Transgenic Plant Journal ©2007 Global Science Books

Molecular Breeding of Novel Yellow Flowers by Engineering the Aurone Biosynthetic Pathway

Eiichiro Ono[1*] • Toru Nakayama[2]

[1] Institute for Health Care Science, Suntory Ltd., Suntory Research Center, 1-1-1 Wakayamadai, Shimamoto, Mishima, Osaka 618-8503, Japan
[2] Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, 6-6-11 Aoba, Sendai, Miyagi 980-8579, Japan
Corresponding author: * **Eiichiro_Ono@suntory.co.jp**

ABSTRACT

Aurone flavonoids confer a bright yellow color to flowers, such as snapdragon (*Antirrhinum majus*). *A. majus* aureusidin synthase (AmAS1), a polyphenol oxidase, was identified as the key enzyme catalyzing the oxidative formation of aurones from chalcones. To date, all known PPOs have been found to be localized in plastids, whereas flavonoid biosynthesis is thought to take place on the cytoplasmic surface of the endoplasmic reticulum. Interestingly, AmAS1 is transported to the vacuole lumen, but not to the plastid, via ER-to-Golgi trafficking. A sequence-specific vacuolar sorting determinant is encoded in the 53-residue N-terminal sequence of the precursor, demonstrating the first example of the biosynthesis of a flavonoid skeleton in vacuoles. Transgenic flowers overexpressing *AmAS1*, however, failed to produce aurones. The identification of *A. majus* chalcone 4'-*O*-glucosyltransferase (UGT88D3) showed that the glucosylation of chalcone by cytosolic UGT88D3 followed by oxidative cyclization by vacuolar aureusidin synthase is the biochemical basis of the formation of aurone 6-*O*-glucosides *in vivo*. Co-expression of the *UGT88D3* and *AmAS1* genes was sufficient for the accumulation of aurone 6-*O*-glucoside in transgenic flowers, suggesting that glucosylation facilitates vacuolar transport of chalcones. Furthermore, their co-expression, combined with the knockdown of anthocyanin biosynthesis by RNAi, increased aurones and decreased other flavonoids, resulting in yellow flowers. These findings not only demonstrate that aurones, flavones, and anthocyanins are derived from the same chalcone pool but also open transgenic strategies to generate yellow flowers for major ornamental species lacking this color variant, such as geraniums, cyclamens, campanulas, and saintpaulias, beyond genetic constraints.

Keywords: *Antirrhinum majus*, flavonoid, flower color, glucosyltransferase, polyphenol oxidase
Abbreviations: 3GT, anthocyanidin 3-*O*-gluocosyltransferase; **Am**, *Antirrhinum majus*; **ANS**, anthocyanidin synthase; **AS**, aureusidin synthase; **C4'GT**, chalcone 4'-*O*-glucosyltransferase; **CHI**, chalcone isomerase; **CHS**, chalcone synthase; **DFR**, dihydroflavonol 4-reductase; **ER**, endoplasmic reticulum; **EMS**, ethylmethanesulfonate; **F3H**, flavanone 3-hydroxylase; **F3'H**, flavonoid 3'-hydroxylase; **F3',5'H**, flavonoid 3',5'-hydroxylase; **FNS**, flavone synthase; **GFP**, green fluorescent protein; **HPLC**, high-performance liquid chromatography; **IFS**, isoflavone synthase; **mRFP**, monomeric red fluorescent protein; **PHC**, 2',3,4,4',6'-pentahydroxychalcone; **PPO**, polyphenol oxidase; **RNAi**, RNA interference; **RT-PCR**, reverse transcription-polymerase chain reaction; **Th**, *Torenia hybrida*; **THC**, 2',4,4',6'-tetrahydroxychalcone; **UGT**, UDP-glycosyltransferase

CONTENTS

INTRODUCTION

Flower color is one of the most important characters in consumer selection between ornamental varieties. Three top-selling cut flowers, i.e., roses (*Rosa* spp.), carnations (*Dianthus caryophyllus*), and chrysanthemums (*Dendranthema* x *morifolium*), have no blue varieties, and several top-selling pot flowers, such as cyclamens (*Cyclamen persicum*), and geraniums (*Pelargonium graveolens*), have no clear yellow varieties. By means of classical breeding techniques, the creation of novel varieties of unknown flower color has been achieved only with limited success because of the constraints of the gene pool in a single plant species. However, through a genetic engineering approach, it is now possible to modify flower colors beyond such genetic constraints (Forkmann and Martens 2001; Tanaka *et al*. 2005). Thus, transgenic carnations with bluish flowers created by means of this strategy have been marketed in North America, Australia, and Japan for 10 years, and, more recently, transgenic bluish roses have successfully been generated through similar approaches (Tanaka *et al*. 2006).

Aurones are a minor class of plant flavonoids that are responsible for the bright yellow color of some important ornamental flowers, such as snapdragon (*Antirrhinum*

Received: 28 February, 2007. Accepted: 16 April, 2007.

Invited Review

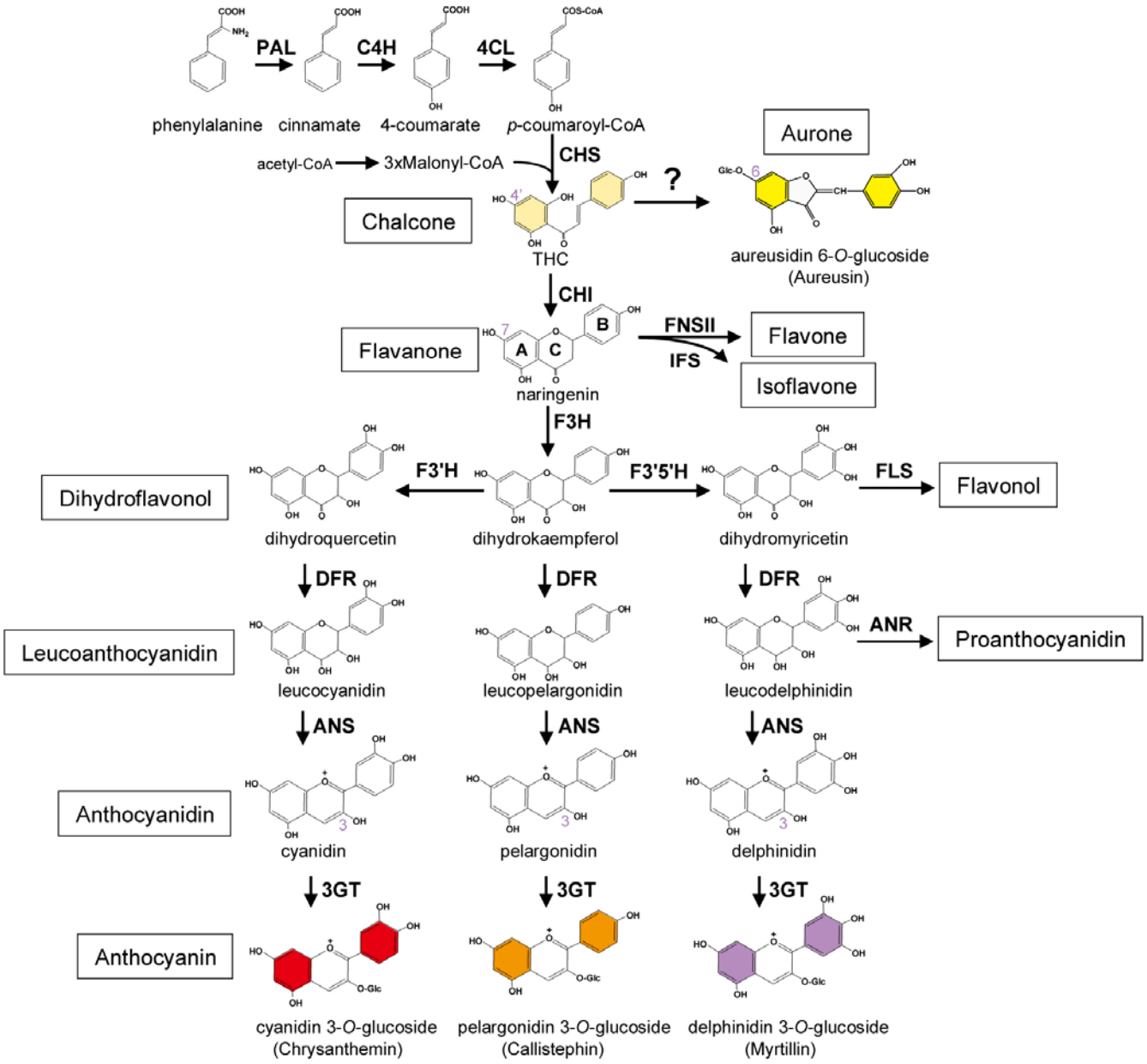


Fig. 1 Schematic representation of the flavonoid biosynthetic pathways. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-coenzyme A ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3',5'H, flavonoid 3',5'-hydroxylase; FNSII, flavone synthase II; IFS, isoflavone synthase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; 3GT, anthocyanidin 3-*O*-glucosyltransferase; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; THC, 2',4',6',4-tetrahydroxychalcone.

majus), cosmos (*Cosmos bipinnatus*), and dahlia (*Dahlia variabilis*). Although the aurone biosynthetic gene has been regarded as an attractive tool to engineer novel yellow flowers, the biochemical and genetic details of aurone biosynthesis remained to be clarified until recently. In 2000, aurone biosynthetic genes were identified, and this achievement enabled the clarification of the unique features of aurone biosynthesis (Nakayama *et al*. 2000; Ono *et al*. 2006a) and the establishment of a method for molecular breeding of novel yellow flowers (Ono *et al*. 2006b). In this review, we describe the genetic, biochemical, and molecular biological aspects of aurone biosynthesis in yellow snapdragon flowers and their application to the molecular breeding of novel yellow flowers.

FLAVONOID BIOSYNTHESIS AND FLOWER COLOR MODIFICATION

Flavonoids, a water-soluble class of secondary metabolites, have a common C_6-C_3-C_6 backbone structure and are widespread in the plant kingdom (Harborne and Baxter 1999). They have a diverse array of physiological roles, as exemplified by those in flower coloration for attracting pollinators and the protection of plants from ultraviolet (UV) light (Winkel-Shirley 2001; Grotewold 2006). Anthocyanins are the largest subclass of flavonoids and are responsible for the majority of the pink, orange, magenta, red, purple, and blue colors of flowers, which are directly related to plant reproduction and survival in nature because pollinators show preference for certain colors (Bradshaw and Schemske 2003; Zufall and Rausher 2004).

Flavonoid biosyntheses have been well characterized and excellently described elsewhere (**Fig. 1**) (Holton and Cornish 1995; Tanaka *et al*. 2005; Grotewold 2006; Yu *et al*. 2006). Chalcone synthase (CHS), a type III polyketide synthase, catalyzes the first committed step of flavonoid biosynthesis, i.e., the consecutive condensation of three molecules of malonyl-CoA with one molecule of *p*-coumaroyl-CoA, producing a chalcone (2'4,4',6-tetrahydroxychalcone, THC), which is the common precursor of all flavonoids. One of two aromatic rings of chalcones and other flavonoids is derived from three malonyl-CoA molecules and is referred to as the A-ring, whereas another aromatic ring, arises from *p*-coumaroyl-CoA molecule, is referred to as the B-

ring. Chalcone itself exhibits yellow color, and its 2'-*O*-glucoside is responsible for the yellow color of carnation. Genetic and biochemical evidences suggested that chalcones are direct precursors of aurones in several aurone-containing plant species (e.g., snapdragon, coreopsis, and soy seedlings; see below). In most plant species, however, THC undergoes stereospecific isomerization to produce (*2S*)-flavanone (e.g., naringenin), a colorless subclass of flavonoids, and this reaction is catalyzed by chalcone isomerase (CHI). A non-enzymatic isomerization of chalcones into flavanones also occurs at around a neutral pH, resulting in a racemic mixture of flavanones including biologically inactive (*2R*)-isomers. The crystal structures of CHS and CHI were solved, providing detailed information for insights into their specificity and catalytic mechanisms (Ferrer *et al*. 1999; Jez *et al*. 2000).

(*2S*)-Flavanone then serves as a direct precursor of three different subclasses of flavonoids – flavones, isoflavones, and dihydroflavonols. The syntheses of flavones and isoflavones from flavanones are catalyzed by flavone synthase (FNSII/CYP93B) and isoflavone synthase (IFS/CYP93C), respectively, which are microsomal cytochrome P450 enzymes; on the other hand, the conversion of flavanones into dihydroflavonols (e.g., dihydrokaempferol, dihydroquercetin, and dihydromyricetin) is catalyzed by flavanone 3-hydroxylase (F3H), which is a soluble, 2-oxoglutarate-dependent dioxygenase. Dihydroflavonol then serves as a substrate for dihydroflavonol 4-reductase (DFR), which catalyzes the conversion of dihydroflavonols to leucoanthocyanidins – the entry step of anthocyanin biosyntheses. The resulting leucoanthocyanidins are subsequently converted into anthocyanidins by the action of anthocyanidin synthase (ANS), another 2-oxoglutarate-dependent dioxygenase. After completion of the anthocyanidin skeleton biosynthesis, anthocyanidins undergo glycosylation and acylation in a species-specific manner. The most common modification of anthocyanidins is 3-*O*-glycosylation (Harborne and Baxter 1999), which is catalyzed by flavonoid 3-*O*-glucosyltransferases (3GT). In general, the glycosylation and acylation of flavonoids play a role in increasing the stability, water solubility, and inter/intra-molecular stacking of flavonoids (Lim *et al*. 2003a; Grotewold 2006). Specifically, the multiple aromatic acylation of anthocyanin makes the pigment bluer, and this effect greatly depends on the position and number of aromatic acyl groups. Finally, anthocyanins are transported in vacuoles after species-specific modifications.

