Available online at www.sciencedirect.com
SCIENCE DIRECT®
PERGAMON
Phytochemistry 64 (2003) 367–383
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Review

New perspectives on proanthocyanidin biochemistry and molecular regulation

M.A. Susan Marles[1], Heather Ray[2], Margaret Y. Gruber*

Saskatoon Research Centre, Agriculture and Agri-Food Canada, 107 Science Place, Saskatoon, SK, Canada S7N 0X2

Received 28 January 2003; received in revised form 26 May 2003

Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Our understanding of proanthocyanidin (*syn*. condensed tannin) synthesis has been recently extended by substantial developments concerning both structural and regulatory genes. A gene encoding leucoanthocyanidin reductase has been obtained from the tropical forage, *Desmodium uncinatum*, with the latter enzyme catalyzing formation of (+)-catechin. The *BANYULS* gene in *Arabidopsis thaliana*, previously proposed to encode leucoanthocyanidin reductase or to regulate proanthocyanidin biosynthesis, has been shown instead to encode anthocyanidin reductase, which in turn converts anthocyanidins (pelargonidin, cyanidin, or delphinidin) into 2,3-*cis*-2*R*,3*R*-flavan-3-ols (respectively, (−)-epiafzelechin, (−)-epicatechin and (−)-epigallocatechin). However, the enzyme which catalyzes the polymerization reaction remains unknown. Nevertheless, a vacuolar transmembrane protein TT12, defined by the Arabidopsis *tt12* mutant, is involved in transport of proanthocyanidin polymer into the vacuole for accumulation. Six different types of regulatory elements, e.g. TFIIIA-like, WD-40-like, WRKY-like, MADS-box-like, myb-like, and bHLH (myc-like), have been cloned and identified using mutants from Arabidopsis (*tt*1, *ttg*1, *ttg*2, *tt*2, *tt*16, *tt*2, *tt*8) and two other species (*Hordeum vulgare* [*ant*13] and *Lotus* spp [*tan*1]). Accordingly, increases in proanthocyanidin levels have been induced in the the world's major forage, alfalfa. These advances may now lead to a detailed understanding of how PA synthesis is controlled and to useful alterations in proanthocyanidin concentration for the improvement of forage species, pulses, and other crop plants.

Keywords: *Arabidopsis thaliana* (L.) Heynh. (Arabidopsis, Brassicaceae); *Hordeum vulgare* L. (barley, Poaceae); *Lotus japonicus* L.; *Medicago sativa* L. (alfalfa); Fabaceae; Pasture bloat; Flavonoid; Condensed tannin; (+)-Catechin; (−)-Epicatechin

Contents

* Corresponding author. Tel.: +-1-306-956-7263; fax: +1-306-956-7247.
E-mail address: gruberm@agr.gc.ca (M.Y. Gruber).
[1] Present address: Institute of Biological Chemistry, Washington State University, PO Box 646340, Pullman, WA 99164-6340, USA.
[2] Present address: Plant Biotechnology Institute, National Research Council, 110 Gymnasium Place, Saskatoon, SK., Canada S7N 0W9.

0031-9422/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0031-9422(03)00377-7

1. Introduction

Proanthocyanidins (e.g. **11**) (PA, *syn*. condensed tannins) are synthesized as one of several branch products of the flavonoid pathway, which arises from one segment of the phenylpropanoid-acetate pathway (via naringenin chalcone [**1**]) (Fig. 1). Naringenin chalcone (**1**) is modified by chalcone isomerase (CHI) to form a variety of flavanones [e.g. naringenin (**2**) and eriodictyol (**3**)]. Additional closely-related flavonoids, 3-hydroxy-*trans*-flavanones (**5**) [*syn*. the dihydroflavonols (**5**), dihydrokaempferol (**5a**), dihydroquercetin (**5b**), dihydromyricetin (**5c**)], are formed by flavanone 3-β-hydroxylase from flavanone (**2–4**) substrates (Fig. 1). Dihydroflavonol reductase (DFR) reduces flavanones (**2**–**4**) to form 2*R*,3*S*,4*S*-leucoanthocyanidin (**7**) molecules, leucopelargonidin (**7a**), leucocyanidin (**7b**) and leucodelphinidin (**7c**), as well as synthesizing precursors for phlobaphenes (PB) (Fig. 1). Leucoanthocyanidin (**7**) molecules are the substrates for (+)-flavan-3-ol (**10**) compounds [e.g. (+)-catechin (**10a**) and (+)-gallocatechin (**10b**)], a 2*R*,3*S*,4*S*- isomer of PA (**11**) (Fig. 1) and anthocyanin, via the anthocyanidin molecule (**12**) (Fig. 2).

In the past 3 years, a host of new biochemical and regulatory elements that govern PA (**11**) biosynthesis has emerged. This review highlights these events while drawing attention to the stereochemistry, substrate specificity and localization of PA (**11**) biosynthesis. Recent landmarks in PA (**11**) biosynthesis include the isolation of the gene for leucoanthocyanidin reductase (LAR, Fig. 1) (Tanner et al., 2002) and the functional identification of *BANYULS* (*BAN*) (Xie et al., 2003), the gene for anthocyanidin reductase (ANR, Fig. 2). Since several authors have comprehensively reviewed anthocyanin biosynthesis and regulation in a variety of taxa (Harborne and Williams, 1995; Holton and Cornish, 1995; Mol et al., 1998; Winkel-Shirley, 2001a) and phlobaphene biosynthesis for maize (*Zea mays* L.) (Styles and Ceska, 1989; Grotewold et al., 1998), these flavonoids are included only where they pertain to PA (**11**) accumulation.

Other research highlights include the identification of a mechanism of vacuolar transport, the isolation of several genes with a role in the regulation of PA (**11**) synthesis, and the accumulation of PA (**11**) in alfalfa forage. While these developments do not provide a complete picture of PA (**11**) biosynthesis and deposition, nevertheless the progress is substantial. Such developments have provided a more detailed and accurate representation of PA (**11**) assembly and have shown that the manipulation of PA (**11**) in valuable crops is nearly at hand.

2. Occurrence and nutritional significance of proanthocyanidins (11)

The presence or absence of PA (**11**) and the degree of polymer extension varies with species and tissue, occuring as soluble, colourless compounds in many non-seed tissues (Koupai-Abyazani et al., 1992; Lees et al., 1993, 1995). Species that produce PA (**11**) in flowers and foliage synthesize polymer having a wide molecular mass range (Koupai-Abyazani et al., 1992; Skadhauge et al., 1997). For example, the model legume, *Lotus japonicus* L., accumulates PA (**11**) in flowers (molecular masses >30 kDa) but not in leaves (Skadhauge, 1996). Its near relative, *L. angustissimus* L., produces oligomers from 850 to 2265 Da in leaves and the *L. japonicus* leaf-mutant, *tan*1, produces PA (**11**) oligomers at the lower end of this size range (Gruber et al., 1998). In addition, the seed coat of many plant species accumulate PA (**11**), which frequently causes poorly-soluble, brown or red-brown pigmentation once the seed has matured (Leung et al., 1979; Shirley et al., 1992, 1995; Nozzolillo and Ricciardi, 1992; Marles et al., 2003).

Proanthocyanidin (**11**) occurrence has a historically-recognized role in the prevention of pasture bloat and the enhancement of bypass protein (Goplen et al., 1980; Lees, 1992). The induction of PA (**11**) biosynthesis in alfalfa (*Medicago sativa* L.) as a solution to pasture bloat and protein loss has been elusive using strategies such as induced mutation, somaclonal variation and somatic hybridization with a PA-accumulating forage species (Goplen et al., 1980; Gruber et al., 1999). Most recently, PA (**11**) has received considerable attention as a putative chemoprotective agent in the prevention of human ailments such as urinary tract infections (Foo et al., 2000) and cardiovascular disease (Porter et al., 2001). However, PA (**11**) content has been difficult to

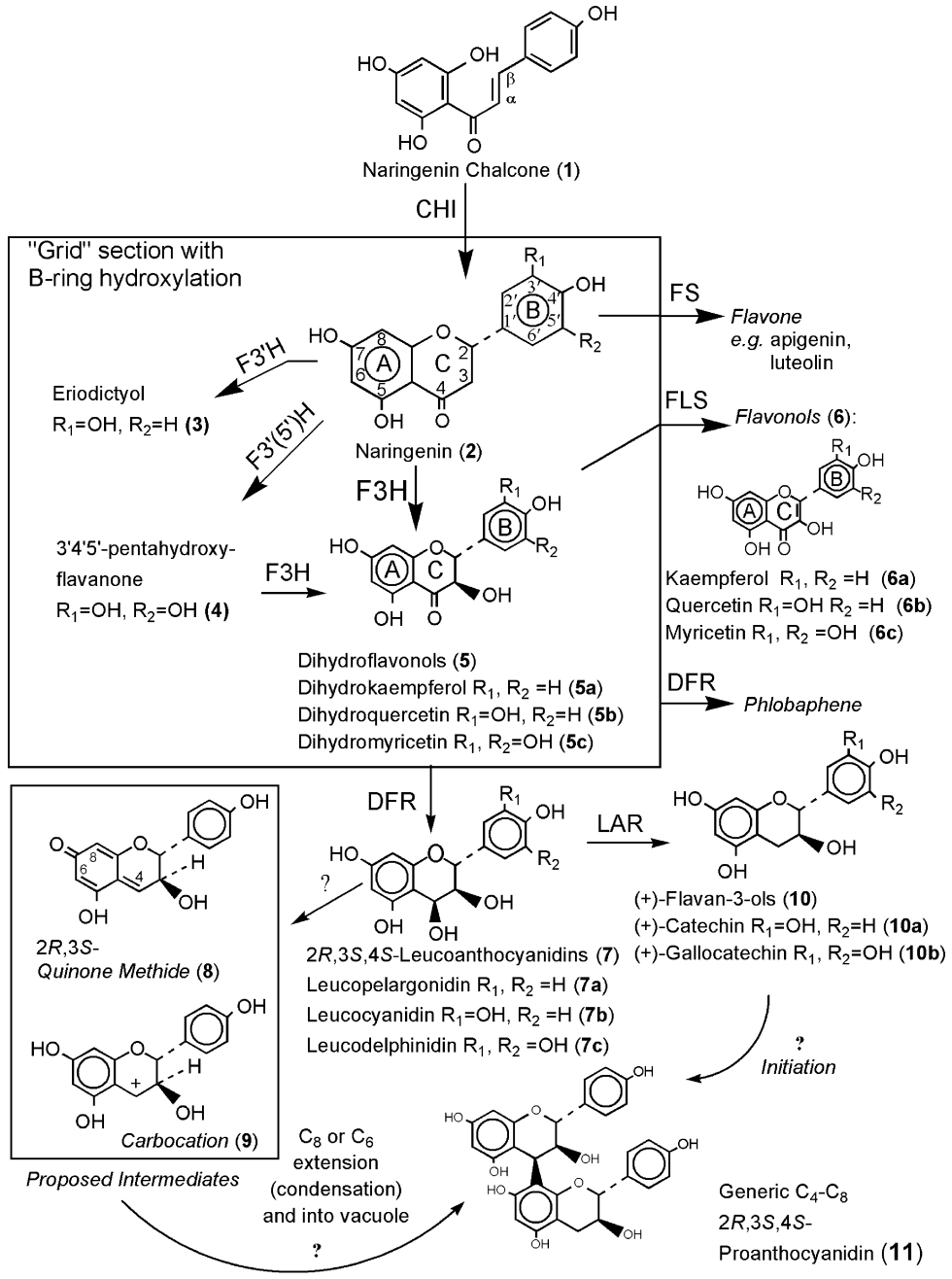

Fig. 1. Flavonoid pathway leading to (+)-flavanols (**10**) and 2*R*,3*S*,4*S*-proanthocyanidins (e.g. **11**). The figure indicates a generic C_4–C_8-linked 2*R*,3*S*,4*S* PA (**11**) dimer as an example, but longer polymers and C_4–C_6 linkages are also found. CHI, chalcone isomerase; F3′H, NADPH-cytochrome P450 flavonoid 3′-hydroxylase; F3′5′H, NADPH-cytochrome P450 flavonoid 3′5′hydroxylase; F3H, flavanone 3-β-hydroxylase; DFR, dihydroflavonol reductase; FS, flavone synthase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase. Reactive 2*R*,3*S* intermediates (quinone methides [**8**] and carbocations [**9**]) are likely formed from 2*R*,3*S*,4*S*-leucoanthocyanidins (**7**) and form extension units at C_8 and C_6 on the flavan-3-ol (**10**) initiator molecule or on the growing PA (**11**) polymer. The question mark (?) indicates steps for which proteins and genes have not been isolated or demonstrated except by mutation studies. "Grid" section indicates formation of eriodictyol (**3**) which initiates the pathway leading to di-hydroxy B-ring subunits in procyanidin-containing PA (**11**). Formation of 3′4′5′-pentahydroxy-flavanone (**4**) initiates the pathway leading to tri-hydroxy B-ring subunits.

manipulate in crops by conventional plant breeding and tissue culture strategies.

