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Anthocyanin pathway in rice (*Oryza sativa* L.): identification of a mutant showing dominant inhibition of anthocyanins in leaf and accumulation of proanthocyanidins in pericarp

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Abstract The present study has surveyed a collection of indica rice (*Oryza sativa*) lines for tissue-specific anthocyanin pigmentation pattern, which has also been used for a genetically meaningful classification. This classification helped predict probable genotypes of rice lines and, in the process, a leaf blade-specific dominant inhibitor of pigmentation (*Ilb*) was predicted and its presence later confirmed in two lines. We ascribe most tissue-specific accumulation of anthocyanins to the presence of a different set of *Pl* alleles. Cyanidin, as a major pigment, and peonidin, as a minor pigment, were detected in purple-pigmented tissues. Further, the floral organ-derived tissues always contained a higher level of anthocyanins and, correspondingly, a relatively increased proportion of peonidin. One line, N22B, with a brown pericarp was identified and shown to accumulate proanthocyanidins, but with no anthocyanins, in the pericarp. We propose that the accumulation of proanthocyanidins is due to a block in the anthocyanin biosynthetic pathway in rice at the anthocyanidin synthase-mediated conversion of leucoanthocyanidin to anthocyanidin.

Key words *Oryza sativa* · Inhibitor · Anthocyanins · Proanthocyanidins · Pigmentation

Introduction

Anthocyanins are a class of flavonoid pigments synthesised by a secondary metabolic pathway from the amino acid phenylalanine, and they impart purple, red and blue coloration in plants. This pathway has been studied in detail in a few plant species such as maize, barley, *Petunia*, and *Antirrhinum*. The enzymes, and the corresponding genes, involved in the synthesis of anthocyanins and in the regulation of the pathway are also well characterised. Consequently, this pathway has been extensively exploited in these species to study a variety of genetic and molecular phenomena such as gene interaction, markers for linkage and mapping, gene isolation, studies on gene organisation, expression and regulation, the characterisation and isolation of transposable elements, and transformation for many useful purposes (McClintock 1950; Peterson 1986; Coe et al. 1988; Dooner et al. 1991; Lloyd et al. 1992).

Although the genetics of anthocyanin pigmentation has been studied in rice (Ramaiah and Rao 1953; Nagao et al. 1962; Dhulappanavar et al 1975; Dhulappanavar 1979; Kadam 1974; Takahashi 1982; Kinoshita and Maekawa 1986; Maekawa and Kita 1987; Kinoshita and Takahashi 1991), a systematic approach to establish a relationship between identified genes and their specific role in the anthocyanin biosynthetic pathway is lacking. Thus, a powerful tool in rice genetics and molecular biology can be developed if this pathway and its genes are characterised in detail and put in a proper perspective. This objective becomes more significant in view of the importance of rice as a major world food crop.

The intermediate compounds in the anthocyanin pathway have been convincingly demonstrated to be involved in plant defence response to pathogens (Hagerman and Butler 1981; Jambunathan et al. 1986; Scalbert 1991), and in abiotic stress response (Beggs et al. 1986; Reddy et al. 1994). In rice, we are interested in studying the role of anthocyanins and other flavonoid pigments in biotic and abiotic stress response, including the involvement of anthocyanins in defence mechanisms against plant pathogens, together with developing anthocyanin-gene markers for transposon-tagging purpose.

To this end, we have begun an investigation into the genetic and biochemical basis of the anthocyanin pathway in rice in order to achieve the above goals. As a first step, we

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have visually screened a rice germplasm collection and classified the selected lines accumulating anthocyanins and related pigments in different tissues to identify anthocyanin-pathway genes responsible for the variation present in these lines. A genetic determinant associated with a leaf blade-specific inhibition of pigmentation has been uncovered. Further, the major anthocyanin pigments of rice have been identified and plants accumulating proanthocyanidins as a result of a lesion at an important enzymatic step have also been described.

Materials and methods

Screening of rice germplasm for pigmentation phenotype

The list of rice lines used in this study is given in Table 1 and includes both land races and cultivars of indica and a few genetically defined japonica types. They were selfed for several generations and made true breeding. The indica lines were selected from a rice germplasm collection at the Directorate of Rice Research, Hyderabad, India, after an extensive visual screening for the pigment phenotypes of various plant parts in the monsoon crop season (July–November) of 1989. The experimental plants were grown either in the field or in pots in the net-house. The plants experienced the following environmental conditions during the scoring of the anthocyanin-pigmentation phenotypes. At Hyderabad, India (longitude 78°4′ E; latitude 17°3′ N; altitude 600 m above mean sea level) the plants were subjected to an average day/night temperature of 30.1/20.7°C with an RH ranging between 76 and 60%. At midday, sunlight intensity was about 2800 μmole m^{-2} s^{-1}. All the experimental plants were grown in black-loam soil under continuous irrigation. The field or net-house grown plants were scored for pigment phenotypes at four stages during the life cycle of the plants: seedling [10 to 50 days after germination (dag)], vegetative (51 to 70 dag), pre-anthesis (71–80 dag) and post-anthesis (81 dag to maturity or 25 days after pollination) stages. Tissues showing a full purple coloration were scored as positive and families with ambiguous phenotypes were discarded. Further, the stable inheritance of these phenotypes was observed for at least three generations. The visual-pigment phenotypes of various plant parts were photographed using a Wild Photomakroskop, M 400 without filters (see Fig. 1).

Table 1 Source of rice lines

Line	Type	Source[a]
H 126	japonica	HU, Sapporo, Japan
A 5	japonica	HU, Sapporo, Japan
A 58	japonica	HU, Sapporo, Japan
A 136	japonica	HU, Sapporo, Japan
H 113	japonica	HU, Sapporo, Japan
Purpleputtu	indica	TNAU, Coimbatore, India
Whiteputtu	indica	TNAU, Coimbatore, India
TN1013	indica	TNAU, Coimbatore, India
G 2237	indica	DRR, Hyderabad, India
G 962	indica	DRR, Hyderabad, India
Crossa	indica	DRR, Hyderabad, India
R27PW	indica	DRR, Hyderabad, India
N22W	indica	DRR, Hyderabad, India
N22B	indica	DRR, Hyderabad, India
Hamsa	indica	DRR, Hyderabad, India

[a] DRR, Directorate of Rice Research; HU, Hokkaido University; TNAU, Tamilnadu Agricultural University

Crosses and embryo rescue

The F_1 hybrid plants were obtained from reciprocal crosses of three different rice lines: Purpleputtu (class I), N22W (class II) and N22B (class IV). Emasculation (5–6 PM) and pollination (9–11 AM) were done successively with a standard bagging method. The pollinated panicles were harvested 15 days after pollination and the F_1 seeds were embryo-rescued by culturing on 1/2 MS medium. The cultured embryos were incubated in darkness at 25±1°C until germination and subsequently transferred to light. The young seedlings at the three-leaf stage were transferred to 1/2 MS liquid solution and were grown under laboratory conditions. After 7 days, the seedlings were transplanted into soil along with the parents. The F_1 plant phenotype was scored for purple pigmentation in various tissues/organs.

