THE NATURE AND GENETIC CONTROL OF A RED ANTHOCYANIN PIGMENT IN THE ROOT MERISTEMS OF *PHALARIS*

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Summary

A red anthocyanin pigment which occurs in the root meristems of certain *Phalaris* species has been identified as a glycoside of pelargonidin. The pigment has been observed only in plants of the three polyploid species *P. minor*, *P. tuberosa*, and *P. arundinacea*, and is absent in the diploid members of the genus. Genetic studies indicate that the character is simply inherited, involving a single major dominant gene controlling the production of the pigment, and a series of modifier genes influencing the level of its expression. In *P. minor*, a self-pollinating annual, the gene is widespread and homozygous, but in *P. tuberosa* and *P. arundinacea*, both cross-pollinating perennials, it occurs at low frequency largely as the heterozygote, and is restricted to certain areas within the range of the distribution of these species. The origin of the gene in *Phalaris*, and its possible adaptive significance is discussed. Also its value as a single gene marker in breeding studies is indicated.

I. Introduction

Red anthocyanin pigment has been recorded in the root tips of a number of plant families including the Crassulaceae, Saxifragaceae, Melostomaceae, and Compositae (Molish 1928). In the Gramineae, a similar red pigment occurs in the root tips of certain *Phalaris* species. It is most obvious in *P. minor*, a widespread annual, and has been recorded in numerous taxonomic descriptions of this species. Bloch (1943) has described differentiation in the red root tips of *P. arundinacea*, and the pigment has also been recorded in the hexaploid race of this species (McWilliam and Neal-Smith 1962). In addition to these species, red root tips have been observed in Mediterranean ecotypes of *P. tuberosa* grown at Canberra. This character has not been reported in any other grasses and appears to be confined entirely to certain species of the genus *Phalaris*.

The striking appearance and ease of identification of this character suggested that it might be of value as a genetic marker. This paper reports the results of a study to investigate the distribution of red root tip within the genus, its genetic control, and the chemical nature of the pigment responsible for its expression.

II. Material and Methods

The red pigment in the root tips of *Phalaris* can be detected readily when the seed is germinated on moist filter paper. This method was used to screen for the presence of the pigment in a number of *Phalaris* species including the Mediterranean group and some of the lesser known American species.

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The location and distribution of pigment in the meristematic region of the root tips were examined in freshly cut hand-sections. The effect of different environmental conditions on the expression of the character and on the growth of "red" and "white" seedlings was also studied in populations grown under controlled conditions of temperature and photoperiod.

Extracts of the red pigment for chemical analyses were prepared from freshly harvested root tips of three species: *P. minor*, *P. tuberosa*, and *P. arundinacea*. Root tips were covered with 70% (v/v) ethanol containing 1% HCl and left for several days at 5°C. While some pigment was extracted, the bulk of the colour remained in the tissues. Concentrated hydrochloric acid was then added to give a final value of 6N HCl and the mixture was heated at 70°C for 5 min. After cooling, the mixture was centrifuged for 20 min at 2200 *g* and the supernatant was extracted with n-amyl alcohol until no further colour remained in the aqueous layer. An orange-red solution of the anthocyanidins was obtained.

A second sample of root tips was extracted by boiling for 1 min with 80% ethanol containing a small amount of calcium carbonate. After cooling and centrifuging, a bluish red solution containing the anthocyanins was obtained.

The inheritance of red root tip was studied in *P. tuberosa* and the hexaploid race of *P. arundinacea*. Plants of each phenotype (red and white) were first characterized genetically on the basis of the segration ratios observed in their self-pollinated progeny. Crosses were then made between different genotypes and the segregation ratios compared with those expected using the χ^2 analysis. Interspecific crosses were also made between *P. tuberosa* and red genotypes of *P. arundinacea* to determine the suitability of this character as a marker for identifying hybrid progeny.

Because of the difficulty of emasculating *Phalaris*, all crosses reported in this study were made by the method of mutual pollination. This technique relies on the self-incompatibility of the ovule parent to ensure maximum cross-pollination. All parents were selected on this basis, but in no instance were they completely self-incompatible.

III. Results

(*a*) *Occurrence of Red Root Tip within the Genus*

In a recent review of the taxonomy of the genus *Phalaris*, Anderson (1961) has described 13 well-defined species. Five of these (New World species) are restricted in their natural distribution to the American continent. A further seven (Old World species) are natives of the Mediterranean region, including the Middle East and parts of Northern India. The remaining species, *P. arundinacea*, has a wide distribution throughout the northern hemisphere, including Europe, Asia, and North America.

Only three of the five New World species were available for examination: *P. lemmonii*, *P. caroliniana*, and *P. californica*, and none showed any evidence of the red pigment. All of the Old World species including *P. arundinacea* were available, and in most cases a wide range of ecotypes, covering the range of the distribution of the particular species, were examined. The results of this survey are given in Table 1.

