

BIOCHEMICAL BASIS OF FLORAL COLOR POLYMORPHISM IN A HETEROCYANIC POPULATION OF *TRILLIUM SESSILE* (LILIACEAE)¹

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ABSTRACT

A study of flavonoids occurring within a heterocyanic population of *Trillium sessile* was made to determine the chemical basis of a common floral color polymorphism in this species. In the study population, three floral color phenotypes (red, pink, yellow) are determined primarily by the presence or absence of anthocyanin compounds in the petal tissue, and secondarily by quantitative differences in the concentration of several flavonol glycosides. Petals of red phenotypes contain both cyanidin 3-arabinoside and 3-diarabinoside, petals of pink phenotypes contain only cyanidin 3-arabinoside, and petals of yellow phenotypes lack cyanidin entirely. Quercetin 3-O-glucoside, quercetin 3-O-arabinoglucoside, quercetin 3-O-arabinogalactoside, and quercetin 3-O-arabinogalactosyl, 7-O-glucoside occur in petals of all three phenotypes but differ in relative amounts. Petals of the red phenotype have mostly 3-O-biosides, but lesser amounts of both quercetin 3-O-glucoside and the 3,7-O-triglycoside. Petals of the pink phenotype contain relatively equal amounts of quercetin mono-, di-, and triglycosides. Petals of the yellow phenotypes contain mostly quercetin 3,7-O-triglycosides, and less mono- and di-glycosides. Small amounts of a quercetin tetraglycoside were detected in petals of both yellow and pink phenotypes, but not in red phenotypes. The enhancement of quercetin polyglycoside biosynthesis in yellow petal phenotypes is attributed to the shunting of dihydroflavonol precursors to synthesis of quercetin compounds when their conversion to anthocyanins is blocked genetically.

FLORAL COLOR POLYMORPHISMS are common in the angiosperms (Harborne, 1967) and occur widely within the genus *Trillium*. Sessile-flowered trillium (*Trillium sessile* L.) is an eastern North American species of mesic woodlands which typically has maroon or dark-red colored petals. Occasional individuals with yellow or yellow-green petals have been recognized taxonomically as forma *viridiflorum* Beyer (Fernald, 1950), although little is known about the genetic basis of the color polymorphism in this species.

Petal color variants in *Trillium* can be categorized as "conspicuous polymorphisms" which are generally believed to have minor evolutionary roles (Wright, 1978). Petal color, however, has been shown to have profound effects on gene flow and mating systems (Levin,

1972; Brown and Clegg, 1984; Levin and Watkins, 1984; Schoen and Clegg, 1985) and these polymorphisms deserve further evolutionary study. Indeed, intrapopulational genetic subdivision in *T. sessile* has been documented with respect to heterocyanic floral color phenotypes (Whitkus et al., 1987). Genetically, flower color in plants may involve serial gene systems and epistasis (Grant, 1975); however, the presence or absence of a particular floral pigment is usually a single-gene difference (Epling, Lewis, and Ball, 1960; Kamsteeg, Van Brederode, and Van Nigtevecht, 1980; Ennos and Clegg, 1983).

Several years ago, we observed a population of *Trillium sessile* characterized by heterocyanic individuals, i.e., plants varied with respect to the expression of anthocyanin pigmentation in their stems, leaves, and petals. Upon closer study, we were able to discern three phenotypes which we categorized by their petal color as "red" (heavily red-pigmented), "pink" (diffusely pink or yellowish-pink) or "yellow" (deep yellow without a trace of red or pink). We hypothesized that the three phenotype classes resulted from the three allelic configurations of a single gene with incomplete dominance. This genetic basis has been doc-

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TABLE 1. Characterization of phenotype classes recognized by differential anthocyanin expression among heterocyanic individuals of *Trillium sessile*

	Petal color		
	Red	Pink	Yellow
Petals	++ ^a	+ ^b	- ^c
Sepals	+	-	-
Leaves	++	+	-
Stems	++	++	-

^a (++) pigments present in high concentration.

^b (+) pigments present in low concentration.