The 3'- and 5'-hydroxylation of the B-ring of flavonoids is catalyzed by microsomal cytochrome P450 enzymes (e.g., F3'H/CYP75B and F3',5'H/CYP75A). Importantly, the B-ring hydroxylation pattern is one of the major determinants of the color and hues of anthocyanin-based pigments. When the modifications on the anthocyanin molecule are equivalent, delphinidin-based pigments (with hydroxy groups at 3'-, 4'-, and 5'-positions) are bluer than cyanidin-based pigments (with hydroxy groups at 3'- and 4'-positions), which are bluer than pelargonidin-based pigments (with the 4'-hydroxy group). Roses and carnations are genetically devoid of F3',5'H genes and unable to produce cyanic delphinidin-based anthocyanins (Tanaka *et al*. 1998), thus lacking bluish flower color varieties. For instance, transgenic carnations and roses expressed heterologously with an F3',5'H gene of petunia (*Petunia hybrida*) and pansy (*Viola wittrockiana*), respectively, actually produced delphinidin-type anthocyanin pigments, resulting in successfully achieved bluish flower color (Tanaka *et al*. 2005, 2006).

AURONE, A YELLOW FLAVONOID

Aurone is a highly yellow-colored subclass of flavonoids with a 2-benzylidene-coumaranone structure ($C_{15}H_{10}O_2$) (Bate-Smith and Geissman 1951; Asen *et al*. 1972; Bohm 1994). The structure contains a five-membered heterocyclic ring, which is characteristic among flavonoid pigments (**Fig. 2A**). The name “aurone” comes from the Latin word “aurum (=gold)” because the pigments show golden yellow color (Bohm 1989). Aurone confers yellow bright color on flowers, such as snapdragon, cosmos, dahlia, linaria (*Linaria bipartite*), coreopsis (*Coreopsis grandiflora*), oxalis (*Oxalis variabilis*), limonium (*Limonium bonduellei*), and bidens (*Bidens pilosa*) (**Fig. 2B-D**) (Shimokoriyama and Hattori 1952; Harborne and Baxter 1999; Venkateswarlu *et al*. 2004). However, it has also been found in fern (*Asplenium kaulfussii*) and liverworts (*Marchantia berteroana* and *Conocephalum supradecompositum*).

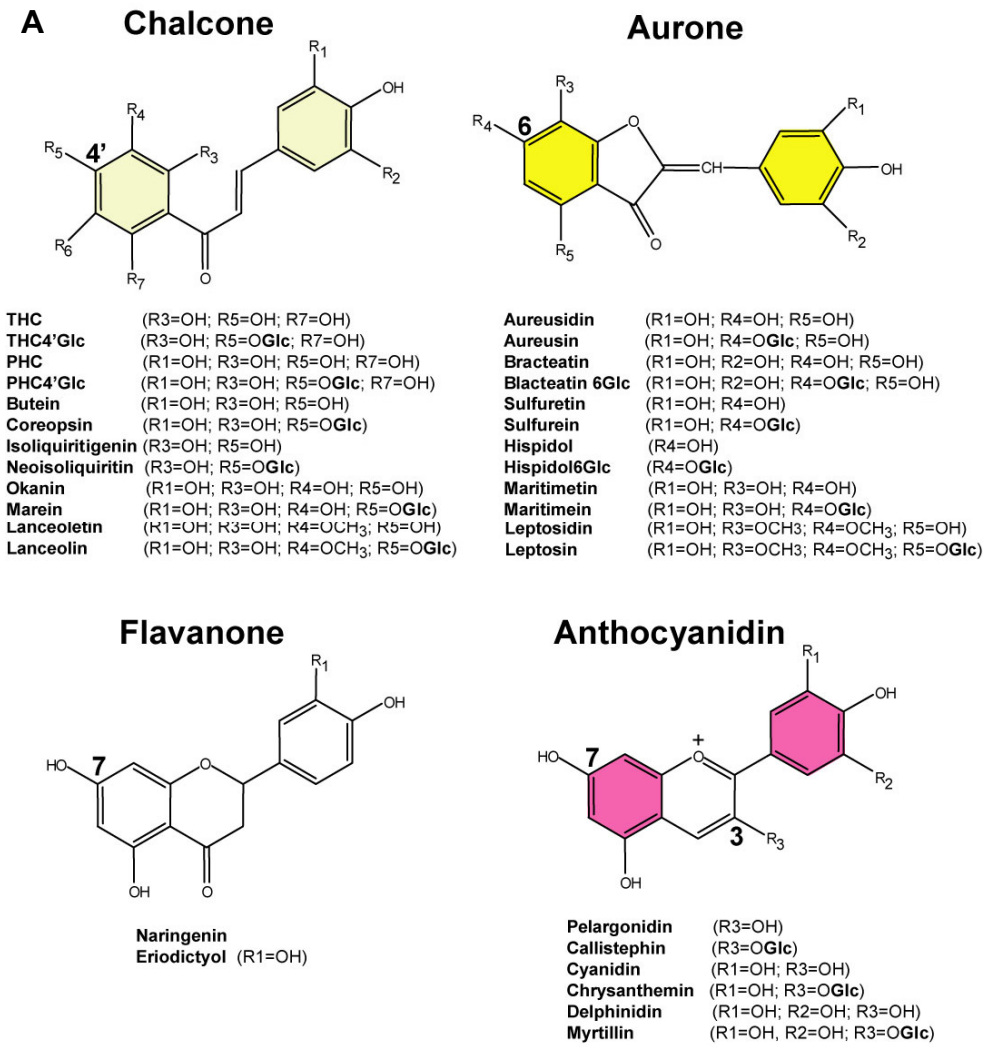
The aurone structures are classified on the basis of the position and number of hydroxy groups on aglycone (**Fig. 2A**). By the year 2000, 18 kinds of aurone aglycones, including aureusidin, bracteatin, sulfuretin, hispidol, and maritimetin and 27 kinds of their *O*-glycosides had been identified (Iwashina 2000). Aurones have at least two hydroxyl groups at the C6 and C4' positions that correspond to the C7 and C4' positions in flavanones/anthocyanins and to the C4' and C4 positions in chalcones, respectively. The aurone with the simplest structure is hispidol (6,4'-hydroxyaurone), which is found in soybean (*Glycine max*). Most naturally-occurring aurones are *O*-glycosylated at their C6 position, e.g., aureusidin 6-*O*-glucoside (aureusin), sulfurtin 6-*O*-glucoside (sulfurein), hispitol 6-*O*-glucoside, maritimetin 6-*O*-glucoside (maritimein), and leptosidin 6-*O*-glucoside (leptosin), indicating that 6-*O*-glycosylation is a common type of aurone modification, corresponding to the 3-*O*-glycosylation of anthocyanidin (Harborne and Baxter 1999). In most cases, the glucosyl group is used as a glycon part of aurone glycosides in higher plants, but, in liverworts and woody plants, rhamnosyl, glucuronosyl, and arabinosyl groups are also identified (Harborne and Baxter 1999).

Since aurone emits yellow-green fluorescence under UV light (Bohm 1989), it is considered to be involved in the fluorescent pattern formation on the petals, which likely serves as an attractant for pollinators (**Fig. 2E-H**) (Thompson and Meinwald 1972; Niovi-Jones and Reithel 2001; Gandia-Herrero *et al*. 2005). However, considering the occurrence of aurones in fern and liverworts, the earliest to colonize on land, the biosynthetic machinery of aurone 6-*O*-glycosides might predate flower evolution (Bahm 1994; Harborne and Baxter 1999; Iwashina 2000). Therefore, the primary physiological role of aurone might be to protect plants from damage from UV light rather than to attract pollinators (Rausher 2005).

Since major ornamental plant species, such as cyclamen and geranium, lack yellow varieties, the ability to confer aurone biosynthesis to those ornamentals would be of considerable commercial interest. In addition to flower coloration, it is also known that aurone possesses useful biological activities; therefore, it is an interesting natural compound from the viewpoint of medical and agricultural researches (Martin-Calero *et al*. 1996; Lawrence *et al*. 2003; Morimoto *et al*. 2007). However, the biosyntheses of aurones have been absolutely unknown for a long time. Thus, yellow is the last color of flavonoids whose mystery is yet to be unveiled.

GENETIC STUDIES OF AURONE BIOSYNTHESIS IN SNAPDRAGON AND COSMOS

Snapdragon is not only a popular garden plant but also a model plant that has made a significant contribution to the establishment of the genetic ABC model of floral organ development (Coen and Meyerowitz 1991). Snapdragon shows a wide variety of flower colors – magenta, orange, red, pink, yellow, cream, and white – which are expressed by a combination of anthocyanins, aurones, and flavones. Yellow and magenta are predominant colors of snapdragon in natural populations, probably due to pollinating bumblebee preference (Niovi-Jones and Reithel 2001; Whibley *et al*. 2006). Thus, snapdragon plays a crucial role in genetic studies on flower coloration (Efremova *et al*. 2006), unlike arabidopsis (*Arabidopsis thaliana*), which is a model plant



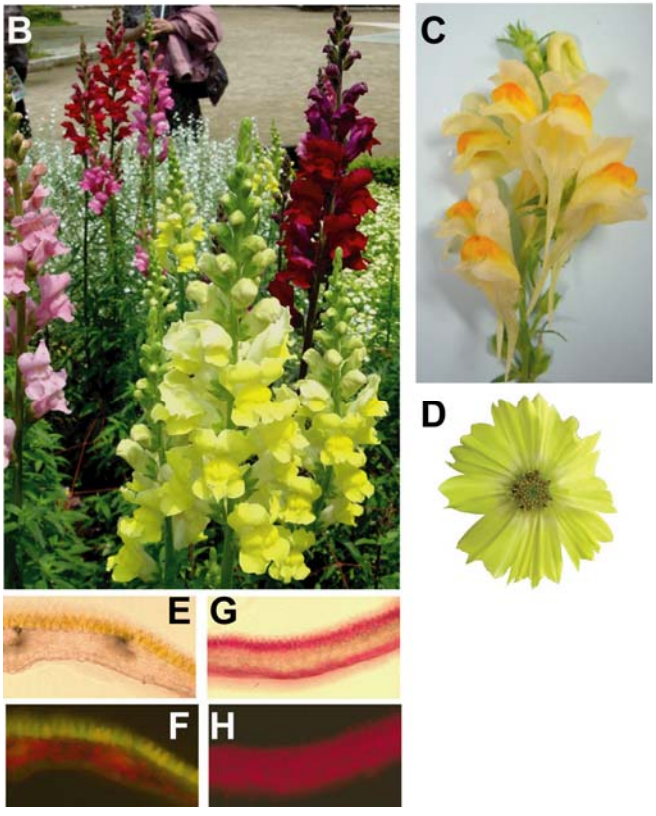


Fig. 2 Yellow flowers by aurones and structures of flavonoids. (**A**) Structures of chalcone, aurone, flavanone, and anthocyanin. The 4' position of chalcone is equivalent to the 6 position of aurone and the 7 position of flavanone/anthocyanin. (**B**) Snapdragon flowers. Yellow (aurone) and pink/magenta (anthocyanin) cultivars were grown at the Kyoto Botanical Garden in the spring of 2006. (**C**) Yellow linaria flowers. (**D**) Yellow cosmos flower of cultivar Yellow Garden (Sakata Seed Corporation Ltd. Kanagawa, Japan). Another yellow cosmos cultivar, Yellow Campus, is also available. Cross sections show fluorescence in the petal epidermal cell layer of an aurone-yellow cultivar (**E, F**), whereas no fluorescence is observed in an anthocyanin-magenta cultivar (**G, H**). (**F**) and (**H**) are fluorescent microscopic images.

producing only colorless flowers (Almeida *et al*. 1989; Luo *et al*. 1991; Martin *et al*. 1991; Schwarz-Sommer *et al*. 2003). The genetic aspects of aurone biosynthesis have also been intensively studied in this plant, whose yellow varieties abundantly produce aureusin (aureusidin 6-*O*-glucoside) and bracteatin 6-*O*-glucoside in the flower.

Genetic loci altering flower color of snapdragon have been genetically identified, and some of them were successfully cloned. *Nivea* (Niv), *Incolorata* (Inc), *Enosinea* (Eos), *Pallida* (Pal), and *Candica* loci are responsible for CHS, F3H, F3'H, DFR, and ANS, respectively (Martin *et al*. 1985; Martin and Gerats 1993). Generally, loss-of-function mutations of these structural genes give rise to acyanic (or colorless) flowers, or, in the case of *eos*, flowers produce only pelargonidin-type of anthocyanin pigments (Forkmann and Stotz 1981). The structural genes, including F3H, DFR, ANS, and 3GT, are positively regulated by the bHLH transcription factor *Delilla* (Del) and the MYB-class transcription factors *Rosea* (Ros) 1, 2 and *Venosa* with different specificities (Goodrich *et al*. 1992; Schwinn *et al*. 2006).

Snapdragon has, at least, two CHS-related genes – AmCHS1, which is identical to Niv, and AmCHS2, which is a homolog of type III polyketide synthase and has an unknown physiological function (Bonas *et al*. 1984; Hatayama *et al*. 2006). Since the recessive *niv* mutant results in a white flower, floral chalcone synthesis is responsible for AmCHS1. 3',4'-Hydroxylated flavonoids (luteolin, quercetin, and cyanidin) are formed in the presence of the dominant *Eos* allele (Forkmann and Stotz 1981). Interestingly, recombinant AmCHS1 produces 2',3,4,4',6'-pentahydroxychalcone (PHC), which is a 3',4'-hydroxylated chalcone, from one cafferoyl-CoA and three malonyl-CoA molecules as well as THC from one *p*-coumaroyl-CoA and three malonyl-CoA molecules *in vitro* (Hatayama *et al*. 2006).

Therefore, it is likely that AmCHS1 serves 3,4'-hydroxylated flavonoids. However, the recessive *eos* mutant lacks cyanidin, suggesting that PHC does not serve as a substrate for anthocyanin and flavone pathways.

Sulfurea (Sulf) is a major locus affecting the distribution of the yellow aurone pigment (Whibley *et al*. 2006). Dominant *Sulf* spatially restricts aurone biosynthesis in the region of the face and throat of flowers (Davies *et al*. 2006). It is, therefore, suggested that Sulf is likely to be a negative regulatory factor for aurone biosynthesis rather than a biosynthetic enzyme. Aurone biosynthesis conferred by recessive *sulf* is required for the *Niv* allele, but not for *Inc*, indicating that aurone is derived from chalcones. A full yellow color is tightly linked to a lack of anthocyanin, such as recessive *inc* and *ros* mutations, because the co-existence of aurone and anthocyanin in petal cells results in an 'anthocyanic' flower color (Forkmann and Stotz 1981; Spribille and Forkmann 1982; Whibley *et al*. 2006).