Identification of anthocyanidin pigments

The various plant organs/tissues, like leaf blade, leaf sheath, ligule, auricle, collar, node, internode, stigma, apiculus, husk, pericarp, etc., were frozen in liquid N^2 and stored at –20°C for further analysis. The stored or freshly harvested tissue was extracted with (25 mg/ml) acidified methanol (1% v/v) for 24 h at 4°C in the dark. To 1 ml of the extract, 0.75 ml of water and 2 ml of chloroform were added which resulted in a Folch partition. The anthocyanin concentration in the upper phase of this partition was calculated from A525 using a millimolar extinction coefficient of 31.6. An aliquot of the methanol-HCl extract was hydrolysed with 2N HCl for 40 min in a boiling water bath. After washing with ethyl acetate, the hydrolysates were extracted into iso-amyl alcohol and evaporated to dryness. The final residue was re-dissolved in methanol-HCl and separated by thin layer chromatography (TLC) on cellulose plates using three different solvent systems: (1) AHW (acetic acid:HCl:water, 30:3:10, v/v/v); (2) AAW (n-amyl alcohol:acetic acid:water, 2:1:1, v/v/v); (3) MHW (methanol:HCl:water, 190:1:10, v/v/v). After drying, the chromatogram was scanned on a densitometer. The separated compounds were identified on the basis of RF values, fluorescence under visible and UV light with or without ammonia, colour reaction to 5% (w/v) Na_2CO_3 solution, and the characteristic absorption maxima (Harborne 1965, 1967; Reddy 1974). Anthocyanidin pigments from pericarp tissue of Purpleputtu were purified by preparative TLC and subjected to proton NMR spectroscopy in CD_3OD solvent (200 MHz, Brucker-Ac-200 Spectrometer). The delta (ppm) values were compared with those of marker pigments, cyanidin and peonidin.

Identification of proanthocyanidins in pericarp

Mature de-hulled seeds were extracted with 10% aqueous methanol (1 ml/50 mg) for 24 h at room temperature with occasional shaking. To 1 ml of the pooled extract, 0.75 ml of water and 2 ml of chloroform were added and the aqueous-methanolic fraction containing flavonoids was subjected to standard analytical tests as described above. In addition, specific tests, such as butanol HCl assay (Bate-smith 1975; Waterson and Butler 1983), a 1% vanillin in HCl test (Price et al. 1978) and 0.5% vanillin in ethanol as TLC spraying reagent, were also used. Chemical tests such as the $NaBH_4$ reduction assay for flavanones, Zn^{+2}/HCl for dihydroflavonols and Mg^{+2}/HCl for flavonols which distinguish them from proanthocyanidins, were also performed (Harborne 1965).

Results

Genetic variability in tissue-specific accumulation of pigments in rice germplasm

We screened a germplasm collection of more than 600 lines for pigment distribution in different plant parts. Initially,

Table 2 Pigmentation pattern in rice lines (japonica – known genotypes in bold; indica – predicted genotype)

Line	Class	Genotype	Phenotype[a]									
			Lb	Ls	Co	Au	Lg	No	In	Ap	Hu	Pe
H 126	*II*	***C^B,A,Pl***	P	P	P	P	RP	G	P	R	C	W
A 58	*II*	***C^B,A,Pr,Pn***	G	G	P	G	G	P	G	G	DP	W
H 113	*II*	***C^+,A^d,P,Pl^w,I-Pl***	G	G	P	P	P	G	P	R	C	BP
A 136	*III*	***C^+,A^d,P***	G	G	G	G	G	G	G	G	C	W
A 5	*IV*	***C^{Br},A,RcRd***	G	G	G	G	G	G	G	G	C	B
Purpleputtu	*I*	*C,A,P,Pl^w*	P	P	P	P	RP	G	P	R	P	BP
G2237	*I*	*C,A,P,Pl^w*	P	P	P	P	RP	G	P	R	P	BP
R 27PW	*II*	*C,A,P,Pl*	P	P	P	P	RP	G	P	R	C	W
G 962	*II*	*C,A,P,Pl^i*	P	P	G	G	RP	G	P	R	C	W
TN1013	*II*	*C,A,P,Pl^i*	P	P	G	G	RP	G	P	R	C	W
Crossa	*II*	*C,A,P,Pl^i*	P	P	G	G	RP	G	P	R	C	W
N22W	*II*	*C,A,P,Pl^+,IIb*	G	G	G	G	G	G	G	R	C	W
Whiteputtu	*III*	*C,A,P^+,Pl^+*	G	G	G	G	G	G	G	G	C	W
Hamsa	*III*	*C,A,P^+,Pl^+*	G	G	G	G	G	G	G	G	C	W
N22B	*IV*	*C,A,P^+,RcRd,IIb*	G	G	G	G	G	G	G	G	C	B

[a] BP, blackish-purple; RP, reddish-purple; DP, deep purple; P, purple; R, red; B, brown; G, green; C, colourless and W, white. Lb, leaf blade; Ls, leaf sheath; Co, collar; Au, auricle; Lg, ligule; No, node; In, internode; Ap, apiculus; Hu, hull; Pe, pericarp;

it was noticed that different lines show a different combination of pigmented tissue. A representative sample of pigmentation phenotypes of specific organs of different rice lines, including F_1 plants, is shown in Fig. 1. The purple/red phenotype is vivid and shows a sharp localisation and an even distribution in the given tissue. The intensity of colour of a tissue/organ varies between lines, pericarp being the strongest.

Although the selected rice lines exhibit purple/red colour in a variety of organs, we concentrated our visual observations on leaf sheath, leaf blade, midrib, leaf margins, leaf tip, ligule, auricle, collar, node, internode, sterile glumes, husk, apiculus, stigma and pericarp. None of these lines showed anthocyanin pigmentation in roots and anthers. In our screening, three distinct types of pigmentation were observed, viz., purple, red, or brown, in addition to colourless. Distinct variation among the lines showing a different combination of tissues accumulating these pigments is apparent from Table 2.

Classification of rice lines based on pigmentation pattern and nature of pigments

Based on the observations from Table 2, we classified the selected lines into four distinct types. Class-I lines, Purpleputtu and G2237, exhibit purple colour in all parts except the node. Class II includes a majority of the tested lines, H-126, A58, H113, R27PW, G-962, TN1013, Crossa, and N22W, and exhibits purple colour only in certain plant organs. Class-III lines, A 136, Hamsa, and Whiteputtu, do not exhibit purple colour in any of the plant parts. Class IV lines, A5 and N22B, exhibit brown colour in the pericarp tissue and thus are distinct from the rest.

Detection of a dominant leaf-colour inhibitor

We have already established variability in our lines with respect to pigmentation pattern. Since we have verified these lines as true breeding, a genetic difference is the most likely cause of this variation. To investigate the genes controlling this tissue-specific pigment variation and to detect the possible occurrence of inhibitors of pigmentation (see discussion), reciprocal crosses among appropriate lines were made. Initially we focused on three lines, viz., Purpleputtu (class I), N22W (class II) and N22B (class IV). The parental (Purpleputtu and N22B) phenotypes along with the phenotypes of the corresponding F_1s are shown in Fig. 1. A summary of the phenotypic comparison of the three parents and their corresponding F_1s in ten different tissues is given in Table 3.