^c (-) pigments absent (undetectable).

umented for similar petal color polymorphisms in many plant species including the "classical" example of incomplete dominance displayed by the red, pink, and white floral phenotypes of snapdragon (Burns, 1980). We set out to determine the genetic basis of the polymorphism in *T. sessile* by carrying out the appropriate crossing experiments. Although we were able to obtain F₁ hybrid seed from the crosses, we reached an impasse when we were unable to germinate them. As an alternative to the crossing experiments, we conducted this study of the biochemical basis of floral color polymorphism in *T. sessile* with the expectation that the chemical data might provide insight into the genetic basis of petal color phenotypes in this species.

MATERIALS AND METHODS—The study site, an 11-hectare woodlot located at the northwest corner of The Ohio State University, Columbus, is described in detail by Horn (1985). Although *Trillium sessile* occurs throughout the site, the heterocyanic population is restricted to the northeast corner of the woods. Within this approximately 35 × 35 m site, about 18,000 individuals of *T. sessile* occur with phenotype proportions of 93.8% reds, 5.1% pinks, and 1.1% yellows. The phenotype classes in the population were delimited by differential pigmentation patterns of leaf, stem, and petal tissue (Table 1). Assignment of individuals to either red or yellow classes was unambiguous; assignment to the pink class was based upon the presence of yellowish pink or diffusely pink petals together with the other array of features listed in Table 1. Fifty individuals of each color class were collected for laboratory analysis.

Analysis of anthocyanins—For each phenotype, equal amounts of stem, leaf, sepal, and petal tissue were extracted in acidic methanol (Arditti and Dunn, 1969), spotted onto Whatman 3MM paper, and chromatographed using the following solvent systems: BAW, TBA, and

HOAc (Markham, 1982). Extracts were also analyzed by thin-layer chromatography (TLC) on Polyamide 6 precoated plastic plates using a 70:20:10; H₂O : ETOH : MEK (water : ethanol : methyl ethyl ketone) aqueous system and a 50:25:21:4; 1,2-dichloroethane : MEOH : MEK : H₂O organic system. Purification of the two anthocyanins detected by these methods was carried out by paper chromatography (PC) using an initial descending separation in 1% HCl, an elution with 2% methanolic acetic acid (MEOH : HOAc; 98:2 v/v), and secondary descending separations in BAW (Harborne, 1967; Asbury, 1973). Purified bands were eluted with 0.01% HCl in MEOH and characterized using UV spectral analysis (Harborne, 1984). Portions of the purified anthocyanins were hydrolyzed in trifluoroacetic acid (TFA) for two hours (Mabry, Markham, and Thomas, 1970), rinsed, and the hydrolyzate taken up in ethyl acetate (EtOAc) and water. Following evaporation of the EtOAc fraction, the aglycones (anthocyanidins) were resuspended in a small amount of acidic MEOH and chromatographed on paper using the FORESTAL system (Harborne, 1984). The spectral and R_f data were used to identify the anthocyanidin moieties. For sugar analysis, the aqueous phase of each extract was evaporated to dryness, resuspended in a small amount of water, and spotted along with standards onto Avicel Microcrystalline Cellulose MN 400 TLC plates. Sugars were identified by comparing R_f values with standards in 6:3:2; pyridine : EtOAc : H₂O using a Fotodyne high-performance radial chromatography (HPRC) chamber. Plates were developed by spraying with a solution of aniline phthalate and warming. The combined spectral, color, R_f, and hydrolysis data were used for final identification of compounds (Harborne, 1967).

Analysis of nonanthocyanin flavonoids—Nonanthocyanin flavonoids were analyzed only from petal tissues. Equal amounts of petal tissue from each phenotype were extracted under agitation first in 85% MEOH, and then in 50% MEOH for 24-hr periods. Extracts for each sample were combined, taken to dryness, and chromatographed on paper in two dimensions using TBA and HOAC systems (Mabry et al., 1970). Compounds resolved on paper were analyzed spectrally (Mabry et al., 1970) and their R_f values computed. Compounds were hydrolyzed in 0.5 ml of water with 7 drops of TFA at 100 C. Hydrolyzates were monitored at 15-min intervals using the polyamide-aqueous TLC system. After complete hydrolysis, aglycones were analyzed by co-chromatography with standards using the

TABLE 2. Biochemical characterization of anthocyanin variation in tissues of *Trillium sessile* floral color phenotypes (R = red, P = pink, Y = yellow)

	Petals			Stems			Leaves		
	R	P	Y	R	P	Y	R	P	Y
Cyanidin									
3-arabinoside	++ ^a	+ ^b	- ^c	++	++	-	+	+	-
Cyanidin									
3-diarabinoside	++	-	-	++	++	-	-	-	-

^a (++) present in high concentration.