Despite its beneficial qualities, PA (**11**) content has a negative impact on non-ruminant livestock and humans, when present in nutritional supplements derived from seed meal. Proanthocyanidin (**11**) contributes to undesirable food qualities such as astringent flavours and discoloured meal, as well as non-specific reactions with protein that create nutritionally-unavailable products (Sarwar et al., 1981; Simbaya et al., 1995). If reductions could be made to the PA (**11**) content in a variety of seed crops, then seed meals such as canola (*Brassica napus* L.)

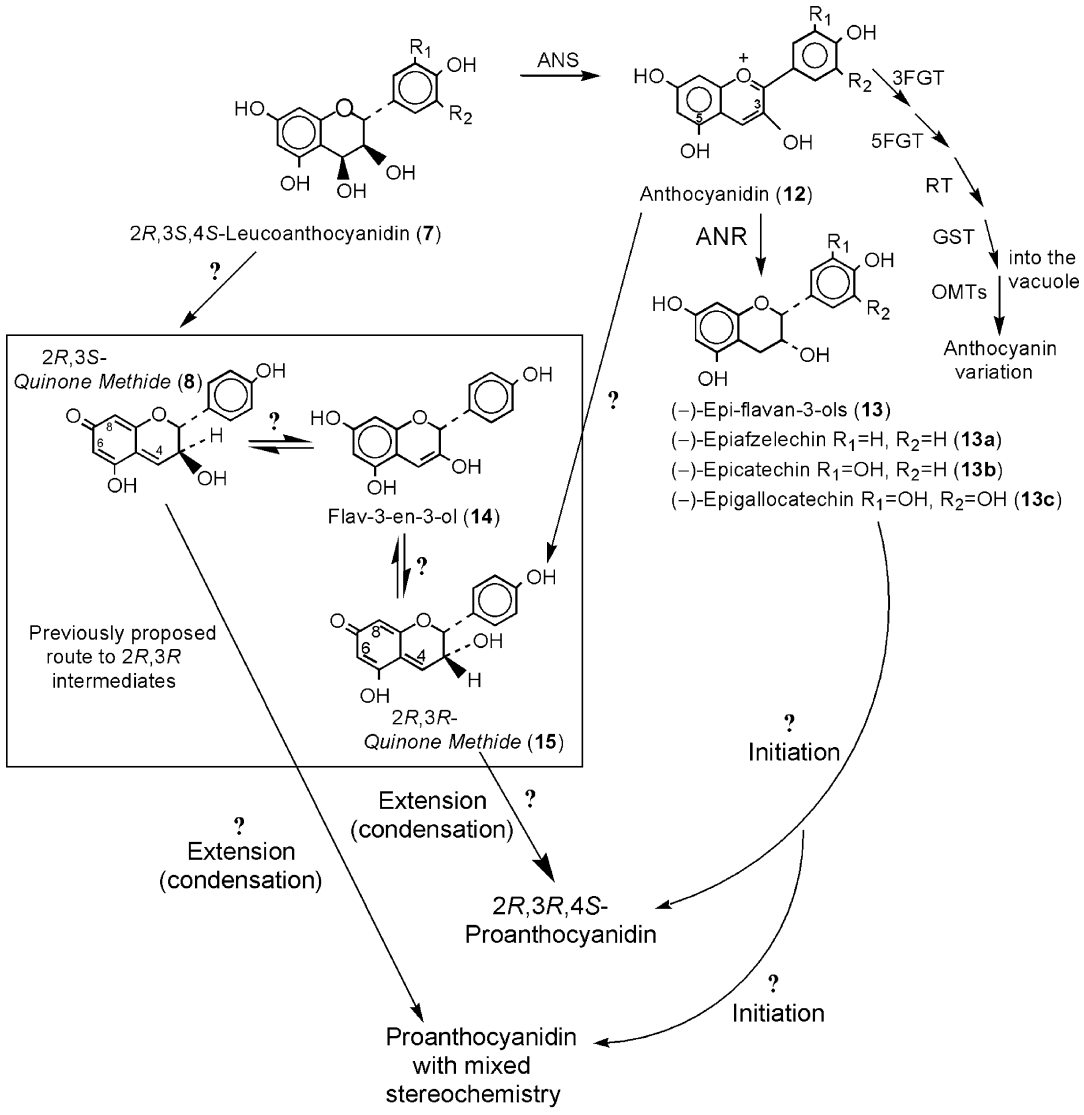

Fig. 2. Derivation of proanthocyanidin containing 2*R*,3*R* stereochemistry. ANS, anthocyanin synthase (also called LDOX) forms anthocyanidins (**12**) from leucoanthocyanidins (**7**). Anthocyanidins (**12**) are converted into (−)-*epi*-flavan-3-ols (**13**) by ANR (anthocyanidin reductase, also called BANYULS). Reactive 2*R*,3*R* extension intermediates (2*R*,3*R* quinone methides [**8**]) could be formed from the planar anthocyanidin (**12**) molecules via an enzyme that can form a stereochemically-correct reactive extension unit rather than a stable product like (−)-epicatechin (**13B**) that is generated by ANR. Alternatively, 2*R*,3*R* extension units could form via a flavan-3-en-3-ol (**14**) intermediate originating from 2*R*,3*S* quinone methides (**8**) that arise from 2*R*,3*S*,4*S*-leucoanthocyanidins (**7**). The 2*R*,3*R* extension units (**15**) would form at C_8 and C_6 on the (−)-*epi*-flavan-3-ol (**13**) initiator molecule or on the growing 2*R*, 3*R*,4*S*-PA polymer. Polymers of mixed stereochemistry may originate from combinations of 2*R*,3*R*,4*S* and 2*R*,3*S*,4*S* initiator and extension units. The question mark (?) indicates steps for which proteins and genes have not been isolated or demonstrated or for which the biosynthetic route is unclear. R_1(OH)/R_2(H), indicates molecules leading to di-hydroxy polymers. R_1(OH)/R_2(OH), indicates molecules leading to tri-hydroxy polymers. Genes which transport and modify substituents on anthocyanin molecules include: FGTs, UDP-flavonoid glycosyl transferases; RT, rhamnosyl transferase; GST, glutathione S-transferase; OMT, *O*-methyl transferases.

could compete favorably in the market place with soybean (*Glycine max* L.) for non-ruminant livestock feed or as a gluten-free supplement in human nutrition.

3. Stereochemistry of proanthocyanidin (11) synthesis

Stereochemical, compositional and biosystematic characterization of PA (**11**) was outlined over two decades ago (Porter, 1988; Haslam, 1989; Stafford, 1990) (Fig. 1). Stafford (1990) skillfully described flavan-3-ol (**10**) stereochemistry, and Porter (1988) reviewed the widespread occurrence of 2,3-*cis*-flavan-3-ols (**13**) [e.g. (−)-epicatechin (**13b**) and (−)-epigallocatechin (**13c**)] and their related polymers. PA (**11**) was usually illustrated as being derived from the enzymatic reduction of dihydroflavonol (**5**) to 2*R*,3*S*-flavan-3*S*,4*S*-diols (**7a–7c**) [generically named 'leucoanthocyanidins' [b], Fig. 1], or from the epimerization of 3-hydroxy-*trans*-flavanones to 3-hydroxy-*cis*-flavanones, followed by reduction to

2*R*,3*R*-flavan-3*R*,4*S*-diols (not shown) (Stafford, 1990). These reactions were followed by a reduction to 2*R*,3*S*-(+)-flavan-3-ols (**10**) [e.g. (+)-catechin (**10a**) or (+)-gallocatechin (**10b**)] (Fig. 1) or 2*R*,3*R*-(−)-flavan-3-ols (**13**) [e.g. (−)-epicatechin (**13b**) and (−)-epigallocatechin (**13b**)] (Fig. 2). Although enzyme-mediated condensation was postulated, biomimetic chemical synthesis of the polymeric form of 2*R*,3*S*,4*S* PA (**11**) under mild acid or alkaline conditions provided supporting evidence for the formation of linear C_4–C_8 linked chains [dominant form of PA (**11**)] or branched C_4–C_6 linked chains (not shown) (Botha et al., 1981; Delcour et al., 1983). This occurred via a 'lower' initiating unit derived from a (+)-flavan-3-ol (**10**) and 'upper' extension units derived from 2*R*,3*S*-quinone methide (**8**)/ carbocation (**9**) intermediates arising from 2*R*,3*S*-flavan-3*S*,4*S*-diols (**7**) (Fig. 1). Polymer hydrolysis in the presence of phenylmethanethiol supported the formation of 2*R*,3*R*-quinone methide (**15**) addition units from 2*R*,3*S*-quinone methides (**8**) via a flav-3-en-3-ol (**14**) intermediate (Fig. 2) (Hemingway and Laks, 1985). The initiating unit of the PA (**11**) polymer was referred to as a 'terminal' unit to indicate that the polymer could not be extended below it (reviewed in Stafford, 1990).

All possible chiral arrangements were usually not recovered in the polymerization processes described in early studies, nor were they recovered in equivalent amounts. For example, the 2,3-*trans*-isomer of (+)-catechin (**10a**) was recovered as the predominant isomer when characterizing the flavan-3-ol (**10**) and flavan-3,4-diol (**7**) monomers leading to the simple and well characterized PA (**11**) polymers in barley (*Hordeum vulgare* L.) (Kristiansen, 1986). Purification of all six PA (**11**) polymers from barley testa indicated that 2,3-*trans* stereochemistry was maintained throughout the two dimers and four trimers (Jende-Strid, 1988). This ''targeted'' stereochemistry was not reflective of PA (**11**) polymers in many of the other taxa examined, particularly since stereoisomer composition can be mixed and dynamic during tissue development. For example, extension units from polymers isolated from sainfoin (*Onobrychis viciifolia* Scop.) leaves contain (+)-catechin (**10a**), (+)-gallocatechin (**10b**), and (−)-epigallocatechin (**13c**) in proportions that change developmentally, and the only terminal unit identified was (−)-epicatechin (**13b**) (Koupai-Abyazani et al., 1993). However, the *ent* form of flavonoids is rarer and can be isolated as an extraction artifact (Lundgren and Theander, 1988).

The stereochemistry of PA (**11**) formation has received considerable attention in recent years since the initial characterization of leucocyanidin reductase (LCR) activity (reviewed in Jende-Strid, 1993) and the demonstration of leucodelphinidin reductase (LDR) activity (Stafford and Lester, 1985; Singh et al., 1997). Both of these enzymes are members of the LAR class of enzymes that use di-hydroxy and tri-hydroxy flavan-3,4-diol (**7b**, **7c**) substrates, respectively. Assays of LAR activity have most frequently used the di-hydroxy substrate [*cis*-leucocyanidin (**7b**)]. Until recently, there were no enzymatic data to support the biosynthesis of (−)-epicatechin (13b) or (−)-epigallocatechin (**13c**), despite their existence in many PA (**11**) polymers due to the chirality of the flavonoid C-ring [e.g. naringenin (**2**), Fig. 1]. Even so, enzyme-mediated synthesis had been postulated for more than a decade (Stafford, 1990). Indeed, racemic mixtures of both (+)-catechin (**10a**) and and its diastereoisomer, *ent*(−)-catechin (2*S*,3*R*-*trans*-catechin [not shown]), as well as (−)-epicatechin (**13b**) and *ent*(+)-epicatechin (not shown), have been isolated from plants, thereby suggesting that biochemical pathways do exist for all the isomers and diastereoisomers of catechin (Nahrstedt et al., 1987; Karimdzhanov et al., 1997).

