Indian J. Genet., **56** (3): 229–247 (1996)

THE R LOCUS IN MAIZE—A VERSATILE MARKER SYSTEM FOR PROBING DIFFERENTIAL GENE EXPRESSION

B. M. PRASANNA AND K. R. SARKAR

Division of Genetics, Indian Agricultural Research Institute
New Delhi 110012

(Received: June 5, 1995; accepted: October 22, 1995)

ABSTRACT

The R locus is a key regulatory gene in the maize anthocyanin biosynthetic pathway. Extensive allelic diversity of R gene and tissue specific patterns of anthocyanin pigmentation governed by the R gene and its displaced repeats (collectively termed as the R gene family) provide an ideal system for the analysis of differential regulation of gene expression. Elegant genetic experiments on R locus have enriched the understanding of tissue-specific gene expression and the regulation mechanisms in higher plants. The progress made in using the R gene as a marker system to study differential gene expression is reviewed here.

Key words: Maize, R gene family, tissue specificity, allelic interaction, gametic imprinting.

An amazing degree of cell and tissue heterogeneity characterizes the differentiated organisms, particularly higher plants. The postulate that the processes of development and differentiation involve sequential and differential expression of genes was first asserted by Haldane [1]. Regulation of gene action is an important requirement of development and differentiation. In particular cell types, certain genes are expressed and others repressed. Even within a single cell type, a particular gene may be allowed to express only in some parts of the cell type and repressed in others. A more trenchant expression of this concept of 'differential gene expression' was made by Wardlaw [2], who stated that "all of the genes may function some of the time and some of them all of the time, but not all of them all of the time." The progress in understanding differential gene expression in maize has come through studies on the regulation of biosynthesis of specific chemical substances.

The anthocyanin pigments represent an ideal subject for such investigations, since these chemicals are well-characterized at the biochemical level [3]; they respond to numerous extrinsic physiological influences; they occur in organisms whose tissues are complex, yet amenable to culture in vitro; and these nonvital pigments are visible to naked eye and can be scored at all times in the course of plant life. The anthocyanin 'markers' have contributed

enormously to maize genetics in unraveling diverse phenomena such as chromosome breakage–fusion–bridge cycle, transposition of the mobile genetic elements [4], analysis of complex loci [5] and paramutation [6]. Among the many genes that are involved in anthocyanin biosynthesis in maize (see [7]), we review here the work on R locus (located on the long arm of chromosome 10).

THE R GENE FAMILY AND ITS MEMBERS

The gene symbol R, first coined by East and Hayes [8], signifies red plant colour. Emerson [9] subsequently identified multiple alleles of both R and a functionally duplicate locus, B. After Fogel [10] determined that the R alleles control organ-, tissue-, and cell-type specificity of anthocyanin pigmentation in maize, intensive studies on the structural and functional aspects of the R locus were taken up.

Genetic analysis of the fine structure of R locus and biochemical-genetic studies of its function since the 1950s revealed the structural and functional complexity of this locus. The important conclusions from these investigations are summarized below.

The R locus consists of two separable units responsible for pigmentation in different tissues of the maize plant. The R(P) component is responsible for pigmentation in vegetative parts of the plant, while the R(S) component confers colour in the aleurone layer of the endosperm [5, 11].

Some of the members of the R gene family, R(Lc) and R(Sn), are displaced repeats, about two map units away from R locus on chromosome 10 and have distinct tissue-specific expression [12–14]. For instance, R(Lc) causes pigmentation in tissues such as leaf midrib, ligule, auricle and glume. Sn, a light- inducible gene, governs pigmentation in pericarp, glume, scutellar node, root etc., only under specific light conditions unlike Lc. The unlinked B (Booster) gene, located on chromosome 2, rarely conditions aleurone or coleoptile colour but frequently governs extensive colour in mature photosynthetic tissues, notably the leaf sheath, leaf blade and husk leaves [7]. R and B, which display duplicate factor inheritance, were also found to be homologous, allowing the isolation of B gene using R genomic sequences as probe [15].

The R alleles from different geographic regions may vary in pigmentation characteristics [16, 17]. More than 50 diverse patterns of gene expression can be attributed to the genetic constitution of this gene family (unparalleled by any other known locus in higher plants) and its variants affect over 20 tissue types differentially [7]. The 'pattern alleles' at the R locus — R-marbled (R-mb), R-stippled (R-st) and R-Navajo (R-nj) — are each specific in the distribution of pigmented areas in the aleurone tissue: R-mb displays coarse, coloured sectors with well-defined borders; R-st produces small, sharply defined coloured

spots; and R-nj confers colour with diffused, graded borders in the crown portion of the kernel [18].

Early work on the genetic analyses of R locus (till 1976), was reviewed by Gavazzi [19]. Significant progress in the understanding of the function of the R locus was made after the biochemical analysis which showed that the R gene product is necessary for the normal enzymatic activities of the structural genes A1, C2 and Bz1 [20]. Cloning and characterization of the R gene confirmed its role in anthocyanin biosynthesis; the R gene product is a transcriptional activator of at least three structural genes: C2 — encoding chalcone synthase, A1 — encoding dihydroquercetin reductase, and Bz1 — encoding UDP glucose:flavonol 3-O-glucosyl transferase [20, 21].