^b (+) present in low concentration.

^c (-) absent (undetectable).

polyamide-organic TLC system. Sugars were identified using TLC/HPLC as described above.

RESULTS—Anthocyanin variation was observed among the three phenotype classes and for leaf, stem, and petal tissues of red and pink phenotypes (Table 2). Tissues of yellow phenotypes lacked anthocyanins. Tissues of red and pink phenotypes contain two cyanidin (CY) anthocyanins, a CY 3-arabinoside and CY 3-diarabinoside. Stems of red and pink phenotypes contain both cyanidin compounds. Leaves of both red and pink phenotypes contain only CY 3-arabinoside. Petals of red phenotypes contain both cyanidin compounds, whereas, those of pink phenotypes contain only the CY 3-arabinoside. Five quercetin (Q) flavonols were detected in *T. sessile*, but varied in occurrence and relative concentrations among the three phenotypes (Table 3). Although Q 3-0-glucoside, Q 3-0-arabinoglucoside, Q 3-0-arabinogalactoside, and Q 3-0-arabinogalactosyl, 7-0-glucoside occurred in all plants examined, the red phenotypes contained mostly 3-0-biosides and less 3-0 monoside or 3,7-0-triglycoside, the pink phenotypes contained relatively equal proportions of mono-, di-, and triglycosides, and the yellow phenotypes have mostly 3,7-0-triglycosides but less mono- and diglycosides (Table 3). A compound identified tentatively (due to insufficient quantities for sugar analysis) as Q 3,7-0-tetraglycoside occurred only in the pink and yellow phenotypes.

DISCUSSION—The isolation of cyanidin 3-diarabinoside in this study population of *Trillium sessile* corroborates earlier reports of the compound from *T. sessile*, *T. cuneatum*, and *T. stamineum* from Tennessee (Asbury, 1973). Although many sessile-flowered trilliums (subgenus *Phyllantherum* Raf.) reportedly contain two cyanidin glycosides (Asbury, 1973; Adams, 1975), this is the first charac-

terization of a second cyanidin compound, cyanidin 3-arabinoside, from *T. sessile* or other *Trillium* species. Because of the uniformity of R_f values reported for the two cyanidin compounds among species of this group, it is likely that all cyanic trilliums contain the same two compounds (Asbury, 1973) namely, the 3-0-mono- and di-arabinosides of cyanidin. With respect to anthocyanin occurrence, this heterocyanic population may differ from other populations by an enhanced synthesis of cyanidin 3-arabinoside. Asbury (1973) reported the di-arabinoside to be in much greater concentration in the species which she investigated.

The highly glycosylated flavonol compounds isolated in this study are similar to those found by Adams (1975) in a study of *Trillium cuneatum* and *T. luteum*, both members of subgenus *Phyllantherum*. Although Adams (1975) reported both quercetin and kaempferol glycosides, only quercetin compounds were found in this study. The observation of a wide range of flavonol glycosides from mono- to tetraglycosides in other *Trillium* species (Adams, 1975) is consistent with the data reported here. Furthermore, the prevalence of arabinose in the glycosides identified by Adams (1975) coincides with the wide occurrence of the sugar observed in both the flavonol and anthocyanin compounds of heterocyanic *Trillium*. The kaempferol 3-0-arabinogalactoside reported for *T. tschonoskii* by Nakano et al. (1983) is also similar with respect to glycosylation to the quercetin 3-0-arabinogalactoside found in this study. From these reports, we estimate that the flavonoid chemistry of many *Trillium* species is based on the flavonols kaempferol and quercetin with common arabinose glycosylation. The identification of the quercetin analog cyanidin as the anthocyanin constituent of sessile-flowered trilliums provides useful information regarding the biosynthetic relationships between these classes of flavonoids.

