cannot yet be completely ruled out. Independent control of proanthocyanidin and anthocyanin formation could be explained by the operation of specific enzyme complexes through which intermediates destined for anthocyanin or proanthocyanidin are differentially channeled (Stafford, 1983; Jaakola *et al.*, 2002). Although metabolic channeling has recently received considerable attention in relation to the control of flavonoid biosynthesis (Winkel-Shirley, 1999; Liu & Dixon, 2001), there is as yet little direct experimental evidence to support this model as regards proanthocyanidin biosynthesis. Knowing the subcellular localization of LAR, ANR and anthocyanidin glycosyltransferase(s) would be helpful in this regard, as would further information on the biochemistry of the several transporter systems that have been implicated, primarily on the basis of genetic evidence (Marrs *et al.*, 1995; Debeaujon *et al.*, 2001; Kitamura *et al.*, 2004), in the movement of monomeric annthocyanidins and (epi)-catechins from cytosol to vacuole (see below). These questions could best be addressed in systems that produce both anthocyanins and proanthocyanidins at the same time, such as some developing fruits (Kennedy *et al.*, 2001; Jaakola *et al.*, 2002) or the genetically engineered systems described in more detail below. Control of the two pathways in systems where there is a clear temporal or spatial separation between anthocyanin and proanthocyanidin biosynthesis could be accounted for simply in terms of differential temporal or cell-specific gene expression. For example, proanthocyanidins and (epi)catechins appear early during fruit development in bilberry, then decrease to almost undetectable levels before the accumulation of anthocyanins; these changes correlate with levels of transcripts encoding PAL, CHS, DFR and ANS during fruit ripening (Jaakola *et al.*, 2002).

To date, enzymological studies have demonstrated pathways for production of 2,3-*trans*-2R,3S-(+)-catechin (via LAR) and 2,3-*cis*-2R,3S-(–)-epicatechin (through ANR). However, there are four potential stereoisomers of the flavan-3-ol nucleus (Fig. 4), including the powerful allelochemical 2S,3R-(–)-catechin (Bais *et al.*, 2003). How is (–)-catechin formed biosynthetically? During *in vitro* incubations of ANR with cyanidin, (–)-catechin appears as a minor product (Xie *et al.*, 2003). Likewise (–)-afzelechin occurs as a minor product formed from pelargonidin, whereas ANR converts delphinidin into equal amounts of (–)-epigallocatechin and (–)-gallocatechin (Xie *et al.*, 2003). The question arises as to whether the epimeric forms are true products of the enzyme, or are formed postenzymatically by epimerization at C_2 under the reaction conditions used *in vitro*. It would be most unusual for a single enzyme to be able to reduce a molecule such as anthocyanidin by hydride transfer to either face of the molecule, thereby accounting for production of opposite epimeric forms. Incubation of recombinant ANR with (–)-epicatechin under optimal conditions for anthocyanidin reduction failed to show any production of (–)-catechin. However, low levels of conversion of (–)-epicatechin to (–)-catechin can occur nonenzymatically in the presence of 100 mM Tris–HCl (pH 7.0) at temperatures above 30°C (Xie *et al.*, 2004b). Such epimerization at C_2 is consistent with the higher thermodynamic stability of the 2,3-*trans*- compared with the 2,3-*cis*-flavan-3-ol, but is unlikely to take place nonenzymatically in plants that produce (–)-catechin, where perhaps an epimerase is involved.

Both ANR and LAR are members of large families of oxidoreductases. The ANR enzymes from *M. truncatula* and *A. thaliana* share only 60% amino acid sequence identity (Xie *et al.*, 2003) but, although exhibiting interesting differences in kinetic properties and substrate preferences, appear to have

