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PRECURSORS AND THE GENETIC CONTROL OF PIGMENTATION

V. INITIATION OF ANTHOCYANIN SYNTHESIS IN *ANTIRRHINUM MAJUS* BY *BOTRYTIS CINEREA*

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Summary

The late block in the biosynthetic pathway to anthocyanin production in the corolla tubes of *Antirrhinum majus* caused by the mutation *delila* (*del*) can be circumvented by inoculation with live *Botrytis cinerea*. Pelargonidin or cyanidin was synthesised depending on the *eosinea—Eosinea* (*Eos*) constitution of the host. Other blocks in anthocyanin synthesis have not been overcome in this way. *B. cinerea* produced tube pigmentation in homozygous *pallida-recurrens* (*pal-rec*) : *del* flowers only where *pal-rec* had mutated to *Pal*. Inoculation of *B. cinerea* to the intermediately blocked acyanic homozygous *incolorata* (*inc*) : *del* only initiated synthesis of cyanidin synergistically after administration of the absent dihydroflavonol, dihydroquercetin. Corolla tubes of *inc Eos* : *del/inc Eos* : *del*, inoculated and later homogenised and filtered to remove contaminating *B. cinerea*, initiated synthesis in *Inc Eos* : *del/Inc Eos* : *del* tubes; the heat labile agent was thus transferable from the non-anthocyanin producing acyanic host to one genetically capable of response, where anthocyanin was produced. Since the evidence was against *B. cinerea* providing an anthocyanin precursor or an enzyme, it is suggested that an enzyme stimulator was either formed in the *A. majus* corolla tubes or transferred from *B. cinerea* as a necessary stimulator for the enzyme that enabled the elaboration of the dihydroflavonol to anthocyanin.

1. Introduction

The pre-flavanone and pre-dihydroflavonol stages in anthocyanin synthesis in *Antirrhinum majus*, when genetically blocked by *nivea/nivea* (*niv/niv*) and *incolorata/incolorata* (*inc/inc*) respectively, can be individually circumvented by the administration of chemically defined precursors (Harrison and Stickland, 1974; Stickland and Harrison, 1974). The third and late major block, the pre-anthocyanin and post-dihydroflavonol, is controlled by the *delila* (*del*) gene, which prevents anthocyanin synthesis only in the corolla tube (plate 1a, *centre*), and to varying degrees by the *pallida* (*pal*) alleles. The *pal/pal* and *del/del* flowers lack the enzyme activity for conversion of the dihydroflavonol to anthocyanin in particular parts of the corolla; thus, no synthesis follows the administration of the last available precursor since it is already present. However, the acyanic corolla tubes of *del/del* flowers can be made to synthesise anthocyanin after inoculation of the tubes with a water suspension of live grey mould, *Botrytis cinerea* (plate 1a, *right*). This reaction is examined further and its relationship to phytoalexin formation discussed.

2. Materials and methods

The homozygous (*inc Eos* : *del*), (*inc Eos* : *Del*), (*pal-recurrens* : *del*), (*eos* : *del*) and (*Eos* : *del*) lines of *Antirrhinum majus* were inbred and maintained at this Institute as standard genetic stock. The (*inc eos* : *del*) line was produced specially for this experimental work. The *Botrytis cinerea* was cultured from naturally infected *A. majus* flowers; the first cultures were grown from single spores plated on yeast RNA culture medium (Harris and Dennis, 1976). Inoculations of *B. cinerea* into the flowers were made from a dense aqueous suspension of spores and mycelia through a hypodermic needle which punctured the corolla tube and left a droplet of the suspension at the inoculation point. Extracts were similarly inoculated.

The inoculated plants were grown in a glasshouse. With *inc* : *del*/*inc* : *del* plants, extracts of the multiply inoculated tubes were prepared 3-4 days after inoculation. About 100 corolla tubes were isolated, ground in liquid N_2, a few ml of water added to form a thin paste and left for an hour before centrifuging for a few minutes. The supernatant was passed through Whatman No. 1 filter paper and subsequently through Millipore (0·45 μ) filters. The filtrate was reduced in volume to 0·5 ml in a rotary evaporator. To ensure that no viable *B. cinerea* had passed through the filter a sample of each extract was plated on yeast RNA culture medium. Bovine albumen was sometimes added to thicken the extract which, when used as an inoculum, would remain in better contact with the punctured corolla tubes. The *B. cinerea* extracts were similarly prepared after the mycelia and spores had been pulverised in a Potter homogeniser. After filtration, some extracts were separated into low and high molecular weight fractions (25,000 mw cut off) by passage through CF25 Centriflo cones (Amicon Corporation).

