CHEMICO-GENETIC STUDIES OF LEUCOANTHOCYANIDIN IN A MAIZE MUTANT

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ABSTRACT

The nature of the accumulated substance in a_2 mutant aleurone was studied in relation to the known gene action order for anthocyanin biosynthesis in maize. Spectrophotometric, chromatographic, and chemical studies clearly suggest that the accumulated substance in a_2 mutant is leucocyanidin (5, 7, 3', 4'-tetrahydroxy flavon : 3, 4 diol). The biogenetic relationship of leucoanthocyanidin to anthocyanin is discussed in the light of the gene-controlled chemical pathway.

IDENTIFICATION of gene-controlled end products has opened new avenues for speculation and experimentation concerning the nature and mode of gene action. This is specially true when contrasting genotypes produce different and easily identifiable substances in an organism. The genetic system of anthocyanin biosynthesis in maize, which is controlled by more than ten genes, is one of the best known examples in higher organisms wherein the biochemical basis of gene action can be analysed.

In maize, the anthocyanin synthesis in aleurone tissue, the outermost layer of endosperm, is controlled by the complementary factors C_1, C_2, A_1, A_2 and R. The dominant inhibitor gene C^I blocks the synthesis whereas the intensifier (*In*), bronze-1 (Bz_1) and bronze-2 (Bz_2) control the intensity of anthocyanin : *Pr/pr* controls the hydroxylation pattern.[1] Reddy and Coe[2] (1962) proposed the following gene action sequence for anthocyanin synthesis using inter-tissue complementation technique with fresh, synthetically-active and genetically-blocked mutant alurone tissue.

C^I C_1 C_2 R *In* A_1 A_2 Bz_1 Bz_2-Anthocyanin
$\rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow$

However, the specific information regarding the chemical nature of intermediates associated with the specific genes in the above gene action sequence is not yet fully available. Coe[3] (1955) has reported the accumulation of a colourless substance in aleurone tissue of a_2 mutant, which upon heating with acid gave coloured anthocyanidin. It has been also stated that the homozygous recessive intensifier (*in*) enhanced the production of this colourless substance, *i.e.*, leucoanthocyanidin by about 10-15 folds in a_2 mutant. Earlier studies have clearly shown that the genes, C_1, C_2, A_1 and R preceding a_2 in the gene action sequence must be present in dominant condition at least in one dose, for the production of leucoanthocyanidin in a_2 mutant.[4] The presence of dominant inhibitor C^I blocks synthesis and *Pr/pr* controls the hydroxylation of leucoanthocyanidin. The present work deals with the nature of the substance accumulated in a_2 mutant aleurone tissue and the specific role of A_2/a_2 gene in anthocyanin biosynthesis.

MATERIALS AND METHODS

Dry mature kernels of a_2 mutant (C_1 C_2 R *In* A_1 a_2 Bz_1 Bz_2 *Pr*) and double mutant *in* a_2 (C_1 C_2 R A_1 *in* a_2 Bz_1 Bz_2 *Pr*), after removal of pericarp, are defatted with petroleum ether (40-60°) for 24 hours and then extracted with cold methanol. After 36 hours, the solvent was removed under reduced pressure on a rotary evaporator and treated with cold ether. Ether insolubles are separated and treated with a small amount of water. The aqueous solution was repeatedly extracted with pure ethyl acetate in a separating funnel. The combined ethyl acetate extract was dried over anhydrous magnesium sulphate. The solvent was removed on a rotary evaporator and a light brown amorphous residue of the substance was obtained. The extracted substance was converted to the corresponding flavylium chloride by boiling with 5% methanolic-2 N hydrochloric acid for 30 min on a steam-bath. The pink solution was extracted repeatedly with isoamyl alcohol. The combined isoamyl alcohol extracts were used for further studies.

Paper chromatography with two different solvent systems BAW (*n*-butanol 4 : acetic acid 1 : water 5) and Forestal (acetic acid 30 : HCl 3 : water 10) on Whatman No. 1 paper was used throughout the investigation. Extracts were kept in the dark during all stages of isolation. The following five techniques were used for the identification of the isolated compound and its flavylium chloride : (1) R_f values ; (2) Visible colours ; (3) Colour reactions ; (4) Reactions to various specific spraying reagents ; (5) Absorption spectra. The pure sample was co-chromatographed with the

extracts for comparison. The chromatographically pure isolated compound and the converted flavylium chloride were taken in methanol and in 5% methanolic hydrochloric acid solution respectively and were subjected to Beckman DB spectrophotometer and readings were taken at intervals of 5 mμ in the UV region of 200-300 mμ and also in the region of 220-560 mμ.