Identification and characterization of these stereochemical variations has important biological implications because stereoisomers can display different biological properties from each other. The allelopathic property of spotted knapweed (*Centaurea maculosa* Lam.) was dependent on the exudation of *ent*(−)-catechin from roots, although the exudate was a racemic mixture (Bais et al., 2002). The co-purifying (+)-catechin (**10a**) was not allelopathic, but had anti-bacterial activity against root pathogens, against which *ent*(−)-catechin was inactive (Bais et al., 2002).

In a landmark study of 2,3-*cis*-flavan-3-ol (**13**) biosynthesis, the enzymatic formation of this type of stereochemistry was demonstrated in vivo by cloning the *Medicago truncatula* Gaertn. and Arabidopsis *BAN* genes and introducing them into transgenic tobacco (*Nicotiana tabacum* L.) and Arabidopsis (Xie et al., 2003). ANR, encoded by the *BAN* gene (Albert et al., 1997; Devic et al., 1999), is a newly characterized enzyme that converts anthocyanidins (**12**) (pelargonidin, cyanidin or delphinidin) to the corresponding 2,3-*cis*-flavan-3-ols [i.e. (−)-epiafzelechin (**13a**), (−)-epicatechin (**13b**), or (−)-epigallocatechin (**13c**)] (Xie et al., 2003) (Fig. 2). Initially, the *BAN* gene of Arabidopsis was tentatively considered to encode LAR due to its homology with DFR and the accumulation of anthocyanins rather than PA (**11**) in the seed coat of the Arabidopsis *ban* mutant (Albert et al., 1997; Devic et al., 1999). This original assignment of function was questioned when both *E. coli* and transgenic Arabidopsis leaf extracts expressing recombinant BAN protein failed to demonstrate LAR activity (Xie et al., 2003) and when (−)-epicatechin (**13b**) was found to be the main flavan-3-ol (**10**) that accumulated in Arabidopsis seeds (Abrahams et al., 2002).

Conversion of anthocyanidins (**12**) to the corresponding 2,3-*cis*-flavan-3-ols (**13**) by ANR and the prevention of PA (**11**) formation in the Arabidopsis *anthocyanin synthase* (*ans*) mutant answer one of the

longer-standing questions about stereochemically-specific PA (**11**) formation, by establishing an enzymatic origin for the widely-occurring (−)-epicatechin (**13b**) and (−)-epigallocatechin (**13c**) units found in procyanidin and prodelphinidin 2,3-*cis* polymers (Porter, 1988; Koupai-Abyazani et al., 1992; Xie et al., 2003). The formation of the ''initiating'' molecule in (−)-*epi*-proanthocyanidin polymers would likely involve a mechanism through (−)-*epi*-flavan-3-ol (**13**) initiation comparable to that found with (+)-flavan-3-ol (**10**) polymer initiation (Figs. 1 and 2). However, the exact route by which stereochemically-correct (−)-*epi* addition units would be generated is still not clear and may invoke formation either through anthocyanidin-derived or leucocyanidin-derived intermediates (Fig. 2). Xie et al. (2003) have recently demonstrated that *BAN* overexpression in tobacco flowers and in Arabidopsis leaves accumulated 2,3-*cis*-flavan-3-ols (**13**). However, identification of PA (**11**) polymers in tissues where these compounds had not previously accumulated requires a more rigorous approach than taken in this study in which PA (**11**) was measured using 4-dimethylaminocinnamaldehyde (DMACA) or *n*-butanol–HCl (*n*-BuOH–HCl) (Xie et al., 2003). On their own, these assays cannot definitively discriminate between unbound flavan-3-ols (**10**) and polymer containing flavan-3-ol (**10**) terminal units (Price et al., 1978; Mole and Waterman, 1987).

4. A cautionary note on extraction and measurement of proanthocyanidin (11)

A large body of literature has accumulated on the chemical analysis of PA (**11**), including polymer extraction protocols and a host of assays to detect or quantify PA (**11**) in a range of tissues. Most of these protocols are easily applied to tissues where PA (**11**) is soluble and readily extracted. However, seed coat and pericarp tissues are particularly difficult to assay accurately (Styles and Ceska, 1989; Marles et al., 2003). During seed maturation, the initially colourless PA (**11**) undergoes secondary changes to form oxidized complexes with cell wall polysaccharides and other phenolics, a process which causes the tissue to darken (Stafford, 1988; Beninger and Hosfield, 1999). This oxidative process may limit the amount of soluble, extractable PA (**11**) and confound the accurate measurement of PA (**11**) content and composition (Styles and Ceska, 1989; Naczk et al., 1998; Marles, 2001).

Co-localization of anthocyanins and PB pigments (derived from 3-deoxy-flavan-4-ol polymerization) with PA (**11**) can interfere with PA (**11**) measurement when using spectrophotometric assays with absorption spectra close to those of the contaminating pigments. Anthocyanin and PA (**11**) co-localization pose particular difficulties for the vanillin–HCl assay (which produces a red vanillin-PA adduct under acidic conditions) and the more sensitive *n*-BuOH–HCl assay (which produces a red [anthocyanidin (**12**)] acid-hydrolysis product) (Sarkar and Howarth, 1976; Price and Butler, 1977; Price et al., 1978; Butler et al., 1982; Watterson and Butler, 1983). Cyanidin-based anthocyanins are a bright red colour, and PB with a very distinctive cherry red colour have been found in floral organs, seed coats and pericarps of some plant species (Bate-Smith and Lerner, 1954; Styles and Ceska, 1975, 1977, 1989; Steenkamp et al., 1985; Lees et al., 1993, 1995; Grotewold et al., 1994).

Some of the genes that influence PA (**11**) biosynthesis in Arabidopsis (discussed in Section 4) were characterized with methods in which pigments in Arabidopsis seed may not have been carefully distinguished using the guidelines reported in the foregoing literature. Frequently, acidic solutions have been used to extract pigment from plants (for example, in Abrahams et al., 2002; Urao et al., 1996; Tanaka et al., 1997). Such treatment can hydrolyze PA (**11**) polymers (similar to the *n*-BuOH–HCl assay) as well as cleave the *O*-glycosidic bonds of anthocyanins to form red anthocyanidin (**12**), especially when heated (Harborne, 1984). Consequently, the biosynthetic origin of the resulting anthocyanidin (**12**) is obscured. Depending on pH, anthocyanidin (**12**) extracts can behave as brightly coloured solutions with very similar spectra to genuine anthocyanins (Mazza and Brouillard, 1990; Davies and Mazza, 1993) and can lead the unwary researcher to draw inaccurate conclusions about the origin of the pigment or the presence of PA (**11**).

Blue staining of PA (**11**) with the reagent DMACA is a way of avoiding confusion with anthocyanin pigments and red condensation adducts. DMACA is specific for flavan-3-ols (**10**, **13**) and PA (**11**) polymers (McMurrough and McDowell, 1978); no response can be detected for hydroxycinnamic acids, the flavonols [kaempferol (**6a**), quercetin (**6b**), myricetin (**6c**)] or dihydroflavonols (**5**). DMACA (like the less-sensitive vanillin) reacts with specific compounds containing *meta*-oriented di- or trihydroxy substituted benzene rings; thus strong reactions occur with leucoanthocyanidins (**7**), catechin (**10a**), gallocatechin (**10b**), epicatechin (**13b**), epigallocatechin (**13c**), procyanidins, prodelphinidins and hydrolyzed PA (**11**) polymer subunits. Since PA (**11**) can occur as dimers, trimers or polymers, not all positions are available for reaction and colour development is reduced unless the polymer is completely acid-hydrolyzed (Price et al., 1978; Mole and Waterman, 1987). These factors can lead to the underestimation of polymer size and concentration, because it is difficult to determine the degree of polymerization remaining after the cleavage reaction (Deshpande et al., 1989; Salunkhe et al., 1990). Furthermore, the assays also do not completely distinguish between flavan-3-ols

(**10**, **13**) and PA (**11**) polymers without prior purification of the polymer.

A reference standard for assays that depend on colour development is most accurate when based on the partial purification of a fresh polymer from the source plant family itself (Mole and Waterman, 1987). The reaction kinetics, such as linearity and reaction times, are different in assays with PA (**11**) standards isolated from different plant sources (Price et al., 1978; Mole and Waterman, 1987). Some authors suggest that dissimilar mixtures of monomeric and polymeric flavans are present in extracts derived from different plant sources (Deshpande and Salunkhe, 1982; Deshpande et al., 1989; Salunkhe et al., 1990).

Anthocyanins and flavan-3-ol (**10**, **13**) monomers can be separated (to a limited degree) from soluble PA (**11**) by binding the PA (**11**) onto PVPP or Sephadex™ LH20 and selectively removing the contaminants with methanol washes, prior to hydrolysis of the bound fraction in *n*-BuOH–HCl (Watterson and Butler, 1983; Porter et al., 1986; Terrill et al., 1992). Where PA (**11**) is insoluble, anthocyanins can be observed qualitatively in tissue mounts as a bright-red colour after minimal exposure ($\leqslant$60 s) to $\leqslant$1% of mineral acid, whereas PA (**11**) must be hydrolyzed extensively with 6 N HCl or 30% HCl in *n*-BuOH in order to be detected (Porter et al., 1986). These types of assays have been used to distinguish PA (**11**) from co-localized contaminating pigments in a variety of legume tissues (Lees et al., 1993, 1994, 1995; Skadhauge et al., 1997) and in a range of Brassicaceae accessions (Marles, 2001; Marles et al., 2003; Ray et al., 2003).

5. Substrate specificity and the enzymology of PA (11) biosynthesis

Enzyme-mediated formation of (+)-catechin (**10a**) and (+)-gallocatechin (**10b**), i.e. LAR activity, and its correlation with PA (**11**) biosynthesis were initially established in developing barley and alfalfa seed, in legume leaves and flowers, and in Douglas fir (*Pseudotsuga menziesii* L.) cell suspension cultures (Stafford and Lester, 1985; Jende-Strid, 1991; Tanner and Kristiansen, 1993; Labesse et al., 1994; Skadhauge et al., 1997; Singh et al., 1997). LAR is an NADPH-dependent enzyme that catalyzes the formation of the 2,3-*trans*-flavan-3-ols (**10**) from 2*R*,3*S*-*trans*-flavan-3*S*,4*S*-*cis*-diols (**7**) (Fig. 1). Extensive efforts to purify LAR from black locust gum (*Robinia pseudoacacia* L.) leaves, big trefoil (*L. uliginosus*) leaves, red-flowering currant (*Ribes sanguineum* Persh.) flowers, sainfoin leaves and silverleaf desmodium leaves (*Desmodium uncinatum* (Jacq.) DC.) indicated that several LAR isoforms could exist within the same tissue (Tanner et al., 2002; Gruber et al., unpublished results). These studies, together with random sequencing of a leaf EST library, have culminated in the isolation of a full-length, functional *LAR* cDNA from *Desmodium* (Tanner et al., 2002) and a homologous cDNA from big trefoil (Yu and Gruber, unpublished results). These cDNAs have high homology to members of a super-family of Reductase/Epimerase/Dehydrogenase (RED) genes (Labesse et al. 1994; Tanner et al., 2002). LAR activity assays with the recombinant protein from *E. coli* extracts indicate that *Desmodium* LAR can utilize 2*R*,3*S*,4*S*-leucopelargonidin (**7a**), 2*R*,3*S*,4*S*-leucocyanidin (**7b**) and 2*R*,3*S*,4*S*-leucodelphinidin (**7c**) (Tanner et al., 2002).