Because of a mutuality in biosynthetic precursors, a biochemical relationship exists be-

TABLE 3. Occurrence and relative concentrations of petal flavonoids in floral color phenotypes of *Trillium sessile*

	Petal color		
	Red	Pink	Yel-low
Cyanidin 3-arabinoside	***a	**	—
Cyanidin 3-diaraboside	**b	— ^d	—
Quercetin 3-0-glucoside	*c	**	*
Quercetin 3-0-arabinoglucoside	***	**	**
Quercetin 3-0-arabinogalactoside	***	**	**
Quercetin 3-0-arabinogalactosyl, 7-0-glucoside	*	**	***
Quercetin 3,7-0-tetraglycoside	—	*	*

^a (***) present in high concentration.

^b (**) present in medium concentration.

^c (*) present in low concentration.

^d (—) absent (undetected).

tween anthocyanins and flavonols in plants (Forkmann, 1977). Both compounds are synthesized from dihydroflavonol, a derivative of flavanone or chalcone (Harborne, 1967; Luckner, 1984). In particular, the chemical relationship between cyanidin and quercetin is intimate as both substances are synthesized directly from dihydroquercetin (Gerats et al., 1982). In *Petunia hybrida*, one gene is known to regulate the conversion of dihydroquercetin into quercetin, and four genes have been identified which affect the conversion of dihydroquercetin into cyanidin. In the latter instance, at least two of the genes alter activity of the enzyme anthocyanidin 3-0-glucosyltransferase (Gerats et al., 1982). In *Petunia*, quercetin is synthesized preferentially to cyanidin; when quercetin synthesis is reduced, cyanidin synthesis is enhanced (Gerats et al., 1982). In *Silene* species, homozygous recessive mutants at gene "C" do not synthesize anthocyanin as do homozygous dominant individuals, and do not accumulate dihydroflavonol precursors (Kamsteeg, Van Brederode, and Van Nigtevecht, 1978). This locus is also presumed to involve activity of UDPG anthocyanidin 3-0-glucosyl transferase (Kamsteeg et al., 1978). The conversion of dihydroflavonols to anthocyanidins presumably passes through an intermediate flavan-3,4-diol (leucoanthocyanidin) stage (probably mediated by an NADPH-dependant reductase) prior to glycosylation (Grisebach, 1985). One or several additional enzymes may be further involved in conversion of leucoanthocyanidins to cyanidins (Grisebach, 1985).

In the case of *Petunia*, the fact that quercetin synthesis is unaffected by mutations altering cyanidin synthesis indicates that the reduction or absence of anthocyanins is not related to a disruption of dihydroflavonol synthesis, but rather to its conversion to cyanidin. Under-

standably, more detailed studies have shown that these mutations often affect the activity of anthocyanidin 3-0-glucosyltransferase, the enzyme responsible for catalyzing a late step in that conversion (Gerats et al., 1982). Genetic analyses of homozygous recessive mutants have shown that different degrees of altered anthocyanin synthesis may occur. Homozygous recessive mutants at some loci result in a complete lack of anthocyanin production, whereas at other loci, the synthesis of anthocyanins is arrested incompletely (Kamsteeg et al., 1978; Gerats et al., 1982). Because these studies did not evaluate heterozygous individuals, the nature of dominance at these loci (i.e., complete or incomplete) cannot be ascertained. It is apparent from these studies, however, that blocking anthocyanin synthesis does not result in accumulation of dihydroflavonol precursors as long as the pathway for flavonol synthesis remains operable. Therefore, it is predictable that an arrested anthocyanin synthesis should be accompanied by an increase in flavonol synthesis due to the greater pool of available dihydroflavonol precursors. Indeed, mutations which block the conversion of dihydroflavonols to anthocyanins in *Matthiola* do result in the accumulation of flavonol glycosides (Forkmann, 1977; Grisebach, 1985).

By analogy, a probable genetic basis of the biochemical variation observed in heterocyanic *Trillium sessile* can be deduced. The lack of detectable anthocyanins associated with continued production of quercetin in yellow phenotypes indicates that a complete genetic block exists at a biosynthetic stage which affects the conversion of dihydroquercetin to cyanidin. The enhanced production of polyglycosylated flavonols (tri- and tetraglycosides) in yellow phenotypes indicates that as in other plants, this genetic block does not result in an accumulation of dihydroflavonol, but results in the shunting of these precursors into production of quercetin glycosides. An expected result is that the accumulation of monoglycosides would provide substrate for further modification into various polyglycosides as sugar substitution occurs in a stepwise fashion. As polyglycosides are synthesized, the pool of monosides (used as substrate) would steadily decrease. As in *Petunia* (Gerats et al., 1982), a smaller pool of quercetin monoglycosides should be produced in red phenotypes (where cyanidin production is not blocked) due to common competition for dihydroflavonol precursors. These expectations are realized in heterocyanic trillium plants, and can be explained by a model invoking a single mutation which affects the activity of an enzyme involved in the conversion