The phenotype of the various tissues of F_1s (NB×PP and NW×PP) is very similar to that of the Purpleputtu parent in all respects except that they failed to show pigmentation in the leaf blade; however, the leaf sheath showed a patchy pigmentation to start with and became uniform at maturity. Similarly, the hull showed a uniformly lighter pigmentation to start with but faded as it matured. All other tissues were uniformly pigmented as described in Table 3. There was no difference in the phenotypic outcome of the reciprocal crosses. In other words, the Purpleputtu parent carries dominant functional alleles for the genes involved in anthocyanin biosynthesis. The lack of pigmentation in the leaf blade of these F_1s can be ascribed to a dominant inhibitor contributed from the other parents, N22B or N22W, in the respective crosses. With regard to the pericarp phenotype, the F_1 seeds invariably exhibited the phenotype of the maternal parent, i.e. the F_1 seeds from the cross of female Purpleputtu×male N22B or male N22W

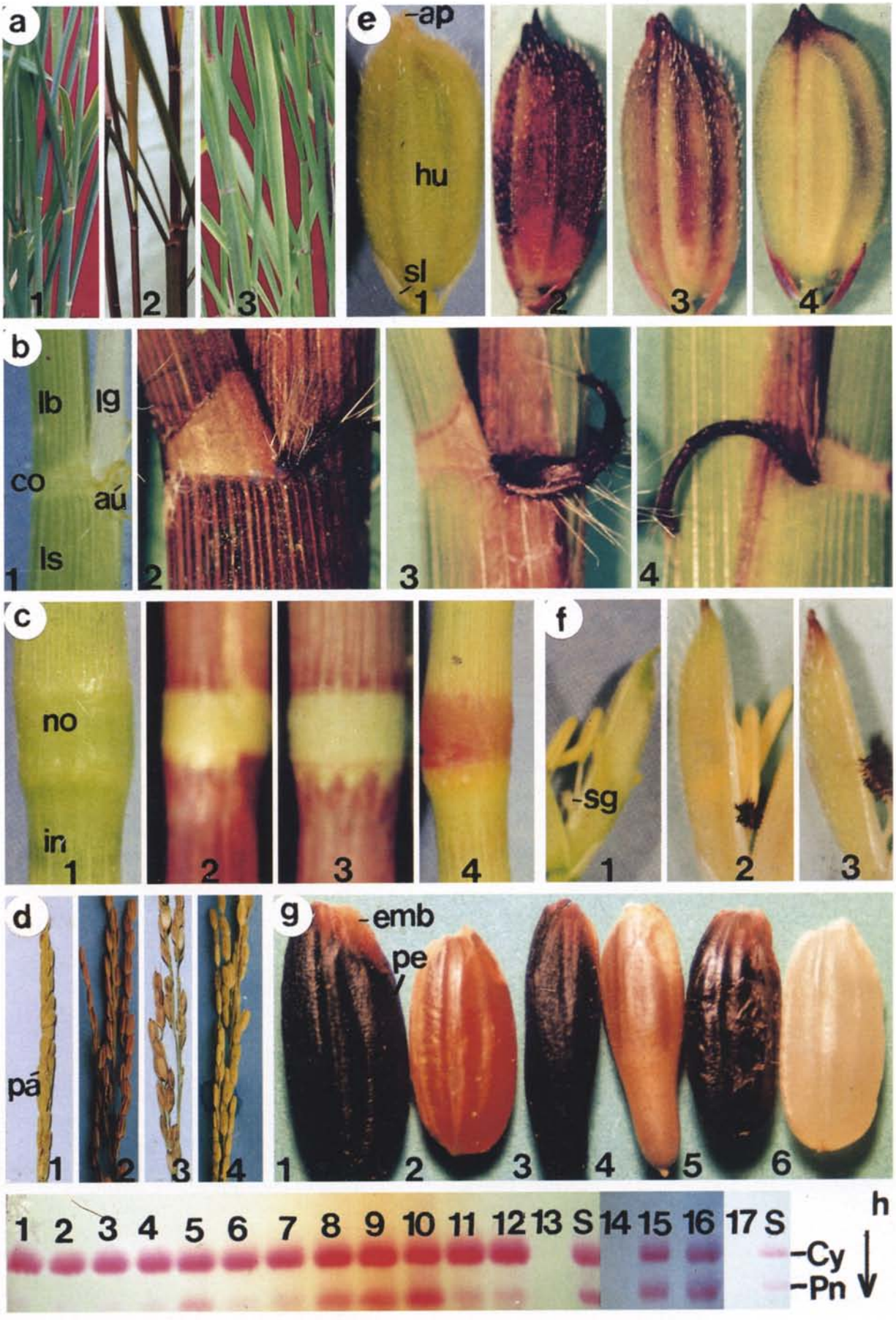
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Table 3 Phenotype comparison of parents and F_1 plants

Parents/F_1[a]	Phenotype[b]									
	Lb	Ls	Co	Au	Lg	No	In	Ap	Hu	Pe
Purpleputtu	P	P	P	P	RP	G	P	R	P	BP
N22W	G	G	G	G	G	G	G	G	C	W
N22B	G	G	G	G	G	G	G	G	C	B
F_1(N22B×PP)	G	P[e]	P	P	RP	G	P	R	P[d]	BP[c]
F_1(N22W×PP)	G	P[e]	P	P	RP	G	P	R	P[d]	BP[c]

[a] The phenotypic outcome of reciprocal crosses is identical
[b] Abbreviations are the same as described in Table 2
[c] Pericarp of F_2 seeds; they are of F_1 genotype as the pericarp is of maternal origin
[d] Young tissue is uniformly light purple and the pigmentation becomes irregular at maturity
[e] At younger stage patchy; but becomes uniformly pigmented later

were purple whereas the seeds from reciprocal crosses are brown (NB×PP) or white (NW×PP). This suggests that the purple or brown or colourless phenotype of seeds is restricted to the pericarp tissue but does not affect the aleurone; the pericarp being maternally derived. This is further substantiated by the observation that the pericarp of F_2 seeds of both crosses was found to be purple. These seeds are heterozygous for the dominant allele from Purpleputtu and the recessive allele from N22B or N22W in the respective crosses (see Table 6).

Identification of anthocyanidin pigments accumulating in purple tissue

As elaborated in the previous section, we have identified three basic types of pigmentation in our lines: purple, red and brown. Further, at the visual level we have noticed variation in the intensity, and a tissue-specific accumulation, of the pigments. We have isolated these pigments from various tissues in order to identify and quantify each of them. The objective of the experiment was to define the nature of the anthocyanin pathway genes that are operative in rice and determine the genetic block in the pathway in the various lines. Toward this goal, the qualitative and quantitative analysis of the pigmented tissue was done using a combination of spectrophotometry, TLC, characteristic chemical tests, and NMR spectroscopy. Spectral analysis revealed that the absorption maxima of the samples corresponded to the visual phenotype of the given tissue. All tissues showing either purple, reddish-purple, deep-purple, blackish-purple, purple-stripes, or red pigmentation always corresponde to λ_{max} 525 for the unhydrolysed samples and λ_{max} 540 for the hydrolysed samples. In contrast, all green-coloured tissue extracts failed to show this peak. Extracts from brown pericarp tissue always showed a peak at λ_{max} 457 for the unhydrolysed samples and two peaks, one at λ_{max} 447 and the other at λ_{max} 540, for the hydrolysed samples (Fig. 2).

