GENETIC CONTROL OF QUERCETIN FORMATION IN THE ALEURONE TISSUE OF MAIZE

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ABSTRACT

The genes *C*, *C2*, *R*, *A*, *A2*, *Bz*, *Bz2* and *Pr* are required for the formation of purple anthocyanin in the aleurone tissue of maize, and the recessive gene(s) result in non-purple (red, bronze and colorless). Aleurone extracts of recessive *a* and certain double recessive combinations were analyzed by paper chromatography, absorption spectra in the ultraviolet (UV) and infrared, mass spectra, and other analytical techniques. Homozygous recessive *a* tissue accumulates the flavonol, quercetin, while the double combinations *a c* and *a r* lack it, suggesting that dominant genes *C* and *R* are required for its formation and act prior to *A* in the synthesis of flavonols, as in the gene action sequence for anthocyanin synthesis. Dominant *C-I* inhibits the formation of quercetin, whereas *Bz*, *Bz2* and *In* do not affect its formation. These results suggest a close biogenetic relationship between quercetin and cyanidin-3-glucoside and also independently confirm the position of *A* in both sequences.

IN maize, *A* is required, in addition to other complementary genes *C*, *C2*, *R*, *A2*, *Bz* and *Bz2*, for the synthesis of anthocyanin pigments in aleurone tissue. Aleurone tissue of homozygous recessive *c*, *c2* and *r* and dominant *C-I* is colorless. Homozygous recessive *a2* accumulates leucocyanidin (COE 1955; REDDY and REDDY 1971); *bz* and *bz2* accumulate luteolinidin and a small quantity of cyanidin-3-glucoside respectively (REDDY and REDDY 1973). The *Pr* locus controls the hydroxylation pattern of the "B" ring of the anthocyanins, leucoanthocyanidins (COE 1955) and flavonols (KIRBY and STYLES 1970). Dominant *C-I* inhibits the formation of leucoanthocyanidins, and recessive *c*, *r* and *a* block its formation (REDDY 1964).

Though the genetic role of the *A* locus in anthocyanin production in aleurone and plant tissue is well understood, the nature of its action has remained obscure. Chemical studies of SANDO, MILNER and SHERMAN (1935) showed the presence of quercetin in homozygous recessive *a* husk tissue, and its corresponding anthocyanin in dominant *AA* husk, which led to the conclusion that *A* may control the oxidation-reduction process; however, later studies could not support this view, since quercetin is also present in *AA* husk (LAUGHNAN 1950). Studies with aleurone tissue of homozygous recessive *a* have revealed the presence of quercetin; double recessive *a pr* accumulates kaempferol, and it was concluded

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that *Pr* acts before *A* in the gene action sequence (KIRBY and STYLES 1970). It has been suggested that the *Bz* gene may be controlling the glycosidation step, based on the evidence of the conversion of quercetin to quercetin-3-glucoside by the enzyme prepared from *Bz* pollen (LARSON and COE 1968). However, the action of the other loci like *C*, *R* and *Bz2* is obscure with relation to the above pigments.

REDDY and COE (1962) proposed the following gene action sequence for seven genes in the synthesis of anthocyanin pigments based on inter-tissue complementation studies:

$$C\text{-}I\text{–}C\text{–}C2\text{–}R\text{–(In)–}A\text{–}A2\text{–}Bz\text{–}Bz2\ .$$

The gene action sequence will help in using certain double recessive combinations of various genes for understanding their role in the synthesis of aleurone pigments and their biogenetic relationships. It would be expected that the recessive genes *c*, *c2*, *r* and dominant *C-I*, which act prior to *A*, must block the accumulation of quercetin in *aaa* tissue, whereas the recessive *a2*, *bz* and *bz2* should not interfere, since they act after *A* in the known gene action sequence for anthocyanin synthesis. The present paper deals with the characterization of the accumulated substances in *aaa* aleurone and action of certain other complementary genes which percede or follow *A* in sequence.

MATERIALS AND METHODS

Homozygous combination stocks *a c*, *a r*, *a C-I*, *a bz*, *a bz2*, *a pr*, triple recessive *a c r* and homozygous single-factor stocks of *a*, *a2*, *c*, *c2*, *r* and *C-I* were used in the present study. These genetic stocks of uniform background (Stock 6×3) were kindly provided by DR. E. H. COE, University of Missouri, Columbia, MO as advanced generation lines of his Stock 6×3 culture. The dry kernels were presoaked in distilled water and the pericarp was peeled off. The aleurone layer was scraped from the endosperm and the powdered aleurone was defatted with cold petroleum ether solvent (60–80). The dried aleurone residue was thoroughly extracted with cold methyl alcohol. The extracts were concentrated under reduced pressure. After washing several times with petroleum ether the residue was redissolved in minimum quantity of methyl alcohol and hydrolyzed with 1N hydrochloric acid for 45 minutes in a water bath at 80°. The concentrated hydrolysate was cooled and extracted with water-free diethylether solvent in a separating funnel. The extraction was repeated four times. The combined extracts were concentrated to dryness and a pale yellow residue was recovered. The extracts were always kept in the dark.