In all cases the anthocyanins formed were identified by TLC on cellulose (Stickland and Harrison, 1974). Although only the aglycone is specified, both pelargonidin and cyanidin were found as the 3-rutinoside.

3. Results

The first observation was made on *Eos* : *del*/*Eos* : *del* plants grown in the glasshouse. Some corolla tubes showed rings of cyanidin which were eventually shown to be caused by entry of *B. cinerea* through lesions produced by insects. Mechanical stimulation alone did not induce pigmentation in this material; nor did inoculation of *Neurospora* and *Penicillium* spp. Cultured *B. cinerea* was purposely inoculated into the tubes of a range of *A. majus* genotypes; the only genotypes that synthesised pigment were those in which *del* blocked corolla tube pigmentation while the lobes were fully pigmented. *Eos* : *del*/*Eos* : *del* 3-4 days after inoculation, synthesised cyanidin around the inoculation point; however, in *eos* : *del*/*eos* : *del* corolla tubes pelargonidin, the normal anthocyanin for this genotype, was formed. This synthesis did not occur when the flowers were kept in darkness after inoculation but within 24 hours of subsequent exposure to light, synthesis commenced.

Attempts to select for *B. cinerea* strains more effective in producing anthocyanin by subjecting the wild type culture to the highly mutagenic *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were ineffective (Harrison and Hopwood, 1969).

PLATE 1a.—*Right:* synthesised cyanidin on *del/del* corolla tubes at the two sites of *B. cinerea* inoculation made four days earlier. *Centre:* uninoculated *del/del* flowers. *Left: pal-rec* : *del/pal-rec* : *del* with the mutant sites of *pal-rec* → *Pal* on the corolla tube only around the points of *B. cinerea* inoculation.

PLATE 1b.—Enlargement of plate 1a, *left*, in which *pal-rec* → *Pal* mutations synthesised cyanidin after inoculation of *B. cinerea* on the *del/del* corolla tube.

Plate II

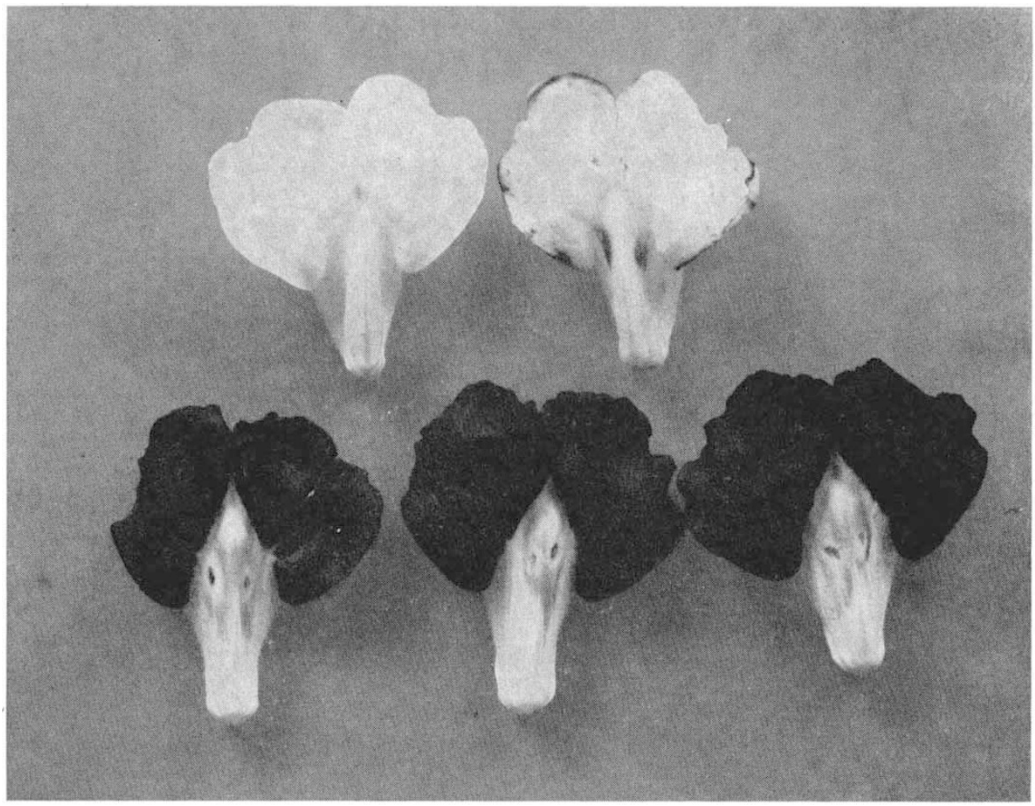
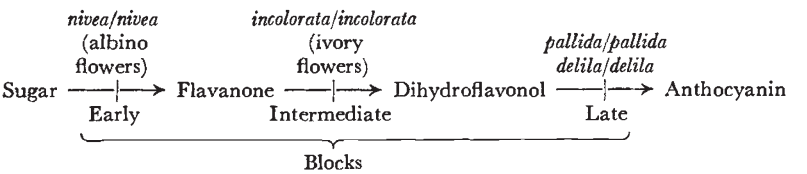