Since anthocyanin pigments correspond to λ_{max} 525 and anthocyanidins to λ_{max} 540, it is proposed that the purple-pigmented parts contain anthocyanin which is converted to anthocyanidins upon acid hydrolysis. Also, as the green parts failed to show any absorption maxima, they evidently do not contain any of the anthocyanin class of pigments. The spectral characteristics of the unhydrolysed extract from the brown tissue correspond to the proanthocyanidin class of compounds (λ_{max} 457) which upon acid hydrolysis, are converted to anthocyanidin with a characteristic peak at λ_{max} 540 in addition to λ_{max} 447 (Fig. 2). In conclusion, the spectral analysis suggests the nature of the pigment that accumulates in various tissues: the purple/red pigments being anthocyanins, and the brown pigments being proanthocyanidins.

The TLC profiles showed clearly that all the tested purple plant parts accumulate the same anthocyanidin pigments, namely cyanidin (RF 0.49) and peonidin (RF 0.69). The composition of anthocyanidins in leaf and pericarp tissues is shown in Table 4. The unhydrolysed samples of the brown-pigmented tissue lack both cyanidin and peonidin. However, as expected, the hydrolysed samples showed a band corresponding to cyanidin. The results of TLC analysis completely matched (Fig. 1 h) with those of spectral analysis. In order to test the presence of other pigments, such as flavonoids, several diagnostic tests were performed

Fig. 1 Tissue-specific distribution of anthocyanin pigments. **a** N22B (*1*), Purpleputtu (*2*), and F_1 plants (*3*). **b** Leaf blade (*lb*), collar (*co*), leaf sheath (*ls*), auricles (*au*), ligule (*lg*) of N22B (*1*), Purpleputtu (*2*), F_1 plant (N22B×Purpleputtu) (*3*), and H 113 (*4*). **c** Node (no), internode (in) of N22B (*1*), Purpleputtu (*2*), F_1 plant (N22B X Purpleputtu) (*3*) and A 58 (*4*). **d** Panicle of N22B (*1*), Purpleputtu (*2*), F_1 plant (N22B×Purpleputtu) (*3*), and R 27PW (*4*). **e** Apiculus (*ap*), hull (*hu*), and sterile lemma (*sl*) of N22B (*1*), Purpleputtu (*2*), F_1 plant (N22B×Purpleputtu) (*3*), and R27PW (*4*). **f** Stigma of N22B (*1*), Purpleputtu (*2*), and F_1 plant (N22B×Purpleputtu) (*3*). **g** Pericarp of Purpleputtu (*1*), N22 B (*2*), F_1 seed (Purpleputtu×N22B) (*3*), F_1 seed (N22B×Purpleputtu) (*4*), F_2 seed (N22B×Purpleputtu) (*5*), and N22W (*6*). **h** TLC separation (Each lane, 1–10, contains acid hydrolysed extract from 50 mg tissue). *Arrow* indicates the direction of chromatogram (developed in AHW solvent). **Lanes:** leaf blade (*1*), leaf sheath (*2*), collar (*3*), auricles (*4*), ligule (*5*), internode (*6*), sterile glumes (*7*), apiculus (*8*), hull (*9*), and pericarp (*10*) tissues of Purpleputtu plant (Note the variation in Cy/Pn ratio). Pericarps of N22B (*11*) and F_1 seed of N22B×PP (*12*). Leaf blade of N22B (*13*), standard pigment markers (*S*), leaf blade of F_1 plant (N22B×Purpleputtu) (*14*), pericarps of F_1 seed (PP×N22B) (*15*), F_2 seed (N22B×PP) (*16*) and N22W (*17*). Standard pigment markers (*S*), Cyanidin (*Cy*) and Peonidin (*Pn*)

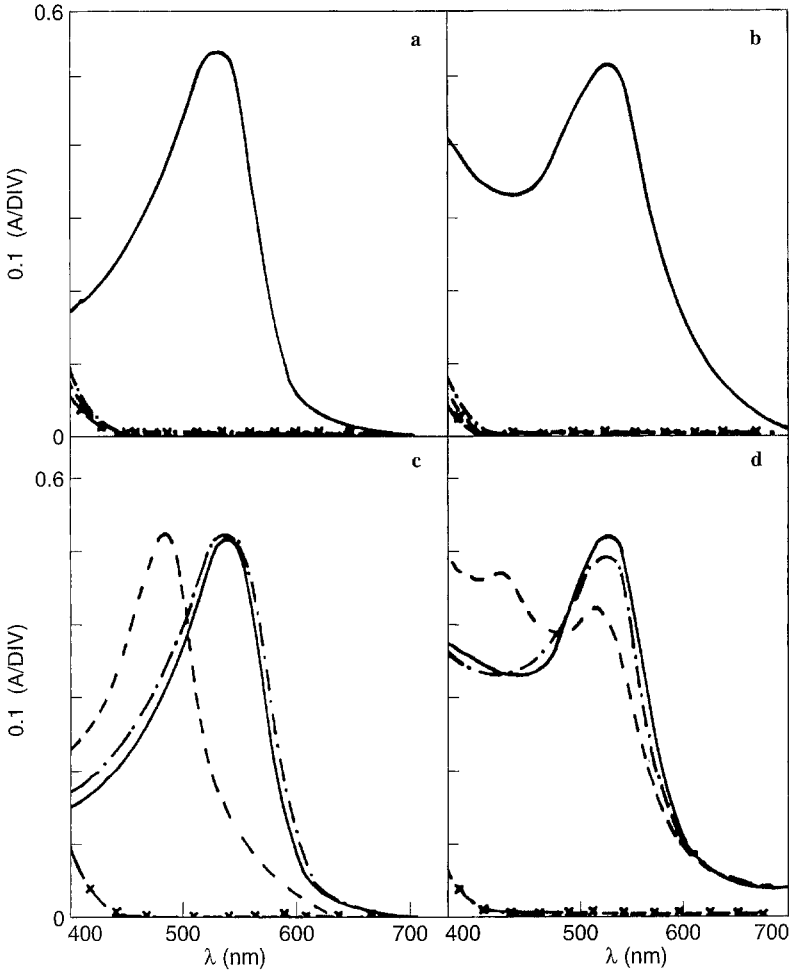

Fig. 2a–d Spectrophotometry of anthocyanins, proanthocyanidins and anthocyanidins. **a** Unhydrolysed and **b** hydrolysed leaf-blade extracts of N22B(- - -), Purpleputtu (—), F_1 plant (N22B×Purpleputtu) (-·-·), and N22W (-×-×). **c** Unhydrolysed and **d** hydrolysed pericarp extracts of N22B(- - -), Purpleputtu (—), F_2 seed (N22B×Purpleputtu) (-·-·) and N22W (-×-×)

Table 4 Occurrence of anthocyanin pigments in leaf and pericarp tissue extracts of different rice lines

Line	Purpleputtu	G-2237	H 126	H 113	TN 1013	Crossa	N22W	A-136	Hamsa	N22B
Leaf blade	+	+	+	–	+	+	–	–	–	–
Pericarp	+	+	–	+	–	–	–	–	–	–

+, presence and –, absence of anthocyanidin (cyanidin and peonidin) pigments

on the tissue extracts. These included $NaBH_4$ for flavanones, the Zn^{+2}/HCl heat test for dihydroflavonols, and the Mg^{+2}/HCl heat test for flavonols. None of the samples tested positive.