The specificity of an individual enzyme or the divergence of activity among multiple copies of the same gene will have an impact on the composition of flavonoid intermediates available to contribute to PA (**11**) composition. Enzymes within the anthocyanin and PA (**11**) biosynthetic pathways have been shown to utilize a range of substrates and are typified by DFR, the step between LAR and flavanone 3-β-hydroxylase (F3H), an enzyme which lies within the "grid" section of flavonoid metabolism where B-ring hydroxylation takes place (Fig. 1). In contrast to LAR, ANR and ANS, which were tested on a range of substrates in vitro or in *E. coli* (Nakajima et al., 2001; Tanner et al., 2002; Xie et al., 2003), substrate specificity for DFR was determined primarily by chemical analysis of anthocyanin-accumulating plants or their mutant lines after the introduction of a *DFR* transgene. For example, the maize *DFR* gene (*A1*) stimulated the accumulation of both kaempferol (**6a**) and quercetin (**6b**) glycosides (but not the respective anthocyanins) when introduced into an Arabidopsis DFR-reduced mutant, *tt*3, compared with the wild type (cv Landsberg *erecta*) and untransformed mutant plants (Dong et al., 2001), indicating that the *A*1 gene can use both mono-hydroxylated [dihydrokaempferol, (**5a**)] and di-hydroxylated [dihydroquercetin (**5b**)] substrates. In maize, the expression of *A*1 normally results in the accumulation of both pelargonidin and cyanidin, anthocyanins formed from equivalent substrates (Grotewold et al., 1994). In another mutant affected in the flavanoid 3′-hydroxylase step (F3′H), *tt*7, the *A*1 transgene stimulated the accumulation of pelargonidin, the mono-hydroxylated anthocyanin, since only dihydrokaempferol (**5a**) was available to the DFR enzyme.

In contrast to the maize *A*1 gene, petunia (*Petunia hybrida* Hort. ex Vilm.) DFR had a more limited substrate specificity and was unable to utilize dihydrokaempferol (**5a**) under any circumstances, consistently resulting in a pink flower phenotype (Johnson et al., 2001). This narrow substrate specificity was altered by the introduction of a chimeric *DFR* encoding the portion of the Gerbera (*Gerbera hybrida*) *DFR* that specifies dihydrokaempferol (**5a**) utilization (Elomaa et al., 1998). As a result, transgenic petunia plants expressing this chimeric *DFR* gene successfully utilized dihydro-

kaempferol (**5a**) to produce pelargonidin and a brick-orange flower phenotype. Enzymes such as DFR with narrow substrate specificity also appear active in transgenic tomato (*Lycopersicum esculentum* L.) fruit (Bovy et al., 2002). Fruits from transgenic tomato plants transformed with the maize anthocyanin regulatory genes *Lc* and *C*1 accumulated high levels of kaempferol (**6a**) and small amounts of naringenin flavanone (**2**) rather than a mixture of different flavonols (**6**) and flavanones [e.g. eriodictyol (**3**)], although transcript analysis showed that most of the flavonoid structural genes (with the exception of F3′H and F3′5′ hydroxlase [F3′5′H]) were induced strongly (Bovy et al., 2002). The preference of the tomato DFR for dihydromyricetin (**5c**) in combination with insufficient expression of F3′5′H resulted in the accumulation of low amounts of the corresponding petunidin and malvidin, rather than other anthocyanins (Bovy et al., 2002). The impact of narrow substrate specificity on product composition can also be found in licorice, (*Glycyrrhiza echinata* Lepech.) which produced 5-deoxyflavonoids in response to the environmental stimulation of a specific isoform of CHI (Kimura et al., 2001). Demonstration of broad substrate specificity with both the recombinant *Desmodium* LAR and *Medicago* BAN enzymes has resolved a second question about PA (**11**) biochemistry, specifically that there may not be a need to introduce several different hydroxylation-specific structural genes in order to tailor PA (**11**) composition in transgenic plants.

6. Structural mutations that affect proanthocyanidin (11) synthesis

Historically, research with plant mutants has been largely responsible for the identification of steps in the PA (**11**) pathway. A comprehensive collection of over 700 mutant phenotypes was developed in barley lines at the Carlsberg Research Laboratory, Denmark, to provide genetic resources to study PA (**11**) biosynthesis. The *ant* mutations cause modifications in PA (**11**) and/or anthocyanin biosynthesis throughout the flavonoid pathway and in several different tissues besides the caryopsis. Diallelic crosses have localized a majority of the mutants to 28 different complementation groups, the *ant* genes (Jende-Strid, 1991, 1993; Gruber et al., 1999). In at least ten of the *ant* mutants, PA (**11**) biosynthesis in the testa layer was affected (Table 1).

Barley mutations have been found that affect *CHI* (*ant*30), *F3H* (*ant*17 and *ant*22) and *DFR* (*ant*18). No mutations have identified *CHS*, *F3′H* or *F3′5′H* as target loci (Table 1). Only *ant*18 has been complemented with a cloned gene (Wang et al., 1993). *Ant*19 was previously thought to be *LAR*; however, the mutation can be partially rescued by growth in fertilizer-supplemented soil (Jende-Strid et al., 1993; Tanner et al., 1992). More recently, PA-reduced mutations have been developed in the dicot Arabidopsis, named for their unpigmented seed coat, *transparent testa* (*tt*) and tannin-deficient seeds (*tds*) (Shirley et al., 1995; Abrahams et al., 2002) (Table 1). The only tissue in Arabidopsis which normally accumulates PA (**11**) is the endothelial layer of the seed coat (Albert et al., 1997; Devic et al., 1999). Arabidopsis mutants are particularly effective genetic systems in which to examine flavonoid metabolic perturbation, since all but one of the structural genes in the core flavonoid pathway appear to be encoded by single-copy genes (Winkel-Shirley, 2001b). Flavonol synthase (FS) is the exception with six gene copies, two of which may not be expressed (Winkel-Shirley, 2001b). A larger

Table 1
Barley and Arabidopsis genes and mutations defining the PA/anthocyanin pathway and their homologues identified in maize[a]

Identified step[b]	Plant species and Locus[c]		
	Barley	Maize	Arabidopsis
Structural proteins			
CHS	(+)	*c2* (+)	*tt*4 (+) *whp*
CHI	*ant*30	(+)	*tt*5 (+)
F3H	*ant*17(+)		*tt6* (+)
		*ant*22	
DFR	*ant*18(+)	*a*1 (+)	*tt*3 (+)
F3′H		*Pr* (+)	*tt*7 (+)
F3′5′H			
FS			*fls*1 (+)
LAR[d]			
ANR[e]			*ban* (+) *ast*
Condensing step	*ant*26		
Vacuolar transport			*tt*12[f]
AS (LDOX)		*a*2 (+)	*tt*19 (+), *ans*
Regulatory proteins			
Myc-like	*ant*13 (+)	*Sn*, *Lc* (+) *R* (+)	*tt*8 (+)
Myb-like		*C*1 (+)	*tt*2 (+)
MADS			*tt*16 (+)[f]
WIP			*tt*1 (+)[g]
WD40			*ttg*1 (+)[h]
WRKY			*ttg*2 (+)[i]
	*ant*25 (development) *ant*19,21,27,28 (Cat$^-$, PA$^-$)		

Cat$^-$, no catechin (**10a**); PA$^-$, no proanthocyanidin (**11**).

[a] Information on barley, (Gruber et al., 1999); maize, (Mol et al., 1996; van der Meer et al., 1993); Arabidopsis, except where noted, (Winkel-Shirley, 2001b);

[b] See Fig. 1 for explanation of enzymatic abbreviations;

[c] (+), cDNA cloned;

[d] (Tanner et al., 2003);

[e] (Xie et al., 2003);

[f] (Nesi et al., 2002);

[g] (Sagasser et al., 2001);

[h] (Walker et al., 1999);

[i] (Johnson et al., 2002).

number of steps in the flavonoid biosynthetic grid has been detected among the Arabidopsis *tt* mutants compared with barley, including mutations affecting *CHS* (*tt*4), *CHI* (*tt*5), *F3H* (*tt*6), *DFR* (*tt*3) and *F3′H* (*tt*7) (Table 1). However, to date no mutants have been found for *LAR*. Rather, mutations have been found that affect *ANR* (*ban*, *ast*) and *ANS* (*LDOX*) (*tt*19) (Table 1).

7. Membrane association, condensation and vacuolar transport of PA (11)

A model developed by Stafford (1990) on the formation of PA (**11**) envisioned dihydroflavanol reductases (*trans*-DFR or *cis*-DFR) as integral membrane proteins on the cytoplasmic face of the endoplasmic reticulum (ER) in close association with a *trans*-diol-reductase, a *cis*-diol-reductase and a condensing enzyme. These latter three factors were illustrated as intrinsic membrane proteins on the lumen face of the ER membrane. In this model, an epimerase enzyme was embedded less deeply on the cytoplasmic face of the ER, and synthesis of flavan-3-ols (**10**, **13**) occurred on the lumen face. Growing polymer chains emerged from the condensing protein into the vacuole, with the initiating unit in the PA (**11**) polymer derived from (+)-flavan-3-ols (**10**), and the extension units derived from 2*R*,3*S*,4*S*- or 2*R*,3*R*,4*S*-leucoanthocyanins (**7**) (Stafford, 1990). A later model showed the enzymes of the early and middle stages of flavonoid biosynthesis to DFR as a cluster on the cytosolic face of the ER, but did not show the final steps of flavan-3-ol (**10** or **13**) formation and polymer assembly (Winkel-Shirley, 2001b).

These previous models and new data can now be reworked into a new picture of PA (**11**) biosynthesis in which all of the enzymes up to the formation of both types of flavan-3-ol (**10**, **13**) stereochemistry (but excluding polymer assembly) are on the cytoplasmic face of the ER or vacuolar membrane. Most of the purified or recombinant enzymes leading up to the flavan-3-ols (**10**, **13**) are soluble or do not display significant transmembrane domains (e.g. F3H, DFR, LAR, ANR/BAN), while the polymerized product is localized in the vacuole (Debeaujon et al., 2001; Winkel-Shirley, 2001b; Spelt et al., 2002). Chalcone synthase was shown to be localized on the cytoplasmic face of the endoplasmic reticular membrane (Hrazdina and Wagner, 1985; Hrazdina et al., 1987). Physical associations have been found in a yeast 2-hybrid assay between DFR, CHS and CHI, although not in the metabolic order of reaction (Burbulis and Winkel-Shirley, 1999). Substrate channeling, which would require physical associations between enzymes, has been demonstrated previously with PA-specific DFR and LDR activities in sainfoin leaf extracts (Singh et al., 1997).

Several of the barley *ant* mutants and Arabidopsis *tds* mutants affect post-DFR steps and are PA-specific (Table 1). So far, the recently-reported *tds* mutations have only been characterized in a preliminary fashion (Abrahams et al., 2002) and their assignment to specific steps may be premature at this time. At this time, both ANR as well as ANS appear necessary to synthesize the (−)-epicatechin flavan-3-ols (**10**), but it has not been demonstrated conclusively whether ANR is involved in the formation of (−)-*epi*-polymer extension units (Xie et al., 2003). Although no gene or enzyme has been demonstrated for the polymerization step, *ant*26 has been suggested as a mutation in the condensing step of barley PA (**11**) biosynthesis, since seed coat of this mutant accumulates (+)-catechin (**10a**) and does not accumulate PA (**11**) (Jende-Strid, 1993). This step was recently found to be dependent on intact membranes based on substrate/product labelling experiments in vivo and in vitro using *ant*26 seed tissue (Skadhauge, unpublished results). Several other PA-specific *ant* mutations (Table 1) have also been assigned tentative function and physical position in PA (**11**) assembly in the barley model and could be located in or associated with the tonoplast.

Tonoplast or ER localization of PA (**11**) biosynthesis has been known for some time, due to detailed histochemical studies in a variety of plant species (Parham and Kaustinen, 1977; Rao, 1988; Lees et al., 1995). However, genes specifying vacuolar localization were not cloned until recently. The Arabidopsis *TT*12 gene has been shown to be involved with transport into the vacuoles of the seed coat epithelium (Debeaujon et al., 2001). Seeds of the *tt*12 mutant appeared dull brown compared to wild type, and PA (**11**) precursors [reported as catechin (**10a**) and leucocyanidin (**7b**)] accumulated in the cytoplasm (Debeaujon et al., 2001). *TT*12 was expressed early in seed development, but the transcript was undetectable later when the vacuoles were filled with PA (**11**) polymer (Debeaujon et al., 2001). The sequence of *TT*12 appeared to encode a protein with twelve transmembrane regions and was identified as a putative secondary transporter protein (Debeaujon et al., 2001). Its transport mechanism is not yet known, but its sequence resembles a MATE protein family (Multidrug And Toxic compound Extrusion) (Debeaujon et al., 2001). The *tt*12 mutant may well be the Arabidopsis analogue of *ant*26 in barley. The availability of the collections of PA-specific mutations in Arabidopsis and barley should facilitate the isolation and characterization of additional genes that code for the condensation and vacuolar deposition steps of PA (**11**) in monocots and dicots. Fluorescence-labelled antibodies to recombinant proteins, increased attention to the exact structure of the

polymer formed and differential substrate labelling experiments that include intact vacuoles will also enable a more accurate and detailed characterization of these events.