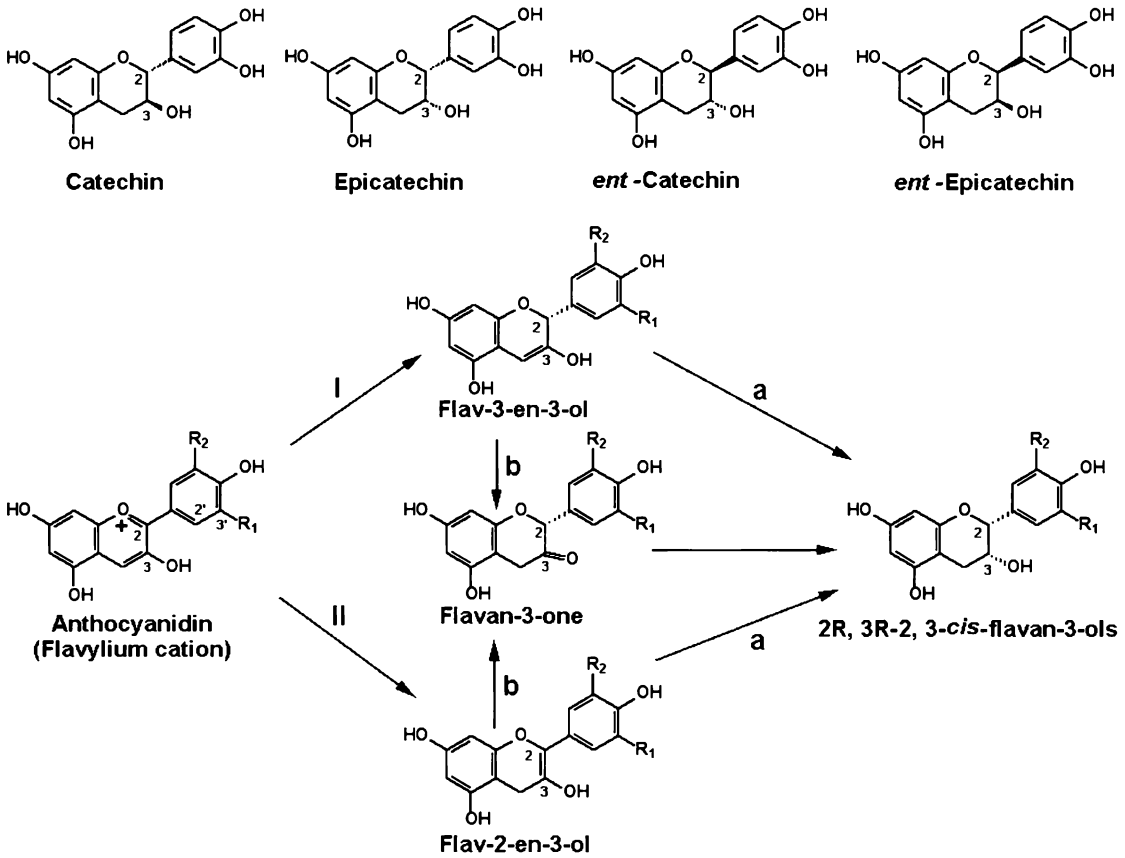

Fig. 4 Four catechin/epicatechin isomers and potential mechanisms of the ANR reaction. Note that the proposed flav-2-en-3-ol and flav-3-en-3-ol intermediates could theoretically be converted to the 2,3-*trans* isomer.

strict stereospecificity leading to 2,3-*cis*-flavan-3-ols (Xie *et al.*, 2003; 2004b). Can the reaction pathways shown in Fig. 4 account for the various stereochemistries encountered in flavan 3-ols and proanthocyanidins? Members of the IFR-like gene family with more than 60% sequence identity can catalyze very different reactions (Gang *et al.*, 1999b). Although preliminary results indicate that ANR activity in crude extracts from *Desmodium*, *Lotus*, grape and barley produces only the 2,3-*cis*-flavan-3-ol, it is nevertheless possible that alternative forms of ANR or LAR might exist with different stereochemical outcomes, and that these could be identified by *in silico* sequence data mining coupled with expression profiling.

2. Intracellular transport of flavan-3-ols

Generally speaking, our understanding of inter- and intracellular transport of plant natural products is still in its infancy (Lin *et al.*, 2003). However, it is likely that transport from the site of synthesis to the site of storage, the vacuole, is a critical process in proanthocyanidin biosynthesis and assembly. Genetic analysis of proanthocyanidin accumulation in the *Arabidopsis* seed coat has resulted in the identification of two genes that appear to be involved in transport processes required for proanthocyanidin synthesis and/or assembly. Mutations in either the *TT12* or *TT19* genes result in a transparent testa caused by reductions in the level of proanthocyanidin pigment (Debeaujon *et al.*, 2001; Kitamura *et al.*, 2004). *TT12* encodes a protein with 12 transmembrane domains exhibiting similarity to prokaryotic and eukaryotic MATE (multidrug and toxic compound extrusion) transporters, and is expressed specifically in the endothelial layer of the developing seed coat (Debeaujon *et al.*, 2001). *TT19* is a member of the *Arabidopsis* glutathione S-transferase (GST) multigene family (Kitamura *et al.*, 2004). Analogies can therefore be made with the vacuolar transport of anthocyanins in maize, brought about by the product of the *BRONZE-2* gene, a GST homolog (Marrs *et al.*, 1995). Although glutathione conjugates of plant polyphenols are 'substrates' for uptake by the vacuolar glutathione conjugate pump (Li *et al.*, 1997; Edwards *et al.*, 2000), it is also possible that the role of GSTs in anthocyanin uptake is one of flavonoid binding rather than enzymatic conjugation (Mueller *et al.*, 2000). The petunia *AN9* gene, encoding a GST involved in anthocyanin transport, can complement the *Arabidopsis tt19* mutation with respect to allowing vacuolar uptake of anthocyanin, but does not restore proanthocyanidin accumulation (Kitamura *et al.*, 2004). The functional relation between TT12 and TT19 is not clear, and TT12 and TT19 have yet to be characterized biochemically.