Confirmation of cyanidin- and peonidin-based anthocyanins as major pigments

Using NMR analysis we confirmed the pigments identified through TLC as cyanidin and peonidin. The proton delta (ppm) values of the major pigment (RF, 0.49) are 8.80 (H-4), 6.94 (H-6), 6.68 (H-8), 7.84 (H-2′), 7.06 (H-5′), 8.23 (H-6′), while those of the minor pigment (RF, 0.69) are 8.81(H-4), 6.94 (H-6), 6.68 (H-8), 7.68 (H-2′), 6.88 (H-5′), 8.17 (H-6′) and 3.84 for the O-CH_3 group (Fig. 3). The delta values were comparable to those of the marker pigments. These data lead to the conclusion that the major pigment of the pericarp of Purpleputtu extract is cyanidin and the minor pigment is peonidin.

Line- and tissue-specific differences in pigment quantity

Although two tissues may be accumulating the same pigments, the intensity may vary between them and this difference is not always detectable visually (Fig. 1). Also, visual assay would fail to detect any difference, if present, in the composition of the pigments. This is important since we found two different anthocyanin pigments, viz., cyanidin and peonidin, accumulating in the purple/red tissues. Purpleputtu, which accumulates pigments in most plant

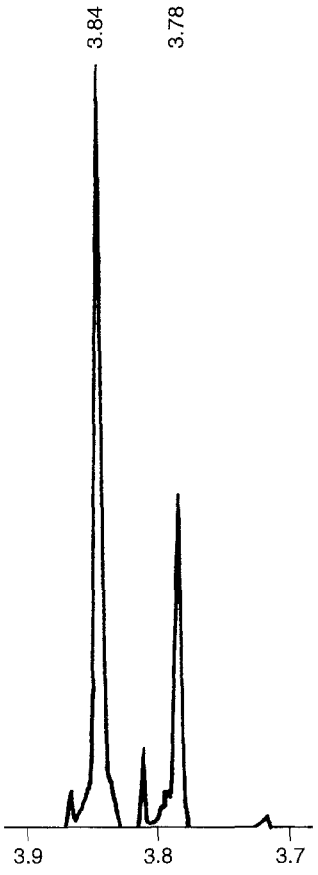

Fig. 3 Proton NMR spectroscopy of peonidin. The delta ppm values are 3.78 (CD_3OD solvent) and 3.84 for the O-CH_3 group of peonidin present at the 3′ position. This peak is absent in cyanidin proton-NMR spectroscopy

parts, is the ideal material for this kind of assay. A quantitative assay was performed on partially purified pigment extracts from each of the tissues (Fig. 1 h, lanes 1–10). The extent of anthocyanidin pigments accumulating in different plant parts of Purpleputtu is given in Table 5 and can be compared with the corresponding visual phenotypes (Fig. 1). Pigment accumulation is the highest in the pericarp tissue (693.9 n moles/mg f.wt); the vegetative tissues of the plant accumulate less than 300 n moles/mg f. wt. It is interesting to note that peonidin, the minor pigment of leaf and other vegetative parts (about 3 n moles/mg f.wt.) except in the case of the ligule (about 28 n moles/mg f.wt.), occurs in relatively high amounts in the pericarp (about 293 n moles/mg f.wt.), a floral-derived organ. Further, there is an apparent correlation between the increased levels of cyanidin and peonidin.

Nature of the brown pigment

In our phenotypic survey we came across some lines showing brown pigmentation in the pericarp tissue of N22B and A5; no other tissue accumulated the brown pigments. During the qualitative assay we detected proanthocyanidin compounds in this tissue. Moreover, we rule out leucoanthocyanidin being accumulated in the brown pericarp because this tissue is not colourless and an unhydrolysed extract of this showed λ_{max} 457 (Fig. 2) instead of the λ_{max} 280 characteristic of the leucoanthocyanidin pigment. Although this supports the fact that the brown pigment is proanthocyanidin, not anthocyanidin or anthocyanin, it needed confirmation by further tests. The unhydrolysed (aqueous or acidified) methanol extract of brown pericarp showed a positive response to the butanol-HCl heat test (red), the 1% vanillin in HCl test (red) and also with 0.5% vanillin in ethanol, used as a spraying solution on TLC plates (the brown-colour bands turn red). The unhydrolysed extracts do not contain either anthocyanidins or anthocyanins as evident from the spectral data (no peak at λ_{max} 540 or 525). On the other hand, the hydrolysed methanolic (aqueous or acidified) extract from the brown pericarp showed a peak at λ_{max} 540 (Fig. 2). This compound in the hydrolysed extract was shown to be cyanidin because after TLC (Fig. 1 h) it co-migrated with the cyanidin marker pigment with an RF value of 0.49 (AHW). In addition, a minor pigment with an RF of 0.69 has also been observed (Fig. 1 h). Thus the red band represents cyanidin pigment derived from proanthocyanidins after hydrolysis. The compound(s) in the brown streak is most likely responsible for the peak at λ_{max} 447 observed in the spectral curve for this sample [this may be the oxidised and polymerised compounds of catechin, the initiating unit for the synthesis of proanthocyanidins (Fig. 2)]. The compound that co-migrated with the marker pigment, cyanidin, on the TLC plate was indeed confirmed to be cyanidin on the basis of a variety of tests. In summary, the brown colour of the pericarp of the tested class-IV rice lines (Table 2) is predominantly due to the accumulation of procyanidins together with a residual amount of propeonidins, the proanthocyanidin class of compounds (Table 6).

Table 5 Qualitative and quantitative analysis of the anthocyanidin pigments of different plant parts of Purpleputtu

Plant organ	Phenotype	Anthocyanidin (in n moles/mg f.wt)[a]		
		Cyanidin	Peonidin	Ratio[b]
Leaf blade	Purple	174.2 ± 15.6	2.09 ± 0.09	0.012
Leaf sheath	Purple	129.0 ± 12.9	1.29 ± 0.02	0.010
Collar	Purple	121.3 ± 13.2	1.21 ± 0.04	0.009
Auricle	Purple	134.6 ± 11.4	1.34 ± 0.06	0.009
Internode	Purple	155.5 ± 15.2	1.71 ± 0.09	0.010
Ligule	Reddish-purple	275.7 ± 09.7	27.57 ± 1.09	0.100
Apiculus	Red	317.8 ± 13.8	20.00 ± 8.40	0.063
Hull	Purple	351.4 ± 18.4	82.10 ± 15.2	0.233
Pericarp	Blackish-purple	401.0 ± 22.0	292.9 ± 18.9	0.730

[a] Anthocyanidin pigments were quantified using a millimolar extinction coefficient of 31.6
[b] Ratio of peonidin to cyanidin

Discussion

Genetic basis of variation in anthocyanin pigment distribution in indica rice

This study has provided a good starting point to probe the anthocyanin pathway in rice in terms of its final products.

