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The Origin of Novel Flavonoids in *Phlox* Allotetraploids

(glycosidating enzymes/gene repression)

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ABSTRACT **The flavonoids of two diploid species of *Phlox* and two of their allotetraploid derivatives were characterized. The tetraploids accumulate five flavonoids not observed in the parental species. Generally, novel compounds are less highly glycosidated than, but otherwise similar to, parental compounds that they partially or totally replace. We propose that hybridity and polyploidy have repressed or suppressed the activity of certain ancestral genes responsible for the production of glycosidating enzymes. Accordingly, the novel compounds are merely the accumulated or modified precursors of parental compounds.**

The development of a specific morphological, physiological, or biochemical expression is dependent upon appropriate genes, the external environment, and the genetic environment. An alteration in the latter may disrupt the processes that permit normal gene activity, so that a novel expression emerges. Interspecific hybridization is one means of subjecting coadapted gene complexes to a manifestly different genetic milieu. The disruptive nature of one or more generations of hybridization on the developmental feedback and regulatory devices in plants is seen in aberrant and unstable development (1), cleistogamy (2), tumor formation (3), variegation (3), breakdown of self-incompatibility (4), imbalance in phenolic aglycone production and glycosidation (5), elimination of organ-specific differences in phenolic compounds (6), and the formation of multiple nucleoli during meiosis (7). An increase in ploidal level also alters the genetic milieu, as seen in the repression of genes coding for ribosomal RNA or enzymes (8). Redundancy of less than the entire genome may have a similar effect (9).

The disturbance of regulatory mechanisms due to a change in genetic environment could cause allopolyploids to experience disruptions in the biosynthesis of secondary constituents such as flavonoids, which have been useful in documenting reticulate evolution. Although such disruption has not been demonstrated, a recent chromatographic survey of flavonoids in *Phlox* suggests that it may have occurred (10). The present investigation was undertaken to characterize the flavonoids of two diploid phloxes and their common allopolyploid derivatives, determine the presence of novel compounds in the latter, and decide whether such compounds might owe their origin to modifications of the ancestral genetic milieu. The species in question are *P. pilosa* L. (2n), *P. drummondii* Hook. (2n), *P. villosissima* (Gray) Whitehouse (4n), and *P. aspera* E. Nels. (4n). Consideration of morphology, ecogeography, karyology, and seed protein chemistry indicates that both tetraploids were derived from the aforementioned diploids (11). The flavonoid data are compatible with this view.

MATERIALS AND METHODS

Twenty-five flowering plants of each species were individually chromatographed to confirm previously described chromatographic patterns and determine which non-anthocyanin flavonoids were reliable genomic markers. Single compounds were isolated and purified by ascending two-dimensional paper chromatography. The first dimension was developed in butanol–acetic acid–water 4:1:2, and the second in acetic acid–water 1:10. 300–700 chromatograms were used to obtain an adequate amount of material for analysis (about 3 mg). In order to determine the oxygenation pattern of each compound, we recorded absorption spectra in spectral methanol alone, and with sodium methoxide, neutral and acidic aluminum chloride, sodium acetate, and boric acid, before and after acidic hydrolysis of oxygen-linked sugars. The procedure is described by Mabry *et al.* (12). Compounds were hydrolyzed with 7% sulfuric acid. The hydrolyzable sugars were identified by paper chromatography according to the method of Pridham (13). The C-glycosidic moieties of some compounds have not been completely characterized. However, the equivalence of compounds has been judged by cochromatography.

RESULTS

All the *Phlox* compounds proved to be glycoflavones, i.e., sugars were attached to the flavone nucleus by carbon linkages rather than oxygen linkages. All of the compounds were glycoflavone derivatives of either apigenin (4′,5,7,-trihydroxyflavone) or luteolin (3′,4′,5,7-tetrahydroxyflavone). The tentative determinations of the glycoflavones are presented in Table 1. *O*-Rhamnosyl-6-*C*-xylosyl derivatives of luteolin and apigenin have been identified in a *P. drummondii* cultivar by UV, NMR, and mass spectroscopy (14). The presence of these compounds in the native *P. drummondii* was supported by cochromatography with the known xylosyl derivatives.