3. Formation of B-type proanthocyanidin oligomers and polymers

The debate concerning the operation of an enzymatic or nonenzymatic condensing mechanism for proanthocyanidin condensation still continues. Unambiguous resolution of the problem will require solid biochemical and genetic support for one or more enzymes involved in proanthocyanidin condensation. Over 25 yr ago Stafford (1988) commented on similarities between proanthocyanidins and lignin, primarily with respect to their common origins as polymers of phenylpropanoid-derived units and potential functions in plant defense. It might be instructive to speculate further concerning potential commonalities in their mode of assembly as, at the time of Stafford's article, the mechanisms of oxidative polymerization of monolignols involving peroxidases and laccases were not fully understood. Furthermore, recent studies on lignan coupling have indicated how stereochemistry can be imposed on reactions involving free radical oxidation of phenolic compounds (Gang *et al.*, 1999a). Following Haslam's example (Haslam, 1977), we provide here some highly speculative views concerning the 'black box' of proanthocyanidin coupling, with examples of potential mechanisms given in Fig. 5.

Quinone methides or carbocations of leucoanthocyanidin derivatives have been generally accepted as the precursors of the extension units of proanthocyanidins (Fig. 5). The *in vitro* chemical condensation of either catechin or epicatechin with leucocyanidin derived from dihydroquercetin gave fundamental support to this hypothesis (Creasey & Swain, 1965), but this biogenetic sequence does not explain why the predominant extension units in proanthocyanidin oligomers or polymers are in the 2R, 3R-2, 3-*cis* configuration, and leucocyanidin, leucopelargonidin or leucodelphinidin have not been isolated from plant tissues producing proanthocyanidins.

Anthocyanins have also been considered as potential substrates for proanthocyanidin polymerization. Condensation between anthoycanins and proanthocyanidin monomers (flavan-3-ols), oligomers or polymers is one of the main processes contributing to the changes in color and astringency as wines age (Haslam, 1980; Bishop & Charles, 1984). Under acidic conditions, anthocyanidins are mainly present as the corresponding flavylium ion, which may undergo polyphenol oxidase (PPO)-catalyzed conversion to a quinone methide and then to a carbocation (Fig. 5). The nucleophilic epicatechin or catechin could theoretically attack the carbocation derivative to yield a proanthocyanidin (Fig. 5).

(+)-Catechin is generally regarded as the most common starter unit in proanthocyanidins. However, in 58 structurally characterized procyanidin oligomers (including 44 trimers, 11 tetramers, two pentamers and one hexamer, consisting of

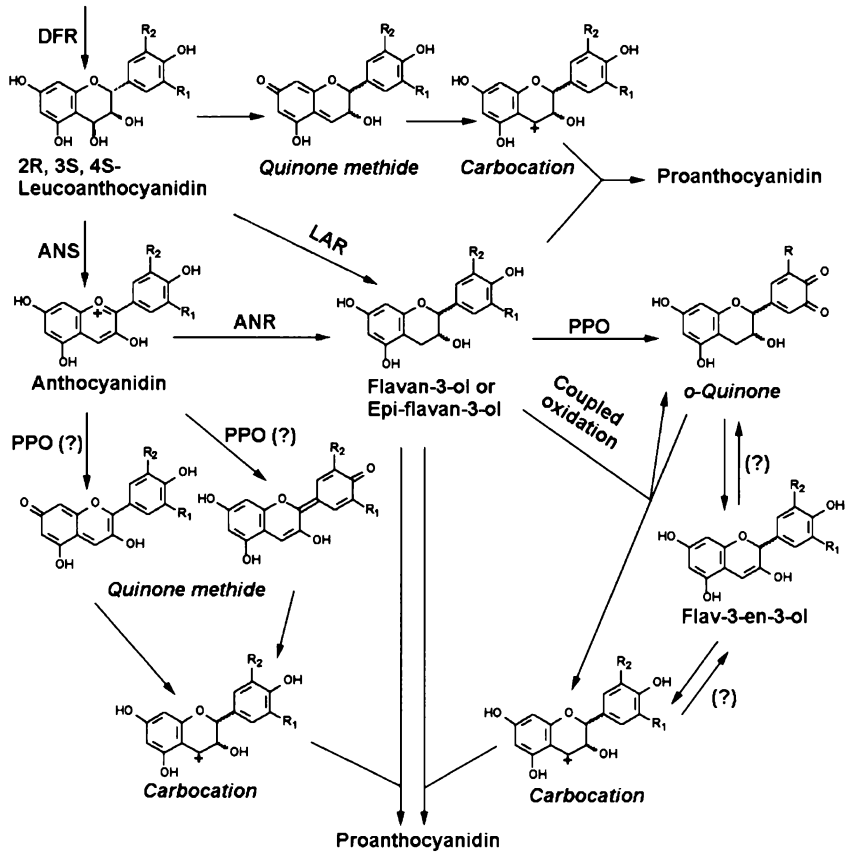

Fig. 5 Speculative schemes for polymerization of proanthocyanidins. See Section VI.3 for details. PPO, polyphenol oxidase.