Table 6 Tissue-specific accumulation of proanthocyanidins and anthocyanins

Parents/F_1	Proanthocyanidins		Anthocyanins	
	Leaf blade	Pericarp	Leaf blade	Pericarp
N22B	–	+	–	–
Purpleputtu	–	–	+	+
N22W	–	–	–	–
NB×PP (F_1 seed)	NA	+	NA	–
PP×NB (F_1 seed)	NA	–	NA	+
NB×PP (F_1 plant)	–	–	–	+
NW×PP (F_1 seed)	NA	–	NA	–
PP×NW (F_1 seed)	NA	–	NA	+
NW×PP (F_1 plant)	–	–	–	+
A 5	–	+	–	–
Hamsa[a]	–	–	–	–

NA, not applicable; +, presence and –, absence of pigments
[a] Colourless control

It has also established true breeding genetic lines which can be further investigated to clearly identify the genes involved in the pathway. We have catalogued and established the existing variation in the tissue-specific expression of anthocyanin pigmentation in ten rice lines of the indica sub-species. The difference between the lines is mainly due to the combination of tissues expressing the pigments.

Anthocyanin pigmentation has been studied in rice and many genes have already been reported. These studies have largely focused on the identification and mapping of loci contributing to pigment synthesis and its distribution in plant organs. A summary of the known genes is presented in Table 7. Particularly noteworthy are the alleles at the *Pl* locus, as well as the *P*, *Pn* and *Prp* loci, because they specify the appearance of pigmentation in various tissues; the combination of alleles determining the pattern of pigment distribution. They have been appropriately termed distributive or localiser genes (regulatory genes of the pathway conditioning tissue specificity). These are in addition to the basic genes (structural genes), *C* and *A*, that determine pigment synthesis in the plant. Several inhibitory genes which inhibit the function of *Pl* alleles have also been described (Takahashi 1982): *IPl*–1, *IPl*–2 and *IPl*–3 inhibit the action of both $Pl^w$and Pl^i alleles of the *Pl* locus; *IPl*–4 and *IPl*–5 inhibit the action of the *Prp* (purple pericarp) gene; and *IPl*–6 inhibits the action of the Pl^i allele (summarised in Table 7).

Genotype prediction from phenotype

As this study is the first systematic attempt to decipher the genetic and biochemical basis of anthocyanin pathway in the indica sub-species, classification of the various lines became necessary to predict their genotypes. This prediction is based on known genes and their alleles in the japonica group. The four classes, identified on the basis of pigment accumulation in ten different plant organs, differ mainly in the allelic composition at the *C*, *A*, *P*, and *Pl* loci (Table 2).

A simplified key to predicting genotypes from pigmentation phenotypes is presented in what follows: the presence of *C* and *A* are mandatory for pigment production; the presence of *P* is confirmed if the apiculus is pigmented; and pigmentation in other aerial tissues determins the status of the specific *Pl* allele present (Table 7). The Pl^w allele, in addition to *C*,*A* and *P*, contributes to pigmentation in the pericarp as well as in most other plant organs, and hence would be present in our class-I lines which show pigmentation in most aerial tissues.

The class-II lines would differ from class-I lines in carrying a different set of *Pl* alleles. The *Pl* and Pl^i alleles, in contrast to the Pl^w allele, condition a non-pigmented pericarp; *Pl* contributes to pigmentation in all shoot tissues and Pl^i to all except the collar and the auricle. In the class II line R27PW, tissue-specific distribution is most likely specified by the *Pl* allele, since it shows pigmentation in most aerial tissues. One subgroup of class II, viz., G 962, TN 1013 and Crossa, are similar except that the pigment distribution is most likely determined by the Pl^i allele as these lines fail to produce pigmented collars and auricles. The genotype of N22W is not easily explained merely by the presence of *C*, *A*, *P* and *Pl* loci. It is therefore considered separately below.

Class-III lines being non-pigmented, either carry null alleles of the regulatory genes, i.e. P^+ and Pl^+, or more likely the non-pigment producing recessive alleles of the structural genes.

Detection of a leaf blade-specific inhibitor of pigmentation

The N22W line is atypical because only the apiculus tissue is pigmented. Since anthocyanin is produced in this line, it carries all the basic genes, and its tissue-specific distribution is hard to explain by considering only the known alleles at the *Pl* locus. It is very likely that it carries one of the alleles at the *Pl* locus (difficult to predict the specific *Pl* from the known information) in addition to an inhibitor that suppresses pigmentation in the tissues specified by the corresponding *Pl* allele.

If the N22W line truly contains the predicted inhibitor of pigmentation, it should be detectable in a cross between appropriate genotypes. The ideal tester parent would be Purpleputtu since it shows pigmentation throughout the plant and the presence of tissue-specific inhibitors present in rice (described above, and in Table 7) has more chances of being detected with this genotype than with any other. Indeed, inhibition of pigment production in leaf blade was detected in the F_1 of the cross between N22W and Purpleputtu (Table 3). The F_1 plant should mimic the Purpleputtu parent since it has all the dominant allelic combinations involved in pigment production and distribution. Failure of pigment production in the leaf blade, even in the presence of the Pl^w allele from the Purpleputtu parent, clearly sug-

Table 7 Description of known genes of anthocyanin gene-pigment system and their phenotypic effects in rice (Chang and Jordan 1963; Takahashi 1982; Kinoshita and Takahashi 1991)

Gene	Phenotypic effect
C (Chromogen)	Responsible for anthocyanin production Alleles: C^B, C^{Br}, C^+ (null), etc
A (Activator)	Activation of *C* gene; essential for anthocyanin production Alleles: A^s, A^E, A, A^+ (null), etc
P (Purple)	Distributor of anthocyanin in apiculus Alleles: P, P^K, P^+ (null), etc
Pl (Purple leaf)	Localizer of anthocyanins in leaf Alleles: Pl^w - leaf blade, leaf sheath, auricles, ligule and pericarp *Pl* - leaf blade, leaf sheath, collar, auricles, ligule, node and internode Pl^i - leaf blade, leaf sheath, ligule and inter node Pl^+ - null allele resulting into colourless phenotype of tissue
Pn (Purple node)	Localizer of anthocyanins in node
Prp (Purple pericarp)	Localizer of anthocyanins in pericarp
Rc (brown pericarp)	Synthesis of brown pigments in pericarp
Rc and *Rd* (brown pericarp)	Synthesis of brown pigments in pericarp
I-Pl (Inhibitor to purple leaf)	Dominant inhibitor of purple anthocyanin pigments
I-Pl–1, *I-Pl*–2, *I-Pl*–3	Inhibit the action of both Pl^w and Pl^i alleles
I-Pl–4, *I-Pl*–5	Inhibit the action of the *Prp* locus; and
I-Pl–6	Inhibits the action of *Pli* allele

gests the involvement of a leaf-colour inhibitor contributed from the N22W parent.