Violacea is another locus regulating aurone biosynthesis (Stubbe 1974). The recessive *violacea* mutant lacks aurone in the face region of the flower, suggesting that it is a region-specific positive regulator of aurone biosynthesis (Davies *et al*. 2006). Recently, a new recessive trait (designated CFR1011) altering aurone biosynthesis was identified from progenies of ethylmethanesulfonate (EMS)-mutagenized *sulf*/*inc* (Davies *et al*. 2006). CFR1011 specifically lacks aurone but not flavones. In the presence of a recessive *inc* mutation, CFR1011 flowers show a cream color that is conferred by flavones. Considering the genotypic and phenotypic behaviors of CFR1011, its corresponding gene is the most likely candidate for the structural genes of aurone biosynthesis. However, neither the CFR1011 nor the *Violacea* gene has been cloned.

Cosmos originally exhibit pink color in the peripheral ray florets, which is conferred by anthocyanin pigments. In contrast, they exhibit yellow color in the central tubular florets, which is conferred by yellow flavonoids, such as coreopsin, a chalcone 4'-*O*-glucoside, and sulphurein, an aurone 6-*O*-glucoside (Shimokoriyama and Hattori 1952; Harborne and Baxter 1999). After over 30 years of cross-breeding, a brilliant yellow cultivar, called Yellow Garden (Sakata Seed Ltd., Kanagawa, Japan), was successfully produced (**Fig 2D**; Inazu 1993). The yellow cultivar has coreopsin and sulphurein as its major yellow pigments in the mature ray florets. The accumulation of both of these yellow flavonoid glucosides is tightly linked to recessive mutations in (y) genes, suggesting that (Y) genes inhibit the yellow flavonoid biosyntheses in the ray florets (Inazu 1993). As seen in snapdragons, aurone and anthocyanin biosyntheses do not appear to alternate because both flavonoid pigments coexist in some cosmos cultivars. It is noteworthy that a recessive *c* locus causing a defect of ANS activity is required for full yellow expression in the ray florets (Inazu 1993). These previous genetic studies of snapdragon and cosmos suggest not only the negative regulations of aurone biosynthesis but also the requirement of depletion of anthocyanins for full yellow coloration.

IDENTIFICATION OF SNAPDRAGON AUREUSIDIN SYNTHASE

Although chalcones were thought to be precursors of aurones, the identity of the enzyme(s) responsible for aurone synthesis from chalcones was elusive. One mechanism proposed for aurone biosynthesis in plants (e.g., soy seedling) was a two-step pathway, in which an H_2O_2-dependent peroxidase-catalyzed oxidation of THC yields a 2-(α-hydroxybenzyl)coumaranone derivative, a hydrated form of aurone, which is then dehydrated to produce hispidol (**Fig. 3A**) (Wong 1967; Rathmell and Bendall 1972). However, the existence of such a pathway remained inconclusive, and no further characterization of aurone biosynthesis had been reported.

In 2000-2001, the metabolic relationship between aurones and chalcones in the yellow snapdragon flowers was clarified (Nakayama *et al*. 2000; Sato *et al*. 2001). In these flowers, the 6-*O*-glucosides of aureusidin and bracteatin occur as major and minor aurone components, respectively, along with small amounts of the 4'-glucosides of THC and PHC (Sato *et al*. 2001). Incubation of THC with the crude extract in the presence of H_2O_2, an enzyme activator, gives rise to aureusidin as the sole aurone product (**Fig. 3B**, arrow *a*), whereas incubation of PHC with the crude extract in the absence of H_2O_2 yields both aureusidin and bractea-

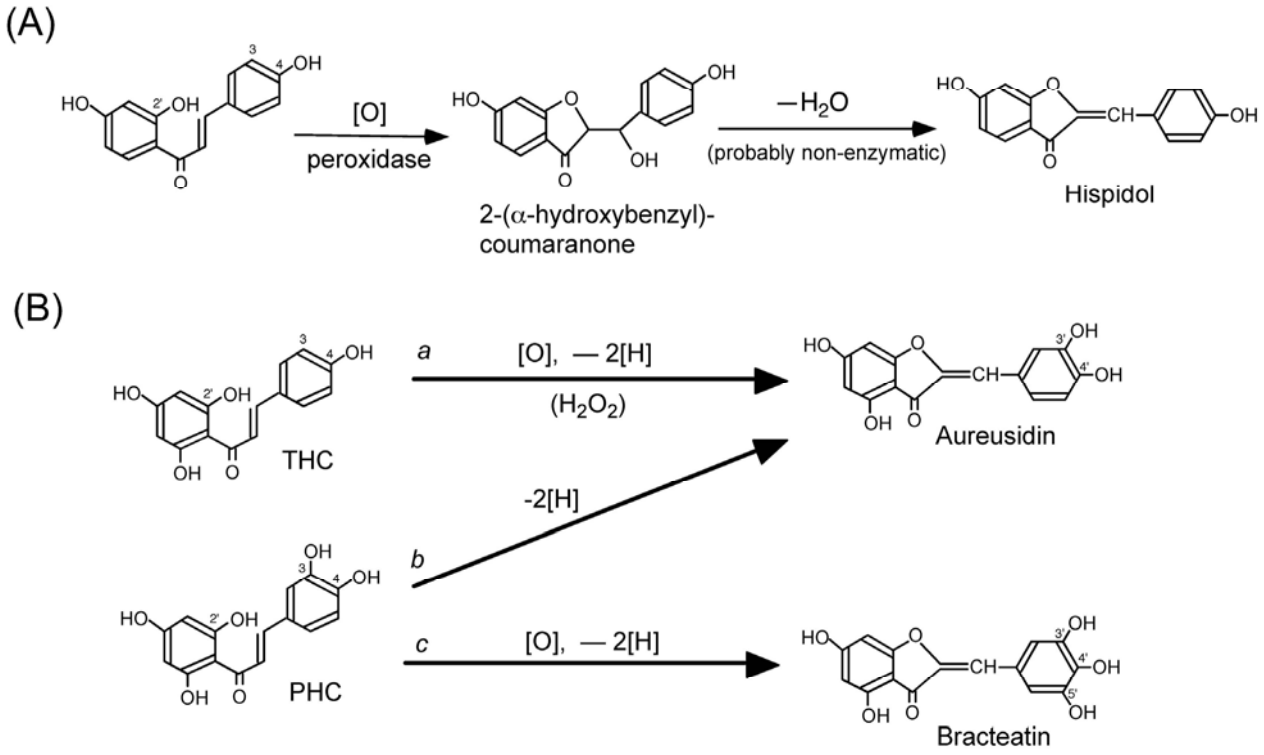


Fig. 3 Possible pathways for aurone biosynthesis from chalcones. (**A**) Proposed pathway of hispidol synthesis in *Phaseolus vulgaris* (Rathmell and Bendall 1972) and *Soja hispida* (Wong 1967). (**B**) Pathway for aurone biosynthesis in yellow petals of snapdragon. Each thick arrow, shown in *a*, *b*, and *c*, is a single enzymatic process and catalyzed by AS.

tin at a 6:1 ratio (mol/mol) (**Fig. 3B**, arrows *b* and *c*). The incubation of aureusidin with the crude extract does not yield bracteatin; therefore, bracteatin is not formed as a result of the 5'-hydroxylation of aureusidin. Thus, in yellow snapdragon flowers, aureusidin can be derived from either THC or PHC, whereas bracteatin arises solely from PHC (Sato *et al*. 2001). It was observed that the formation of the aurone structure (i.e., the oxidative cyclization of chalcone) is usually accompanied by hydroxylation of the B-ring moiety. This hydroxylation should not, however, take place by the action of F3'H, because snapdragon F3'H (the Eos product, see above) is unable to act on THC (Forkmann and Heller 1999). Several lines of evidence suggest that the formation of aurones from chalcones in the flower is a single enzymatic process, in which an enzyme, which we call aureusidin synthase (AS), catalyzes both of these transformations (Nakayama *et al*. 2000; Sato *et al*. 2001). This is inconsistent with the proposed peroxidase-catalyzed process of aurone synthesis (see above), which requires additional F3'H activity for the production of aureusidin from THC.

Attempts were then made to isolate this novel enzyme from the crude extracts of yellow snapdragon buds. A preliminary examination revealed that the amount of AS in the buds was unexpectedly small and the enzyme activity was very unstable. To overcome these difficulties, a large amount (32 kg) of snapdragon buds, which show the highest AS activity, was collected from 16,600 stems of snapdragon plants as starting materials for enzyme purification. In addition, THC was added to the purification buffers to enhance the enzyme stability. Finally, the enzyme (90 μg) was purified to homogeneity after 9 purification steps (Nakayama *et al*. 2000). It was a monomeric, copper-containing glycoprotein with a molecular mass of 39 kDa. Some other enzymological properties of the purified AS are summarized in **Table 1**. Specificity analyses show that the enzyme specifically acted on chalcones with a 4-monohydroxy or 3,4-dihydroxy B-ring to produce aurones (Nakayama *et al*. 2001). The analysis also suggests that the oxidative cyclization of chalcones should be preceded by B-ring hydroxylation. The 4'-*O*-glucosides of THC and PHC are very good substrates for the enzyme, showing twice-higher specific activities than the corresponding aglycons, even in the absence of added H_2O_2, an activator of the enzyme (Nakayama *et al*. 2001).

Table 1 Properties of aureusidin synthase purified from *A. majus* flowers.

Molecular mass	39 kDa (SDS-PAGE and gel filtration)
Subunit structure	Monomeric
Presence of sugar chain	Glycoprotein
Metal cofactor	Binuclear copper (atomic absorption spectrometry)
Activator	H_2O_2 (with THC as a substrate)
Inhibitor	Phenylthiourea (competitive; K_i, 1 μM)
Optimum pH for activity	pH 5.4 (THC), pH 6.8 (PHC)
Stability[a]	up to 40°C (at pH 5.4 for 30 min) pH 5 – 8 (at 30°C for 1 h or at 4°C for 24 h)
Substrate specificity	Highly specific for chalcones with both 2' and 4-hydroxy functions (see **Fig. 3B** for details).

[a] Unpublished results.

The cDNA cloning of AS was completed as follows.

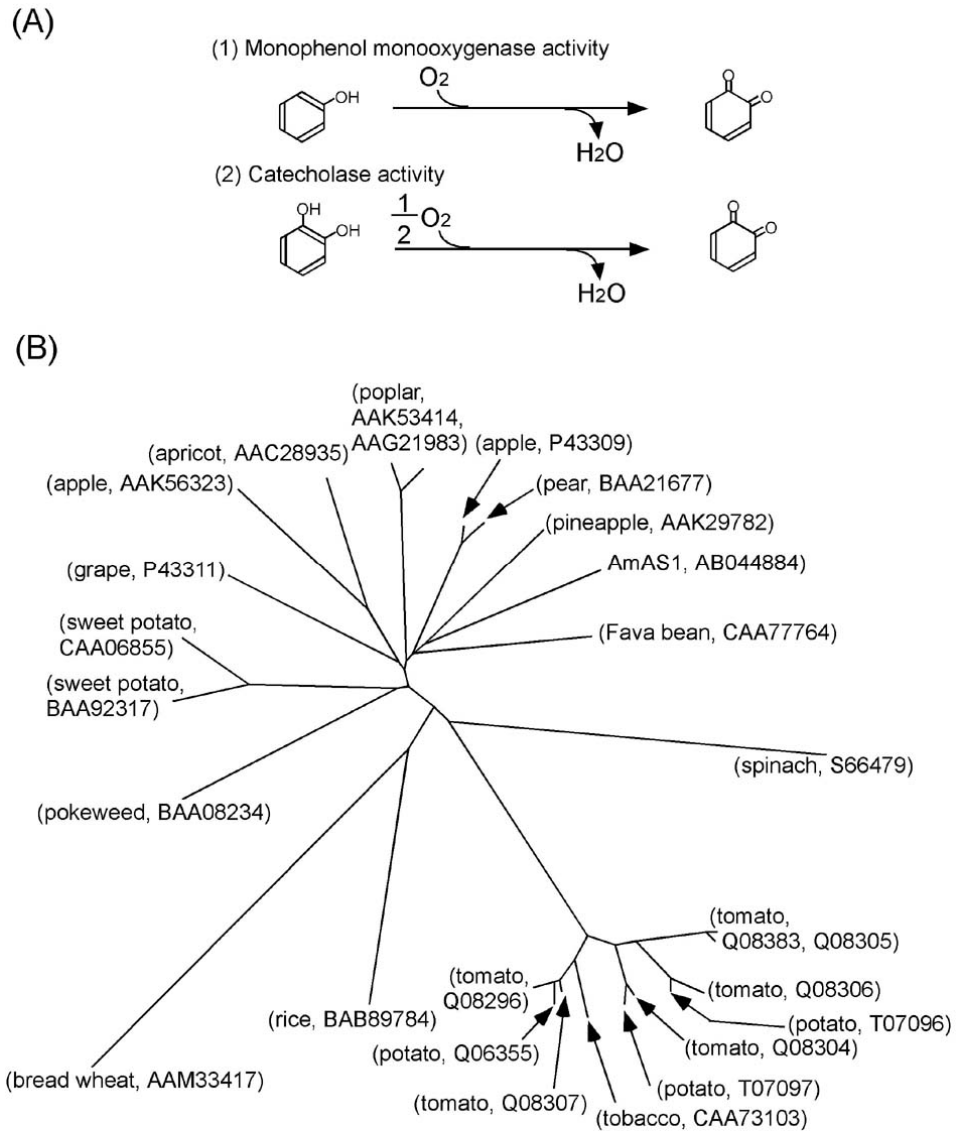


Fig. 4 Reactions and phylogenetics of plant PPOs. (**A**) PPO-catalyzed oxidation of phenol (phenol monooxygenase activity) and catechol (catecholase activity). (**B**) A molecular phylogenetic tree of plant PPO family proteins was constructed by the neighbor-joining method. The lengths of lines indicate the relative distances between nodes. Genbank/DDBJ accession numbers of PPO sequences are shown with plant names in parentheses.