Paper chromatography with BAW (n-butyl alcohol : acetic acid : water, 4:1:5, upper layer) and Forestal (glacial acetic acid : conc. hydrochloric acid : water, 30:30:10) solvents on Whatman No. 1 paper were used to separate and purify the pigments. Two-dimensional preparative paper chromatography with BAW and 10% acetic acid solvents were also employed. The developed chromatograms were dried and examined under UV (long range) light. Average *Rf* values were calculated from eight or more chromatograms and compared with pure quercetin (control) developed on the same chromatogram. Finally, the chromatographically pure pigment was redissolved in spectroscopic grade methyl alcohol and its absorption spectrum in UV and visible range was obtained with a UV Speccord VIS (Carl Zeiss—Automatic). Where chelation was required, one ml of the reagent solution, i.e., 1% aluminium chloride in methyl alcohol, aluminium chloride in hydrochloric acid and sodium acetate, prepared according to MABRY, MARKHAM and THOMAS (1970) were added to three ml of the extract. Average λ max (absorption maxima) were calculated from at least three recordings. The pigment was finally checked for purity by thin layer chromatography on silica gel with chloroform : ethyl acetate mixtures and subjected

to infrared and mass spectral analysis. In addition, several characteristic chemical tests, such as those involving magnesium hydrochloric acid, zinc hydrochloric acid, sodium borohydride reduction and sodium borohydride-HCl : 2,3 dichloro-5,6 dicyano-1,4 benzoquinone, have been carried out with the recessive aleurone tissue extracts. The isolated flavonol was characterized by *Rf* values, visible colors of the pigment, UV fluorescence, reaction to various chemical and spraying reagents, and absorption spectra.

RESULTS

Flavonols are pale yellow pigments which fluoresce under UV light, and the extracts when treated with magnesium-hydrochloric acid turn pink to red. Chromatograms of homozygous recessive *a* aleurone (*aaa* as the endosperm is triploid) hydrolysates consistently yielded a pale yellow pigment which showed a vivid fluorescence under UV light (long range). Table 1 shows the λ max average *Rf* values and effect of chelating agents on the spectral pattern of the isolated pigment.

The pale yellow pigment of *aaa* gave an abs. max at 369 mμ and average *Rf* values of 0.63 (BAW) and 0.42 (Forestal), which are in close agreement with pure quercetin. It gave the characteristic bathochromic spectral shifts with chelating reagents $AlCl_3$ and $AlCl_3$/HCl indicating the presence of orthodihydroxy substituents. The infrared spectrum of the *aaa* pigment was superimposable on that of pure quercetin. Mass spectral analysis further confirmed that the accumulated pigment is quercetin. Another faint yellow pigment with higher *Rf* values (0.80 to 0.9) that fluoresces under UV was also observed on the *a* chromatograms. This was present in trace amounts and was inconsistent in behavior.

Double combinations: The chromatograms of the aleurone hydrolysates of homozygous *a C-I*, *a c*, *a r* and triple recessive *a c r* were colorless, and no pigment was visible under UV and after spraying with certain reagents such as vanillin-toluene-p-sulphonic acid, vanillin hydrochloric acid and 10% Sulphuric acid. Also, none of these extracts gave a positive response for the presence of any flavonoid pigments with certain diagnostic chemical tests and reagents, as mentioned in MATERIALS AND METHODS. However, the hydrolysates of the double

TABLE 1

Characterization of the pigments extracted from aleurone tissue of aaa *and some double combinations**

Genotype	Rf values BAW	Rf values Forestal	λ Max. (mμ)	UV†	$AlCl_3$‡ +Δλ(mμ)	$AlCl_3$/HCl‡ +Δλ(mμ)	NaOAc‡ +Δλ(mμ)
aaa	0.63	0.42	269, 369	+	87	56	20
a bz	0.62	0.42	269, 369	+	86	56	20
a bz2	0.62	0.43	269, 370	+	87	54	19
a pr	0.82	0.54	268, 366	+	—	—	—
a in	0.62	0.43	269, 369	+	86	56	20
Quercetin	0.62	0.42	269, 370	+	85	56	21

* The extracts of combinations *a c*, *a r*, *a C-I* and *a c r* did not reveal this pigment.
† Yellow fluorescence (+).
‡ Bathochromic shifts of λ 369.

combinations *a in*, *a bz* and *a bz2* yielded a yellow pigment which behaved identically to that of *aaa* (Table 1). No significant differences between *aaa* and double recessive *a in* in terms of quantity of the pigment were observed. The hydrolysates of the double mutant *a pr* revealed a faint yellow pigment with *Rf* values 0.82 (BAW) and 0.54 (Forestal) and λ max. at 366 mμ, values which closely agree with the standard values of kaempferol (HARBORNE 1967).