PLATE 2.—*Top left: inc* : *del/inc* : *del* flowers. *Top right: inc* : *del/inc* : *del* inoculated with *B. cinerea* on tube. After 24 hours imbibition of dihydroquercetin synthesis occurred patchily over the lobes but only around the inoculation areas on the corolla tube. *Bottom row:* sterile extract of *inc* : *del/inc* : *del* corolla tubes (inoculated 4 days previously with *B. cinerea*) inoculated into *Inc* : *del/Inc* : *del* corolla tubes. Large areas around inoculation points synthesised cyanidin.

(i) *Hypostatic instability of* pallida-recurrens

When the unstable *pal-rec* allele " mutates "* to *Pal*, anthocyanin is synthesised only in those cells affected by the functional change, provided there is no other block in the anthocyanin pathway. In an *inc/inc* plant, anthocyanin synthesis is blocked at an intermediate stage; this single block can be overcome by administering the missing dihydroflavonol precursor, dihydroquercetin (Harrison and Stickland, 1974). In *inc* : *pal-rec/inc* : *pal-rec* flowers, the mutations *pal-rec*→*Pal* are unexpressed but, after administration of dihydroquercetin, anthocyanin is synthesised in those cells affected by the mutations.



When acyanic corolla tubes of *pal-rec* : *del/pal-rec* : *del* were inoculated with *B. cinerea*, pigmented sites developed around the inoculation points after 3 to 4 days. These sites indicated the cells in which *pal-rec*→*Pal* mutations had occurred but were unexpressed until the pigment pathway was unblocked by *B. cinerea* (plate 1a, *left*; plate 1b). The late block of *del* and the early and intermediate blocks of *niv* and *inc* all prevented the expression of mutant sites; but when circumvented by *B. cinerea* (for *del*) and by dihydroquercetin (for *niv* and *inc*), the instability of *pal-rec* was shown to be unaffected by these epistatic genes.

(ii) *The synergistic effect of* B. cinerea *and dihydroquercetin*

The acyanic *inc/inc* flowers synthesise cyanidin after administration of dihydroquercetin. When the *inc/inc* flower is *Del* then synthesis occurs in the whole flower, but when *del/del*, cyanidin is formed only in the corolla lobes. Inoculations of *B. cinerea* into *inc* : *del/inc* : *del* flowers produced no cyanidin. Subsequent feeding of dihydroquercetin yielded cyanidin around the inoculation points in the corolla tube, and more generally in the lobes as expected (plate 2, *upper right*). In the corolla tube, we assume that *B. cinerea* has stimulated activity of the *Del* enzyme which enabled the elaboration of the imbibed precursor to form cyanidin. Thus, only when both the enzyme stimulation by *B. cinerea* and the precursor, dihydroquercetin, were provided was cyanidin synthesised in the corolla tube.