Class IV (brown pigmentation) is a special case and is represented by a single indica line, N22B. The phenotype of N22B is similar to that of N22W in not accumulating anthocyanin pigments in any of the aerial tissues, but differs from N22W as it shows brown pigmentation in the pericarp and has a colourless apiculus. Incidentally, both have a common origin. It is therefore no coincidence that we detected the leaf blade-specific inhibitor of pigmentation in this line too. The phenotype of various tissues of parents (Purpleputtu and N22B) and the F_1 plant are shown in Fig. 1. Their corresponding pigmentation pattern is presented in Table 3. Spectrophotometry (Fig. 2 and Table 7) and TLC (Fig. 1 h, lanes 11–17) data confirmed the lack of anthocyanin pigments in the leaf blade. All other tissues of the F_1 plants and the pericarp of F_2 seeds (i.e. F_1 genotype, pericarp being a maternal tissue) accumulate the same kind of anthocyanidin pigments (cyanidin and peonidin). In summary, the pattern of anthocyanin pigmentation in F_1 plants is identical with that of the Purpleputtu parent except in the leaf blade. This result agrees with that obtained in the case of the related N22W line with respect to the presence of the inhibitor.

Inhibitors of pigmentation have previously been reported in rice (Table 7; Kadam 1936; Dhulappanavar 1973; Kinoshita and Takahashi 1991; Takahashi 1982). The inhibitor detected in the present study is unique because its inhibitory action is specific to the leaf blade and is unlike the previously reported inhibitors of leaf pigmentation, *I-Pl-1, I-Pl-2, I-Pl-3* and *I-Pl-6*, whose inhibitory effects extend over a range of tissues. We term this unique inhibitor *Ilb* (*Inhibitor of leaf-blade* pigmentation).

Given the presence of *Ilb* in N22W and N22B, it is now possible to reconstruct their genotypes for the specific *Pl* allele present. We rule out the presence of alleles like Pl^w, *Pl* or Pl^i of the *Pl* locus as these alleles are known to contribute to pigmentation in tissues that *Ilb* cannot suppress, being a leaf blade-specific inhibitor. N22W and N22B most likely contain the null allele, Pl^+, that does not contribute to pigmentation in any of the tissues. The apiculus pigmentation in N22W is easily explained by the action of the *P* locus (Table 7; Takahashi 1957).

The unresolved part in genotyping the lines is in determining the genetic factor(s) responsible for brown pigmentation in the class-IV line, N22B. To resolve this issue, identification of the brown pigment accumulating in the pericarp of N22B became necessary.

Nature of pigments

The results obtained in our study indicate that the biosynthesis of anthocyanin pigments contributes red/purple colour to various tissues of tested rice plants except for the brown-pigmented pericarp in N22B line. There are three common types of anthocyanidin pigments encountered in plants, viz., -4′ (pelargonidin), -3′,4′ (cyanidin) and -3′,4′,5′ (delphinidin) hydroxylated forms. Several modifications, such as methylation, acylation, glycosylation, malonylation etc., of these three basic pigments give rise to a variety of pigments (Harborne 1965). In rice, the data from spectrophotometry (Fig. 2), TLC (Fig. 1 h) and NMR spectroscopy (Fig. 3) confirmed that the major pigment of purple-coloured tissues is cyanidin and the minor pigment is peonidin, a 3′-methoxy cyanidin derivative (Fig. 4). Plants are known to accumulate a combination of anthocyanin pigments, such as pelargonidin and cyanidin, in maize (Coe et al. 1988; Styles and Ceska 1977), cyanidin, delphinidin and their methylated forms , peonidin, petunidin and malvinidin, in *Petunia* (Gerats et al. 1984), and cyanidin and delphinidin in barley (Meldgard 1992).

The N22B line is unusual in that it accumulates brown pigment in the pericarp and we have unambiguously identified it as proanthocyanidin (its implication with respect to the anthocyanin pathway in rice is discussed in a later section).

The anthocyanin pathway is controlled by many genes with diverse alleles, and interaction between them is expected to lead to both qualitative and quantitative differences in the tissue-specific distribution of pigments. Pigment-composition studies have therefore been carried out on various pigment-producing tissues.

Tissue-specific variation in pigment composition

Purpleputtu (class 1) accumulates anthocyanin pigments in almost all tissues except node and exhibits significant quantitative differences (Table 5 and Fig. 1 h lanes 1–10). The total anthocyanidin content increases by about 5-fold in pericarp tissue compared to most vegetative tissues. Interestingly, the peonidin content is higher by several orders of magnitude in floral-derived organs, viz., apiculus, hull and pericarp, compared to the vegetative tissues.

It is likely that when anthocyanin concentration increases in mature pericarp tissue, the conversion of cyanidin to peonidin is increasingly efficient, which is also true in UV-B-induced rice seedlings found in our earlier studies (Reddy et al. 1994). Four-day-old etiolated rice seedlings after exposure to sunlight (UV-B) synthesise cyanidin which begins to accumulate from 8 h and reaches a maximum at 24 h. Conversion to the minor pigment peonidin does not occur until a minimum level of cyanidin is synthesised at 16 h . The petunia $an^{+/p}$ mutants at the *An1* locus differ from one another in anthocyanin-pigment composition. From the ratio of peonidin to cyanidin in mature flowers of these mutants, it was concluded that the methylation of cyanidin to yield peonidin is determined primarily by allelic state, but also to some extent by the concentration of anthocyanin pigments (Gerats et al. 1984).

Accumulation of proanthocyanidin

The N22B line in our collection and the A5 line from the japonica group have been classified separately solely because they exhibit a brown-pigmented pericarp due to the accumulation of proanthocyanidins. Proanthocyanidins are distributed in vegetative tissues of *Vicia faba* (Crofts et al. 1980), *Phaseolus vulgaris* (Ma and Bliss 1978), and *Lotus corniculatus* (Sakar and Howarth 1976), in seed coats of *Glycine max* (Todd and Vodkin 1993), and *Hordeum vulgare* (Aastrup et al. 1984), and in leaves and seed coats of sorghum (Paroda et al. 1975; Haskins and Gorz 1986). The pericarp tissue of *Zea mays* in the *a2* and *bz* mutants accumulates 3-deoxy proathocyanidins and its oxidised forms, phlobaphenes (Styles and Ceska 1972, 1989; Coe et al. 1988). In seed coats of *Glycine max*, the *R* genotypes accumulate proanthocyanidins as well as anthocyanidins, but recessive *r* genotypes contain only proanthocyanidins (Todd and Vodkin 1993). Genetic analysis of japonica rice has identified two genes, *Rc* and *Rd*, whose presence confers brown pigmentation on the pericarp (Takahashi 1982); this is in contrast to the purple pericarp pigmentation conferred by the *Prp* locus.