Fifteen compounds were isolated from the four species. *Phlox drummondii* contained eight flavonoids, four of which occurred exclusively in that species. *Phlox pilosa* contained four flavonoids, one of which was species-specific. *Phlox aspera* and *P. villosissima* accumulate compounds of their diploid progenitors, but not all of them. The former species also accumulates three non-parental compounds, and the latter species four non-parental components (Table 1). Some of the non-parental compounds were shared by both tetraploids, others were not, so that when both tetraploids are considered we find five non-parental (herein referred to as novel) compounds. Four of the five are *C*-monoglycosides; the fifth is an *O*-*C*-diglycoside. The parental complement contains only one *C*-

TABLE 1. *Flavonoids of diploid and tetraploid Phlox*

Apigenin derivatives	Species*	Luteolin derivatives	Species*
Vicenin (6,8-di-*C*-glycoside)	D,P,A,V	Lucenin (6,8-di-*C*-glycoside)	D,P,A,V
6-*C*-xylosyl-*O*-rhamnoside	D,A,V	6-*C*-xylosyl-*O*-rhamnoside	D
6-*C*-xylosyl-*O*-glucoside	A†,V†	6-*C*-glycoside$_1$-*O*-rhamnoside	D,A,V
6-*C*-glycosyl$_3$-*O*-rhamnoside‡	D	6-*C*-glycoside$_1$	A†,V†
		8-*C*-glycoside$_1$	A†
6-*C*-glycosyl$_4$-*O*-glycoside	D	6-*C*-glycosyl$_2$-di-*O*-xyloside§	P
6-*C*-glycoside$_3$	V†	6-*C*-glycosyl$_2$	V†
		6-*C*-glycosyl$_4$-*O*-rhamnoside	D
		6-*C*-glycoside$_5$	P,A

* Species designations: D = *P. drummondii*; P = *P. pilosa*; A = *P. aspera*; V = *P. villosissima*.

† Novel compound.

‡ Numerical subscripts refer to type of sugar. Glycosides with the same subscript are judged equivalent.

§ May simply be 6-*C*-glycosyl$_2$-*O*-xyloside.

monoglycoside (Table 1). The novel compounds have not been detected in minor concentrations or in rare variants in either *P. pilosa* or *P. drummondii*.

DISCUSSION

The presence of novel glycoflavones has been demonstrated in the allotetraploids *P. aspera* and *P. villosissima*. All of these compounds are closely related in biogenesis to compounds found in their progenitors. The principal difference between the novel and parental compounds lies in the number of oxygen-linked sugars attached to the basic glycoflavone. The novel compounds usually are of less complex structure, and may replace certain parental compounds. The situation in *Phlox* is in contrast to the additive profiles typical of allopolyploids in other genera (15). The question thus arises as to the origin of the novel products.

As a prelude to a specific hypothesis, let us consider the biosynthesis and genetics of flavonoids. Flavonoids are constructed from a condensation of phenylpropanoid and polyacetate-derived C_6 moieties (16). *In vivo* and *in vitro* studies have demonstrated that *C*-linked sugars are added to the developing flavonoid before the ring is formed, and that *O*-linked sugars are added after ring formation, as are other substituents (17). Moreover, it is clear that flavonoids are assembled along a stepwise biosynthetic pathway which may branch and which may be blocked at various points. The genetic control of glycosidation also has been demonstrated. One of the best-known studies involves *Streptocarpus hybrida*, which has five genes controlling anthocyanidin glycoside synthesis (18). Four of the genes independently mediate specific steps in the biosynthetic pathway. The simple genetic control of steps in flavonoid biosynthesis seems to be the rule rather than the exception (19).