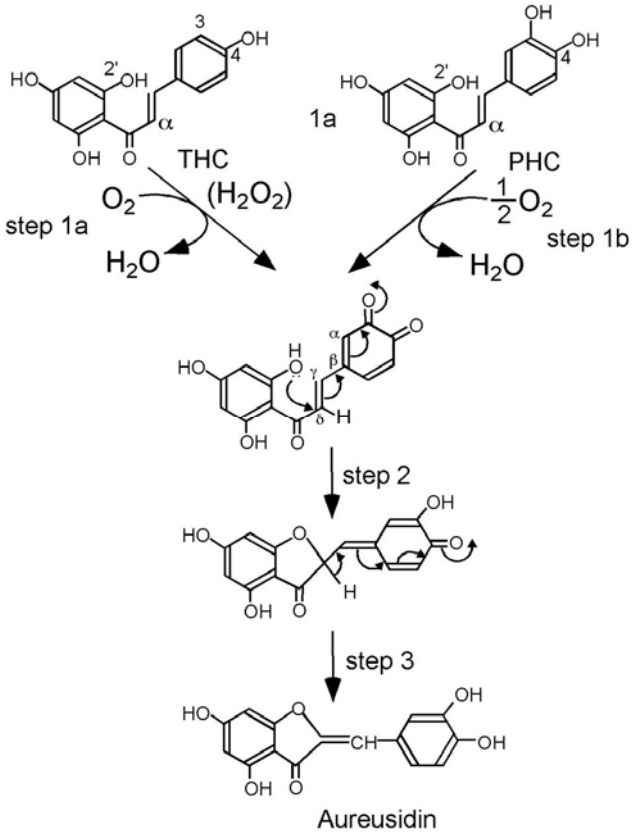


Fig. 5 PPO-catalyzed synthesis of aurones from THC and PHC. AS is a chalcone-specific homolog of PPO. Steps 1a and 1b are the PPO-catalyzed processes (see also **Fig. 4A**), whereas steps 2 and 3 may take place non-enzymatically. This mechanism consistently explains the facts that the formation of the aurone structure (i.e., the oxidative cyclization of chalcone) is usually accompanied by the hydroxylation of the B-ring moiety of THC and the oxidative cyclization of chalcones is preceded by B-ring hydroxylation (Nakayama *et al*. 2001).

Subtraction was performed to identify cDNA clones that were specifically expressed in the yellow petals but not in the aurone-lacking pink ones. Among many yellow-specific clones selected, the deduced amino acid sequence of one clone, termed *AmAS1*, was found to contain the partial amino acid sequences determined with the purified enzyme (Nakayama *et al*. 2000). Northern blot analyses using this clone as a probe show that *AmAS1* was specifically expressed in the aurone-containing yellow (or pink) petals, allowing us to confirm that *AmAS1* codes for AS (Nakayama *et al*. 2000).

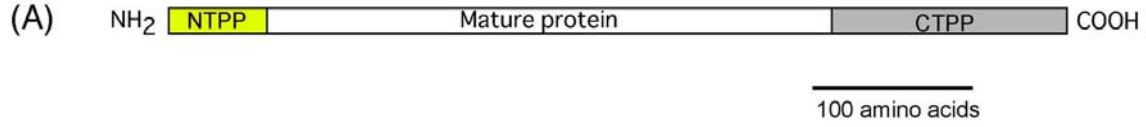
The primary structure analysis of AmAS1 revealed that AS is a homolog of plant polyphenol oxidase (PPO) (**Fig. 4**) (Nakayama *et al*. 2000). PPO occurs ubiquitously in higher plants and generally catalyzes the hydroxylation of monophenols to *o*-diphenols (monophenol monooxygenase activity; EC1.14.18.1) and the subsequent oxidation of *o*-diphenols to *o*-quinones [*o*-diphenol: oxygen oxidoreductase (also called catecholase) activity; EC1.10.3.1] (**Fig. 4A**) (Mayer 1987; Pourcel *et al*. 2007). In plant cells, these oxidation products are further reacted to give high-molecular-weight compounds and cause the browning of plant tissues (Vaughn *et al*. 1988). Plant PPOs are generally encoded as a precursor protein of 550-600 amino acids, which produces a mature form following proteolytic removal of the N-terminal propeptide (NTPP) of 80-100 amino acids and/or the C-terminal propeptide (CTPP) of approximately 150 amino acids (Vaughn *et al*. 1988). The molecular mass calculated from the deduced amino acid sequence of AmAS1 (56 kDa) is significantly higher than the value estimated with the purified AS (39 kDa), and this can be explained in terms of the proposed biogenesis of plant PPOs mentioned above (**Fig. 6A**). Plant PPOs contain a pair of type-3 copper atoms (Mayer 1987), and potential copper ligands are also identified in the amino acid sequence of AmAS1. Most importantly, the specificity and mechanism of AS-catalyzed aurone synthesis can be consistently described in terms of PPO-catalyzed reactions (**Fig. 5**) (Nakayama *et al*. 2001). Because AS cannot act on non-chalcone phenolics, it is concluded that AS is a chalcone-specific PPO, which is specialized for aurone biosynthesis in the snapdragon flowers. It must be mentioned that, according to this PPO-catalyzed mechanism of aurone synthesis, the product aurone must have a 3',4'-dihydroxy or 3',4',5'-trihydroxy B-ring. Consistently, nearly all aurones found so far in nature possess the 3',4'-dihydroxy or 3',4',5'-trihydroxy B-ring moiety or their *O*-substituted derivatives (Harborne and Baxter 1999). However, some aurones have been reported to have a B-ring moiety with no hydroxyl function or only one (Harborne and Baxter 1999) [e.g., hispidol found in soy seedlings (Wong 1967; Rathmell and Bendall 1972)], suggesting that these may be produced through an alternative mechanism (e.g., **Fig. 3A**), which would require further examination.

SUBCELLULAR LOCALIZATION OF AURONE BIOSYNTHESIS

It has been shown that PPOs are generally localized in plastids (or chloroplasts) in plant cells (Mayer 1987; Vaughn *et al*. 1988). However, AS shows several unusual features that argue against its localization in plastids. For example, although plant PPO precursors share the primary structural characteristics of NTPP (see above) that are targeted to plastids, NTPP of the AmAS1 protein does not share such features (**Fig. 6B**). Moreover, the fact that AS is a glycoprotein and displays the maximum activity at acidic pH (5.4) also contradicts its localization in plastids (Nakayama 2002).

The cellular localization of AS in the petal cells of yellow snapdragon was first analyzed by sucrose-density gradient centrifugation and differential centrifugation analyses (Ono *et al*. 2006a). The organelles of the cells of yellow petals of snapdragon were separated from each other on the basis of their isopycnic density, and a correlation between the distribution of aureusidin synthase with those of organelle markers was examined. AS activity was not correlated with the markers of plastids, mitochondria, Golgi bodies, or ER. The AS activity was detected in the lowest isopycnic-density fractions, suggesting that AS is localized in the cytosol or vacuoles of petal cells of yellow snapdragon. The localization of AS was then conclusively established by means of transient assays (Ono *et al*. 2006a), where green fluorescent protein (GFP) chimeras with the putative NTPP and CTPP of AmAS1 were expressed in the epidermal cells of onion (**Fig. 7**). The results clearly show that AS is localized in the vacuole lumen, and the information required for the vacuolar targeting of this PPO homolog is encoded within the 53-residue N-terminal sequence, but not in CTPP, of the precursor AmAS1 protein. The molecular and catalytic properties of AS, such as the optimum pH for catalytic activity and the occurrence of sugar chain(s) in the enzyme molecule, are all consistent with the vacuolar localization of this enzyme. Mutational analyses revealed that the sequence -Asn^{48}-Ser-Leu-Ala-Tyr^{52}- of NTPP serves as the sequence-specific vacuolar-sorting determinant of AmAS1 (Matsuoka and Neuhaus 1999; Ono *et al*. 2006a). Moreover, it was shown by means of two independent approaches that intracellular translocation of the AmAS1 precursor to vacuoles takes place through ER-to-Golgi trafficking (Ono *et al*. 2006a). On the basis of the results presented above, the subcellular compartmentation of the aurone biosynthetic pathway in petal cells of snapdragon flowers is proposed to be as shown in **Fig. 8** (Ono *et al*. 2006a).

AS is able to utilize both 4'-glucoside and aglycon forms of chalcones, and it remained to be determined which form serves as the physiological substrate of the enzyme in petal cells. The establishment of the cellular localization of



(B)

```
AmAS1     M--FKNP--- -NIRYHKLSS ---KSND--- --NDQESSHR C---KHILLF I---I---TL  37
Grape     MASL-PWSLT TSTAIANTTN ISAFPPSPLF QRASHVPVAR NRSRRFAPSK VSCNSANGDP  59
Pokeweed  MASLSSPPTS TTTSATTLAT ---NISNPLF PKTSQFSLPG CRNRRHVMPK ISCKVDKDEL  57

AmAS1     F--------- ---------- -LL-IVGLYI ANSLAYAR-F ASTSTGPIAA PDVTKCGQP-  74
Grape     NSDSTSDVRE TSSGKLDRRN VLLGIGGLYG AAGGLGATKP LAF-GAPIQA PDISKCGT-A 117
Pokeweed  YPKTTN--DD GNTNKLDRRN MLIGLGSIYG A-GTTLGGGP A-TLAAPLIT PNPT-EFGAS 112
```

Fig. 6 Propeptides of AmAS1. (**A**) Organization of prosequences [NTPP (yellow rectangle) and CTPP (gray rectangle)] and mature protein (white rectangle) of AmAS1. (**B**) Alignment of the N-terminal amino acid sequence of AmAS1 with those of the plastid PPO precursors [Grape, PPO of *Vitis vinifera*, Genbank accession number S52629; Pokeweed, PPO of *Phytolacca americana*, BAA08234]. Hydroxylated amino acid residues in the 30-residue N-terminal portion are shown in blue. Amino acid residues identical to those of AmAS1 are shown in red. The N-terminal processing site identified in the PPO precursors is indicated by a vertical line in the grape PPO sequence. Sequences representing the "thylakoid transfer domain" (Joy *et al*. 1995) of plastid PPOs are shown with green boxes. The putative sequence-specific vacuolar-sorting determinant of AmAS1 is double-underlined.

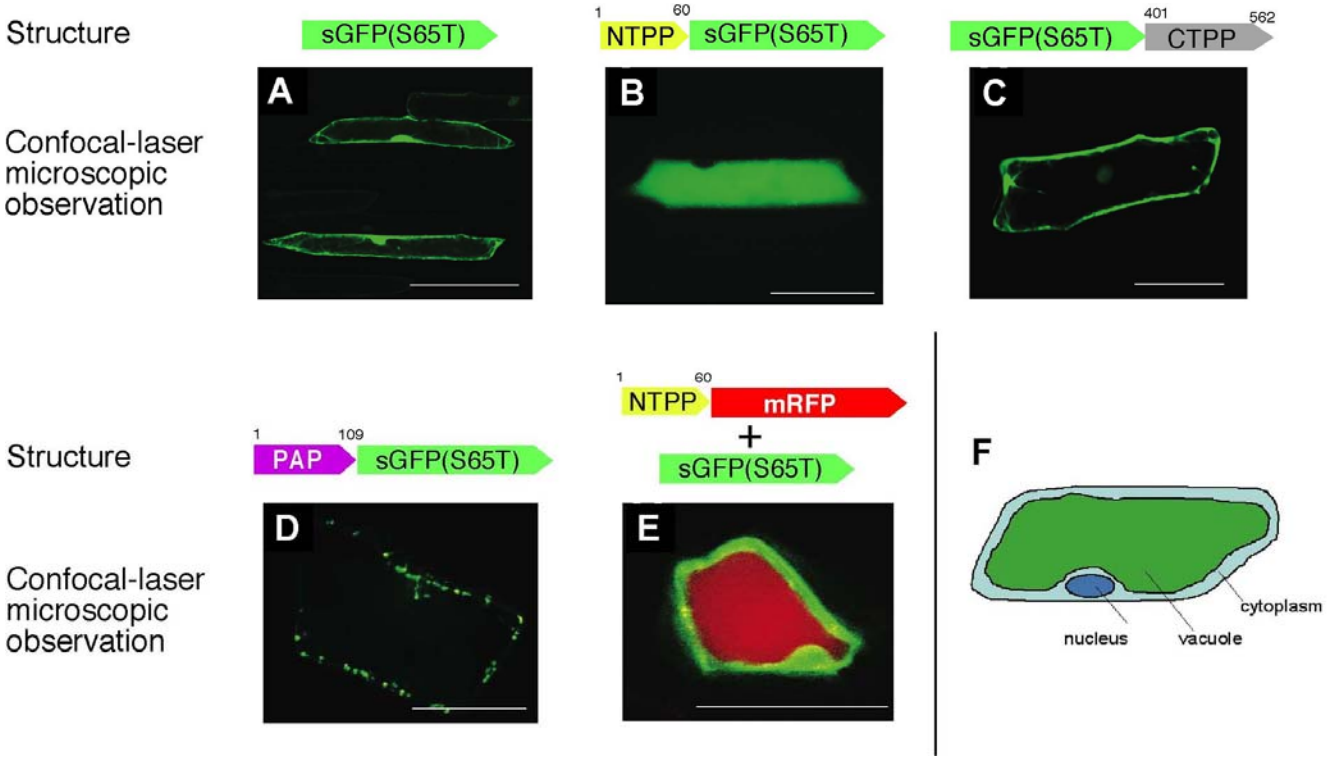


Fig. 7 Results of transient assays for the prosequence-dependent intracellular sorting of fluorescent protein chimeras. At 48 h after the particle-gun bombardment of a plasmid encoding the chimeric protein on epidermal cells of onion (panels A-D) or petal cells of snapdragon (panel E), transformed cells were observed by a confocal-laser fluorescence microscopy. The structures of the expressed chimeric proteins are shown above the panels, with pentagonal boxes representing NTPP of AmAS1 (yellow), sGFP(S65T) (green), CTPP of AmAS1 (gray), NTPP of PAP1 (purple), and mRFP (red), each shown with an N terminus (left) → C terminus (right) orientation. The portion of NTPP and CTPP utilized is shown, above boxes, by amino acid numbering in the corresponding PPO precursors (see also **Fig. 6** for details). (**A**) In the absence of the fused NTPP or CTPP, the expressed GFP was localized in the cytoplasm and nuclei of the cells. (**B**) When an N-terminal fusion of GFP with NTPP was expressed, the fluorescence signal was observed in vacuoles, indicating that the expressed GFP was exclusively localized in the vacuole lumen. (**C**) When a C-terminal fusion of GFP with CTPP was expressed, the fluorescence signals appeared to be localized in the cytoplasm and nuclei of the cells. (**D**) When a GFP chimera with an NTPP of *Phytolacca americana* (pokeweed) PPO (a plastid enzyme) (Joy *et al*. 1995) at the N-terminus of GFP was expressed, the fluorescence signal was found to be localized in granular organelles (probably plastids) distributed in the cytoplasm. (**E**) Similar transient assays were also carried out with petals of yellow snapdragon as host cells by using the red fluorescent protein (RFP) as a fluorogenic reporter, and the results were analogous to those obtained with onion epidermal cells (panel **B**). Panel (**F**) shows a schematic representation of the epidermal cells of onion. Bars, 100 μm for panels (**A**)~(**D**), and 20 μm for panel (**E**).