The red pigment was found only in root tips of the three polyploid species *P. minor*, *P. tuberosa*, and *P. arundinacea*, and was absent in the 12- and 14-chromosome diploids. It is widespread in *P. minor*, and, with the exception of one introduction from Tripoli, the pigment was present in all plants of the other Mediterranean ecotypes, and in a high proportion of the plants from three localities in India. From a representative collection of ecotypes of *P. tuberosa*, eight were found to contain a varying proportion of red progeny. These, without exception, were all derived from the western end of the Mediterranean region, from Algeria, Morocco, Portugal, and Spain. The majority of the ecotypes of the more widespread tetraploid race of *P. arundinacea*, and in particular those from North America, lack the red pigment.

TABLE 1

DISTRIBUTION OF RED ROOT TIP IN THE OLD WORLD SPECIES OF THE GENUS PHALARIS

Species	Chromosome No. (2*n*)	Life Cycle	Natural Distribution	Ecotypes Examined	Ecotypes Segregating Reds
P. canariensis	12	Annual	Mediterranean	4	0
P. brachystachys	12	Annual	Mediterranean	2	0
P. truncata	12	Perennial	Mediterranean	3	0
P. paradoxa	14	Annual	Mediterranean	2	0
P. coerulescens	14	Perennial	Mediterranean	10	0
P. tuberosa	28	Perennial	Mediterranean	28	8
P. minor	28	Annual	Mediterranean +India	17	16
P. arundinacea	28	Perennial	Eurasia+ N. America	30	2
P. arundinacea	42	Perennial	Mediterranean (Spain and Portugal)	13*	13

* Including four introductions from North America thought to be derived originally from the Mediterranean (McWilliam and Neal-Smith 1962).

It was found in two introductions from Botanic Gardens in Europe, but in view of the pollen contamination experienced in such circumstances the validity of these observations must remain in some doubt. Introductions of the hexaploid race of *P. arundinacea*, on the other hand, all possess the red pigment, which is usually strongly developed and present in a high proportion of the individuals of any one population. Apart from four bred strains of this species obtained from U.S.A., all other ecotypes were from Spain and Portugal, the only region where this race of *P. arundinacea* is known to occur naturally.

(*b*) *General Features of the Pigment*

The red pigment is localized in the apical meristematic region of the root tip and is confined to the cytoplasm of the epidermal cells in this region. Beyond this zone (1–2 mm) the colour fades and finally disappears as the cells become progressively vacuolated.

The pigment is not visible in the dry seed, and is synthesized, following imbibition, in the apex of the primary root as this emerges from the embryo still within the coleorhiza. Thereafter, it is produced in all actively growing root meristems throughout the life of the plant. The colour expression is relatively constant within a particular genotype, but can vary in intensity between different genotypes. This difference does not appear to be a function of the environment in which the plants are grown. No difference was observed in the intensity of pigmentation in plants from three populations of *P. tuberosa* grown over a range of controlled temperatures varying from 9 to 31°C and day lengths from 8 to 16 hr. Also, the presence or absence of the pigment had no effect on the growth rate and general development of plants in these environments.

(c) *Chemical Nature of the Pigment*

Preliminary examination by descending chromatography on Whatman No. 1 paper, using n-butanol–acetic acid–water (3 : 1 : 5 v/v) as the developing solvent (Forsyth and Simmonds 1954), indicated the presence of only one anthocyanidin, with an R_F of approximately 0·56.

Further examination of the extract containing the anthocyanidin was carried out by the method of Robinson and Robinson (1931). The tests used and results obtained are as follows:

(1) In order to extract the pigment completely into 1% HCl, an amount of benzene equal to 12 volumes of the amyl alcohol extract had to be added.

(2) When a small amount of sodium acetate crystals was added to the amyl alcohol extract, the colour changed from orange-red to bluish red. No further colour change was observed following the addition of a drop of 2% ferric chloride solution.

(3) A sample of the amyl alcohol solution was shaken vigorously with an equal volume of 5% NaOH in air. The solution was then acidified with conc. HCl and shaken again. No colour change was observed in the amyl alcohol layer, indicating the pigment to be resistant to oxidation. This was confirmed by shaking the alcohol extract with an equal volume of hydrogen peroxide (20 volumes), when no colour change was observed.

(4) The pigment from the amyl alcohol solution was extracted into 1N HCl and from this solution it was completely extracted by an equal volume of the "delphinidin reagent".

The results of the above tests indicated that the anthocyanidin present was pelargonidin. This was confirmed by spectrophotometric examination of the pigment in 70% ethanol, where absorption maxima were observed at 493 and 400 mμ, with a shoulder at 480 mμ and a trough at 438 mμ (Geissman 1955).