8. The regulation of proanthocyanidin (11) biosynthesis

Within the past few years, PA (**11**) regulatory genes have been cloned and characterized using several mutants. These mutations partially define the regulation of PA (**11**) biosynthesis and have been developed in barley and Arabidopsis (Jende-Strid, 1993; Shirley et al., 1995; Albert et al., 1997; Tanaka et al., 1997; Abrahams et al., 2002) (Table 1). Most of these mutations show reductions in PA (**11**) content, while some show reductions in both anthocyanin and PA (**11**) (Jende-Strid, 1993; Shirley et al., 1995). Regulatory mutations have also been identified in the model legume *Lotus japonicus*, a species in which PA (**11**) is strongly accumulated in floral organs, seeds and stems, but normally not accumulated in leaves (Skadhauge et al., 1997). The *tan* mutants of *L. japonicus* accumulate PA (**11**) ectopically in leaves (Gruber et al., 1998; Ray and Gruber, 2001). The cloned genes include basic helix-loop-helix (bHLH, *myc*-like) genes, a *myb* gene (*TT*2), a MADS homeodomain gene (*BSISTER*), a WD-40 transcription factor (*ttg*1), a gene for a new type of zinc finger protein (WIP; *tt*1), and a WRKY transcription factor, *ttg*2 (Table 1) (de Vetten et al., 1997; Walker et al., 1999; Sagasser et al., 2001; Nesi et al., 2002; Johnson et al., 2002). The majority of these genes have been recently cloned from Arabidopsis.

Myc genes have been found to regulate PA (**11**) biosynthesis, and together with other regulatory genes such as *myb* genes, they make a wide array of control possible. Both types of genes are known to regulate other flavonoid end-products such as anthocyanins and PB, as demonstrated by yeast two-hybrid interaction assays, gel mobility shift data and genetic crosses (Cone et al., 1986; Styles and Cseska, 1989; Ludwig and Wessler, 1990; Tonelli et al., 1991; Goff et al., 1992; Grotewold et al., 1994). A bHLH gene, *TAN*1, has been cloned from PA-accumulating *L. uliginosus* forage, and the expression pattern of its homologues correlates with PA (**11**) accumulation in PA-accumulating *Lotus* species (Ray and Gruber, 2001). In *L. japonicus*, where leaves do not normally accumulate PA (**11**), disruption of the *TAN*1 homologue is associated with accumulation of moderate amounts of PA (**11**) (Ray and Gruber, unpublished results). These data suggest that the wild type allele may act as a repressor in *L. japonicus*, while the alleles in *L. uliginosus* and *L. corniculatus* activate PA (**11**) gene expression.

The bHLH gene, *TT*8, from Arabidopsis also controls PA (**11**) accumulation and regulates the expression of *BAN* and *DFR* (Nesi et al., 2000). However, an additional factor must regulate *DFR* since transcripts for *DFR* disappeared well before *TT8* expression declined in developing seeds of Arabidopsis. Alternately, *TT*8 may also regulate a later step in PA (**11**) formation. Ectopic expression of *TT*8 does not induce two other Arabidopsis PA (**11**) regulatory genes, *TTG*1 and *TT*2, which are also required for normal expression of *BAN* and *DFR*. *TT*8 is expressed in other tissues besides seed coat endothelium; therefore, it affects other processes in addition to PA (**11**) biosynthesis (Walker et al., 1999; Nesi et al., 2000).

*TAN*1 and *TT*8 are regulatory genes with a significant degree of homology to *ANT*13, a third *myc*-like PA-related gene and the first isolated from a monocot. *ANT*13 was isolated from barley based on differential expression between the seed testa-pericarp layers of the *ant*13 mutant and a parental line; the *ant*13 mutant produces a truncated RNA (Ray and Gruber, unpublished results). The Arabidopsis genome contains additional theoretical genes with a stronger resemblance to *TAN*1 and *ANT*13 than to *TT*8. Development of a targeted set of disruption mutants (Bouché and Bouchez, 2001) may define a function for one or more of these genes in PA (**11**) biosynthesis.

*TT*2 is a plant *myb* gene from Arabidopsis with two blocks of conserved repeats, R2 and R3 (each about 50 amino acids long) (Nesi et al., 2001). *TT*2 is expressed early in seed development, and to a lesser degree in flower tissue, but not elsewhere. Functional *TT*2 is required for the later genes of PA (**11**) synthesis: *DFR*, *LDOX*, *BAN*, and *TT*12. Direct interaction between *TT*8 and *TT*2 has not been documented. It seems likely, however, that *TT*2 with its more restricted expression pattern, interacts with *TT8* and is limiting for PA (**11**) synthesis in wild type Arabidopsis. Expression of *TT*8 is independent of *TT*2 in wild type Arabidopsis, but ectopic expression of a *TT*2 transgene induced additional *TT*8 in seedlings and roots (Nesi et al., 2001). In addition, *BAN* was induced in both tissues and *DFR* in roots of transgenic plants (Nesi et al., 2001). This suggests the involvement of another factor, as yet unidentified, controlling the expression or activity of *TT*2 and/or *TT*8. *TT*2 is the only *myb* gene found to date that appears to regulate PA (**11**) biosynthesis. In several forage legumes (alfalfa, *Lotus* species and sainfoin), *myb*-like genes did not correlate with PA (**11**) biosynthesis, suggesting that in these species, the *myc* regulator may be limiting (Ray and Gruber, unpublished results).

A third type of protein found to regulate PA (**11**) synthesis is a WD40-like protein, *TTG*1, which so far has not been shown to interact directly with myc or myb proteins or gene promoters. *TTG*1 strongly resembles the anthocyanin regulator *AN*11 of petunia (80% identical amino acids), but has more widespread effects (de

Vetten et al., 1997). *TTG*1 is a protein of multiple roles. Functional *TTG*1 is required for normal expression of *BAN* and *DFR*, and therefore, for anthocyanin and PA (11) accumulation in Arabidopsis (Walker et al., 1999); however, it is also expressed in several plant tissues which lack PA (11). *AN*11 only conditions petal pigmentation (de Vetten et al., 1997), while *TTG*1 affects root hair and trichome formation as well as seed coat colour (Walker et al., 1999). It appears to act upstream of both myc-like and myb-like proteins or to control their interaction (Walker et al., 1999), but its mode of action is not clear.

Another gene, *TTG*2, affects trichome morphology, as well as PA (**11**) accumulation. *TTG*2 is a member of a fourth class of regulatory genes, the WRKY transcription factors (Johnson et al., 2002). WRKY acts downstream of *TTG*1, but its interaction with later structural genes of PA (**11**) synthesis has yet to be examined.

A MADS homeodomain gene *TT*16 (*BSISTER*) has recently been identified from an Arabidopsis mutant and represents a fifth class of transcription factors that control PA (**11**) biosynthesis (Nesi et al., 2002). *TT*16 appeared to act upstream of *BAN* and probably of other regulatory genes. It was expressed in buds, flowers, and early in seed coat development. Like some of the *tds* mutants (*tds*1, 2, and 5), *TT*16 did not affect PA (11) accumulation in the chalazal and micropylar regions; rather, this specific area of Arabidopsis seed coat appeared to be regulated independently (Abrahams et al., 2002). Spatial control of seed coat pigmentation in these areas has also been described in many other legume cultivars (e.g. *Phaseolus* spp.; *Glycine max*) (Bernard and Weiss, 1973; Todd and Vodkin, 1996; Beninger et al., 2000).

*TT*1 from Arabidopsis is a member of a sixth class of PA (**11**) regulatory genes, the zinc-finger proteins. While it is most similar to the TFIIIA proteins, it differs in some key features and therefore was placed in a new subclass, termed 'WIP' (Sagasser et al., 2001). *TT*1 is expressed in flowers and developing seeds, with the exception of the chalazal and micropylar areas. The *tt*1 mutant has yellow (unpigmented) seed, but the structural genes upstream of *BAN* are all expressed normally. However, (+)-catechin or (−)-epicatechin (**10**, **13**) (based on material staining positively with vanillin-HCl) accumulated in the developing seed (Sagasser et al., 2001), suggesting that *TT*1 is a regulatory gene that specifically controls PA (**11**) polymer assembly, rather than the formation of monomeric flavan-3-ols (**10**, **13**). Hence, *TT*1 may be related to or control a gene that is similar to *ant*26 gene of barley (Jende-Strid et al., 1999). *TT*1 may also interact with developmental genes in addition to controlling PA (**11**) synthesis, since its over-expression caused aberrant organ morphology (Sagasser et al., 2001).

Additional factors contributing to PA (**11**) synthesis, both of a structural and regulatory nature, remain to be elucidated. For example, the six *tds* mutants of Arabidopsis all act downstream from *BAN* (Abrahams et al., 2002). Some of these mutants accumulate PA (**11**) monomers early in development, but lose the PA (**11**) before maturity. Two mutants of barley, *ant*25 and *ant*26, show the same accumulation pattern (Jende-Strid et al., 1999). This group may contain a gene for the condensation step.

Within two or three years, six classes of regulatory genes affecting PA (**11**) accumulation have been identified. As yet we have little understanding of their interactions. Intercrossing of the Arabidopsis mutants and detailed examination of the resulting phenotypes will reveal many new gene interactions that can be assigned a role in PA (**11**) biosynthesis; however, as was demonstrated in anthocyanin biosynthesis, the details are certain to vary from species to species.

9. Genetic manipulation of proanthocyanidins (11) in crop species

Attempts to manipulate PA (**11**) concentration in different forage and edible crop species have preceded a thorough understanding of the genes involved. Mutations in a gene can sometimes be restored by ectopic expression of a related gene, but we cannot yet predict whether introduced genes will interact appropriately with the genes normally expressed in their new environment. Cases of unexpectedly high or low expression or ectopic effects have been found by different researchers. For example, a plant breeding strategy dependent on interspecific crosses between yellow-seeded *Brassica carinata* A. Braun. (Ethiopian mustard) and *B. napus* to produce yellow-seeded *B. napus* cultivars was not entirely successful at incorporating an unidentified gene that controlled the dominant unpigmented seed coat trait of *B. carinata* (Rashid et al., 1994). *Brassica napus* progeny from such crosses produced seed that was either heavily mottled or still completely pigmented.

The genetics and enzymology of phenylpropanoid and flavonoid biosynthesis have been studied intensively using seed, foliage and flower colour mutants of petunia, snapdragon (*Antirrhinum majus* L.), maize, barley, Arabidopsis and other genera, and a highly regulated, biochemically complex pathway is emerging (Jende-Strid, 1993; Harborne and Williams, 1995; Grotewold et al., 1998; Gruber et al., 1999; Quattrocchio et al., 1998; Ray and Gruber, 2001). Targeted metabolic profiling of flavonoids in near-isogenic lines of yellow- and brown-seeded *B. carinata* was used to identify a dominant genetic block in seed coat and seedling leaf pigment biosynthesis (Marles et al., 2003). Gene expression studies indicated that down-regulation of *DFR* occurred in both seedling and developing seed tissue of the yellow-seeded *B. carinata* line. This research identified

DFR as an important locus of control for PA (**11**) biosynthesis for the Brassicas, and will contribute to development of a yellow-seeded, high oil-low fibre phenotype in canola (Marles et al., 2003).