(iii) *Inactivated* Botrytis cinerea *and concentrated filtrates*

No formation of anthocyanin followed corolla tube inoculations made with an aqueous suspension of *B. cinerea* killed by boiling. A similarly killed suspension, from four petri dishes of *B. cinerea* filtered and reduced in volume in a rotary evaporator to 0·5 ml, also failed to stimulate pigmentation in

* The term *mutates* here does not imply a near permanent structural change to the gene but a change in gene function that could be reversible and possibly results from a transposition of controlling elements (Harrison and Carpenter, 1979).

Eos : *del*/*Eos* : *del* corolla tubes. However, when the same procedure was adopted with an unboiled and Millipore filtered suspension a very slight synthesis involving only a few cells around the inoculation points was discernible, *i.e.*, there was a small amount of a heat-labile factor present in the *Botrytis*.

(iv) *Transfer of stimulator*

Corolla tubes of *inc Eos* : *del*/*inc Eos* : *del* were multiply inoculated with an aqueous suspension of live *B. cinerea*. After 4 days a *B. cinerea*-free extract of the corolla tubes was prepared (see Methods). The extract was inoculated into *Eos* : *del*/*Eos* : *del* corolla tubes and after 3 days larger diffuse patches of cyanidin appeared (plate 2, *bottom row*) which indicated an overall absorption and not a spread of synthesis from the inoculation point. The pigment stimulating property of the extract was heat labile.

(v) *Low and high molecular weight fractionation*

When the *B. cinerea*-free extract was separated into low and high molecular weight fractions, the " low " fraction inoculation gave large patches of cyanidin of the same type as whole extract, whereas the " high " fraction gave a very poor production of pigment which could well have arisen from contamination with the " low " fraction.

Live *B. cinerea* thus stimulated the formation of a heat labile factor of low molecular weight, thus unlikely to be a protein, which is capable of activating the *Del* enzyme.

(vi) *Transfer of* del *corolla tube precursor and* Del *enzyme*

Complementation tests in *A. majus* using flower homogenates or extracts, successfully initiated pigment synthesis where the recipients had an earlier block (Harrison and Stickland, 1974; Harrison and Stickland, 1978). For example, a flower homogenate of ivory *inc*/*inc* administered to albino *niv*/*niv* will initiate anthocyanin synthesis.

An ethyl acetate extract of *Inc eos* : *del*/*Inc eos* : *del* corolla tubes was administered as an aqueous solution to *inc Eos* : *Del*/*inc Eos* : *Del* in which it initiated a little synthesis of cyanidin in corolla lobes and tubes. The *del* corolla lobes thus contained an extractable precursor (presumably dihydrokaempferol) although the synthesis achieved was less than from a comparable amount of lobe tissue. The recipient added an hydroxyl group and converted the pelargonidin precursor to cyanidin.

All attempts to transfer the *Del* enzyme, using an aqueous homogenate of corolla tubes from *inc* : *Del*/*inc* : *Del* or *Inc* : *Del*/*Inc* : *Del* to *Inc* : *del*/*Inc* : *del*, failed since no synthesis was initiated.

4. Discussion

The inoculation of *A. majus* genotypes with *B. cinerea* initiates anthocyanin synthesis only in corolla tubes where pigment was genetically blocked solely by *del*/*del*. If the synthesis resulted from the insertion of a precursor then flowers blocked earlier, *i.e.*, solely by *inc*/*inc*, should respond since the *Del* enzyme would be present, but they did not. There must therefore be an

increase in the activity, possibly a *de novo* induction, of this *Del* enzyme or stimulation of an inactive enzyme already present. Transference and imbibition of large enzyme molecules from the growing *B. cinerea* into the corolla tube cells would be unlikely, as would be the possession by *B. cinerea* of an enzyme capable of metabolising dihydroflavonol to anthocyanin. It is proposed that either a specific *Del* enzyme stimulator occurs naturally in *B. cinerea* or, perhaps more probably, is induced by it in the *A. majus* corolla tubes where the precursors already present can be elaborated to form the anthocyanin relevant to the genotype. A heat labile substance has been extracted from infected *inc* : *del*/*inc* : *del* corolla tubes which initiated synthesis in *Inc* : *del*/*Inc* : *del* tubes.