Observations and Discussion

The average R_f values and absorption maxima of converted anthocyanidins along with pure cyanidin chloride are presented in Table I. The chromatograms (circular) of the isolated compound when sprayed with different specific spraying reagents (and heated for 30 min at 80° C in a hot air-oven whenever it was necessary) gave the following: (*a*) characteristic green colours with ferric reagent; (*b*) positive reaction to ammoniacal silver nitrate solution; (*c*) intense violet-red colours with vaniline-toluene-*p*-sulphonic acid; (*d*) purple colouration with 10% sulphuric acid. It was soluble in water, ethylacetate and insoluble in ether. When subjected to various characteristic chemical reactions, it gave the following: (*a*) dark green colouration with neutral ferric chloride, (*b*) positive reaction to aqueous sodium hydroxide (fresh) and sulphuric acid, (*c*) pink colouration with hydrochloric acid on heating for a few minutes on steam-bath, (*d*) negative reaction to Mollish test for carbohydrates, hence an aglycone, (*e*) absorption maxima at 286 mμ.

The converted anthocyanidin was fairly stable and gave positive reactions to the various above-mentioned specific spraying reagents. In addition, it gave the characteristic R_f values and absorption maxima (*see* Table I) and also showed the positive reaction to cyanidin reagent.

The chromatographic, spectrophotometric and chemical studies suggest that the isolated compound is leucocyanidin or 5, 7, 3′, 4′-tetrahydroxy flavan : 3, 4 diol. The exact role of leucoanthocyanidin in anthocyanin biosynthesis is not yet clear. Chemico-genetic studies with flower petals of Impatiens suggested a close relationship between the leucoanthocyanin and anthocyanin biosynthesis.[5]. Later studies with seed coats of Phaseolus[6] and more recently labelled studies using phenylalanine-1-C^{14} with Impatiens[7] lead to the conclusion that both leucoanthocyanin and anthocyanin are synthesised by parallel biosynthetic pathways.

The double recessive combinations of a_2 bz_1, a_2 bz_2 and *in* a_2 in the gene action sequence accumulate leucoanthocyanidin whereas it was absent in double mutants of C^r a_2, c_1 a_2, c_2 a_2, r a_2 and a_1 a_2 alerone suggesting that bz_1, bz_2 and *in* do not interfere with the synthesis of leucocyanidin whilst the C^r and recessive c_1, c_2, r and a_1 block it.[4] Though *Pr/pr* controls the hydroxylation pattern of the leucoanthocyanidin and anthocyanidin its exact position in the known gene action sequence was not clear. The studies on the accumulated substance in the double mutants of a_1 *Pr* (quercetin), and a_1 *pr* (Kaempferol) and a_2 *Pr* (leucocyanidin) and a_2 *pr* (leucopelargonidin) suggest that *Pr/pr* acts prior to A_1 and A_2 in the known gene action sequence.[8] These observations emphasise a common biosynthetic pathway for leucoanthocyanidin and anthocyanin (*see* Fig. 1).

If leucocyanidin is a precursor in anthocyanin biosynthesis, then at least two steps such as chromogenation and glycosidation are necessary for conversion of leucocyanidin to