only catechin and epicatechin), isolated from over 25 different species (Porter, 1988, 1993), the ratio of proanthocyanidins with catechin as starter unit was only 45%. Among the 134 extension units in the above oligomers, epicatechin accounted for 81%. Thus 2,3-*cis*-(–)-epicatechin is the predominant extension unit among oligomeric proanthocyanidins, and also a very common starter unit. Despite this, (–)-epicatechin has not been viewed as a potential precursor of proanthocyanidins, other than as a starter unit. ANR converts achiral cyanidin into (–)-epicatechin, and ectopic expression of *Medicago* ANR in tobacco flowers results in accumulation of proanthocyanidin oligomers (Xie *et al.*, 2003; Fig. 3c). Crude enzyme preparations from barley, grape, *Desmodium uncinatus* and *L. corniculatus*, species that accumulate high levels of proanthocyanidins, all exhibit high ANR activity (unpublished results). This provides indirect evidence for the proposal that ANR could supply the monomers for proanthocyanidin formation.

In one model of proanthocyanidin formation from a flavan-3-ol, epicatechin or catechin are converted to the corresponding quinones by PPO. The quinones are then converted to carbocations via flav-3-en-ols, or reduced to carbocations through coupled nonenzymatic oxidation (Fig. 5). Nucleophilic attack on the carbocations by epicatechin or catechin could produce dimers and then oligomeric proanthocyanidins linked through C_4–C_8 or C_4–C_6. In partial support of such a mechanism, formation of procyanidins B1 and B2 was observed when sorghum procyanidin polymers were added to white or red wines in the presence of catechin or epicatechin (Haslam, 1980), and tyrosinase has been shown to oxidize catechin to quinones accompanied by formation of procyanidin B3 (Oszmianski & Lee, 1990).

Flav-3-en-3-ol has been proposed to be an intermediate in the biogenesis of flavan-3-ol and anthocyanidin from leucoanthocyanidin (Haslam, 1977). It has also been suggested that flav-3-en-3-ols may be intermediates in the enzymatic conversion of anthocyanidins into flavan-3-ols by ANR (Xie *et al.*, 2004b). Figure 5 also includes a scheme in which flav-3-en-3-ol is derived from catechin or epicatechin *o*-quinones by nonenzymatic reduction and then rearranged to form the corresponding carbocation. Alternatively, the flav-3-en-3-ol involved in condensation might be the intermediate of the ANR reaction converting anthocyanidin into flavan-3-ol (Xie *et al.*, 2004b).

4. Formation of A-type proanthocyanidins

The A-type proanthocyanidins are characterized by linkage between C_2 of the upper unit and the oxygen at C_7 of the starter unit, in addition to linkage between C_4 of the upper unit and positions 6 or 8 of the lower unit (Fig. 1). Presumably, linkage to C_4 of the upper unit proceeds first as in the biosynthesis of B-type proanthocyanidins, and A-type proanthocyanidins can be formed from the B-type compounds *in vitro*. For example, treatment of proanthocyanidins B1 or B2 (Fig. 1) with the oxidizing agent 1,1-diphenyl-2-picrylhydrazyl radical resulted in formation of proanthocyanidins A1 or A2 (Kondo *et al.*, 2000). On the basis of experiments on the effects of incubation of epicatechin with banana extracts, it has been proposed that the A-type linkages result from PPO-mediated oxidation of the B-ring to a quinoidal structure, with subsequent addition of the C_7 hydroxyl to C_2 (Tanaka *et al.*, 2000). In the case of the larger oligomeric A-type proanthocyanidins, it is not known whether an A-linked dimer is formed first, with subsequent addition of further B-linked extension units, or whether the additional C_2–C_7 bond is formed at a later stage.