AS in vacuoles provides important information relative to the real *in vivo* substrate of the enzyme as well as the biochemical significance of the cellular compartmentation of the metabolic pathway. To date, flavonoid biosynthesis has been proposed to generally occur in the cytoplasm or on the cytoplasmic surface of ERs (Burbulis and Winkel-Shirley 1999). In cells of yellow snapdragon flowers, the biosynthesis of chalcones (THC and PHC and their 4'-*O*-glucosides) should also take place in the cytoplasm. The cytoplasmic milieu (pH~7) entails a very rapid enzymatic and non-enzymatic isomerization of chalcones to produce flavanones (Sato *et al*. 2001) (see also **Fig. 8**), which do not serve as substrates for AS (Nakayama *et al*. 2001). Thus, for the efficient accumulation of aurones in the flower, a

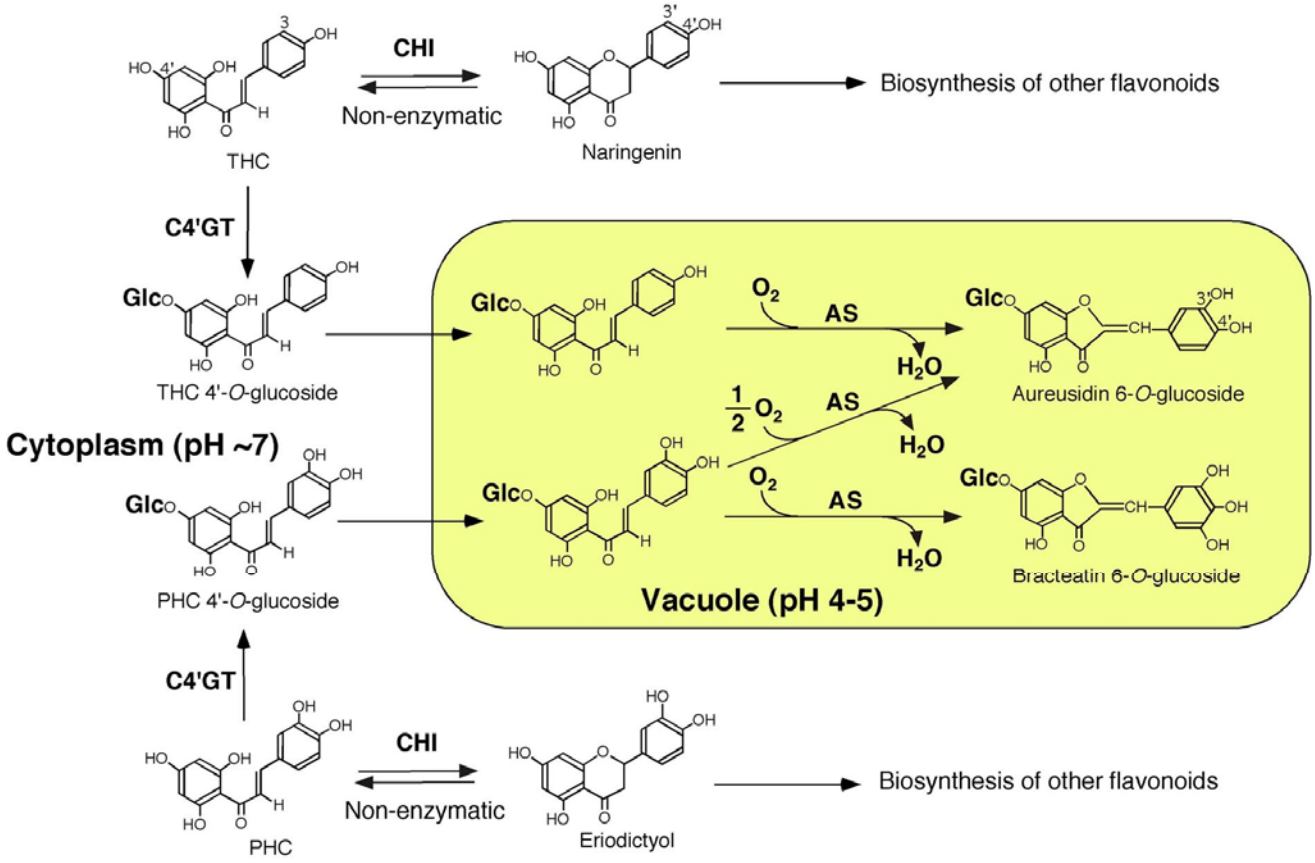


Fig. 8 Proposed intracellular compartmentation of the aurone biosynthetic pathway in petal cells of snapdragon flowers. Synthesis, glycosylation, and isomerization of chalcones may take place on the cytoplasmic surface of the ER. Aureusidin can be produced from either THC or PHC, whereas bracteatin arises solely from PHC. Moreover, the 4'-*O*-glucosides of these chalcones serve as very good substrates for enzymatic aurone synthesis (Sato *et al*. 2001; Nakayama *et al*. 2001). AS, aureusidin synthase; CHI, chalcone isomerase; and Glc, β-D-glucopyranosyl. This figure was reproduced from Ono *et al*. (2006a) *The Plant Journal* **45**, 133-143, with kind permission of Blackwell Publishing, Oxford, UK.

mechanism that circumvents such a non-productive pathway must be operative. The compartmentation of AS in vacuoles could serve as one such strategy. Namely, in the vacuole, the isomerization of chalcones would be very slow due to its acidic milieu (pH 4~5) as well as the absence of chalcone isomerase (CHI). It is generally accepted that flavonoids are glycosylated before translocation from the cytoplasm to the vacuole (Tanaka *et al*. 2005). Thus, in petal cells of snapdragon flowers, chalcones, once produced, should be 4'-*O*-glucosylated in the cytoplasm and immediately incorporated into vacuoles. The chalcone 4'-*O*-glucosides would be subsequently oxidized in vacuoles by the action of AS, resulting in the accumulation of aurone 6-*O*-glucosides therein (**Fig. 8**). Therefore, the real *in vivo* substrates for AS appear to be the 4'-*O*-glucosides of chalcones and not the unglycosylated forms. Thus, aurone biosynthesis in snapdragon provides the first example of the biosynthesis of a flavonoid skeleton in the vacuole lumen after glycosylation. An interesting issue to be addressed in future studies would be how this PPO has acquired its unique subcellular localization and biological role (flower coloration) during its evolution.

IDENTIFICATION OF SNAPDRAGON CHALCONE 4'-*O*-GLUCOSYLTRANSFERASE

AmAS1 is a vacuolar PPO, so that aurone biosynthesis should occur in vacuoles of snapdragon petals. Consequently, the substrate chalcones have to be transported, from cytoplasm, to the lumen of vacuoles in petal cells. One key event required for the translocation of plant metabolites into vacuoles is their "glycosylation". Glycosylation is a major modification of endogenous and xenobiotic compounds that enhances their water solubility and stability and facilitates their incorporation into vacuoles (Jones and Vogt 2001; Bowles *et al*. 2005; see also Section FLAVONOID BIOSYNTHESIS AND FLOWER COLOR MODIFICATION). Glycosylation is generally catalyzed by a superfamily of enzymes, called UDP-dependent glycosyltransferases (UGTs) (Ross *et al*. 2001), which consists of highly divergent members in terms of primary structure and physiological function. For example, the *Arabidopsis* genome is predicted to contain over 100 UGT-related sequences, most of whose physiological roles remain to be clarified (Li *et al*. 2000; Ross *et al*. 2001). In the case of aurone biosynthesis in snapdragon flowers, chalcones have to be specifically glucosylated at the 4' position for their translocation to vacuoles. Consistently, small amounts of chalcone 4'-*O*-glucosides were identified in yellow snapdragon petal cells (see Section IDENTIFICATION OF SNAPDRAGON AUREUSIDIN SYNTHASE, Sato *et al*. 2001). In addition to snapdragon, chalcone 4'-*O*-glycosides are also found in various plant species (Harborne and Baxter 1999), suggesting that 4'-*O*-glycosylation is a common modification of chalcones. However, no chalcone 4'-*O*-glycosyltransferase had been identified.

Chalcone 4'-*O*-glucosyltransferase (C4'GT) activity was detected in the crude extracts of yellow snapdragon buds (Ono *et al*. 2006b). In order to identify snapdragon C4'GT, an extensive screening of its cDNA was carried out on a snapdragon petal cDNA library, and a strong candidate of the C4'GT cDNA was identified (Accession AB 198665). The expression of this cDNA, termed *UGT88D3* according to the UGT nomenclature guidelines, paralleled that of *AmAS1* during flower development and, thus, UGT 88D3 was expected to be involved in yellow flower coloration. The UGT88D3 is 40% identical to a UGT of *Arabidopsis* (UGT88A1; function unknown) in its primary structure and contains a sequence motif (called "PSPG" box) that is highly conserved among plant secondary metabolism-

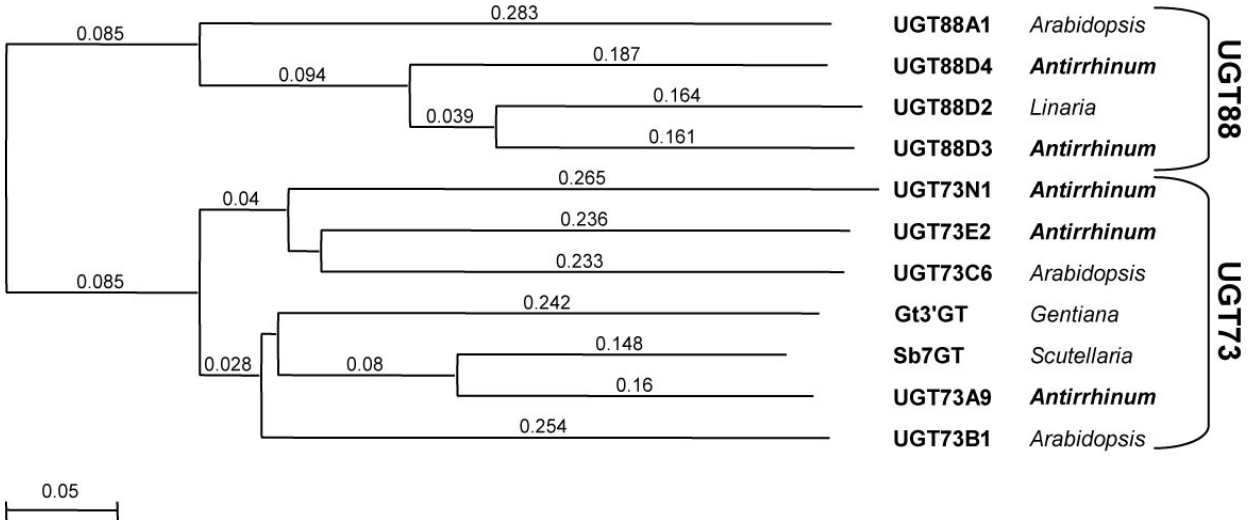


Fig. 9 Phylogenetic analysis of UGT88 and UGT73 family. A phylogenic tree was constructed by the neighbor-joining method. The number on the branches indicates a sequence difference (0.05 corresponds to a 5% change). Gt3'GT is *Gentiana triflora* anthocyanin 3'-*O*-glucosyltransferase (Fukuchi-Mizutani *et al*. 2003). UGT88D4 is a snapdragon UGT whose biochemical activity remains to be determined (Ono *et al*. 2006b).

related UGTs (i.e., plant members of family 1 of the UGT superfamily; Vogt and Jones 2000). As expected, recombinant UGT88D3 that was heterologously expressed in *Escherichia coli* cells displayed C4'GT activity against THC and PHC (Ono *et al*. 2006b). Noticeably, the calculated pI value (6.82) of UGT88D3 is uniquely higher than those of other snapdragon UGTs (4.73-5.89), which is consistent with the unique chromatographic behavior of the native AmC4'GT activity in the crude extract of yellow snapdragon petals – the activity was exclusively identified in the flow-through fractions of anion-exchange chromatography executed at neutral pH (Ono *et al*. 2006b). All of these observations led to the conclusion that UGT88D3 is responsible for the 4'-*O*-glucosylation of chalcones and is involved in aurone biosynthesis in the yellow petal cells of snapdragon.

The UGT88D3 was the first characterized member of this UGT88 family, and the specificity and biochemical role of this class of enzymes are further corroborated by the following observations. Yellow petals of linaria (a Scrophulariaceae plant) also contain aurone 6-*O*-glucosides (aureusin and bracteatin 6-*O*-glucoside) (**Fig. 2A, 2C**) (Harborne and Baxter 1999). A UGT88D3 homolog (UGT88D2: accession AB198666) was identified in the yellow petals of linaria by means of a homology-based strategy using the UGT88D3 sequence (unpublished data). The UGT88D2, which is 65% identical to UGT88D3 in its primary structure (**Fig. 9**), shows a high pI value (6.20) and displays C4'GT activity. Thus, C4'GT genes appear to be structurally and functionally conserved among the aurone-containing Scrophulariaceae plants.