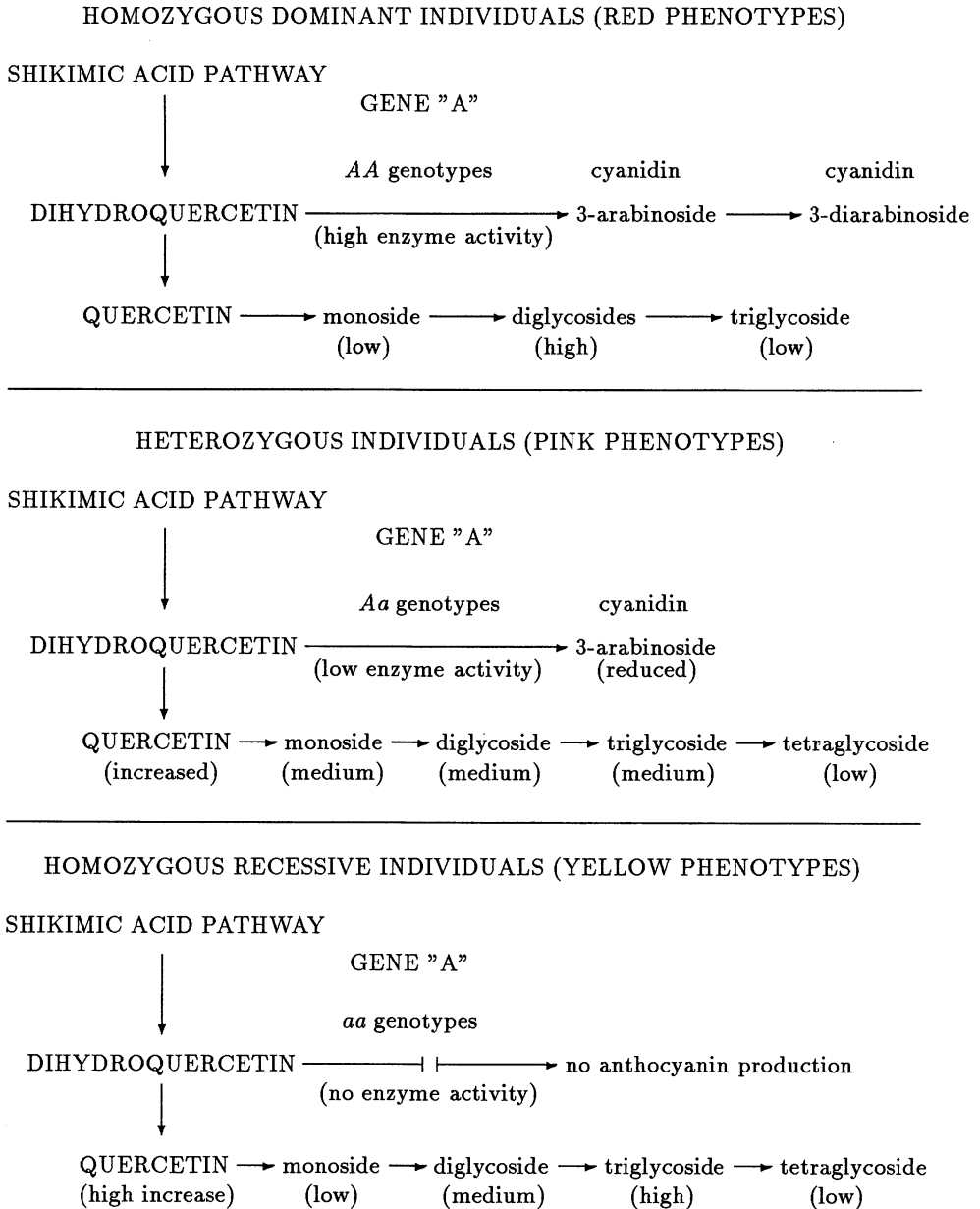


Fig. 1. Proposed genetic model to account for observed biochemical differences among floral color phenotypes of *Trillium sessile*.

of dihydroquercetin to the corresponding 3,4-flavan-diol, or the conversion of the leucoanthocyanidin to cyanidin. We tentatively refer to this uncertain enzyme locus in *T. sessile* as gene "A."