DISCUSSION

The data obtained from various sources in the present study confirm that quercetin is the major pigment of homozygous *aaa* aleurone hydrolysates. Homozygous double recessive *a pr*, which shows clear differences in spectral maxima and *Rf* values when compared with extracts of other double combinations, accumulates kaempferol as reported earlier (KIRBY and STYLES 1970). Homozygous double recessive *a bz*, *a bz2* and *a in* also accumulated quercetin, whereas double combinations *a C-I*, *a c*, *a r* and triple recessive *a c r* did not show this pigment.

From these results it is concluded that the basic complementary genes *C* and *R* must be present in dominant condition for the synthesis and accumulation of quercetin in *aaa* aleurone tissue and that their recessive alleles block its formation. The *bz* and *bz2* genes do not interfere in the synthesis of quercetin, whereas dominant *C-I* inhibits, as evidenced by absence of quercetin in the double combination *a C-I*. These observations lead to the conclusion that *C-I*, *C* and *R* act prior to *A*, whereas *Bz* and *Bz2* follow *A* in the synthesis of quercetin as well as anthocyanin pigments. Thus, the present studies independently confirm the position of *A* in the gene action sequence of REDDY and COE (1962). It is interesting to note that homozygous recessive intensifier (*in*), which enhances anthocyanin formation in *AAA* background, seems to have no effect on flavonol synthesis since there is no significant increase in the quantity of quercetin in the double combination *a in* compared to *a*, as shown by optical density values (REDDY and REDDY, manuscript in preparation). The presence of quercetin in *aaa* aleurone tissue and the requirement of common genes *C* and *R* for its synthesis suggest that anthocyanins and quercetin may have a close biogenetic relationship. KIRBY and STYLES (1970) suggested that recessive *a* blocks the anthocyanin pathway after flavanonol in HARBORNE's scheme (1967). Though this appears to be most probable, it may not be unequivocal because a metabolic block, caused by a recessive mutation in a biosynthetic pathway, does not necessarily result in the accumulation of the substance immediately preceding the block (BIRCH 1972). In the absence of direct evidence on the step controlled by recessive *a* it may be assumed that the recessive *a* allele is an amorph, having no action. However, since quercetin accumulates as a result of the *a* block, and assuming that flavanonols (rather than flavonols) are the most natural precursers of anthocyanins and flavonols (GRISEBACH 1968), it would be reasonable to postulate that the dehydrogenation of flavanonol to flavonol, a minor reaction in dominant tissue, becomes a major one in *aaa* tissue, as suggested by KIRBY and STYLES (1970).

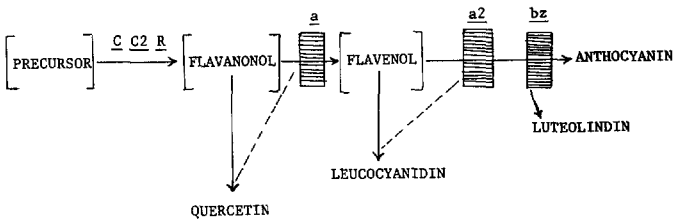

FIGURE 1.—Postulated genetic blocks in anthocyanin biosynthesis, based on the genetic and chemical analysis of single and double recessive combination stocks.

The fact that aleurone tissue of dominant *AAA* also accumulates trace amounts of quercetin supports the above view.

With respect to the action of *A* in the synthesis of anthocyanin pigments, it has generally been considered that dominant *A* gene action is involved in the reduction at the C-4 position of quercetin to chrysanthemin or a common precursor in the husk tissue of maize (SANDO, MILNER and SHERMAN 1935). However, later studies of LAUGHNAN (1950) suggested that *A* gene action may not be solely concerned with the oxidation-reduction step since four or five phenolic pigments were present in *A A* and *a a* husk tissue. The *A* gene action also seems to be required for the production of 3-deoxyflavonoid pigments such as luteoforol and luteolinidin in husk tissue of maize (STYLES and CESKA 1972). In the absence of clear evidence of a single step reduction *in vivo* of quercetin or its immediate precursor to anthocyanin, no such direct effect—i.e., an oxidation-reduction step —can be attributed to the *A* gene. The present studies clearly suggest that quercetin results from the *a* block and that the dominant genes *C* and *R* are required for its accumulation. Preliminary studies also suggest that *A2* and *Bz* follow the *a* block (Figure 1), and that the corresponding recessive types accumulate leucocyanidin and luteolinidin respectively (REDDY 1974).

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