It seems reasonable to assume that flavonoid glycosidation in *Phlox* occurs in well-defined steps, most of which are mediated by single genes. If we accept this assumption, we may construct putative biosynthetic pathways showing the biogenetic relationships of the novel and parental compounds. As seen in Fig. 1, novel compounds may be only a step removed from parental compounds. *Phlox villosissima* and *P. aspera* accumulate relatively large quantities of luteolin 6-*C*-glycoside$_1$ and luteolin 6-*C*-glycosyl$_1$-*O*-rhamnoside, the former being absent and the latter being present in *P. drummondii* (Fig. 1, I). *Phlox villosissima* accumulates luteolin 6-*C*-glycoside$_2$, whereas it is absent from *P. pilosa*, which contains luteolin 6-*C*-glycosyl$_2$-*O*-diglycoside (Fig. 1, II). *Phlox villosissima* also accumulates apigenin 6-*C*-glycoside$_3$, whereas *P. drummondii* contains apigenin 6-*C*-glycosyl$_3$-*O*-glycoside (Fig. 1, III). In these cases, perhaps the enzymes necessary for the terminal step in biosynthesis are not functioning or are doing so at undetectable levels.

Other novel compounds are not simply precursors of parental compounds. Both allotetraploids accumulate apigenin 6-*C*-xylosyl-*O*-glucoside and apigenin 6-*C*-xylosyl-*O*-rhamnoside. *Phlox drummondii* accumulates the latter but not the former (Fig. 1, IV). Ostensibly, a new enzyme is present in the tetraploids which is capable of effecting an *O*-sugar linkage with xylose. The tetraploids have a branched biosynthetic pathway with apigenin 6-*C*-xyloside serving as a common precursor. The allotetraploids and *P. drummondii* contain luteolin 6-*C*-glycosyl$_1$-*O*-rhamnoside, although it is present in low concentration in the former. On the other hand, *P. aspera* and *P. villosissima* are accumulating relatively large quantities of the putative precursor of this glycoside; and *P. aspera* has opened a new pathway leading from a precursor to luteolin 8-*C*-glycoside$_1$ (Fig. 1, V).

The partial or complete blocks in flavonoid biosynthesis inferred thus far have been late in the biosynthetic pathway. Blocks ostensibly exist at earlier stages of flavonoid biosynthesis as well, for parental compounds are not invariably replaced by less complex ones in the allotetraploids. Two compounds not having close relatives in the latter are luteolin 6-*C*-xylosyl-*O*-rhamnoside and luteolin 6-*C*-glycosyl$_4$-*O*-rhamnoside, both of which are produced by *P. drummondii* (Fig. 1, VI and VII).

We propose that the complete or partial blocks in the aforementioned pathways may be due to repression or suppression of gene action brought about by the interaction of disparate genomes and polyploidy. Suppression is evident in the nearly complete failure of some biosynthetic steps to be accomplished and in the accumulation of the immediate precursor. Repression can only be inferred. We cannot discount the possibility that genes were lost prior to or after chromosome doubling. However, the chance that genes were lost prior to chromosome doubling actually is remote, for F_1 hybrids between *P. pilosa* and *P. drummondii* are sterile (10). Accordingly, the first steps in the evolution of the tetraploids must not have included reassortment of genome components at the diploid level.

The new biosynthetic capabilities of the tetraploids (Fig. 1, IV and V) may be due to derepression of parental genes, the reconstruction of ancestral biosynthetic pathways through the complementation of related genomes, or the evolution of new biosynthetic pathways. If the first alternative is applicable, the genes in question must have been contributed by *P. drummondii* because its biosynthetic pathways have substrates (apigenin 6-*C*-xyloside and luteolin 6-*C*-glycoside$_1$) upon which the activated genes may act. *Phlox pilosa* lacks these substrates.