5. Transcription factors controlling the biosynthesis of proanthocyanidins

In the past 5 yr major advances in our understanding of proanthocyanidin regulatory genes have been made in *Arabidopsis*. The regulatory circuit seems complex and requires coordinated regulation of several genes involved in (1) the general flavonoid pathway to anthocyanidins; (2) the proanthocyanidin branch pathway controlling the biosynthesis of the subunits, their transport into the vacuole, and their subsequent polymerization into proanthocyanidin polymers; and (3) developmental processes for the generation of the specialized cells in which proanthocyanidins are deposited. The transcriptional regulators currently believed to be involved in the control of proanthocyanidin biosynthesis and accumulation in *Arabidopsis* are summarized in Table 3.

Homologs of the maize *R* and *C1* genes are the most likely candidates for regulating general flavonoid biosynthesis up to anthocyanidins, but so far no mutant defective in these genes has been characterized which affects the regulation of most of the genes of the flavonoid pathway and proanthocyanidin biosynthesis in *Arabidopsis*. There appear to be multiple flavonoid pathway transcription factors in *Arabidopsis*, and overexpression of the *Arabidopsis C1* orthologs *PAP1* or *PAP2* results in constitutive anthocyanin production in *Arabidopsis* and tobacco (Borevitz *et al.*, 2001). Similarly, expression of two *R* gene orthologs of *Arabidopsis*, *GLABRA3* (*GL3*) and *MYC-146/ENHANCER OF GLABRA3* (*EGL3*), which encode basic helix–loop-helix (bHLH) proteins, can activate biosynthesis of anthocyanins in petals of a white-flowered mutant of *Matthiola incana* (Ramsay *et al.*, 2003; Zhang *et al.*, 2003). In addition to *C1* (MYB) and *R* (MYC/bHLH) orthologs, *TTG1* is also required for anthocyanin biosynthesis because ectopic *PAP1* expression does not result in production of anthocyanin in the *ttg1* mutant (Borevitz *et al.*, 2001). TTG1, a WD domain protein, is known to interact physically with MYB (PAP1 and PAP2) and MYC (GL3 and EGL3) transcription factors in the yeast two-hybrid system (Payne *et al.*, 2000; Zhang *et al.*, 2003).

A second class of regulatory genes that modulate late flavonoid biosynthetic genes [encoding dihydroflavonol reductase

Table 3 Transcription factors involved in proanthocyanidin biosynthesis in *Arabidopsis*

Gene	Characteristics of transcription factor	Genes regulated	Reference
TT1	WIP domain-containing plant zinc-finger protein involved in regulation of seed endothelium development	*BAN*	Sagasser *et al.* (2002)
TT2 (MYB123)	R2–R3 MYB domain-type nuclear protein	*TT8, TT12, BAN, DFR, LDOX*	Nesi *et al.* (2001); Debeaujon *et al.* (2003)
TT8	Basic helix–loop–helix (bHLH) domain protein	*BAN and DFR*	Nesi *et al.* (2000)
TT16	BSISITER MADS domain protein required for development and pigmentation of seed endothelium	*BAN*	Nesi *et al.* (2002)
TTG1	WD40 repeat protein which regulates trichome differentiation, root hair formation, seed mucilage production, PA and anthocyanin biosynthesis	*TT8, DFR, GL2 TTG2, FUS*	Walker *et al.* (1999)
TTG2	A zinc finger-like protein of the plant-specific WRKY family which modulates trichome differentiation, seed mucilage production and PA biosynthesis	*BAN, TT2*	Johnson *et al.* (2002)
PAP1 & 2 (MYB75 & 90)	R2R3MYB domain protein involved in anthocyanin biosynthesis	*PAL, CHS, DFR, LDOX*	Borevitz *et al.* (2000)
GL3 & EGL3 (MYC146)	bHLH domain protein involved in anthocyanin production, trichome differentiation and seed mucilage production		Ramsay *et al.* (2003); Zhang *et al.* (2003)
ANL2	Homeodomain protein belonging to the HD-GLABRA2 group which affects anthocyanin biosynthesis	*DFR*	Kubo *et al.* (1999)
FUSCA3	B3 domain containing member of ABI3/VP1 transcription factor family	*TTG1*	Luerssen *et al.* (1998)

(DFR) and leucoanthocyanidin dioxygenase (LDOX) as well as the proanthocyanidin-specific genes *BAN* and *TT12*] includes *TT2*, *TT8* and *TTG1*, which encode R2–R3 MYB, MYC/bHLH and WD40 repeat domain proteins, respectively (Walker *et al.*, 1999; Nesi *et al.*, 2000, 2001). TT2 is a master regulator as its mutation affects the expression of *DFR*, *LDOX* and *BAN* in developing siliques while its ectopic expression can induce *BAN* and *TT8* (the latter in roots only) and upregulate *DFR* and *LDOX* (Nesi *et al.*, 2000, 2001; Debeaujon *et al.*, 2003). It appears that the above three transcription factors work in concert and positively regulate the expression of *BAN*, *DFR* and *LDOX*. In fact no *BAN* transcript was detected in developing siliques of *tt2*, *tt8* and *ttg1* mutants, consistent with their homogenously yellow seed phenotype caused by total lack of proanthocyanidin deposition. Probable DNA sequences where TT2 and TT8 may bind within the *BAN* promoter have been described, and deletion of this sequence element has been reported to abolish the endothelium-specific expression of BAN (Debeaujon *et al.*, 2003).