It must be mentioned that the 7-position of flavones, flavonols, and anthocyanins, and the 6-position of aurones, are all equivalent to the 4'-position of chalcones (see **Fig. 2A**). In this regard, the UGT73 family of enzymes appears to be similar to the UGT88 family of enzymes in terms of the regiospecificity of glycosyl transfer. For example, *Arabidopsis* UGT73C6 catalyzes the 7-*O*-glucosylation of flavonol 3-*O*-rhamnoside (Jones *et al*. 2003). *Arabidopsis* UGT73B1 is 40% identical to UGT73C6 in its primary structure and was recently characterized as flavonoid 7-*O*-glucosyltransferase (7GT) (Kim *et al*. 2006). Moreover, *Scutellaria baicalensis* flavone 7GT is 42% identical to *Arabidopsis* UGT73C6 and likely to be a member of the UGT73 family of enzymes, judging from the sequence similarity (Hirotani *et al*. 2000; Nagashima *et al*. 2000). During the course of our screening program of cDNAs encoding snapdragon UGT, three UGT73 family genes (*UGT 73A9*, *UGT73E2*, and *UGT73N1*) were found to be expressed in snapdragon petals (**Fig. 9**) (Ono *et al*. 2006b; unpublished results). Because snapdragon flowers accumulate large amounts of flavone 7-*O*-glucosides, some of these snapdragon UGT73 proteins may be involved in the 7-*O*-glucosylation of flavones (Asen *et al*. 1972). It is noteworthy that UGT73A9 is identical to a UGT that was originally regarded as an aurone 6-*O*-glucosyltransferase (Davies *et al*. 2006). However, considering the established vacuolar localization of aurone synthesis in yellow snapdragon petal cells, it is highly unlikely that UGT73A9 catalyzes the 6-*O*-glucosylation of aurones in vacuoles because UGT73A9 is likely to be a cytoplasmic enzyme, judging from its primary structural characteristics.

It is generally accepted that each flavonoid 3-*O*-glucosyltransferase (3GT), 5-*O*-glucosyltransferase (5GT), and 7GT separately forms a phylogenic clade in the family 1 of a UGT superfamily, illustrating the correlation between phylogenetics and biochemical function (i.e., glycosyl-acceptor specificity and regiospecificity) of flavonoid UGTs (Gachon *et al*. 2005; Tohge *et al*. 2005; Yonekura-Sakakibara *et al*. 2007). However, despite the similarity of the C4'GTs to the previously known flavonoid 7GTs in the regiospecificity of glycosyl transfer, UGT88D3 shares only 22%, 24%, and 24% sequence identity with UGT73C6, UGT73B1, and *S. baicalensis* 7GT, respectively, indicating that C4'GTs and these flavonoid 7GTs are distantly related to each other in phylogenetic terms. Therefore, these two UGT families seem to be good examples for addressing the relationship among the substrate specificity, regioselectivity, and structure of UGTs (Lim *et al*. 2003b). Further crystallographic and biochemical studies using these UGTs and mutants will provide important information to address this issue (Offen *et al*. 2006).

MOLECULAR BREEDING OF YELLOW FLOWERS

Yellow is the long-awaited character for major ornamental flowers lacking this color – geranium, cyclamen, sweet pea (*Lathyrus odoratus*), verbena (*Verbena hybrida*), campanula (*Campanula medium*), saintpaulia (*Saintpaulia ionantha*), impatiens (*Impatiens walleriana*), hydrangea (*Hydrangea macrophylla*), and morning glory (*Ipomoea nil*). These plant species seem not to have acquired or to have lost the machinery of yellow pigment biosyntheses during plant evolution. Aurone biosynthetic genes have been clarified and now serve as promising tools to generate yellow varieties of these ornamental flowers. As the first step, transgenic plants heterologously expressing *AmAS1* were generated. Torenia (Scrophulariaceae *Torenia hybrida* cv. Summerwave Blue, Suntory Flowers, Ltd., Tokyo, Japan) was used as the host plant because this plant is closely

Fig. 10 Phenotype of triple transgenic *Torenia* producing aurone 6-*O*-glucoside. Transgenic lines of torenia coexpressing *UGT88D3* (AmC4'GT) and *AmAS1*, with the knockdown of endogenous *F3H* by RNAi, produce yellow flowers in which aurone 6-*O*-glucoside is dominantly produced due to the suppression of anthocyanin biosynthesis (right). The host plant for transformation is the torenia cultivar Summerwave blue (Suntory Flowers, Ltd., Tokyo, Japan; left). ox; overexpression.

related to snapdragon (also Scrophulariaceae) and has been used for many transgenic studies due to the availability of the well-improved *Agrobacterium* method for its transformation, unlike snapdragon (Aida *et al*. 2000). The transgenic lines of torenia expressing *AmAS1* did not produce any aurones, and their flower color was not changed (Ono *et al*. 2006b). These observations were consistent with our conclusion that 4'-*O*-glucosides, but not the non-glycosylated form of chalcones, are the real substrates for AS *in vivo* (see above). Transgenic lines of torenia co-expressing *UGT88D3* and *AmAS1* were then generated. The resulting flowers displayed a yellow color on their stamens and carpels and in the region between petal robes and throat and emitted significant cellular fluorescence (Ono *et al*. 2006b). The analysis showed that significant amounts of aureusidin 6-*O*-glucoside (aureusin) as well as THC 4'-*O*-glucoside accumulated in their petals, with a slight decrease in the amounts of anthocyanins and flavones. This result clearly demonstrated that coexpression of *UGT88D3* and *AmAS1* is sufficient to produce aurone 6-*O*-glucoside *in vivo* (Ono *et al*. 2006b). Moreover, the UGT88D3-GFP chimera protein localizes in the cytoplasm, where chalcones are synthesized, whereas AmAS1-mRFP chimera protein localizes in the vacuole of snapdragon petal cells (Burbulis and Winkel-Shirley 1999; Ono *et al*. 2006b). Therefore, it is strongly emphasized that the glucosylation of chalcones facilitates the vacuolar transport of chalcones.

However, the transgenic flowers coexpressing *UGT 88D3* and *AmAS1* did not show a full yellow color due to the coexistence of aurones and anthocyanins in petals (Ono *et al*. 2006b), which is consistent with the fact that the biosynthesis of aurones does not alternate with those of anthocyanins, as shown by previous genetic studies of aurone biosynthesis in snapdragon and cosmos (see above). Thus, in order to generate entirely yellow torenia flowers, the production of anthocyanins in the flower has to be suppressed. To achieve this, triple transgenic lines of torenia were designed, where *UGT88D3* and *AmAS1* were co-expressed and one of the endogenous anthocyanin biosynthetic genes was suppressed by RNA interference (RNAi). CHI is not a suitable target of RNAi for the inhibition of anthocyanin biosynthesis because flavanones, a precursor of anthocyanins, can inevitably be derived from THC in cytoplasmic *milieu* (Forkmann and Dangelmayr 1980; Davies *et al*. 1998; see above). Instead, F3H, which is an enzyme immediately downstream of CHI in the pathway and catalyzes the conversion of flavanone to dihydroflavonol (see Section FLAVONOID BIOSYNTHESIS AND FLOWER COLOR MODIFICATION), was a good target, judging from the occurrence of the anthocyanin-less phenotype of snapdragon with a single recessive *inc* mutation. In fact, single transgenic lines of torenia with RNAi of F3H showed a decreased level of endogenous *F3H* mRNA, resulting in white flowers (Ono *et al*. 2006b). Thus, triple transgenic lines of torenia were generated, where *UGT 88D3* and *AmAS1* were co-expressed and F3H was specifically suppressed by RNAi. The resulting transgenic lines successfully produced fully yellow flowers with enhanced cellular fluorescence (**Fig. 10**) (Ono *et al*. 2006b). These flowers contained only negligible amounts of anthocyanins but produced approximately twice the amount of aureusidin 6-*O*-glucoside as flowers of the double-transgenic lines. These results, in turn, corroborate that aurones, anthocyanins, and flavones are all derived from the same pool of chalcones. Importantly, the inhibition of endogenous anthocyanin biosyntheses allows the efficient utilization of the precursor chalcone for aurone biosynthesis, causing the enhancement of yellow flower color in addition to the elimination of the cyanic color from the flower. Fully yellow torenia flowers were also generated by means of specific inhibition of endogenous DFR by RNAi in transgenic lines co-expressing *UGT88D3* and *AmAS1* (Ono *et al*. 2006b). Moreover, the fact that the RNAi of ANS effectively inhibits anthocyanin accumulation in torenia to result in white flower color (Nakamura *et al*. 2006) implies that ANS can also serve as a target of RNAi for the molecular breeding of yellow flowers, in addition to F3H and DFR. Generally, the strength of coloration is related to the total amount of pigments. Soybean *CHS7* and *CHS8* are thought to be the most likely key enzyme genes for increased isoflavone production in soybean seeds (*Glycine max*) (Dhaubhadel *et al*. 2007). In this context, the increase of CHS activity may also effectively contribute molecular breeding of yellow flowers via increased production of aurone 6-*O*-glucosides.

It would be noteworthy that the accumulation of chalcones in the flower has also been regarded as a strategy for molecular breeding of yellow flowers because chalcones themselves are pale yellow in color and occur in almost all plant species (Tanaka *et al*. 2005). However, breeding of yellow flowers by means of this approach has been attained with only limited success, mainly because of biochemical instability of chalcones – they are easily isomerized, both

enzymatically and non-enzymatically, to produce colorless flavanone (Davies *et al*. 1998; see above). Transgenic torenia expressing *UGT88D3* showed the accumulation of THC 4'-*O*-glucoside (but not PHC 4'-*O*-glucoside, probably due to lack of PHC in this torenia flower) (Ono *et al*. 2006b). However, no color changes occurred in the transgenic flowers. 2'-*O*-Glucosylation of chalcones is considered to inhibit their isomerization, and, in fact, chalcone 2'-*O*-glucoside stably exists in the flowers of carnation and aster (*Callistephus chinensis*) and is responsible for their yellow color (Forkmann and Dangelmayr 1980; Harborne and Baxter 1999). Carnation chalcone 2'-*O*-glucosyltransferase (C2'GT) genes appear to be a promising tool for the molecular breeding of yellow flowers; however, no transgenic flowers expressing *C2'GT* have yet been reported (Ogata *et al*. 2004). It is also considered that 6'-deoxychalcones, found in Asteraceae and Fabaceae, are stable species of chalcone (Davies *et al*. 1998). The biosynthesis of 6'-deoxychalcones requires chalcone reductase (CHR) (Bomati *et al*. 2005), which is an aldoketo reductase superfamily enzyme. In fact, it was observed that the glycosides of 6'-deoxychalcones stably occur in yellow flowers of cosmos, bidens, dahlia, and coreopsis (Harborne and Baxter 1999). Therefore, the CHR gene was also expected to serve as an attractive tool for the molecular breeding of yellow flowers. However, the transgenic petunia expressing alfalfa (*Medicago sativa*) *CHR* results in only faint yellow buds in spite of the accumulation of 6'-deoxychalcone glucosides (Davies *et al*. 1998).

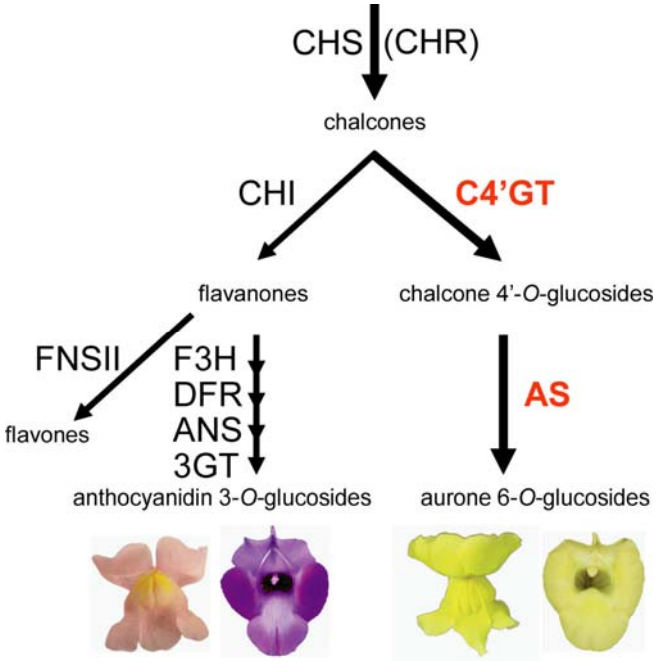


Fig. 11 Proposed aurone biosynthetic pathway. The 4'-*O*-glucosylation of chalcone by cytosolic C4'GT followed by oxidative cyclization by vacuolar AS is the biochemical basis of aurone 6-*O*-glucoside biosynthesis *in vivo*. Chalcone is the branching point to aurone pathway from the flavone/anthocyanin pathway.

PERSPECTIVES

In the cells of snapdragon petals, the flavonoid biosynthetic pathway is partitioned at chalcones into aurone and anthocyanin/flavone biosyntheses, and CHI catalyzing the first committed step of anthocyanin/flavone biosynthesis should compete for the substrate with C4'GT catalyzing the first committed step of aurone biosynthesis (**Fig. 11**). In the cells of arabidopsis seedlings, it was suggested that CHI should directly interact with CHS to form a complex, a metabolon, which allows the instantaneous, stereospecific isomerization of chalcones to the (*2S*) isomer of flavanones, thereby preventing the non-enzymatic formation of a non-productive (*2R*) isomer (Burbulis and Winkel-Shirley 1999; Jorgensen *et al*. 2005). Such metabolic channeling through the formation of metabolon among flavonoid biosynthetic enzymes has also been predicted for other plant species. It is known that chalcone accumulation in yellow varieties of carnation occurs as a result of the loss of CHI (Forkmann and Dangelmayr 1980). However, aurones and anthocyanins coexist in petals of some varieties of snapdragon, cosmos, and the triple transgenic lines of torenia in the presence of the endogenous CHI. Moreover, the yellow coloration of flowers of snapdragon, cosmos, and the triple transgenic torenia does not require loss-of-CHI function. Taken together, these findings indicate that C4'GT-dependent aurone biosynthesis and CHI-dependent anthocyanin/flavone synthesis are not alternative pathways. C4'GT should also interact with CHS to overcome the CHI-mediated channeling of the substrate to anthocyanin/flavone biosynthesis and direct it to aurone biosynthesis. Further studies on protein-protein interactions are needed to address these issues.

