HORTSCIENCE 28(6):659-660. 1993.

Characterization of the Flavonoids from *Petunia ×hybrida* Flowers Expressing the *A1* Gene of *Zea mays*

R.J. Griesbach
Florist and Nursery Crops, U.S. Department of Agriculture, Beltsville, MD 20705

Additonal index words. gene transfer, genetic engineering, flower color

Abstract. **The flavonoids from flowers of transgenic *Petunia ×hybrida* Vilm. plants containing the Al gene from *Zea mays* L. were characterized. The *A1* gene encodes the enzyme dihydroflavonol reductase and was introduced into a mutant petunia defective for this gene. Control, nontransgenic plants produced flowers that contained ≈ 50 ng anthocyanin/ 100 mg tissue dry weight. Anthocyanin distribution was 63% cyanidin, 28% delphinidin, and 9% pelargonidin. In contrast, the transgenic plants produced flowers that contained ≈ 500 ng anthocyanin/100 mg tissue dry weight, with 34% as cyanidin, 12% as delphlnidin, and 54% as pelargonidin. The increase in anthocyanin production in the transgenic plants resulted in a corresponding molar decrease in flavonol accumulation.**

The biochemistry and genetics of the flavonoid biosynthetic pathway in plants is probably the most thoroughly understood of any metabolic pathway (Harborne, 1988). This pathway has been extensively studied in the genus *Petunia* (Fig. 1) (de Vlaming et al., 1984). In *Petunia,* the anthocyanin pigments and flavonol copigments have been characterized (Griesbach et al., 1991). Procedures for various enzyme assays are well developed (Forkmann and Ruhnau, 1987). In addition, many genes have been mapped (de Vlaming et al., 1984), cloned (Mel et al., 1988), and the resulting gene families characterized (Beld et al., 1989).

Transgenic petunia plants have been created that contain several novel flavonoid genes (Krol et al., 1990; Meyer et al., 1987; Napoli et al., 1990). Transgenic petunia expressing the *A1* gene of *Z. mays* produced flowers having an orange color (RHS 40D; Royal Horticultural Society, 1966) not previously seen in the genus (Meyer et al., 1987). The *Al* gene encodes the enzyme dihydroflavonol-4-reductase (DFR) (Linn et al., 1990). This gene corresponds to the *An6* gene of *Petunia* (Beld et al., 1989). The *Petunia* mutant RLO1 was selected as the DNA recipient because of its defective *ht1* and *hf1* genes. The gene *Ht1* encodes the enzyme dihydroflavonoid-3'-hydroxylase, and *Hf2* encodes dihydroflavonoid-5'-hydroxylase (Stotz et al., 1985). In previous reports (Linn et al., 1990, Meyer et al., 1987), no detailed pigment analysis was conducted. In this study, freezed-dried flowers from the host clone and several transgenic clones expressing the *A1* gene were analyzed.

Received for publication 14 Aug. 1992. Accepted for publication 4 Dec. 1992. Freeze-dried plant material was supplied by Peter Meyer and Felicitas Lion, Max Planck Inst., Köln, Germany. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

The flower tissue was extracted twice and its flavonoids isolated and resolved through high-performance liquid chromatography (HPLC) as previously reported (Griesbach and Asen, 1990; Griesbach et al., 1991). Flavonol HPLC was carried out on a 7.8 × 300-mm Bondapak C_{18} column using a 20-min, linear gradient of 0 to 20% (v/v) acetonitrile containing 1% (v/v) aqueous triethylamine at pH 3.0. The acetonitrile concentration was then held at 20% for another 20 min. The flow rate was 1 ml·min^{-1} and the elutant was monitored at 540 nm.