Further confirmation that the anthocyanin present in the root tips is a glycoside of pelargonidin was obtained from the ethanolic extract prepared in the presence of calcium carbonate. This solution was bluish red in colour. An amount of concentrated HCl equal to a quarter of the volume of the solution was then added, and the mixture boiled for 1 min. Extraction with amyl alcohol produced a green fluorescent solution, indicating the presence of pelargonenin.

The results of these tests indicated that the red colouring matter present in the root tips of the three species examined was a glycoside of pelargonidin. Unfortunately, the amount of material available was insufficient for the determination of the identity of the sugar residues present.

(*d*) *Genetics*

The results of selfing red and white phenotypes of both *P. tuberosa* and *P. arundinacea* revealed the existence of three distinct genotypes:

(1) Red phenotypes producing all red progeny;

(2) Red phenotypes segregating red and white progeny in the ratio 3 : 1; and

(3) White phenotypes producing all white progeny.

These ratios strongly suggest that the production of the red pigment is under the control of a single dominant gene (*R*). On this assumption, red phenotypes would be either homozygous or heterozygous dominants (*RR* or *Rr*) and the white phenotypes homozygous recessives (*rr*). These results were consistent for both species, and a summary of the results for *P. tuberosa* is given in Table 2.

TABLE 2

SEGREGATION OF RED ROOT TIPS IN SELF-POLLINATED PROGENIES OF P. TUBEROSA

Pooled data from selfing individual genotypes

Phenotype	Progeny Segregating		Genotype	Expected Ratio	$\chi^2 P\,(n=1)$*
	Red	White			
Red	164	0	*RR*	1 : 0	
Red	941	322	*Rr*	3 : 1	0·50–0·70
White	0	1036	*rr*	0 : 1	

* χ^2 for heterogeneity not significant.

To confirm the genetic interpretation based on the results of segregation in selfed progenies, individual plants of these three genotypes were pair-crossed, involving both intra- and interspecific combinations. The results for *P. tuberosa* and for the interspecific crosses (*P. tuberosa* × *P. arundinacea*) are presented in Table 3.

None of the observed segregation ratios in either species differed significantly from those expected on the hypothesis of a single dominant gene controlling pigment production. The small excess of white seedlings in crosses involving either the heterozygote (*Rr*) or the homozygote recessive (*rr*) as the ovule parent were assumed to be the result of selfing, as in no instance was it possible to obtain completely self-incompatible parent plants. The incidence of selfing was verified in the interspecific crosses, as non-hybrid seedlings were readily identified at the time of flowering.

Variation in the intensity of pigmentation in root tips is under some form of genetic control, but the precise nature of this control is obscure. The most likely

explanation is a system with a major dominant gene controlling the production of the pigment, and a series of modifier genes influencing the level of its expression.

No crosses were attempted with *P. minor* as this species is naturally self-pollinating and is difficult to emasculate. Those plants producing all red progeny are assumed to be homozygous dominants (*RR*), and those producing all white progeny to be homozygous recessives (*rr*).

TABLE 3

SEGREGATION OF RED ROOT TIP IN P. TUBEROSA: INTRA- AND INTERSPECIFIC CROSSES

Pooled data from crosses between individual genotypes

Species	Phenotype	Genotype ♀ ♂	Progeny Segregating Red	Progeny Segregating White	Expected Ratio	$\chi^2_{P\,(n=1)}$*
P. tuberosa	Red × red	*RR* × *RR*	273	0	1 : 0	
	Red × red	*RR* × *Rr*	217	2†	1 : 0	
	Red × red	*Rr* × *RR*	525	21	1 : 0	
	Red × red	*Rr* × *Rr*	988	317	3 : 1	0·50–0·70
	White × red	*rr* × *RR*	208	8	1 : 0	
	White × red	*rr* × *Rr*	402	443	1 : 1	0·10–0·20
	White × white	*rr* × *rr*	0	756	0 : 1	
P. tuberosa × *P. arundinacea* (2*n*=42)	White × red	*rr* × *RR*	1660	46	1 : 0	
	White × red	*rr* × *Rr*	396	437	1 : 1	0·10–0·20

* χ^2 for heterogeneity not significant for all groups.

† Thought to be contaminant seed.

IV. DISCUSSION

The results of this survey suggest that the gene controlling the production of the red pigment (a glycoside of pelargonidin) in the root tips of *Phalaris* is confined to the three Old World polyploid species. In *P. minor*, an annual self-pollinating species, the gene is widespread and homozygous and has been found in all but one of the populations examined. In *P. tuberosa* and *P. arundinacea*, both cross-pollinating perennials, the gene occurs at a lower frequency, largely as the heterozygote, and is confined to populations from certain geographic regions of the distribution of these two species. It was found only in *P. tuberosa* ecotypes from the western end of the Mediterranean region, and in *P. arundinacea* it occurs almost entirely in introductions of the hexaploid race from Spain and Portugal.