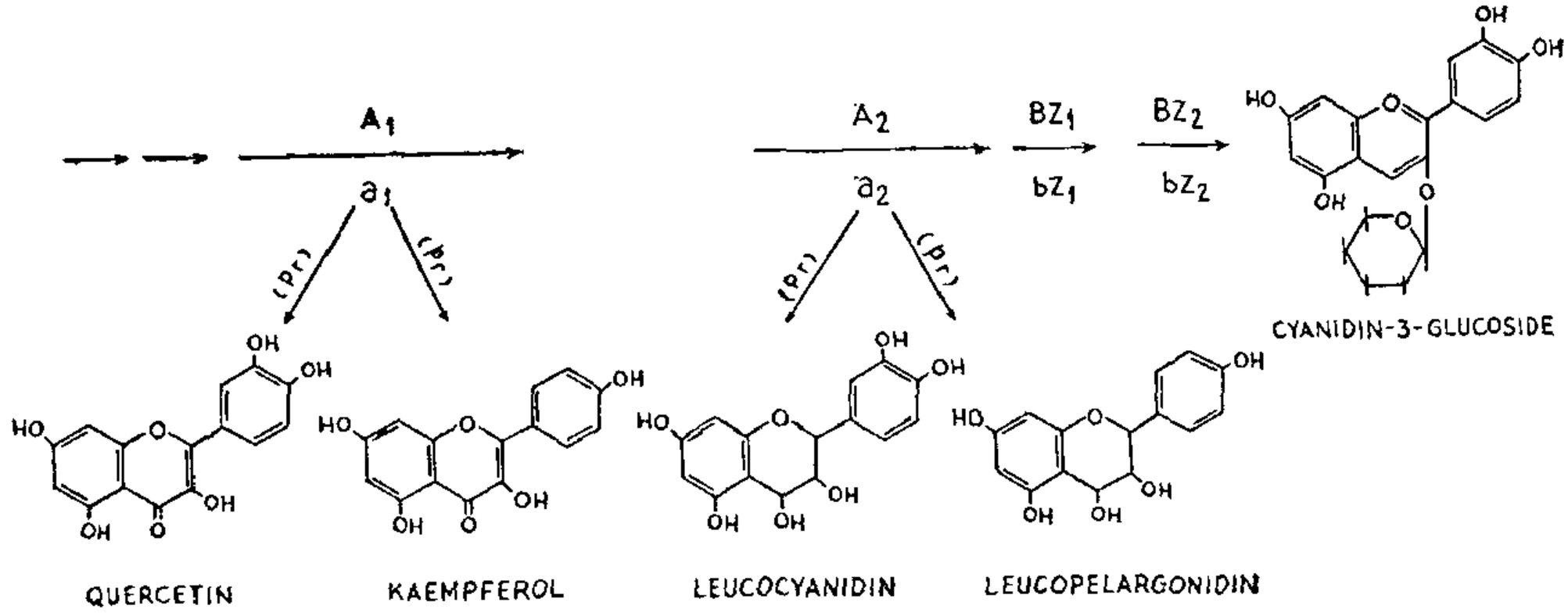

FIG. 1. Gene-controlled chemical pathway of anthocyanin synthesis in maize.

TABLE I

The R_f values and absorption maxima of the converted anthocyanidin and pure cyanidin chloride

Genotype	R_f values		Absorption maxima in mμ	
	Baw	Forestal	UV	Visible
a_2 ..	0·68	0·48	279	541
in a_2 ..	0·68	0·47	278	542
Cyanidin chloride	0·69	0·49	279	543

anthocyanin. In the known gene action sequence, there are only three genes A_2, Bz_1 and Bz_2 which may convert leucocyanidin to anthocyanin. The A_2/a_2 gene could act in two different ways either by direct conversion of leucocyanidin to cyanidin or on a common precursor in the biosynthesis of these pigments. The glycosidation of quercetin to its 3-glycoside (isoquercetin) by an enzyme from Bz_1 pollen extracts suggests indirectly that this gene might control the glycosidation of anthocyanin molecule.[9] Further studies on isolation and characterisation of gene-controlled intermediates in anthocyanin biosynthesis, in maize, may unravel the mechanism of gene action at chemical level.

ACKNOWLEDGEMENTS

The authors thank Professor O. S. Reddi, Head, Department of Genetics, Professor N. V. Subba Rao, Head, Department of Chemistry, Osmania University, for providing facilities, and the Department of Atomic Energy for the grant to carry out this work.

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IN VITRO GROWTH REQUIREMENTS OF MATURE ENDOSPERM OF *RICINUS COMMUNIS*

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SATSANGI and Mohan Ram (1965) succeeded in inducing embryoid formation in the callus of mature endosperm of *Ricinus communis*, but failed to obtain organogenesis. The present investigation was undertaken (*a*) to ascertain the growth requirements, and (*b*) to explore the possibility of induction of root and shoot.

Ripe fruits were collected from the Delhi University Campus. After removal of the fruit-wall and seedcoat, the mature endosperm (with embryo intact) was washed with 'cetavlon' (cetrimide concentrate, diluted to 100 times) and surface-sterilized with chlorine water for 10-12 min. The endosperm was then washed with sterile distilled water and soaked in it for 24 hr. This was then implanted aseptically on modified White's semi-solid (agar 0·8%) medium containing 2% sucrose (WB). The supplements used were: indoleacetic acid (IAA), indolebutyric acid (IBA), indolepropionic acid (IPA), naphthaleneacetic acid (NAA), phenoxyacetic acid (PAA), 2,4-dichlorophenoxy acetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), adenine (AD), adenine sulphate ($ADSO_4$), kinetin (KN), benzyladenine (BA), diphenyl urea (DPU), 6-(γ,γ-dimethylallylamino)-purine (6-γ,γ), SD 8339, triacanthine (TA), zeatin (ZN), myo-inositol, casein hydrolysate (CH), coconut milk (CM), and yeast extract (YE). The effect of various sugars was also tested.

On WB alone, WB + IAA, WB + IBA, WB + IPA, WB + NAA, or WB + KN, after six weeks, in 80% cultures, the embryo developed into a normal seedling (Fig. 1, A). However, on WB + 2, 4-D, germination of the embryo was delayed and swelling of the radicle was observed after 3 weeks. The endosperm showed only slight swelling and cracks but did not callus. On WB + 2, 4-D (2 ppm) + KN (5 ppm) + YE (2,500 ppm), germination of the embryo was altogether suppressed, and the radicle callused. Along the surface in contact with the embryo, the endosperm also proliferated forming a white, fluffy callus (Fig. 1, B). In subcultures maintained on the same medium, the endosperm callus showed satisfactory growth and became compact after four passages (each passage of four weeks) (Fig. 1, C).

Squash preparations of a 4-week-old endosperm callus revealed thin-walled cells of different shape and size, while a 10-week-old