Nagao et al. (1957) reported on the chemical nature of the reddish-brown colour in the pericarp tissue of rice and found it to be a mixture of compounds consisting of catechins, catechol and phlobaphenes. These pigments were considered to be other than anthocyanins (Takahashi 1982). We have unambiguously identified the brown pigment isolated from the pericarps of N22B and A5 in our lines to be proanthocyanidins. This brown pigment does convert to the corresponding anthocyanidins upon acid hydrolysis (a characteristic test for proanthocyanidins) in sharp contrast to the non-conversion of catechins, catechols, and phlobaphenes, which we failed to detect by both the vanillin test and spectrophotometry (λ_{max} 540).

The significance of this result lies in identifying the lesion in the anthocyanin pathway that causes proanthocyanidin to accumulate in these lines instead of anthocyanins when the pathway is driven to the end (Fig. 4 summarises the steps involved in the general anthocyanin biosynthetic pathway). Proanthocyanidins are polymeric flavonoids synthesised from flavan-3-ols (catechins) and leucoanthocyanidin (Porter et al. 1986; and steps 13 and 14 in Fig. 4). Although two loci, *Rc* and *Rd*, in japonica rice have been implicated in the accumulation of brown pigment in the pericarp, their exact role in the pathway is unknown. The genetics of regulation of the proanthocyanidin biosynthetic pathway is known to some extent in seed coats of barley in which both procyanidins and prodelphinidins accumulate (Jende-Strid 1991). The gene *Ant19* encodes dihydroflavononol reductase or leucoanthocyanidin reductase which converts leucoanthocyanidin to catechin (Fig. 4, step 13), thereby initiating the polymerisation into proanthocyanidins (step 14) (Kristiansen 1984). The *A2* gene of maize codes for an NADPH- dependent oxidoreductase (Menssen et al. 1990). This enzyme, now referred to as anthocyanidin synthase, converts leucoanthocyanidin to anthocyanidin (Heller and Forkmann 1988; Fig. 4, step 10). These two enzymes are, therefore, reductases although they differ in their oxidative activity. Based on this information (that proanthocyanidins are reduction products) we conclude that the brown pigmentation is caused by a block in the conversion of leucocyanidin to cyanidin (step 10 in Fig. 4). Most likely the oxidation activity of anthocyanidin synthase is blocked in brown-pericarped rice since we failed to detect the oxidation or oxidoreduction products of this reaction, viz., phlobaphenes or cyanidin, respectively.

Proanthocyanidins are important plant-defence chemicals since they act as feeding deterrents (Scalbert 1991) and anti-fungal agents (Hagerman and Butler 1981; Jambunathan et al. 1986). Their accumulation therefore warrants further study in order to define their usefulness in future rice biotechnology programmes.

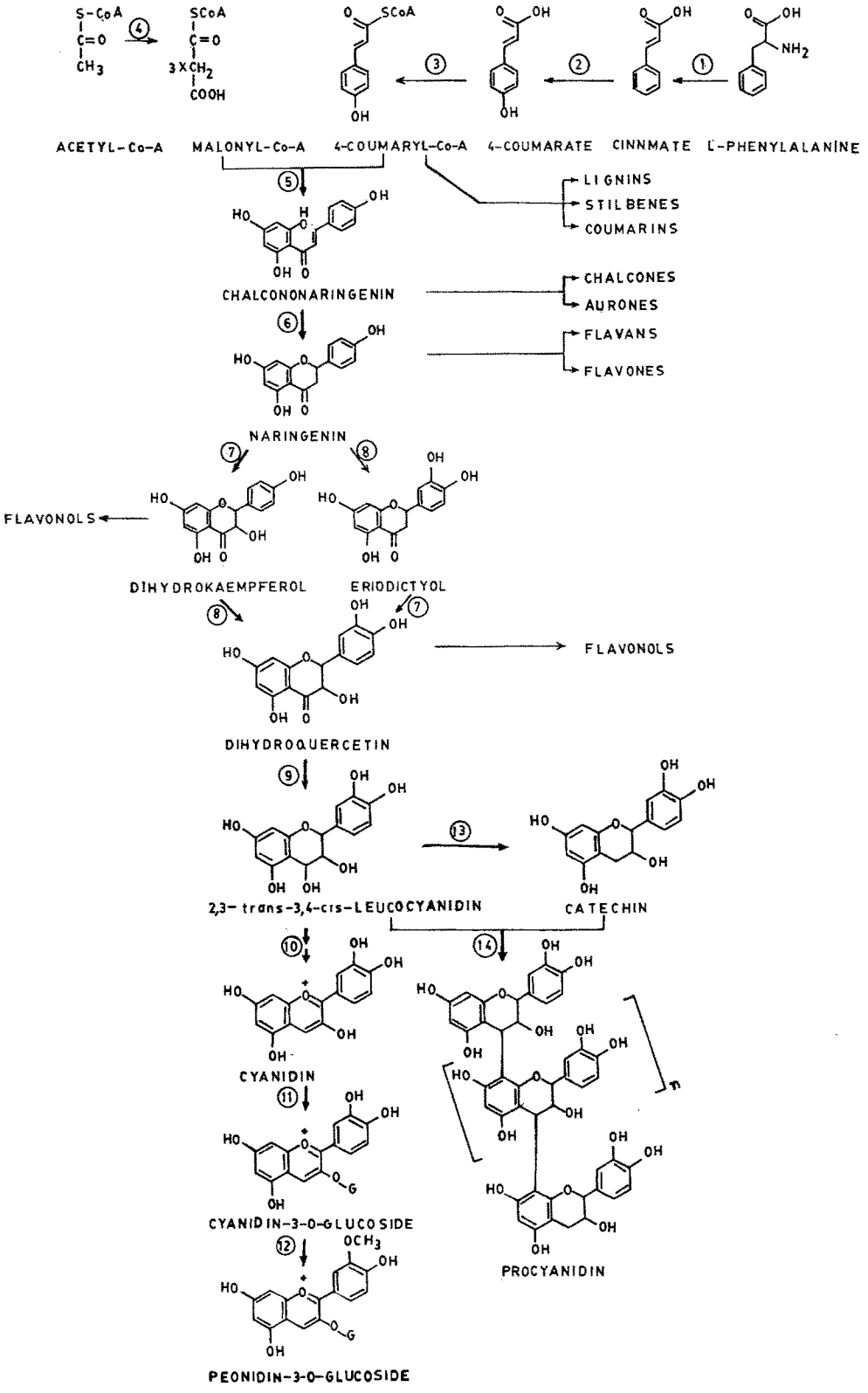

Fig. 4 Generalised anthocyanin biosynthetic pathway. The numbers refer to probable enzymes involved in the pathway. *1* phenylalanine ammonia lyase, *2* cinnamate-4-hydroxylase, *3* 4-coumarate:CoA ligase, *4* acetyl-CoA-corboxylase, *5* chalcone synthase, *6* chalcone isomerase, *7* flavonone-3-hydroxylase, *8* flavonoid-3-hydroxylase, *9* dihydroquercetin reductase, *10* anthocyanidin synthase, *11* flavonoid-3-O-glycosyltransferase, *12* S-adenosyl-L-methionine:anthocyanin-3′-O-methyl transferase, *13* leucoanthocyanidin reductase and *14* Condensing enzyme and/or chain-polymerising enzyme. The structural and functional characterisation of enzymes *12*, *13* and *14* are yet to be established

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