As advances were made in understanding the regulation of anthocyanin biosynthesis, PA (**11**) researchers turned to anthocyanin regulatory genes in the hope that these genes would also regulate PA (**11**). In particular, this strategy was important to those researchers interested in developing PA (**11**) in valuable forage crop species such as alfalfa and clover. Although PA (**11**) was easily down-regulated and the relative proportion of different subunits altered when antisense structural transgenes were tested (Robbins et al., 1999), the function of anthocyanin regulatory transgenes in heterologous species was unpredictable. Difficulties were related to copy number and mismatching of transgenes with the regulatory processes in the recipient plant species. In birdsfoot trefoil (*L. corniculatus*), the PA (**11**) content and composition were altered by introducing a maize bHLH transgene (*Sn* allele) (Damiani et al., 1999; Robbins et al., 2003). In some sets of these transgenic plants there was a consistent reduction of PA (**11**) and silencing of the regulatory process in forage (Damiani et al., 1999), while in others, the accumulation of PA (**11**) was strongly enhanced (Robbins et al., 2003). Plants up-regulated *DFR* and enhanced PA (**11**) when the transgene was present as a single copy (Robbins et al., 2003). Both anthocyanin and PA (**11**) content increased and PA (**11**) was deposited in the spongy mesophyll cells in the single copy transgenic plants, while lignin and flavonols remained unaltered. In contrast, lines containing multiple copies of *Sn* suppressed PA (**11**) compared to untransformed plants (Robbins et al., 2003). In maize, the myc-like *R* gene family of proteins that includes Sn requires the presence of a myb protein to complete DNA binding and anthocyanin gene activation (Grotewold et al., 1998). Clearly the maize Sn protein was able to recruit PA (**11**) myb proteins in *Lotus*.

Although the progress with manipulation of PA (**11**) in *Lotus* was very encouraging, birdsfoot trefoil is a relatively minor forage species. Consequently, two maize regulatory genes, the bHLH *R* homologue *B-Peru* and the *myb*-like gene *C*1, were introduced into alfalfa and white clover (*Trifolium* spp), the world's major forage crop species. While traces of anthocyanin were induced in clover (de Majnik et al., 1998), no detectable changes occurred with alfalfa, although both transgenes were expressed (Larkin, personal commun.; Ray et al., 2003). However, the maize *Lc* gene, a bHLH gene with strong similarity to *B-Peru*, strongly induced anthocyanins and produced red-purple forage in transgenic alfalfa when the plants were exposed to indoor high light intensity or when grown under field conditions (Gruber et al., 2001; Ray et al., 2003). Greenhouse plants displayed an ordinary green phenotype. In addition to a red-purple phenotype, the *Lc*-transgenic alfalfa showed reduced flavone content when field-grown plants were tested. Most importantly, transgenic forage grown under enhanced light in a growth chamber displayed LCR activity at an equivalent level found in developing alfalfa seed of the untransformed parent genotype (Ray et al., 2003), and PA (**11**) and not (+)-catechin (**10**) accumulated in the transgenic alfalfa forage (Ray et al., 2003).

Field-grown *Lc*-transgenic alfalfa was also examined using an in vitro digestibility test with freshly-collected rumen fluid (to indicate forage quality). A decline in the initial rate of dry matter disappearance was correlated with high anthocyanin content in the forage, but the relationship with PA (**11**) has not yet been defined in this material (Y. Wang et al., unpublished results). Plants with the highest anthocyanin content had a digestibility profile that was close to the profile of AC Grazeland, the world's first reduced-bloat alfalfa cultivar that was selected for thickened cell walls (http://www1.agric.gov.ab.ca/general/cropvart.nsf/Varieties/, 23 January 2003). These findings suggest that it may not be necessary to introduce additional genes into alfalfa, except to raise the concentration of PA (11) in the new alfalfa germplasm to levels required for optimum forage quality.

A 2.4 kb *Lc* allele containing a 200 bp untranslated region (UTR) was more effective at inducing anthocyanin in transgenic alfalfa than a 2.2 kb allele with a shorter UTR (Ray et al., 2003). In other plant species, the longer UTR was inhibitory (Wang and Wessler, 1998, 2001). However, introduction of *B-Peru* or either of the *Lc* alleles into *Brassica napus* resulted in equivalent strong induction of leaf anthocyanin under high light intensity (S. Wang et al., unpublished results). These experiments with *myc*-like genes demonstrate that phenotypic responses to regulatory transgenes that have very similar sequences are highly dependent on the recipient genotype, particularly regarding details of their interaction with other regulatory factors.

10. Conclusions

In the past few years, the field of PA (**11**) biochemistry has been changed by the discovery of new biochemical and regulatory genes. *LAR* and *ANR*, two genes that act at the entrance way into the two major stereo-specific pathways of PA (**11**) biosynthesis, have been isolated, and their recombinant proteins have been shown to catalyze the formation of 2*R*,3*S*-flavan-3-ols (**10**) and 2*R*,3*R*-flavan-3-ols (**13**). In addition, the *TT*12 gene was shown to control the transport of PA (**11**) polymer into the vacuole. Six different types of regulatory genes have been reported and cloned from Arabidopsis, barley and trefoil forage. As well, the PA (**11**) and anthocyanin pathways could be stimu-

lated simultaneously by transgenic manipulation of alfalfa, the world's most widely grown forage.

This progress has led us to a new era in PA (**11**) manipulation in valuable crop plants. Recent developments have enabled a more detailed and accurate representation of PA (**11**) formation and pointed to a complex regulatory pathway comparable to that found in anthocyanin biosynthesis. Still, a number of significant questions about how PA (**11**) polymers are actually assembled remain unanswered. The chemical mechanism for the production of stereochemically-correct *2R,3R*-flavan-3-ol (via intermediates **8**, **14**, **15**) addition units is still unproven. The proteins that are required to produce activated initiation units and extension units for both of the major types of stereochemistry remain unidentified. In addition, the localization of polymer assembly proteins to specific regions of the ER/vacuolar membrane and lumen is still outstanding. The availability of mutants specific to PA (**11**) polymer assembly in Arabidopsis and barley should rapidly stimulate the isolation and characterization of genes that code for condensation and vacuolar deposition steps. In the future, fluorescent antibody labelling of their proteins in situ, as well as differential substrate labelling experiments using intact vacuoles, will enable an accurate characterization of condensation proteins and elucidate a more complete picture of the biosynthesis of these intriguing compounds.

Molecular geneticists are now in a position to analyze the role that LAR and ANR play in the development of the two major types of flavan-3-ol (**10**, **13**) stereochemistry, particularly when these genes are introduced into anthocyanin-accumulating or anthocyanin-free tissues. The introduction of regulatory genes from different plant sources should enable us to determine whether these factors control the biosynthesis of only one type or several types of stereochemical isomers and to identify genes which produce appropriate tissue-specific expression patterns. It should also be possible to use combinations of these new regulatory and biosynthetic genes to tailor both PA (**11**) composition and content to suit specific plant species and applications, more so as new genes which control polymer length become available. Finally, the development of isogenic lines with different PA (**11**) composition will enable us to determine whether specific stereoisomers have particular functions in plants and should provide new opportunities to broaden the application of PA (b) to medicine, industry, agriculture and the environment.

Acknowledgements

The authors extend their thanks to R.A. Dixon, P.J. Larkin and P. Morris for sharing information prior to publication. In addition, we are grateful to N.D. Westcott and A. Hannoufa, Saskatoon Research Centre, Agriculture and Agri-Food Canada for helpful suggestions in their review of the manuscript.

References

Abrahams, S., Tanner, G.J., Larkin, P.J., Ashton, A.R., 2002. Identification and biochemical characterization of mutants in the proanthocyanidin pathway in Arabidopsis. Plant Physiology 130, 561–576.

Albert, S., Delseny, M., Devic, M., 1997. *BANYULS*, a novel negative regulator of flavonoid biosynthesis in the *Arabidopsis* seed coat. The Plant Journal 11, 289–299.

Bais, H.P., Walker, T.S., Stermitz, F.R., Hufbauer, R.A., Vivanco, J.M., 2002. Enantiomeric-dependent phytotoxic and antimicrobial activity of (−)-catechin: a rhizosecreted racemic mixture from spotted knapweed. Plant Physiology 128, 1173–1179.

Bate-Smith, E.C., Lerner, N.H., 1954. Leuco-anthocyanins. II. Systematic distribution of leuco-anthocyanins in leaves. Biochemical Journal 58, 126–128.

Beninger, C.W., Hosfield, G.L., 1999. Flavonol glycosides from Montcalm dark red kidney bean: implications for the genetics of seed coat color in *Phaseolus vulgaris* L. Journal of Agricultural and Food Chemistry 47, 4079–4082.

Beninger, C.W., Hosfield, G.L., Bassett, M.J., Owens, S., 2000. Chemical and morphological expression of the *B* and *Asp* seedcoat genes in *Phaseolus vulgaris*. Journal of the Amererican Society for Horticultural Science 125, 52–58.

Bernard, R.L., Weiss, M.G., 1973. Qualitative genetics. VIII. Pigment. In: Caldwell, B.E., Howell, R.W., Judd, R.W., Johnson, H.W. (Eds.), Soybeans: Improvement, Production and Uses. American Society of Agronomy, Madison, WI, pp. 144–154.

Botha, J.J., Ferreira, D., Roux, D.G., 1981. Synthesis of condensed tannins. Part 4. A direct biomimetic approach to [4,6]- and [4,8]-biflavonoids. Journal American Chemical Society Perkins Transactions I 1235–1245.

Bouché, N., Bouchez, D., 2001. *Arabidopsis* gene knockout: phenotypes wanted. Current Opinions in Plant Biology 4, 111–117.

Bovy, A., de Vos, R., Kemper, M., Schijlen, E., Pertejo, M.A., Muir, S., Collins, G., Robinson, S., Verhoeyen, M., Hughes, S., Santos-Buelga, C., van Tunen, C.A., 2002. High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes *Lc* and *C1*. The Plant Cell 14, 2509–2526.

Burbulis, I.E., Winkel-Shirley, B., 1999. Interactions among enzymes of the *Arabidopsis* flavonoid biosynthetic pathway. Proceedings of the National Academy of Sciences USA 96, 12929–12934.

Butler, L.G., Price, M.L., Brotherton, J.E., 1982. Vanillin assay for anthocyanidins (condensed tannins): modification of the solvent for estimation of the degree of polymerisation. Journal of Agricultural and Food Chemistry 30, 1087–1089.

Cone, K.C., Burr, F.A., Burr, B., 1986. Molecular analysis of the maize anthocyanin regulatory locus *C*1. Proceedings of the National Academy of Sciences USA 83, 9631–9635.

Damiani, F., Paolocci, F., Cluster, P.D., Arcioni, S., Tanner, G.J., Joseph, R.G., Li, Y.G., de, Majnik, J., Larkin, P.J., 1999. The maize transcription factor *Sn* alters proanthocyanidin synthesis in transgenic *Lotus corniculatus* plants. Australian Journal of Plant Physiology 26, 159–169.

Davies, A.J., Mazza, G., 1993. Copigmentation of simple and acylated anthocyanins with colorless phenolic compounds. Journal of Agricultural and Food Chemistry 41, 716–720.

de Majnik, J., Tanner, G.J., Joseph, R.G., Larkin, P.J., Weinman, J.J., Djordjevic, M.A., Rolfe, B.G., 1998. Transient expression of maize anthocyanin regulatory genes influences anthocyanin expression in

white clover and peas. Australian Journal of Plant Physiology 25, 335–343.

de Vetten, N., Quattrocchio, F., Mol, J., Koes, R., 1997. The *an11* locus controlling flower pigmentation in petunia encodes a novel WD-repeat protein conserved in yeast, plants and animals. Genes and Development 11, 1422–1434.

Debeaujon, I., Peeters, A.J.M., Leon-Kloosterziel, K.M., Koornneef, M., 2001. The *TRANSPARENT TESTA12* gene of Arabidopsis encodes a multidrug secondary transporter-like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium. The Plant Cell 13, 853–871.

Delcour, J.A., Ferreira, D., Roux, D.G., 1983. Synthesis of condensed tannins. Part 9. The condensation sequence of leucocyanidin with (+)-catechin and with the resultant procyanidins. Journal American Chemical Society Perkins Transactions I, 1711–1717.

Deshpande, S.S., Cheryan, M., Salunkhe, D.K., 1989. Tannin analysis of food products. CRC Critical Reviews in Food Science and Nutrition 24, 401–449.

Deshpande, S.S., Salunkhe, D.K., 1989. Interactions of tannic acid and catechin with legume starches. Journal of Food Science 47, 2080–2083.

Devic, M., Guilleminot, J., Debeaujon, I., Bechtold, N., Bensaude, E., Koornneef, M., Pelletier, G., Delseny, M., 1999. The *BANYULS* gene encodes a DFR-like protein and is a marker of early seed coat development. The Plant Journal 19, 387–398.

Dong, X., Braun, E.L., Grotewold, E., 2001. Functional conservation of plant secondary metabolic enzymes revealed by complementation of Arabidopsis flavonoid mutants with maize genes. Plant Physiology 127, 46–57.