A third category of transcription factors, which includes TT1, TT16 and TTG2, regulates organ and cell development for proanthocyanidin deposition, in addition to transcription of proanthocyanidin-specific genes. *TT1* and *TT16* are necessary for proanthocyanidin biosynthesis and correct cell morphology in the seed body, but not in the chalaza/micropyle region. TT1 is a WIP domain-containing zinc finger protein (Sagasser *et al.*, 2002) whereas TT16 is the ARABIDOPSIS BSISTER (ABS) MADS domain protein (Nesi *et al.*, 2002). Both these proteins modify the spatial pattern of *BAN* gene expression (Debeaujon *et al.*, 2003). Ectopic expression of *TT2* overcomes the proanthocyanidin deposition defects in the *tt16* mutant, suggesting that proanthocyanidin biosynthesis does not require *TT16* in the presence of *TT2*. TTG2 is a zinc finger-like protein of the plant-specific WRKY family which modulates trichome differentiation, mucilage production and proanthocyanidin biosynthesis (Johnson *et al.*, 2002). TTG2 requires TTG1 function for regulating proanthocyanidin biosynthesis. The regulatory cascades currently believed to control proanthocyanidin synthesis and accumulation in the *Arabidopsis* seed coat are summarized diagrammatically in Fig. 6.

The desirability of eliminating proanthocyanidins from barley to improve beer quality has resulted in a number of genetic studies of barley mutants deficient in proanthocyanidin accumulation (von Wettstein *et al.*, 1985; Yadav *et al.*, 2001). Over 600 such *ant* mutants have been described. These mutants define 10 loci: three loci defined by *ant17*, *ant18* and *ant30* encode F3H, DFR and CHI, respectively; and six loci (*ant19*, *ant25*, *ant26*, *ant27*, *ant28* and *ant29*) are defective in conversion of leucoanthocyanidin to proanthocyanidins (Jende-Strid, 1991) (Jende-Strid, 1993). More detailed phenotypic and molecular analysis has indicated that the tenth locus, *Ant13*, is a regulatory gene for the general flavonoid pathway. *Ant21* is probably a regulatory gene affecting the biosynthesis of both anthocyanins and proanthocyanidins (Jende-Strid, 1991). *Ant13* mutants appear to have reduced agronomic performance (Horsley *et al.*, 1991).

In addition to barley, regulatory mutants called *tan* have been characterized in the model legume *Lotus japonicus*, a