The transgenic torenia studies showed that THC 4'-*O*-glucoside is the authentic precursor of aureusin (aureusidin 6-*O*-glucoside) *in vivo*. Yellow cosmos (*C. bipinnatus* cv. Yellow Garden) flowers contain coreopsin (butein 4'-*O*-glucoside) and sulfurein (sulfuretin 6-*O*-glucoside), clearly demonstrating the structural correlation between chalcone 4'-*O*-glucoside and aurone 6-*O*-glucoside, i.e., coreopsin is the precursor of sulfurein. Similarly, coreopsis and bidens have lanceolin (lanceoletin 4'-*O*-glucoside) and marein (okanin 4'-*O*-glucoside), which are likely precursors of leptosin (leptosidin 6-*O*-glucoside) and maritimein (maritimetin 6-*O*-glucoside), respectively (**Fig. 12**) (Harborne and Baxter 1999). Considering that cosmos, coreopsis, and bidens are all Asteraceae plants, these structural correlations imply that chalcone 4'-*O*-glucosylation followed by oxidative cyclization is the general basis of aurone 6-*O*-glucoside biosynthesis in Asteraceae as well as Scrophulariaceae. It is noteworthy that prenylated aurones are rarely found in *Antiaris toxicaria* and *Glycyrrhiza glabra* (Hano *et al*. 1990; Li *et al*. 2000). These unique prenylaurones are not glycosylated, suggesting a glycosylation-independent aurone biosynthesis. It is possible that other classes of PPO rather than vacuolar PPOs as AS are involved in the non-glycosylated aurone biosynthesis (Pourcel *et al*. 2007).

Although aurone 6-*O*-glycosides occur even in fern and liverworts, neither aurone 6-*O*-glycosides nor striking homologs of *UGT88D3* and *AmAS1* are found in arabidopsis and rice (*Oryza sativa*). The occurrence of aurones and their biosynthetic enzymes appears to be limited to a particular range of higher plants, in particular, to Scrophulariaceae, Asteraceae, Fabaceae, and Plumbaginaceae (Harborne and Baxter 1999; Iwashina 2000). This sporadic distribution of aurones implies the parallel-evolution of aurone biosynthetic machineries. Further identification of aurone biosynthetic genes from different plant species will clarify this evolutional issue.

Although glucosylation is sufficient for the vacuolar transport of chalcones, the mechanism transporting chalcone glucosides to vacuoles remains unknown. Maize (*Zea mays*) *Bronze 2*, petunia *Anthocyanin 9* (AN9), and arabidopsis *Transparent Testa 19* (TT19) encode a glutathione *S*-transferase (GST) that appears to serve as a carrier protein for the translocation of anthocyanins to tonoplast (Marrs *et al*. 1995; Alfenito *et al*. 1998; Kitamura *et al*. 2004). Moreover, multidrug resistance-associated protein (MRP) and multidrug and toxic compound extrusion (MATE) protein are also considered to be involved in flavonoid sequestration to vacuoles (Debeaujon *et al*. 2001; Goodman *et al*. 2004). It would be of interest to determine whether chalcone glucosides are also transported thorough these flavonoid-transporting mechanisms from the viewpoint of the substrate specificities of vacuolar transporting system.

Gain-of-function analysis of UGT88D3 and/or AmAS1 clearly showed that both enzymes are indispensable for biosynthesis of aurone 6-*O*-biosynthesis (Ono *et al*. 2006b).

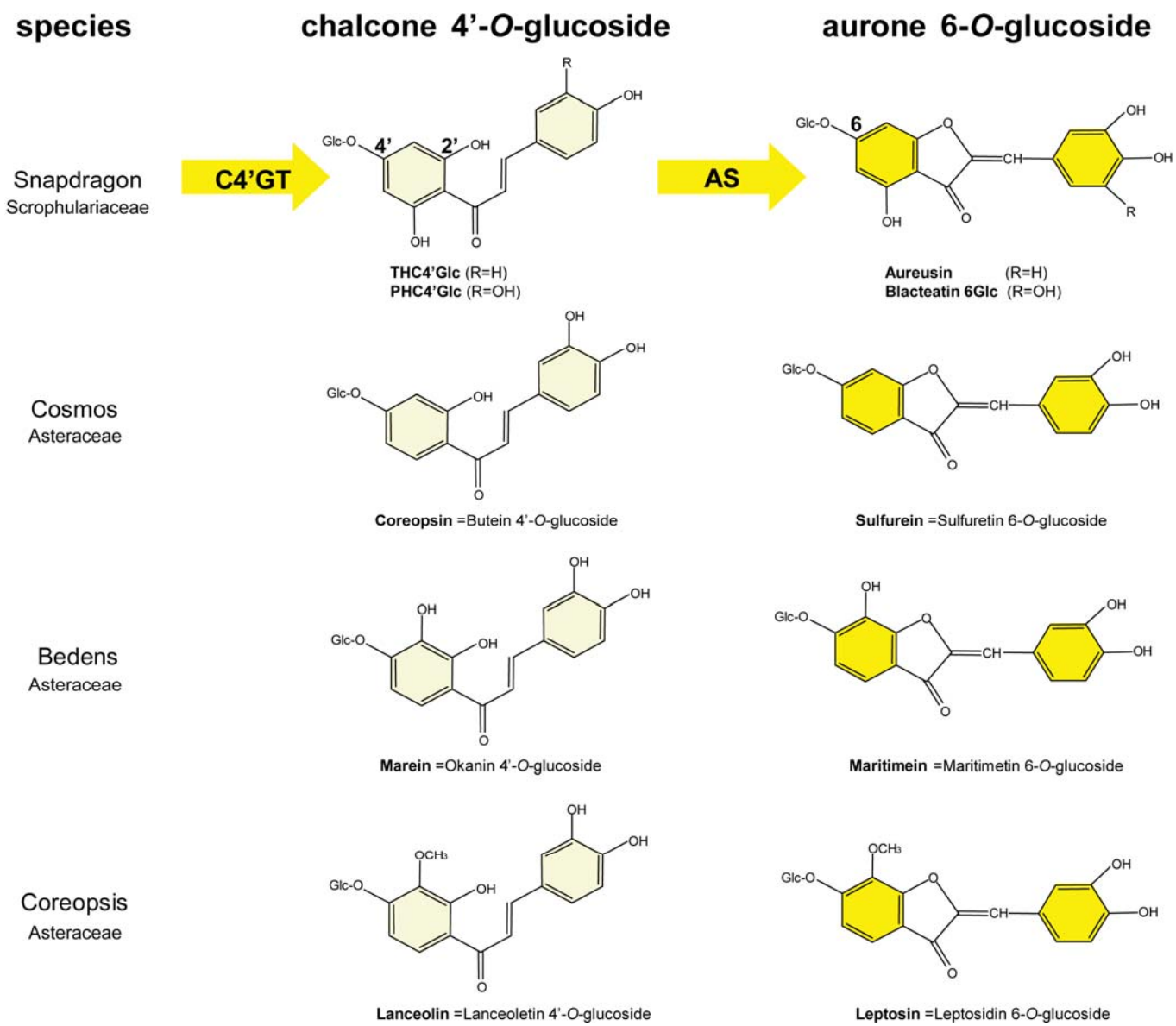


Fig. 12 Structural correlation between chalcone 4'-*O*-glucosides and aurone 6-*O*-glucosides. THC 4'-*O*-glucoside is the authentic precursor of aureusin (aureusidin 6-*O*-glucoside) in snapdragon. Similarly, cosmos, coreopsis, and bidens have coreopsin (butein 4'-*O*-glucoside), lanceolin (lanceoletin 4'-*O*-glucoside), and marein (okanin 4'-*O*-glucoside), which are the most likely precursors of sulfurein (sulfuretin 6-*O*-glucoside), leptosin (leptosidin 6-*O*-glucoside), and maritimein (maritimetin 6-*O*-glucoside), respectively.

In contrast, mutants lacking their enzymatic activities are still unknown. Since only a single copy of *AmAS1* exists in the snapdragon genome (Ono *et al*. 2006b), its loss-of-function will certainly result in a lack-of-aurone phenotype. On the other hand, UGT88D3 is a member of the UGT superfamily, suggesting that functionally redundant UGTs exist in the snapdragon genome.

The coordinated expression and function of *UGT88D3* and *AmAS1* during snapdragon flower development suggest that the both enzyme genes of aurone biosynthesis are under the same transcriptional regulation. Previously identified genetic loci altering aurone biosynthesis are likely to be candidates that are involved in this regulation (Davies *et al*. 2006). However, the *sulf*, *violacea*, and CFR1011 line affect neither the transcript abundance nor the change in the cording region of *AmAS1* (Davies *et al*. 2006). Thus, their targets may be UGT88D3 or the vacuolar transporting machineries of chalcones and AmAS1. Further characterization and identification of these loci will provide new insights of the aurone biosynthesis.

ACKNOWLEDGEMENTS

We thank Drs. Takaaki Kusumi, Yoshikazu Tanaka, Masahiro Nakao, Yuko Fukui, Masako Fukuchi-Mizutani, Yukihisa Katsumoto, Noriko Nakamura, Hiroaki Okuhara (Suntory), Kenichi Suzuki, Yasuyuki Murakami (Suntory flowers), Takaharu Tanaka, Honoo Satake (SUNBOR), Prof. Masaatsu Yamaguchi (Minami-Kyushu University), Prof. Yasuyuki Yamada, Prof. Takashi Hashimoto (NAIST), Prof. Kazufumi Yazaki (Kyoto University) and Prof. Tokuzo Nishino (Tohoku University) for useful comments and discussions.

REFERENCES

Aida R, Kishimoto S, Tanaka Y, Shibata M (2000) Modification of flower color in torenia (*Torenia fournieri* Lind.) by genetic transformation. *Plant Science* **153**, 33-42

Alfenito MR, Souer E, Goodman CD, Buell R, Mol J, Koes R, Walbot V (1998) Functional complementation of anthocyanin sequestration in the vacuole by widely divergent glutathione *S*-transferases. *The Plant Cell* **10**, 1135-1149

Almeida J, Carpenter R, Robbins TP, Martin C, Coen ES (1989) Genetic interactions underlying flower color patterns in *Antirrhinum majus*. *Genes and Development* **11**, 1758-1767

Asen S, Norris KH, Stewart RN (1972) Copigmentation of aurone and flavone

from petals of *Antirrhinum majus*. *Phytochemistry* **11**, 2739-2741

Bate-Smith EC, Geissman TA (1951) Benzalcoumaranones. *Nature* **167**, 688

Bohm BA (1989) Methods in Plant Biochemistry, *Plant Phenolics* **1**, Academic Press (UK) 237-282

Bohm BA (1994) The flavonoids. In: Harborne JB (Ed) *The Minor Flavonoids*, Chapman and Hall, London, UK, pp 387-440

Bomati EK, Austin MB, Bowman M, Dixon RA, Noel JP (2005) Structural elucidation of chalcone reductase and implications for deoxychalcone biosynthesis. *The Journal of Biological Chemistry* **280**, 30496-30503

Bonas U, Sommer H, Seadler H (1984) The 17bp Tam1 element of *Antirrhinum majus* induces a 3 bp duplication upon integration into chalcone synthase gene. *The EMBO Journal* **3**, 1015-1019

Bowles D, Isayenkova J, Lim E-K, Poppernberger B (2005) Glycosyltransferases: Managers of small molecules. *Current Opinion in Plant Biology* **8**, 254-263

Bradshaw HD, Schemske DW (2003) Allele substitution at a flower color locus produces a pollinator shift in monkeyflowers. *Nature* **426**, 176-178

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

Coen ES, Meyerowitz EM (1991) The war of the whorls: Genetic interactions controlling flower development. *Nature* **353**, 31-37

Davies KM, Bloor SJ, Spiller GB, Deroles SC (1998) Production of yellow color in flowers: Redirection of flavonoid biosynthesis in Petunia. *The Plant Journal* **13**, 259-266

Davies KM, Marshall G, Bradley JM, Schwinn KE, Bloor SJ, Winefield CS, Martin CR (2006) Characterization of aurone biosynthesis in *Antirrhinum majus*. *Physiologia Plantarum* **128**, 593-603

Debeaujon I, Peeters AJ, Leon-Kloosterziel KM, 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

Dhaubhadel S, Gijzen M, Moy P, Farhangkhoee M (2006) Transcriptome analysis reveals a critical role of *CHS7* and *CHS8* genes for isoflavonoid synthesis in soybean seeds. *Plant Physiology* **143**, 326-338

Efremova N, Castillo R, Navarro C (2006) The utility of snapdragon (*Antirrhinum majus*) to study flower development and coloration. In: Teixeira da Silva (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol I), Global Science Books, London, pp 12-24

Ferrer JL, Jez JM, Bowman ME, Dixon RA, Noel JP (1999) Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. *Nature Structural Biology* **6**, 775-784

Forkmann G, Dangelmayr B (1980) Genetic control of chalcone isomerase activity in flowers of *Dianthus caryophyllus*. *Biochemical Genetics* **18**, 519-527

Forkmann G, Stotz G (1981) Genetic control of flavanone 3-hydroxylase activity and flavonoid 3'-hydroxylase activity in *Antirrhinum majus* (snapdragon). *Zeitschrift für Naturforschung* **36c**, 411-416

Forkmann G, Heller W (1999) Biosynthesis of flavonoids. In: Sankawa U (Ed) *Polyketides and Other Secondary Metabolites, Including Fatty Acid and Other Derivatives*, Elsevier, Amsterdam, The Netherlands, pp 713-748

Forkmann G, Martens S (2001) Metabolic engineering and applications of flavonoids. *Current Opinion in Biotechnology* **12**, 155-160

Fukuchi-Mizutani M, Okuhara H, Fukui Y, Nakao M, Katsumoto Y, Yonekura-Sakakibara K, Kusumi T, Hase T, Tanaka Y (2003) Biochemical and molecular characterization of a novel UDP-glucose:anthocyanin 3'-*O*-glucosyltransferase, a key enzyme for blue anthocyanin biosynthesis, from gentian. *Plant Physiology* **132**, 1652-1663

Gachon CMM, Langlois-Meurinne, Saindrenan P (2005) Plant secondary metabolism glycosyltransferases: The emerging functional analysis. *Trends in Plant Science* **10**, 542-549