The three tetraploid species of *Phalaris* (*P. minor*, *P. tuberosa*, *P. arundinacea*) are considered to be allopolyploids, largely on the evidence of the regular formation of bivalents at meiosis (Hanson and Hill 1953; Starling 1961; McWilliam 1962). The identity of the ancestral diploids has not been definitely established and it is quite possible that some of these may now be extinct, as only two 14-chromosome diploids (*P. coerulescens* and *P. paradoxa*) are represented in the Mediterranean region. There is evidence that *P. coerulescens* may be one of the progenitors of two of the tetra-

ploids: Hayman (1955) found a considerable degree of homology between the genome of *P. coerulescens* ($2n = 14$) and one of the two genomes of *P. minor* ($2n = 28$), and a similar situation has been found in hybrids between *P. coerulescens* and *P. tuberosa* ($2n = 28$) (McWilliam, unpublished data). The ancestry of *P. arundinacea* ($2n = 28$) is not known, but there is evidence (McWilliam and Neal Smith 1962) that the hexaploid form of this species has arisen through hybridization between *P. tuberosa* and *P. arundinacea* at the junction of their respective distributions in southern Europe.

The presence of the red pigment in the polyploid species and its absence in the diploids suggests either that the gene was present in one of the extinct ancestral diploid forms, or that it arose spontaneously subsequent to the evolution of the polyploid species from the diploids. As there is no evidence of the gene ever having been present at the diploid level, the latter hypothesis seems more likely. Further, because of the widespread occurrence of the gene in *P. minor*, one might speculate that it first arose by mutation in this species, and has subsequently spread through occasional gene exchange to the other two polyploid species.

There is some evidence to support this view. Natural hybrids between *P. minor* and *P. tuberosa* have been recorded (Covas and Cialzeta 1953), and as both species are sympatric over the greater part of their natural range, there is every possibility that gene exchange has occurred, as for example in North Africa, where the gene for red pigment is relatively common in populations of *P. tuberosa*. In this region, and particularly in Morocco, *P. tuberosa* is somewhat distinct with many of the features of an annual species, including vigorous seedling development and early maturity, suggesting affinity with *P. minor*. If the gene has been incorporated into *P. tuberosa* in this way, it may well have spread from Morocco to other populations of *P. tuberosa* in the Western Mediterranean, and ultimately through interspecific hybridization with *P. arundinacea* to the hexaploid race of this species.

The presence of the gene in the hexaploid populations of *P. arundinacea* in Spain and Portugal is thus further evidence for the hybrid origin of this form, and may explain why the gene is common in the hexaploid, but rare in the more widespread tetraploid form of this species.

The adaptive significance of the gene controlling the production of the anthocyanin pelargonidin in the roots of *Phalaris* is not known, but the gene frequency, particularly in *P. minor*, indicates that it conveys some selective advantage. The gene may provide such an advantage under particular environmental conditions, as suggested by its occurrence in certain well-defined geographic races of *P. tuberosa*. However, growth studies with this species in a range of controlled environments failed to demonstrate any measurable effect of the gene in relation to temperature or photoperiod. Also the presence of the gene in a wide range of climatic ecotypes of *P. minor* indicates that its occurrence may be relatively independent of the external environment.

Various glycosides of pelargonidin occur as a red pigment in the flowers of a wide range of ornamental plants (Geissman 1962), but this class of anthocyanins has not been recorded previously in root tissue. It is possible in the case of *Phalaris* that the pigment may have a beneficial influence on the growth and development of roots. Bloch (1943) has shown for *P. arundinacea* that the concentration of the

pigment is highest in epidermal cells that subsequently initiate root hairs. Also a number of workers have demonstrated an association between the rooting ability of cuttings and the level of anthocyanin in the tissue (van Overbeck and Gregory 1945; Robbins 1957; Bachelard and Stowe 1962). There is further evidence that related compounds, leucoanthocyanins, may play an active role in the growth of plant tissues (Steward and Shantz 1959). Any such stimulus leading to a more active or extensive root system may be an advantage to plants during the establishment phase, or in areas where moisture is often limiting during the growing season, as for example in North Africa.

As a single gene marker the red pigment has proved useful for a number of purposes in the *Phalaris* breeding programme. It has been used in pollen competition studies to measure the extent of outcrossing in self-compatible genotypes of *P. tuberosa*, and also to estimate the effectiveness of random mating in polycross populations. It has also been of value for the rapid identification of F_1 hybrids produced from interspecific hybridization in *Phalaris*, particularly where the hybrids are difficult to distinguish from either parent in the seedling stage. For this purpose, the gene is incorporated in the pollen parent, and hybrids identified at the time of seed germination.

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