There is considerable evidence in the literature demonstrat-

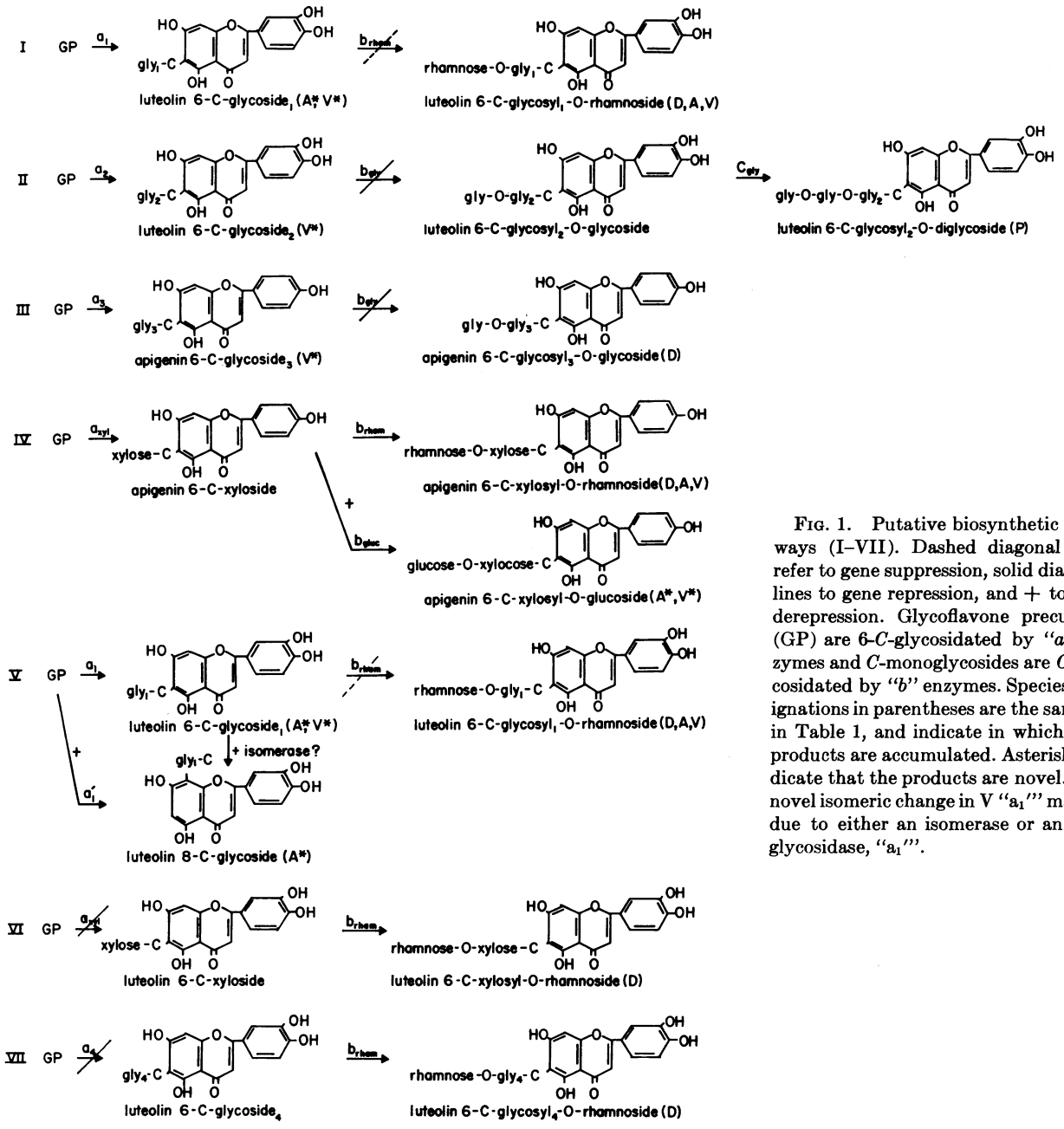

FIG. 1. Putative biosynthetic pathways (I–VII). Dashed diagonal lines refer to gene suppression, solid diagonal lines to gene repression, and + to gene derepression. Glycoflavone precursors (GP) are 6-*C*-glycosidated by "*a*" enzymes and *C*-monoglycosides are *O*-glycosidated by "*b*" enzymes. Species designations in parentheses are the same as in Table 1, and indicate in which taxa products are accumulated. Asterisks indicate that the products are novel. The novel isomeric change in V "a_1'" may be due to either an isomerase or an 8-*C*-glycosidase, "a_1'".