Anthocyanin HPLC was carried out on a 7.8 × 300-mm Bondapak C_{18} column using a 30-min, linear gradient of O to 10% acetonitrile containing 15% acetic acid and 1.5% phosphoric acid, followed by a 10-min, linear increase to 20% acetonitrile, where it was held for another, 10 min. The flow rate was 1 ml·min^{-1}, and the elutant was monitored at 540 nm. The percentage of each flavonoid in the HPLC profile and the total amount of all the flavonoids present were determined. Measurements from the two samples were averaged, and the standard deviation calculated.

The mutant RL01 has a leaky *ht1* gene because its flowers are not white, as previously reported for this mutant, but very pale red (RHS 49C) and contain quercetin derivatives (Tables 1 and 2). In wild-type *Ht1* (with red RHS 43A flowers), there is ≈ 500 ng of anthocyanin/100 mg dry weight (Griesbach et al., 1991). In comparison, the mutant RL01 has a 99% reduction in anthocyanin amount (Table 1). In wild-type *Ht1,* ≈ 85% of the flavonols are quercetin glucosides (Griesbach and Asen, 1990), whereas in RL01, ≈ 10% are quercetin derivatives (Table 2).

The *Petunia* DFR has a greater substrate specificity for the 3', 4'-substituted dihydroflavonol (dihydroquercetin) than the 4'-substituted dihydroflavonol (dihydrokaempferol) (Forkmann and Ruhnau, 1987). Therefore, very little pelargonidin is produced. In *Ht1* wild-type (red flowers), 89% of the anthocyanin is cyanidin, 3% delphinidin, and 8% pelargonidin (Griesbach et al., 1991). In the *ht1* low anthocyanin-producing mutant RL01, 63% of the pigment is cyanidin, 28% is delphinidin, and 9% is pelargonidin, with a

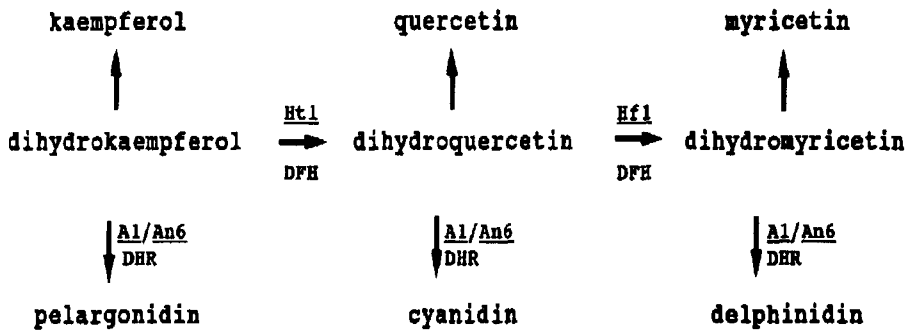

Fig. 1. Part of the *Petunia* flavonoid biosynthetic pathway involved in converting dihydroflavonols into anthocyanin. *Ht1* and *Hf1* genes encode different forms of the enzyme dihydroflavonol hydroxylase (DFH). *An6* and *A1* genes encode different forms of the enzyme dihydroflavonol reductase (DFR).

Table 1. Anthocyanin composition of flowers from the nontransgenic *Petunia ×hybrida* clone RL01 and two homozygous-expressing transgenic *P. ×hybrida* clones, 41-17 and 43-1. Values are reported as the mean (standard deviation) percentage of the total concentration. For example, in RL01, 27.7% of the anthocyanin is delphinidin-3-glucoside, and the total amount of all anthocyanins is 5.53 ng/100 mg dry weight.

	Anthocyanin[z] content			
Clones	del	cya	pel	Total (ng/100 mg dry wt)
RL0l	27.7 (6.59)[y]	62.9 (1.13)	9.11 (7.14)	5.53 (1.73)
41-17	11.3 (2.62)	34.5 (4.74)	54.3 (7.21)	53.1 (14.69)
43-1	11.7 (0.94)	33.9 (2.51)	54.5 (3.49)	59.-1 (3.48)

[z]del = delphinidin-3-glucoside, cya = cyanidin-3-glucoside, pel = pelargonidin-3-glucoside.
[y]Mean (standard deviation) percentage of total.