Gandia-Herrero F, Garcia-Carmona F, Escribano J (2005) Floral fluorescence effect. *Nature* **437**, 334

Goodman CD, Casati P, Walbot V (2004) A multidrug resistance-associated protein involved in anthocyanin transport in *Zea mays*. *The Plant Cell* **16**, 1812-1826

Goodrich J, Carpenter R, Coen ES (1992) A common gene regulates pigmentation pattern in diverse plant species. *Cell* **68**, 955-964

Grotewold E (2006) The genetics and biochemistry of floral pigments. *Annual Review of Plant Biology* **57**, 761-780

Hano Y, Mitsui P, Nomura T (1990) Constituents of the Moraceae plants VI. Two new prenylaurones, antiaurones A and B, from the root bark of *Antiaris toxicaria* Lesch. *Heterocycles* **30**, 1023-1030

Harborne JB, Baxter H (1999) *The Handbook of Natural Flavonoids* (Vol 2), John Wiley and Sons, NY, USA, pp 115-217

Hatayama M, Ono E, Yonekura-Sakakibara K, Tanaka Y, Nishino T, Nakayama T (2006) Biochemical characterization and mutational studies of a chalcone synthase from yellow snapdragon (*Antirrhinum majus*) flowers. *Plant Biotechnology* **23**, 373-378

Hirotani M, Kuroda R, Suzuki H, Yoshikawa T (2000) Cloning and expression of UDP-glucose flavonoid 7-*O*-glucosyltransferase from hairy root cultures of *Scutellaria baicalensis*. *Planta* **210**, 1006-1013

Holton TA, Cornish EC (1995) Genetics and biochemistry of anthocyanin biosynthesis. *The Plant Cell* **7**, 1071-1083

Inazu A (1993) Biochemical and genetic studies on a new cosmos (*Cosmos bipinnatus* Cav.) cultivar with yellow ray petals. *Bulletin of the Faculty of Agriculture, Tamagawa University* **33**, 75-140

Iwashina T (2000) The structures and distribution of the flavonoids in plants. *Journal of Plant Research* **113**, 287-299

Jez JM, Bowman ME, Dixon RA, Noel JP (2000) Structure and mechanism of the evolutionarily unique plant enzyme chalcone isomerase. *Nature Structural Biology* **7**, 786-791

Jones P, Vogt T (2001) Glycosyltransferases in secondary plant metabolism: Tranquilizers and stimulant controller. *Planta* **213**, 164-174

Jones P, Messner B, Nakajima J, Schaffner AR, Saito K (2003) UGT73C6 and UGT78D1, glycosyltransferases involved in flavonol glycoside biosynthesis in *Arabidopsis thaliana*. *The Journal of Biological Chemistry* **278**, 43910-43918

Jorgensen K, Rasmussen AV, Morant M, Nielsen AM, Bjarnholt N, Zagrobelny M, Bak S, Moller L (2005) Metabolon formation and embolic channeling in the biosynthesis of plant natural products. *Current Opinion in Plant Biology* **8**, 280-291

Joy IV RW, Sugiyama M, Fukuda H, Komamine A (1995) Cloning and characterization of polyphenol oxidase cDNAs of *Phytolacca americana*. *Plant Physiology* **107**, 1083-1089

Kim JH, Kim BG, Park Y, Ko JH, Lim CE, Lim J, Lim Y, Ahn J-H (2006) Characterization of flavonoid 7-*O*-glucosyltransferase from *Arabidopsis thaliana*. *Bioscience, Biotechnology and Biochemistry* **70**, 1471-1477

Kitamura S, Shikazono N, Tanaka A (2004) *TRANSPARENT TESTA 19* is involved in the accumulation of both anthocyanins and proanthocyanidins in *Arabidopsis*. *The Plant Journal* **37**, 104-114

Lawrence NJ, Rennison D, McGown AT, Hadfield JA (2003) The total synthesis of an aurone isolated from *Uvaria hamiltonii*: Aurones and flavones as anticancer agents. *Bioorganic and Medicinal Chemistry Letters* **13**, 3759-3763

Li W, Asada Y, Yoshikawa T (2000) Flavonoid constituents from *Glycyrrhiza glabra* hairy root cultures. *Phytochemistry* **55**, 447-456

Li Y, Baldauf S, Lim E-K, Bowles DJ (2000) Phylogenetic analysis of the UDP-glycosyltransferase multigene family of *Arabidopsis thaliana*. *The Journal of Biological Chemistry* **276**, 4338-4343

Lim E-K, Bowles DJ (2003a) A class of plant glycosyltransferases involved in cellular homeostasis. *The EMBO Journal* **23**, 2915-2922

Lim EK, Baldauf S, Li Y, Elias L, Worrall D, Spencer SP, Jackson RG, Taguchi G, Ross J, Bowles DJ (2003b) Evolution of substrate recognition across a multigene family of glycosyltransferases in *Arabidopsis*. *Glycobiology* **13**, 139-145

Luo D, Coen ES, Doyle S, Carpenter R (1991) Pigmentation mutants produced by transposon mutagenesis in *Antirrhinum majus*. *The Plant Journal* **1**, 59-69

Marrs KA, Alfenito MR, Lloyd AM, Walbot V (1995) A glutathione *S*-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2*. *Nature* **375**, 397-400

Martin C, Carpenter R, Sommer H, Saedler H, Coen ES (1985) Molecular analysis of instability in flower pigmentation of *Antirrhinum majus*, following isolation of the *pallida* locus by transposon tagging. *The EMBO Journal* **4**, 1625-1630

Martin C, Prescott A, Mackay S, Bartlett J, Vrijlandt E (1991) Control of anthocyanin biosynthesis in flowers of *Antirrhinum majus*. *The Plant Journal* **1**, 37-49

Martin C, Gerats T (1993) Control of pigment biosynthesis genes during petal development. *The Plant Cell* **5**, 1253-1264

Martin-Calero M, La Casa C, Motilva V, Lopez A, Alarcon de la, Lastra C (1996) Healing process induced by a flavonic fraction of *Bidens aurea* on chronic gastric lesion in rat. Role of angiogenesis and neutrophil inhibition. *Zeitschrift für Naturforschung* **51**, 570-577

Matsuoka K, Neuhaus JM (1999) *Cis*-elements of protein transport to the plant vacuoles. *Journal of Experimental Botany* **41**, 825-835

Mayer AM (1987) Polyphenol oxidase in plants – Recent progress. *Phytochemistry* **26**, 11-20

Morimoto M, Fukumoto H, Nozoe T, Hagiwara A, Komai K (2007) Synthesis and insect antifeedant activity of aurones against *Spodoptera litura* larvae. *Journal of Agricultural and Food Chemistry* **55**, 700-705

Nagashima S, Hirotani M, Yoshikawa T (2000) Purification and characterization of UDP-glucuronate: Baicalein 7-*O*-glucuronosyltransferase from *Scutellaria baicalensis* Georgi. Cell suspension cultures. *Phytochemistry* **53**, 533-538

Nakamura N, Fukuchi-Mizutani M, Miyazaki K, Suzuki K, Tanaka Y (2006) RNAi suppression of the anthocyanidin synthase gene in *Torenia hybrida* yields white flowers with higher frequency and better stability than antisense and sense suppression. *Plant Biotechnology* **23**, 13-17

Nakayama T, Yonekura-Sakakibara K, Sato T, Kikuchi S, Fukui Y, Fukuchi-Mizutani M, Ueda T, Nakao M, Tanaka Y, Kusumi T, Nishino T (2000) Aureusidin synthase: A polyphenol oxidase homolog responsible for flower coloration. *Science* **290**, 1163-1166

Nakayama T, Sato T, Fukui Y, Yonakura-Sakakibara K, Hayashi H, Tanaka Y, Kusumi T, Nishino T (2001) Specificity analysis and mechanism of au-

rone synthesis catalyzed by aureusidin synthase, a polyphenol oxidase homolog responsible for flower coloration. *FEBS Letters* **499**, 107-111

Nakayama T (2002) Enzymology of aurone biosynthesis. *Journal of Bioscience and Bioengineering* **94**, 487-491

Niovi-Jones K, Reithel JS (2001) Pollinator-mediated selection on a flower color polymorphism in experimental populations of *Antirrhinum* (Scrophulariaceae). *American Journal of Botany* **88**, 447-454

Offen W, Martinez-Fleites C, Yang M, Kiat-Lim E, Davis BG, Tarling CA, Ford CM, Bowles DJ, Davies GJ (2006) Structure of a flavonoid glucosyltransferase reveals the basis for plant natural product modification. *The EMBO Journal* **25**, 1396-1405

Ogata J, Itoh Y, Ishida M, Yoshida H, Ozeki Y (2004) Cloning and heterologous expression of cDNAs encoding flavonoid glucosyltransferases from *Dianthus caryophyllus*. *Plant Biotechnology* **21**, 367-375

Ono E, Hatayama M, Isono Y, Sato T, Watanabe R, Yonekura-Sakakibara K, Fukuchi-Mizutani M, Tanaka Y, Kusumi T, Nishino T, Nakayama T (2006a) Localization of a flavonoid biosynthetic polyphenol oxidase in vacuole. *The Plant Journal* **45**, 133-143

Ono E, Fukuchi-Mizutani M, Nakamura N, Fukui Y, Yonekura-Sakakibara K, Yamaguchi M, Nakayama T, Tanaka T, Kusumi T, Tanaka Y (2006b) Yellow flowers generated by expression of the aurone biosynthetic pathway. *Proceedings of the National Academy of Sciences USA* **103**, 11075-11080

Pourcel L, Routaboul J-M, Cheynier V, Lepiniec L, Debeaujon I (2007) Flavonoid oxidation in plants: From biochemical properties to physiological functions. *Trends in Plant Science* **12**, 29-36

Rathmell WG, Bendall DS (1972) The peroxidase-catalyzed oxidation of a chalcone and its possible physiological significance. *Biochemical Journal* **127**, 125-132

Rausher MD (2005) The science of flavonoids: The evolution of flavonoids and their genes. In: Grotewold E (Ed) *The Science of Flavonoids*, Springer-Verlag (US), Inc., pp 175-211

Ross J, Li Y, Lim E-K, Bowles DJ (2001) Higher plant glycosyltransferases. *Genome Biology* **2**, reviews 3004.1-3004.6

Sato T, Nakayama T, Kikuchi S, Fukui Y, Yonekura-Sakakibara K, Ueda T, Nishino T, Tanaka Y, Kusumi T (2001) Enzymatic formation of aurones in the extracts of yellow snapdragon flowers. *Plant Science* **160**, 229-236

Schwarz-Sommer Z, Davies B, Hudson A (2003) An everlasting pioneer: The story of *Antirrhinum* research. *Nature Reviews Genetics* **4**, 655-664

Schwinn K, Venail J, Shang Y, Mackay S, Alm V, Butelli E, Oyama R, Bailey P, Davies K, Martin C (2006) A small family of MYB-regulatory genes controls floral pigmentation intensity and patterning in the genus *Antirrhinum*. *The Plant Cell* **18**, 831-851

Shimokoriyama M, Hattori S (1952) Anthochlor pigments of *Cosmos sulphureus*, *Coreopsis lanceolata* and *C. sazicola*. *Journal of the American Chemical Society* **75**, 1900-1904

Spribille R, Forkmann G (1982) Genetic control of chalcone synthase activity in flowers of *Antirrhinum majus*. *Phytochemistry* **21**, 2231-2234

Stubbe H (1974) New mutants of *Antirrhinum majus*. *Kulturpflanze* **22**, 376

Tanaka Y, Tsuda S, Kusumi T (1998) Metabolic engineering to modify flower color. *Plant Cell Physiology* **39**, 1119-1126

Tanaka Y, Katsumoto Y, Brugliera F, Mason J (2005) Genetic engineering in floriculture. *Plant Cell, Tissue and Organ Culture* **80**, 1-24

Tanaka Y (2006) Flower colour and cytochrome P450. *Phytochemistry Reviews* **5**, 283-291

Thompson WR, Meinwald J (1972) Flavonoids: Pigments responsible for ultraviolet absorption in nectar guide of flower. *Science* **177**, 528-530

Tohge T, Nishiyama Y, Yokota-Hirai M, Yano M, Nakajima J, Awazuhara M, Inoue E, Takahashi H, Goodenowe DB, Kitayama M, Noji M, Yamazaki M, Saito K (2005) Functional genomics by integrated analysis of metabolome and trascriptome of Arabidopsis plants over-expressing an MYB transcription factor. *The Plant Journal* **42**, 218-235

Vaughn KC, Lax AR, Duke SO (1988) Polyphenol oxidase: The chloroplast oxidase with no established function. *Physiologia Plantarum* **72**, 659-665

Venkateswarlu S, Panchagnula GK, Subbaraju GV (2004) Synthesis and antioxidative activity of 3',4',6,7-tetrahydroxyaurone, a metabolite of *Bidens frondosa*. *Bioscience, Biotechnology and Biochemistry* **68**, 183-185

Vogt T, Jones P (2000) Glycosyltransferases in plant natural product synthesis: Characterization of a super gene family. *Trends in Plant Science* **5**, 380-386

Yonekura-Sakakibara K, Tohge T, Niida R, Saito K (2007) Identification of a flavonol 7-*O*-rhamnosyltransferase gene determine flavonoid pattern in *Arabidopsis* by transcriptome coexpression analysis and reverse genetics. *The Journal of Biological Chemistry* **282**, 14932-14941

Yu O, Matsuno M, Subramanian S (2006) Flavonoid compounds in flowers: genetics and biochemistry. In: Teixeira da Silva (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol I), Global Science Books, London, pp 282-292

Whibley AC, Langlade NB, Andalo C, Hanna AI, Bangham A, Thebaud C, Coen E (2006) Evolutionary paths underlying flower color variation in *Antirrhinum*. *Science* **313**, 963-966

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

Wong E (1967) Aurone Biosynthesis II. Formation of 4',6'-dihydroxy-2-(a-hydroxybenzyl)coumaranone from 2',4,4'-trihydroxychalcone by cell-free extracts of soybean. *Phytochemistry* **6**, 1227-1233

Zufall RA, Rausher MD (2004) Genetic changes associated with floral adaptation restrict future evolutionary potential. *Nature* **428**, 847-850