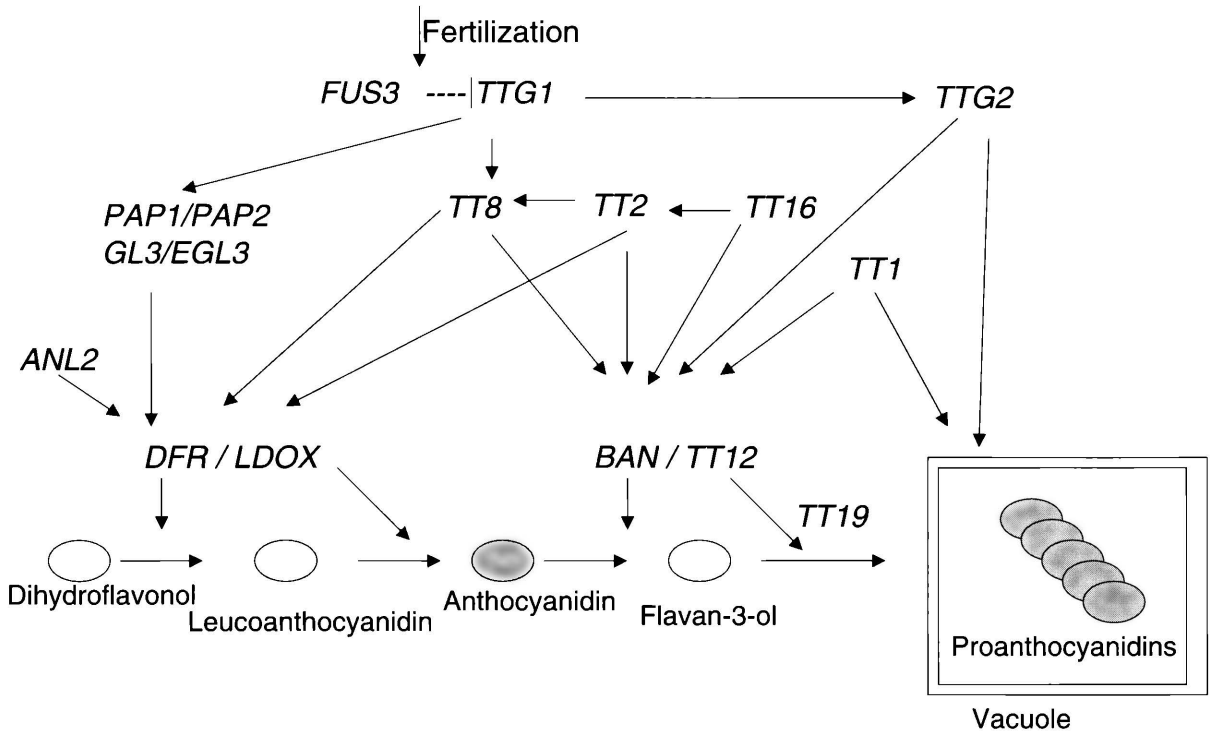

Fig. 6 Genetic model for the transcriptional control of proanthocyanidin biosynthesis in *Arabidopsis*. See Section VI.5 and Table 3 for details.

species in which proanthocyanidins accumulate in floral organs, seeds and stems, but not in leaves. These *tan* mutants ectopically accumulate proanthocyanidins in leaves (Gruber *et al.*, 1998). Furthermore, mutants of indica rice have been described that lack production of anthocyanins in the leaves, but instead accumulate proanthocyanidins in the seed pericarp (Reddy *et al.*, 1995).

One central piece of the puzzle which remains to be resolved is how the above transcription factors are coordinately expressed in the endothelium layer of the seed. It seems that fertilization serves as a key signal as most of the genes specific to proanthocyanidin biosynthesis are switched on soon after fertilization. One set of genes collectively designated as *LEAFY COTYLEDON* (*LEC*), which appear to be major regulators of a variety of embryonic stages (Harada, 2001), may have roles in determining the boundaries of gene expression. The ectopic production of trichomes and anthocyanins in *lec* mutant cotyledons suggests that *LEC* genes regulate epidermal differentiation and anthocyanin production by negatively regulating *TTG1* expression in the protodermal layer of the young embryo (Tsuchiya *et al.*, 2004). To date, three *LEC* genes, namely *LEC1*, *LEC2* and *FUS3*, have been molecularly characterized. *LEC1* encodes a transcription factor related to the *HAP3* gene family, while *LEC2* and *FUS3* encode proteins with close amino acid similarity to the B3 domain of the *ABI3/VP1* transcription factor family (Luerssen *et al.*, 1998; Harada, 2001).

VII. Genetic manipulation of the proanthocyanidin pathway

To date, most attempts to genetically engineer proanthocyanidins or their flavan-3-ol precursors in plants have been directed towards bloat reduction in forage legumes. These studies have used two types of genes, structural pathway genes or transcription factors, and have targeted species that naturally produce proanthocyanidins in the forage (such as birdsfoot trefoil, *L. corniculatus*) and those that do not (such as alfalfa).

Functional identification of the *M. truncatula BAN* (*ANR*) gene was achieved by constitutively expressing the gene in transgenic tobacco (Xie *et al.*, 2003). Analysis of leaf and flower tissue for proanthocyanidins by staining with dimethlaminocinnamaldehyde (DMACA) reagent indicated that only the outer petals of the flowers from transgenic (but not control) plants contained proanthocyanidin-like material. This appeared to be localized to vacuoles (Xie *et al.*, 2003). Analysis of extracts from flowers of MtBAN-expressing tobacco by thin-layer chromatography confirmed the oligomeric/polymeric nature of the DMACA-reactive material (Fig. 3c). The lack of any products of the ANR reaction in the leaves of transgenic plants can be explained by the lack of anthocyanin substrate in this tissue. However, this does not explain the very distinct interface between the staining and nonstaining parts of the flower, both of which contain anthocyanin (Fig. 3b). Furthermore, cotransformation of tobacco with MtBAN and PAP1,

a myb transcription factor from *Arabidopsis* whose ectopic expression results in high-level constitutive anthocyanin production (Borevitz *et al.*, 2001), leads to accumulation of epicatechin and proanthocyanidin in the leaves (unpublished results). The flowers of these plants exhibit more intense anthocyanin pigmentation and DMACA staining, but with the same limited tissue localization (Fig. 3b). Clearly, factors in addition to substrate supply are necessary for successful accumulation of proanthocyanidins in transgenic plants.