Table 2. Flavonol composition of flowers from the nontransgenic *Petunia ×hybrida* clone RL01 and two homozygous-expressing transgenic clones 43-1 and41-17. Vafues are reported as the mean (standard deviation) percentage of total. For example, in RL01, 1.05% of the flavonol is present as quercetin-7-glucoside and the total amount of all flavonols is 56.68 ng/10 mg dry weight.

	Flavonol[z]											
Clone	qu7glu	qu3soph	qu3soph-7glu	qu3,7-diglu	km7glu	km3glu	km3soph-7glu	km3caff-soph7glu	km3soph	km3,7-diglu	km3caff-soph	Total (ng/10 mg dry wt)
RL-01	1.05[y]	6.72	3.33	8.70	0.94	3.46	15.24	11.29	32.81	9.18	7.14	56.68
	(0.68)	(3.13)	(2.39)	(5.40)	(0.79)	(1.47)	(1.15)	(3.97)	(7.28)	(0.18)	(3.48)	(10.05)
41-17	8.31	7.08	5.29	17.48	1.10	0.88	7.32	16.88	24.00	8.08	4.00	23.63
	(1 .42)	(2.71)	(2.27)	(5.27)	(0.70)	(0.90)	(3.62	(3.85)	5.93)	4.12)	(1.13)	(4.79)
43-1	5.54	5.74	4.96	25.38	1.42	5.46	8.55	11.10	27.86	3.35	0.82	18.93
	(0,11)	(3.36)	(0.16)	(0.49)	(1.76)	(0.22)	(0.33)	(3.72)	(1.83)	(1 .46)	(0.16)	(3.78)

[z]qu = quercetin, km = kaempferol, soph = sophoroside, glu = glucoside, caff = caffeic acid.
[y]Mean (standard deviation) percentage of total.

trace of peonidin and petunidin present (Table 1).

Petunia hybrids clone RL01-17 is a primary transgenic plant derived from direct DNA transfer to protoplasts of the RL01 clone. RL01 -17 stably incorporated and expressed the Al gene of *Zea.* This primary transformant was self-pollinated and the two expressing progeny clones (43-1 and clone 41-17) were analyzed. Peter Meyer (personal communication) has determined that clone RL01-17 was heterozygous for the *A1* gene, while clones 43-1 and 41-17 were both homozygous for the *A1* gene.

Flowers of 41-17 and 43-1 produced less than half of the flavonol produced by the nontransgenic clone RL01 (Table 2). In addition, the percentage of flavonol as kaempferol decreased from 80.1% (0.94+ 3.46+ 15.24+ 11.29+32.81+9.18+7.14) in RL0l to 62.3% in clone 41-17 and to 58.6% in clone 43-1.

Both transgenic clones produced ≈ 10 times more anthocyanin than the nontransgenic clone RL01 (Table 1). More than half of the anthocyanin in the transgenic plants was pelargonidin, whereas in the control RL01, <10% was pelargonidin. The amount of cyanidin and delphinidin also increased in the transgenic plants. In flowers of the nontransgenic RL01, there was 3.5 ng cyanidin/100 mg dry weight (62.9% of 5.53 ng) and 1.5 ng delphidin/100 mg dry weight (27.7% of 5.53 ng); whereas in the transgenic plants, there was 18.3 ng cyanidin/100 mg dry weight in clone 41-17 and 28.2 ng in clone 43-1, and 6.0 ng delphidin/ 100 mg dry weight in-clone 41-17 and 9.4 ng in clone 43-1. The total number of anthocyanin and flavonol molecules in the control (1×10^7 M) equaled that in both transgenic plants (1.2×10^7 M). Thus, the introduction of the *A1* gene increased the production of delphinidin, cyanidin, and pelargonidin at the expense of kaempferol.