ing the disruptive effect of hybridity and polyploidy on genetic regulatory mechanisms. Consider first the effect of hybridity on such mechanisms. Interspecific hybrids in *Lilium* fail to glycosylate ferulic acid in a normal fashion, 60% of the ferulic acid in a hybrid seed being free, as compared to 6% in intraspecific seed (5). Interspecific hybrids in *Nicotiana* may manifest positive or negative chemical heterosis for phenol content and for the associated polyphenoloxidase and peroxidase systems (20). An example of regulatory gene suppression comes from *Dicentra*, where hybrids accumulate flavonoids less highly glycosylated than the parental compounds, while the parents show only a trace of the precursor (21). Suppression also is suggested in *Phaseolus*, where major constituents of species hybrids occur as trace components in one species (22). The disruption of regulatory mechanisms in hybrids is not unique to plants. The inhibition of the autosomally inherited gene for liver alcohol dehydrogenase has been noted in chicken–quail hybrids (23). An asynchronous activation of parental alleles at tissue-specific gene loci occurs in trout hybrids during early stages of development (24), and for some enzymes the maternal isozyme pattern may persist through these stages (25). The presence of only maternal gene activity during early development has also been reported in *Rana* hybrids (26). Interactions between genomes affecting the gene activity are evident in somatic cell hybrids as well (27).

Consider next the effect of polyploidy on gene activity. In plants, the alterations of physiological and biochemical properties following chromosome doubling have long been known (28). The effect of such doubling on specific genes is only now coming to light. The repression of redundant loci is suggested in *Triticum*, where the number of isozymes in hexaploid wheat is not a summation of the number of isozymes of the parental

genomes; only one of 12 enzyme systems studied was polymorphic (29). In a similar vein, only the nucleolar organizers of one genome are operative in allotetraploids of several genera (7). Evidence has been presented for the duplication of alcohol dehydrogenase genes in maize, one of which is normally repressed but can be derepressed under stress (8). The biosynthesis of flavonoids has been upset in autotetraploids of raspberries and *Phlox subulata* by the presence of novel compounds (30, and unpublished observations). The compounds have not been characterized, but may well be precursors that are accumulated as a result of gene suppression or repression. Turning to animals, we note that there is repression of an immunohemoglobin in malignant plasma cells of the mouse, the amount of immunohemoglobin produced being an inverse function of ploidal level in cell clones (31).

Schwartz (9) proposes that the repression of redundant loci may be the rule rather than the exception. Ohno (32) contends that all regulated structural genes in a newly arisen tetraploid may be subjected to super-repression, which may be escaped by the simultaneous functional diversification of redundant regulator and structural genes. He maintains that the concordant duplication of a regulatory gene and a structural gene upsets the balance between the two in favor of the former. This view is in accord with that of Britten and Davidson (33), who state the "the likelihood of utilization of new DNA for regulation is far greater than the likelihood of invention of a new and useful amino acid sequence...". Following these arguments, one must conclude that a polyploid contains a very large number of redundant and (or) silent genes.

In conclusion, there is ample evidence that alterations in the genetic environment accomplished via hybridization or polyploidy may repress or suppress gene activity. Moreover, it is clear that flavonoid glycosidation occurs in a stepwise fashion, with most steps being under single gene control. Accordingly, it is feasible to relate the origin of the novel flavonoids in *Phlox* to such altered gene activity in a novel genetic environment.

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