Other attempts to engineer proanthocyanidins using biosynthetic genes have been performed in *L. corniculatus*. Here the objective was to reduce proanthocyanidin levels by antisense downregulation of *DFR* expression (Carron *et al.*, 1994; Robbins *et al.*, 1998). This strategy was successful in hairy root cultures, with reductions in proanthocyanidin levels of up to ≈80% (Carron *et al.*, 1994). However, overexpression of *DFR* in hairy roots led to variable effects on proanthocyanidin content and monomer composition (Bavage *et al.*, 1997). In stably transformed whole plants the expression of antisense *DFR* resulted in a small decrease of proanthocyanidins in root, stem and leaf in some lines, but to increases in others (Robbins *et al.*, 1998). There was no evidence for accumulation of pathway intermediates or default products in the downregulated lines. Earlier studies in *L. corniculatus* had indicated that antisense downregulation of a chalcone synthase gene resulted in a paradoxically increased level of proanthocyanidins in glutathione-elicited hairy root cultures (Colliver *et al.*, 1997). The effects were dependent on the plant genotype as it related to constitutive proanthocyanidin accumulation. Overall, with the exception of antisense *DFR*, manipulation of early pathway enzymes in *L. corniculatus* does not appear to result in a consistent proanthocyanidin phenotype.

It is likely that genes yet to be discovered are involved in proanthocyanidin synthesis and accumulation. Genetic manipulation of the proanthocyanidin pathway by overexpression of transcription factors has been attempted in the hope that this will turn on all the necessary genes. As described above, the transcriptional control of proanthocyanidin deposition in the *Arabidopsis* seed coat is very complex, involving a hierarchical array of interacting factors. Furthermore, although ectopic expression of a single transcription factor may turn on multiple genes within a pathway, PAP1 being a good example (Borevitz *et al.*, 2000), seldom is a complete secondary metabolic pathway from the interface with primary metabolism activated in this way. Nevertheless, two reports claim increased proanthocyanidin accumulation in forage legumes overexpressing transcription factors. In the case of alfalfa, neither the maize *B-Peru* nor *C1* anthocyanin regulatory gene was effective in modulating the pathway, but accumulation of proanthocyanidin was recorded in leaf tissue expressing the maize *Lc* gene that encodes a myc-family transcription factor (Ray *et al.*, 2003). The *Lc*- transgenic alfalfa appeared to require a stress, such as exposure to high light intensity or cold, for the appearance of anthocyanin and proanthocyanidin (Ray *et al.*, 2003). In *L. corniculatus*, constitutive expression of the maize Sn-transcription factor resulted in increased proanthocyanidin accumulation in roots, but unaffected or suppressed levels in leaves (Damiani *et al.*, 1999). Levels of DFR and LAR enzyme activities mirrored the leaf proanthocyanidin phenotypes (Damiani *et al.*, 1999).

In white clover, constitutive expression of the B-Peru myc transcription factor resulted in altered patterns of anthocyanin pigmentation (deMajnik *et al.*, 2000), but no effects on proanthocyanidins were reported.

VIII. Conclusions and future prospects

Considerable progress has been made recently in our understanding of proanthocyanidin biosynthesis and its regulation. However, many important questions remain to be answered, including the following.

- What are the mechanisms for flux control at the interface between the anthocyanin and proanthocyanidin pathways?
- Do LAR and ANR produce only monomeric flavan 3-ols as the starter units for proanthocyanidin biosynthesis, or are the extension units also synthesized via these enzymes? In other words, is the model in which the extension unit is formed from leucoanthocyanidin via a flav-3-en-3-ol or quinone methide intermediate still viable?
- Do LAR and ANR enzymes exist with the ability to generate products with alternative stereochemistries?
- Where are LAR and ANR proteins localized within the cell?
- How many proteins/genes are required for transport of proanthocyanidin monomers to the vacuole? Are leucoanthocyanidins transported to the vacuole (for subsequent polymerization) and, if so, how?
- What are the exact biochemical functions/mechanisms of TT12 and TT19?
- Is there an enzymatic system for proanthocyanidin polymerization? If so, is this localized in the vacuole?
- How are the A-type linkages in proanthocyanidins made?
- How is the stereochemistry of proanthocyanidin linkage controlled?
- How many genes are required to introduce constitutive proanthocyanidin accumulation into plant tissues that do not naturally make these compounds?
- Can the potential health beneficial properties of proanthocyanidins be validated so as to allow definition of a recommended daily intake?

The current status of the proanthocyanidin field is the result of the consecutive application, over some 40 yr, of chemistry, biochemistry and molecular biology. The answers to the above questions will be obtained only by the concerted application of these same approaches, but with the addition of genetics, genomics, cell biology and metabolic engineering. Understanding this 'final frontier' of flavonoid biosynthesis is now within our grasp, and the implications of success for plant improvement and human health make this a worthwhile challenge.

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