Another question, however, is whether such a mutation would be expressed completely or incompletely with regard to dominance. The biochemical profile of pink phenotypes indicates that expression is probably incomplete.

In these phenotypes, cyanidin synthesis is only partially blocked as evidenced by the small amount of cyanidin monoside produced. The absence of cyanidin diglycoside in "pinks" presumably results from insufficient substrate for further glycosylation. Pink phenotypes also produce an intermediate array of quercetin compounds (Table 3) including some tetraglycoside, which indicates that excess dihydroquercetin is being shunted to the production

of quercetin glycosides. In some of the pink phenotypes, we observed that anthocyanins were more prevalent in the veins of the petals rather than diffused throughout. A similar observation was made in *Matthiola* by Forkmann (1977) who noted that administration of precursors to whole petals of acyanic mutants resulted in considerable anthocyanin production in the veins before the pigmentation eventually extended throughout the petals. The conspicuous vein coloration in some pink phenotypes of *T. sessile* is consistent with our hypothesis of a diminished supply of precursors necessary for anthocyanin synthesis in these plants.

These observations lead us to conclude that in this population of *T. sessile*, a simple mutation has occurred at an enzyme locus (which we refer to as gene "A") which regulates an intermediate step in the synthesis of cyanidin from dihydroquercetin. The mutation is probably expressed as incompletely dominant alleles with pink phenotypes representing heterozygotes, and red and yellow phenotypes representing homozygous dominant and recessive individuals respectively. A scheme depicting our proposed model is illustrated in Fig. 1.

Although this interpretation satisfies questions regarding the observed biochemical profiles among petal color phenotypes, we realize that this hypothetical conclusion presents only one possible model which must be confirmed eventually by successful genetic analyses. We cannot exclude the possibility of multiple factor effects and mention in this respect the case of *Petunia* where anthocyanin synthesis is also affected by an independent locus which regulates the synthesis of quercetin (Gerats et al., 1982). Furthermore, in populations of morning glory polymorphic for floral color, the genetic control of stem color is partially influenced by pleiotropy of two loci which are involved in flower color expression (Schoen et al., 1984). Therefore, the ultimate expression of anthocyanins in various tissues of *T. sessile* is likely to be under more complex genetic control as evidenced by the persistence of both cyanidin glycosides in stem tissue of red and pink phenotypes despite the differential expression in petal and sepals of these same individuals (Table 2).

CONCLUSIONS—The basis of floral color polymorphism in *Trillium sessile* is probably similar to that found in other plant species investigated thus far. Biochemical evidence indicates that a relatively simple genetic basis, probably a single gene mutation with incomplete dominance, underlies the observed mor-

phological differences of red, yellow, and pink phenotypes which occur within a single population of the species. This mutation may affect the activity of an enzyme which brings about the conversion of dihydroquercetin to leucoanthocyanin, or activity of an anthocyanidin 3-O-arabinosyltransferase enzyme which presumably brings about the final conversion of cyanidin 3-O-arabinoside from dihydroquercetin in this species. It may be possible to clarify the exact gene involved by conducting complementation experiments with pink and yellow phenotypes using leucoanthocyanin precursors. Such an experiment would at least determine whether or not the genetic block acted prior to or subsequent to the flavan-3,4-diol stage.

Because dihydroquercetin is a common precursor to both anthocyanin and flavonol synthesis, the biochemical effects of this mutation are extensive. Phenotypes vary not only by their differential expression of anthocyanin compounds in tissues, but also with respect to relative concentrations of various quercetin glycosides. The interactions of these biochemical differences result in the variety of polymorphic color hues which have been observed in petals of this species. Because genetic analyses of this population incorporating usual crossing experiments reach an impasse with the inability to successfully germinate hybrid seeds, these data have provided an alternate approach to developing a model predicting the genetic basis of these phenotypes. This information provides us with a useful basis for carrying out further evolutionary studies of the heterocyanic population.

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