Precursors such as dihydroquercetin will initiate cyanidin synthesis in the dark when fed to *inc* flowers grown in the light. However, *del* flowers inoculated with *B. cinerea* and enclosed in a light-proof container failed to synthesis cyanidin. On removal to light after 4 days in the dark, cyanidin production commenced within 24 hours. In comparison plants not darkened needed 3-4 days after inoculation for cyanidin formation to start. There thus appeared to be a " dark " reaction, and on admission of light a rapid stimulation of *Del* enzyme activity occurred. The activity of this enzyme appeared to be similar in its reaction to light to that of many other enzymes involved in flavonoid metabolism (Hahlbrock *et al.*, 1971). The " dark reaction " would be the build-up of the enzyme stimulating system and perhaps accumulation of a factor from *B. cinerea*. Whether the increase in enzyme activity was due to stimulation of an existing enzyme or to *de novo* synthesis (induction) of a missing enzyme has not been resolved.

The stimulation of plants to produce or increase anthocyanin by galls, bacteria, fungi, mechanical damage, plant regimes and temperature was summarised by Onslow (1925). It has been suggested (Harborne, 1967) that flavonoid formation constitutes a defence mechanism and the isoflavonoids have frequently been cited as controllers of infection (Deverall, 1976; Vanetten and Pueppke, 1976). Phenolics in general may have a similar role but acyanic *A. majus* plants do not appear more vulnerable to infection than anthocyanic forms and even *nivea*/*nivea* plants when grown outdoors are not unduly vulnerable to disease and fungal infection. The resistance to *Puccinia antirrhini* is not correlated with the amount of anthocyanin pigmentation in the plants but is controlled by a dominant gene R that is linked between *Inc* and *Eos* (Sampson, 1960).

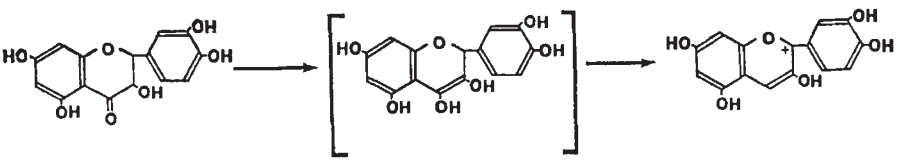
The reactions reported in this paper show some of the properties regarded as characteristic of phytoalexin production since they occur only after fungal invasion of higher plant tissue and the " phytoalexin " produced is confined, or close, to the tissue colonised by the fungus. However, the compounds produced, cyanidin- or pelargonidin-3-rutinoside, are normal constituents in other genotypes of *A. majus* corolla tubes, and even in *del*/*del* flowers they occur in other parts of the same corolla.

It is necessary for both the *pal* and *del* steps to be functioning for anthocyanin synthesis to occur. Thus, in *pal* : *del*/*pal* : *del* there is no synthesis in the corolla tube following *B. cinerea* inoculations but in *Pal* : *del*/*Pal* : *del* synthesis can occur.

It is significant that in *Antirrhinum* only the late block in the anthocyanin pathway can be overcome by the introduction of *B. cinerea* or homogenate extracts of *del*/*del* tubes stimulated by *B. cinerea*. The earlier *niv* and *inc*

blocks are readily circumvented by eriodictyol and by dihydroquercetin respectively for cyanidin and by naringenin and dihydrokaempferol for pelargonidin. Whether the *del* block is before or after *pal* is unresolvable at this stage since there was no synthesis following inter-feeding of *Del* to *pal* and *Pal* to *del*. Attempts to build up the concentration of any intermediate precursor by feeding dihydroquercetin to either genotype, followed by extraction and administration to the other, also gave negative results. Indeed, the two blocks could be similar, their difference being the consequence of a spatial control in certain parts of the corolla. However, if they are identical blocks then synthesis might be expected in *pal*/*pal* blocked lobes following *B. cinerea* inoculations, but this does not occur. It would thus seem probable that the dihydroflavonol to anthocyanin progression (fig. 1) has an intermediary flavenol stem (Harborne, 1967). It would be at this flavenol point in the pathway that the possible separate *pal* and *del* controls occur.

FIG. 1



Dihydroflavonol (dihydroquercetin) Flavenol Anthocyanin (cyanidin)

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