Elomaa, P., Mehto, M., Kotilainen, M., Helariutta, Y., Nevalainen, L., Teeri, T.H., 1998. A bHLH transcription factor mediates organ, region and flower type specific signals on dihydroflavonol-4-reductase (dfr) gene expression in the inflorescence of *Gerbera hybrida* (Asteraceae). The Plant Journal 16, 93–99.

Foo, L.Y., Lu, Y.R., Howell, A.B., Vorsa, N., 2000. The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated *Escherichia coli in vitro*. Phytochemistry 54, 173–181.

Goff, S.A., Cone, K.C., Chandler, V.L., 1992. Functional analysis of the transcriptional activator encoded by the maize *B* gene: evidence for a direct functional interaction between two classes of regulatory protein. Genes and Development 6, 864–875.

Goplen, B.P., Howarth, R.E., Sarkar, S.K., Lesins, K., 1980. A search for condensed tannins in annual and perennial species of *Medicago*, *Trigonella*, and *Onobrychis*. Crop Science 20, 801–804.

Grotewold, E., Chamberlin, P., Snook, M., Siame, B., Butler, L., Swenson, J., .Maddock, S, St Clair, G., Bowen, B., 1998. Engineering secondary metabolism in maize cells by ectopic expression of transcription factors. The Plant Cell 10, 721–740.

Grotewold, E., Drummond, B.J., Bowen, B., Peterson, T., 1994. The *myb*-homologous *P* gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. Cell 76, 543–553.

Gruber, M.Y., Ray, H., Auser, P., Skadhauge, B., Falk, J., Thomsen, K.K., Stougaard, J., .Muir, A.D., Lees, G., Coulman, B., McKersie, B., Bowley, S., von Wettstein, D., 1999. Genetic systems for condensed tannin biotechnology. In: Gross, G.G., Hemingway, R.W., Yoshida, T. (Eds.), Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology. Kluwer Academic, New York, pp. 315–341.

Gruber, M.Y., Skadhauge, B., Stougaaard, J., 1998. Condensed tannin mutations in *Lotus japonicus*. Polyphenol Actualities 18, 4–8.

Gruber, M.Y., Ray, H., Westcott, N., 2001. Regulation of flavonoid expression in alfalfa using maize regulatory genes. PCTCA0101624; USA 60/248581.

Harborne, J.B., 1984. Chapter 2, Phenolic compounds. In: Phytochemical Methods: a Guide to Modern Techniques of Plant Analysis. London, Chapman and Hall Inc., pp. 37–99.

Harborne, J.B., Williams, C.A., 1995. Anthocyanins and other flavonoids. Natural Product Reports 12, 639–657.

Haslam, E., 1989. Plant Polyphenols: Vegetable Tannins Revisited. Cambridge University Press, Cambridge. 230 p..

Hemingway, R.W., Laks, P.E., 1985. Condensed tannins: a proposed route to 2*R*,3*R*-(2,3-*cis*)-proanthocyanidins. Journal of the Chemical Society, Chemical Communications 23, 746–747.

Holton, T.A., Cornish, E.C., 1995. Genetics and biochemistry of anthocyanin biosynthesis. The Plant Cell 7, 1071–1083.

Hrazdina, G., Wagner, G.J., 1985. Metabolic pathways as enzyme complexes: evidence for the synthesis of phenylpropanoids and flavonoids on membrane-associated complexes. Archives of Biochemistry and Biophysics 237, 88–100.

Hrazdina, G., Zobel, A.M., Hoch, H.C., 1987. Biochemical, immunological, and immunocytochemical evidence for the association of chalcone synthase with endoplasmic reticulum membranes. Proceedings of the National Academy of Sciences of the United States of America 84, 8966–8970.

Jende-Strid, B., 1988. Analysis of proanthocyanidins and phenolic acids in barley, malt, hops and beer. In: Linskens, H.F., Jackson, J.F. (Eds.), Modern Methods of Plant Analysis, Vol. 7. Springer-Verlag, Berlin, pp. 109–127.

Jende-Strid, B., 1991. Gene-enzyme relations in the pathway of flavonoid biosynthesis in barley. Theoretical and Applied Genetics 81, 668–674.

Jende-Strid, B., 1993. Genetic control of flavonoid biosynthesis in barley. Hereditas 119, 187–204.

Jende-Strid, B., Lundqvist, U., Franckowiak, J.D., 1999. Desciptions of barley genetic stocks for 1998. Barley Genetics Newsletter 29, 80–123.

Johnson, C.S., Kolevski, B., Smyth, D.R., 2002. *TRANSPARENT TESTA GLABRA*2, a trichome and seed coat development gene of Arabidopsis, encodes a WRKY transcription factor. The Plant Cell 14, 1359–1375.

Johnson, E.T., Ryu, S., Yi, H., Shin, B., Cheong, H., Choi, G., 2001. Alteration of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase. The Plant Journal 25, 325–333.

Karimdzhanov, A.K., Kusnetsova, N.N., Dzhataev, S.A., 1997. Phenolic compounds of the plant *Gossypium hirsutum* and of callus tissue from its anthers. Chemistry of Natural Compounds 33, 187–190.

Kimura, Y., Aoki, T., Ayae, S., 2001. Chalcone isomerase isozymes with different substrate specificities towards 6′-hydroxy and 6′-deoxychalcones in cultured cells of *Glycyrrhiza echinata*, a leguminous plant producing 5-deoxyflavonoids. Plant and Cell Physiology 42, 1169–1173.

Koupai-Abyazani, M.R., McCallum, J., Bohm, B.A., 1992. Identification of the constituent flavonoid units in sainfoin proanthocyanidins by reversed-phase high-performance liquid chromatography. Journal of Chromatography 594, 117–123.

Koupai-Abyazani, M.R., McCallum, J., Muir, A.D., Bohm, B.A., Towers, G.H.N., Gruber, M.Y., 1993. Developmental changes in the composition of proanthocyanidins from leaves of sainfoin (*Onobrychis viciifolia* Scop.). Journal of Agricultural and Food Chemistry 41, 1066–1070.

Kristiansen, K.N., 1986. Conversion of (+)-dihydroquercetin to (+)-2,3-*trans*-3,4-*cis*-leucocyanidin and (+)-catechin with an enzyme extract from maturing grains of barley. Carlsberg Research Communications 51, 51–56.

Labesse, G., Vidal-Cros, A., Chomilier, J., Gaudry, M., Mornon, J.-P., 1994. Structural comparisons lead to the definition of a new superfamily of NAD(P)(H)-accepting oxidoreductases: the single-domain reductases/epimerases/dehydrogenases (the "RED" family). Biochemical Journal 304, 95–99.

Lees, G., Gruber, M.Y., Suttill, N.H., 1995. Condensed tannins in sainfoin. II. Occurrence and changes during leaf development. Canadian Journal of Botany 73, 1540–1547.

Lees, G., Hinks, C.F., Suttill, N.H., 1994. Effect of high temperature

on condensed tannin accumulation in leaf tissues of big trefoil (*Lotus uliginosus* Schkuhr). Journal of Agricultural and Food Chemistry 65, 415–421.

Lees, G.L., 1992. Condensed tannins in some forage legumes: their role in the prevention of ruminant pasture bloat. In: Hemingway, R.W., Laks, P.E. (Eds.), Plant Polyphenols: Synthesis, Properties, Significance, Vol. 59. Plenum Press, New York, pp. 915–934.

Lees, G.L., Suttill, N.H., Gruber, M.Y., 1993. Condensed tannins in sainfoin. 1. A histological and cytological survey of plant tissues. Canadian Journal of Botany 71, 1147–1152.

Leung, J., Fenton, T.W., Mueller, M.M., Clandinin, D.R., 1979. Condensed tannins of rapeseed meal. Journal of Food Science 44, 1313–1316.

Ludwig, S.R., Wessler, S.R., 1990. Maize *R* gene family: tissue-specific helix-loop-helix proteins. Cell 62, 849–851.

Lundgren, N., Theander, O., 1988. *cis*- and *trans*-Dihydroquercetin glucosides from needles of *Pinus sylvestris*. Phytochemistry 15, 829–832.

Marles, M.A.S., 2001. Biochemical and Molecular Studies of the Seed Coat of *Brassica carinata* (A.Braun) and other Brassicaceae. PhD thesis, University of Saskatchewan, Saskatoon, Canada.

Marles, M.A.S., Gruber, M.Y., Scoles, G.J., Muir, A.D., 2003. Pigmentation in the developing seed coat and seedling leaves of *Brassica carinata* is controlled at the dihydroflavonol reductase locus. Phytochemistry 62, 663–672.

Mazza, G., Brouillard, R., 1990. The mechanism of co-pigmentation of anthocyanins in aqueous solutions. Phytochemistry 29, 1097–1102.

McMurrough, I., McDowell, J., 1978. Chromatographic separation and automated analysis of flavonols. Analytical Biochemistry 91, 92–100.

Mol, J., Jenkins, G., Schäfer, E., Weiss, D., 1996. Signal perception, transduction, and gene expression involved in anthocyanin biosynthesis. Critical Reviews in Plant Sciences 15, 525–557.

Mol, J.N., Grotewold, E., Koes, R., 1998. How genes paint plants and seeds. Trends in Plant Science 3, 212–217.

Mole, S., Waterman, P.G., 1987. A critical analysis of techniques for measuring tannins in ecological studies. I. Techniques for chemically defining tannins. Oecologia 72, 137–147.

Naczk, M., Amarowicz, R., Sullivan, A., Shahidi, F., 1998. Current research developments on polyphenolics of rapeseed/canola: a review. Food Chemistry 62, 489–502.

Nakajima, J., Tanaka, Y., Yamazaki, M., Saito, K., 2001. Reaction mechanism from leucocyanidin to anthocyanidin 3-glucoside, a key reaction for coloring in anthocyanin biosynthesis. Journal of Biological Chemistry 276, 25797–25803.

Nahrstedt, A., Proksch, P., Conn, E.E., 1987. Dhurrin, (−)-catechin, flavonol glycosides and flavones from *Chamaebatia foliosa*. Phytochemistry 26, 1546–1547.

Nesi, N., Debeaujon, I., Jond, C., Pelletier, G., Caboche, M., Lepiniec, L., 2000. The *TT*8 gene encodes a basic helix-loop-helix domain protein required for expression of *DFR* and *BAN* genes in Arabidopsis siliques. The Plant Cell 12, 1863–1878.

Nesi, N., Debeaujon, I., Jond, C., Stewart, A.J., Jenkins, G.I., Caboche, M., Lepiniec, L., 2002. The *TRANSPARENT TESTA*16 locus encodes the ARABIDOPSIS BSISTER MADS domain protein and is required for proper development and pigmentation of the seed coat. The Plant Cell 14, 2463–2479.

Nesi, N., Jond, C., Debeaujon, I., Caboche, M., Lepiniec, L., 2001. The Arabidopsis *TT2* gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. The Plant Cell 13, 2099–2114.

Nozzolillo, C., Ricciardi, L., 1992. Proanthocyanidin content of broad bean seeds: relationship of seed coat color. In: Hemingway, R.W., Laks, P.E. (Eds.), Plant Polyphenols: Synthesis, Properties, Significance, Vol. 59. Plenum Press, New York, pp. 911–914.

Parham, R.A., Kaustinen, H.M., 1977. On the site of tannin synthesis in plant cells. Botanical Gazette 138, 465–467.

Porter, L.J., 1988. Flavans and proanthocyanidins. In: Harborne, J.B. (Ed.), The Flavonoids: Advances in Research Since. Chapman and Hall, New York, pp. 21–62.

Porter, L.J., Hrstich, L.N., Chan, B.G., 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry 25, 223–230.

Porter, M.L., Krueger, C.G., Wiebe, D.A., Cunningham, D.G., Reed, J.D., 2001. Cranberry proanthocyanidins associate with low-density lipoprotein and inhibit in vitro Cu^{2+}-induced oxidation. Journal of the Science of Food and Agriculture 81, 1306–1313.

Price, M.L., Butler, L.G., 1977. Rapid visual estimation and spectrophotometric determination of tannin content of Sorghum grain. Journal of Agricultural and Food Chemistry 6, 1268–1273.

Price, M.L., Van Scoyoc, S., Butler, L.G., 1978. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. Journal of Agricultural and Food Chemistry 26, 1214–1216.