Introducing a foreign gene encoding art enzyme that had a new substrate specificity affected the biosynthetic pathway in an unexpected manner (Fig. 1). Adding the *A1* gene did not result in exclusive pelargonidin production, because enzyme concentration is itself controlled by many genes, and genes that affect mRNA stability, protein turnover, transcription and translation rates, and the concentration of cofactors could each have a different effect on endogenous and introduced enzymes (Keightley, 1989). The flow at a particular step in a pathway depends on the concentration of all metabolic intermediates. The activity of enzymes upstream and downstream can affect the concentration of precursors and end products for that particular step. To modify a pathway genetically in a specific manner, information is essential on the relative Km of the foreign enzyme and of the endogenous enzymes upstream and downstream.

Introducing a maize DFR gene into a leaky dihydroflavonol-3'-hydroxylase (DFH) mutant petunia did not create petunias that produced only pelargonidin and that had true-orange flowers. One can create petunias with true-orange flowers in two ways. First, the maize DFR gene could be introduced into a nonleaky DFH mutant. Second, a different DFR gene specific for the dihydrokaemperfol percursor could be introduced into the leaky DFH mutant.

Literature Cited

Beld, M., C. Martin, H. Huits, A.R. Stuitje, and A. Gerats. 1989. Flavonoid synthesis in *Petunia hybrida*- Partial characterization of dihydroflavonol-4-reductase genes. Plant Mol. Biol. 13:491–502.

Forkmann, G. and B. Ruhnau. 1987. Distinct substrate specificity of dihydroflavonol-4-reductase from flowers of *Petunia*. Z. Naturforsch. 42c:1146-1148.

Griesbach, R.J. and S. Asen. 1990. Characterization of the flavonol glycosides of *Petunia.* Plant Sci. 70:49–56.

Griesbach, R.J., S. Asen, and B.A. Leonhardt. 1991. *Petunia hybrida* anthocyanins acylated with caffeic acid. Photochemistry 30:1729–1731.

Harborne, J.B. 1988. The flavonoids, advances in research since 1980. Chapman and Hall, London.

Keightley, P.D. 1989. Models of quantitative variation of flux in metabolic pathways. Genetics 121:869–876.

Krol, A.R., L. Mur, P. Lange, J.N. Mel, and A.R. Stuitje. 1990. Inhibition of flower pigmentation by antisense CHS genes: Promoter and minimal sequence requirements for the anti sense effect. Plant Mol. Biol. 14:457-466.

Linn, F., I. Heidmann, H. Saedler, and P. Meyer. 1990. Epigenetic changes in the expression of the maize Al gene in *Petunia hybrids.* Role of numbers of integrated gene copies and state of methylation. Mol. Gen. Genet. 222:329–336.

Meyer, P., I. Heidmann, G. Forkmann, and H. Saedler. 1987. A new petunia flower colour generated by transformation of a mutant with a maize gene. Nature (London) 330:677-678.

Mel, J.N., A.R. Stuitje, A. Gerats, and R.E. Koes. 1988. Cloned genes of phenylpropanoid metabolism in plants. Plant Mol. Biol. Rptr. 6:274-279.

Napoli, C., C. Lemieux, and R. Jorgensen. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell 2:279–289.

Royal Horticultural Society. 1966. RHS colour chart. Royal Hort. Soc., London.

Stotz, G., P. de Vlaming, H. Wiering, A.W. Schram, and G. Forkmann. 1985. Genetic and biochemical studies on flavonoid-3'-hydroxylation in flowers of *Petunia hybrida.* Theor. Applied Genet. 70:300-305.

de Vlaming, P., A.G. Gerats, H. Wiering, H.J.W. Wijsman, A. Cornu, E. Farcy, and D. Maizonnier. 1984. *Petunia hybrida:* A short description of the action of 91 genes, their origin and their map location. Plant Mol. Biol. Rptr. 2:21-42.