Quattrocchio, F., Wing, J.F., van der Woude, K., Mol, J.N., Koes, R., 1998. Analysis of bHLH and myb proteins: species-specific regulatory differences are caused by divergent evolution of target anthocyanin genes. The Plant Journal 4, 475–488.

Rao, K.S., 1988. Fine structural details of tannin accumulations in dividing cambial cells. Annals of Botany 62, 575–581.

Rashid, A., Rakow, G., Downey, R.K., 1994. Development of yellow seeded *Brassica napus* L. through interspecific crosses. Plant Breeding 112, 127–134.

Ray, H., Gruber, M.Y., 2001. Novel regulatory genes involved in condensed tannin synthesis in plants. International PCT Application, CA01/01091.

Ray, H., Yu, M., Auser, P., Blahut-Beatty, L., McKersie, B., Bowley, S., Westcott, N., Coulman, B., Lloyd, A., Gruber, M.Y., 2003. Expression of anthocyanin and proanthocyanidin following transformation of alfalfa with maize *Lc*. Plant Physiology 132, 1–16.

Robbins, M.P., Paolocci, F., Hughes, J.-W., Turchetti, V., Allison, G., Arcioni, S., Morris, P., Damiani, F., 2003. *Sn*, a maize bHLH gene, modulates anthocyanin and condensed tannin pathways in *Lotus corniculatus*. Journal of Experimental Botany 54, 239–248.

Robbins, M.P., Bavage, A.D., Morris, P., 1999. Designer tannins: using genetic engineering to modify levels and structures of condensed tannins in *Lotus corniculatus*. In: Gross, G.G., Hemmingway, R.W., Yoshida, T. (Eds.), Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology. Kluwer, New York, pp. 301–314.

Sagasser, M., Lu, G.-H., Hahlbrock, K., Weisshaar, B., 2001. *A. thaliana* TRANSPARENT TESTA 1 is involved in seed coat development and defines the WIP subfamily of plant zinc finger proteins. Genes and Development 16, 138–149.

Salunkhe, D.K., Chavan, J.K., Kadam, S.S., 1990. Dietary Tannins: Consequences and Remedies. CRC Press Inc, Boca Raton, FL. 200 p.

Sarkar, S.K., Howarth, R.E., 1976. Specificity of the vanillin test for flavanols. Journal of Agricultural and Food Chemistry 24, 317–320.

Sarwar, G., Bell, J.M., Sharby, T.F., Jones, J.D., 1981. Nutritional evaluation of meals and meal fractions derived from rape and mustard seed. Canadian Journal of Animimal Science 61, 719–733.

Shirley, B.W., Hanley, S., Goodman, H.M., 1992. Effects of ionizing radiation on a plant genome: analysis of two Arabidopsis transparent testa mutations. Plant Cell 4, 333–347.

Shirley, B., Kubasek, W.L., Storz, G., Bruggemann, E., Koorneef, M., Ausubel, F.M., Goodman, H.M., 1995. Analysis of *Arabidopsis* mutants deficient in flavonoid biosynthesis. The Plant Journal 8, 659–671.

Simbaya, J., Slominski, B.A., Rakow, G.F.W., Campbell, L.D., Downey, R.K., Bell, J.M., 1995. Quality characteristics of yellow-seeded *Brassica* seed meals: protein, carbohydrates, and dietary fiber components. Journal of Agricultural and Food Chemistry 43, 2062–2066.

Singh, S., McCallum, J., Gruber, M.Y., Towers, G.H.N., Muir, A.D., Bohm, B.A., Koupai-Abyazani, M.R., Glass, A.D.M., 1997. Biosynthesis of flavan-3-ols by leaf extracts of *Onobrychis viciifolia*. Phytochemistry 44, 425–432.

Skadhauge, B., 1996. Genetics and Biochemistry of proanthocyanidin biosynthesis and their biological significance in crop plants, PhD thesis. Royal Veterinary and Agriculture University, Copenhagen, Denmark.

Skadhauge, B., Gruber, M.Y., Thomsen, K.K., von Wettstein, D., 1997. Leucocyanidin reductase activity and accumulation of proanthocyanidins in developing legume tissue. American Journal of Botany 84, 494–503.

Spelt, C., Quattrocchio, F., Mol, J., Koes, R., 2002. ANTHOCYANINI of petunia controls pigment synthesis, vacuolar pH, and seed coat development by genetically distinct mechanisms. The Plant Cell 14, 2121–2135.

Stafford, H.A., 1988. Proanthocyanidins—and the lignin connection. Phytochemistry 27, 1–5.

Stafford, H.A., 1990. Flavonoid Metabolism. CRC Press, Boca Raton, FL. 298 p..

Stafford, H.A., Lester, H.H., 1985. Flavan-3-ol biosynthesis, the conversion of (+)-dihydromyricetin to its flavan-3,4-diol (leucodelphinidin) and to (+)-gallocatechin by reductases extracted from tissue cultures of *Ginkgo bilboa* and *Pseudotsuga menziesii*. Plant Physiology 78, 791–794.

Steenkamp, J.A., Steynburg, J.P., Brandt, E.V., Ferreira, D., Roux, D.G., 1985. Phlobatannins, a novel class of ring-isomerized condensed tannins. Journal of the Chemical Society, Chemical Communications 23, 1678–1679.

Styles, E.D., Ceska, O., 1975. Genetic control of 3-hydroxy- and 3-deoxy-flavonoids in *Zea mays*. Phytochemistry 14, 413–415.

Styles, E.D., Ceska, O., 1977. The genetic control of flavonoid synthesis in maize. Canandian Journal of Genetics and Cytology 19, 289–302.

Styles, E.D., Ceska, O., 1989. Pericarp flavonoids in genetic strains of *Zea mays*. Maydica 34, 227–237.

Tanaka, A., Tano, S., Chantes, T., Yokota, Y., Shikazono, N., Watanabe, H., 1997. A new *Arabidopsis* mutant induced by ion beams affects flavonoid synthesis with spotted pigmentation in testa. Genes and Genetic Systems 72, 141–148.

Tanner, G.J., Kristiansen, K.N., 1993. Synthesis of ^{3}H-3,4-*cis*-leucocyanidin and enzymatic reduction to catechin. Analytical Biochemistry 209, 274–277.

Tanner, G.J., Watson, J.M., Abrahams, S., Ashton, A.R., Francki, K.T., Larkin, P.J., 2002. Novel gene and uses therefore to modify pasture qualities of crops. Australia. PCT WO02066625 application.

Tanner, G.J., Kristiansen, K.N., Jende-Strid, B., 1992. Biosynthesis of proanthocyanidins (condensed tannins) in barley. Proceedings of the XVI International Conference Groupe Polyphenols, Portugal, July, p. 4.

Terrill, T.H., Rowan, A.M., Douglas, G.B., Barry, T.N., 1992. Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals, and cereal grains. Journal of the Science of Food and Agriculture 58, 321–329.

Todd, J.J., Vodkin, L.O., 1996. Duplications that suppress and deletions that restore expression from a chalcone synthase multigene family. The Plant Cell 8, 687–699.

Tonelli, C., Consonni, G., Dolfini, S.F., Dellaporta, S.L., Viotti, A., Gavazzi, G., 1991. Genetic and molecular analysis of *Sn*, a light-inducible, tissue specific regulatory gene in maize. Molecular and General Genetics 225, 401–410.

Urao, T., Yamaguchi-Shinozaki, K., Mitsukawa, N., Shibata, D., Shinozaki, K., 1996. Molecular cloning and characterization of a gene that encodes a MYC-related protein in *Arabidopsis*. Plant Molecular Biology 32, 571–576.

van der Meer, I.M., Stuitje, A.R., Mol, J.N.M., 1993. Regulation of general phenylpropanoid and flavonoid gene expression. In: Verma, D.P.S. (Ed.), Control of Plant Gene Expression. CRC Press, Boca Raton, pp. 125–155.

Walker, A.R., Davison, P.A., Bolognesi-Winfield, A.C., James, C.M., Srinivasan, N., Blundell, T.L., Esch, J.J., Marks, M.D., Gray, J.C., 1999. The *TRANSPARENT TESTA GLABRA1* locus, which regulates trichome differentiation and anthocyanin biosynthesis in Arabidopsis, encodes a WD40 repeat protein. The Plant Cell 11, 1337–1349.

Wang, L., Wessler, S.R., 1998. Inefficient reinitiation is responsible for upstream open reading frame-mediated translational repression of the maize *R* gene. The Plant Cell 10, 1733–1745.

Wang, L., Wessler, S.R., 2001. Role of mRNA secondary structure in translational repression of the maize transcriptional activator *Lc*. Plant Physiology 125, 1380–1387.

Wang, X., Olsen, O., Knudsen, S., 1993. Expression of the dihydroflavonol reductase gene in an anthocyanin-free barley mutant. Hereditas 119, 67–75.

Watterson, J.J., Butler, L.G., 1983. Occurrence of unusual leucoanthocyanidin and absence of proanthocyanidins in sorghum leaves. Journal of Agricultural and Food Chemistry 3, 41–45.

Winkel-Shirley, B., 2001a. It takes a garden. How work on diverse plant species has contributed to an understanding of flavonoid metabolism. Plant Physiology 127, 1399–1404.

Winkel-Shirley, B., 2001b. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiology 126, 485–493.

Xie, D.-Y., Sharma, S.B., Paiva, N.L., Ferreira, D., Dixon, R.A., 2003. Role of anthocyanidin reductase, encoded by *BANYULS* in plant flavonoid biosynthesis. Science 299, 396–399.

Further reading

Dixon, R.A., Harrison, M.J., 1990. Activation, structure, and organization of genes involved in microbial defense in plants. In: Scandalios, J.G. (Ed.), Genomic Responses to Environmental Stress, Vol. 28. Academic Press, Inc, Toronto, pp. 165–234.

Hagmann, M.L., Heller, W., Grisebach, H., 1983. Induction and characterization of a microsomal flavonoid 3′-hydroxylase from parsley cell cultures [*Petroselinum hortense*]. European Journal of Biochemistry 134, 547–554.

Heller, W., Forkmann, G., 1993. Chapter 11, Biosynthesis of flavonoids. In: Harborne, J.B. (Ed.), The Flavonoids. Chapman and Hall, pp. 399–425.

M.A. Susan Marles holds a PhD (2001) in Plant Sciences from the University of Saskatchewan. She graduated from the University of Victoria, with a BSc Degree (Honors) in Biology (1972) and from the University of Saskatchewan and with an MSc (1995) in Plant Biochemistry. Prior to her return to graduate school, she worked as a Research Associate for several years at the University of Victoria (Biology) and subsequently at the University of Saskatchewan (Crop Science/Plant Biochemistry). As a graduate student, she received scholarships from NSERC (Natural Sciences and Engineering Council of Canada) and the University of Saskatchewan's Doctoral Award from the College of Graduate Studies. In addition, she received a graduate student award from the Phytochemical Society of North America's Joint Meeting with the Phytochemical Society of

Europe (1997). Currently, Dr. Marles works as a Postdoctoral Research Fellow at the Institute of Biological Chemistry, WSU, in Dr. N.G. Lewis' laboratory.

Heather Ray holds a PhD in Genetics from the University of Alberta, Canada, where she carried out a thesis on allelopathy under the supervision of Philip J. Hastings. She then trained in bacterial and fungal plant pathology before moving on to work in the phenylpropanoid pathway, particularly regulation of proanthocyanidins. She now works in signal transduction pathways at the Plant Biotechnology Institute, Saskatoon, Canada.

Margaret Y Gruber is both a Research Scientist in the Molecular Genetics Section of the Saskatoon Research Centre, Agriculture and Agri-Food Canada (Canadian government) since 1989 and a graduate supervisor with the College of Graduate Studies, University of Saskatchewan since 1994. She received her BsC (1974) in biology, and her MSc (1984) and PhD (1989) in plant molecular biology and biochemistry of oxidative stress from The University of Waterloo (Ontario, Canada). From 1992 to 1993 she worked as a Visiting Scientist at The Carlsberg Laboratory, Denmark. She has two research programs in support of Canadian agriculture: biochemistry and molecular breeding of proanthocyanidin-containing alfalfa forage for the value-added beef sector and insect chemical ecology and host plant genomics for the canola production industry.