In short, the R alleles determine not only whether anthocyanin has to be synthesized, but also where, when and how much. The allelic diversity at the R locus makes it an ideal system to probe differential gene expression. There are two major questions that are of interest from the gene regulation point of view:

(i) How the R protein transactivates the structural genes directly responsible for anthocyanin biosynthesis?

(ii) How is the R gene itself regulated?

The coordinated genetic regulation of the anthocyanin structural genes addresses the first question and has been discussed in detail by Dooner [20], Prasanna and Sarkar [22] and Bodeau and Walbot [3]. In this article, we will concentrate on the basis for allele specific differences at R locus and the phenomena by which the regulator itself is regulated, in the light of the recent findings in molecular genetics.

DIFFERENTIAL GENE EXPRESSION AND PHENOTYPIC DIVERSITY OF THE R GENE FAMILY

The regulatory R gene of anthocyanin biosynthesis although present in all the cells of an individual, there is differential expression of the tissue-specific structural genes. Genetic analysis of R locus indicated that the tissue-specific pigmentation may be due to differential regulation of gene expression, where the R gene expresses in certain tissues and not in others [12, 16, 17].

The R gene product eluded the maize geneticists for several decades. R-nj was the first member of the R allelic series to be cloned with the help of transposon tagging [24] using the transposable element Activator (Ac), which subsequently led to the cloning of R(Lc) [21] and helped in understanding the structural and functional complexity of R.

Molecular organization of the R gene complex. Preliminary molecular characterization of the R gene family revealed the following salient features.

The R gene product shares features with many eukaryotic DNA- binding regulatory proteins (the myc family of protooncogenes) including those of *Drosophila* and mammals [21].

R(Lc), R(P), R(S) as well as R(Sn) were found to produce a single 2.5 kb transcript [21, 25–28]. R(Lc) DNA encodes a 610 amino acids long protein with an extensive acidic N-terminal region (220 amino acids) characteristic of a transcriptional activator. Apart from the DNA binding acidic domain, a shorter basic region of 93 amino acids was found at the C-terminus which shares homology with a Helix–Loop–Helix (HLH) motif, and is involved in the formation of dimers with other HLH proteins. The HLH motif is commonly found in different members of the R gene family that are tissue-specific [29].

Tissue specificity of R gene action. There are at least three plausible mechanisms at the transcriptional level by which the same gene may be expressed in different tissues or at different times during development: (i) multiple genes; (ii) multiple promoters; and (iii) multiple regulators. All these three mechanisms appear to have some role to play in the tissue- specific gene expression of the R gene family.

(i) *Multiple genes.* Molecular analysis of the R gene complex supports the earlier observations on the structural complexity of the locus made by several workers through genetic analysis [7]. The R locus has been found to contain two components: the (P) component, conditioning plant colour, consists of a single gene, while (S), the aleurone pigmenting component is a part of a more complex arrangement including two S genes and a third cryptic region of the complex, termed Q, consisting of a truncated R sequence [30]. Regulation of expression of these gene components differentially in various tissues (aleurone and plant parts) is proposed to be responsible for the differences in the expression of anthocyanin genes.

It is established by genetic and molecular analyses that B and R, located on two different chromosomes, share both structural and functional similarities and serve as duplicate genes. However, in comparison with R, the B locus appears simpler in its molecular organization; the B locus has a single coding region unlike the R gene complex. Analysis of several independent transposon insertions indicated that the tissue specific regulation conferred by the B allele is mediated by controlling sequences located upstream of the coding region rather than expression of separate gene components in different tissues as in the case of R [15, 31].

(ii) *Multiple promoters and multiple regulators.* Recent studies indicated that a high degree of sequence similarity exists in the R proteins like the products of S, Lc, B-Peru and B-I.

Most of the differences were found to be localized in the 5'-untranslated region, consistent with the proposal that distinct regulatory sequences are responsible for the tissue- specific expression of these genes [28, 31].

Are various R proteins expressed in different plant tissues functionally equivalent? Particle bombardment studies using chimeric R gene construct (Lc cDNA + constitutive CaMV 35S promoter) showed autonomous pigmentation of tissues that are not normally pigmented even in the presence of dominant gene, Lc [25]. This indicates that pigmentation can be induced in an otherwise nonpigmented tissue by changing the R promoter. Northern analysis of various tissues highlighted a strict correlation between pigment accumulation in different tissues and the expression of the regulatory and structural genes, suggesting that the pattern of pigmentation relies on a mechanism of differential expression of the members of the R gene family [28].

Molecular analysis of two B alleles with distinct tissue-specific anthocyanin pigmentation in plant and seed tissues revealed high sequence identity in the coding segment and its 3'-flanking region (98 and 90% respectively); in contrast, most of the 5'-region of their mRNAs and their 5'-flanking sequences did not show any significant sequence identity. This observation suggested that these alleles diverged from each other by complex genomic rearrangements rather than by simple base pair substitutions. Thus, different leader and promoter sequences of the two B alleles determine distinct tissue specificities of anthocyanin production [32].

Can the diversity in the R gene expression, therefore, be attributed solely to the promoter differences? The answer is not affirmative, as recent reports indicate that allele specific differences may also arise from differential regulation of RNA processing or translation [33]. While the results of molecular analysis so far suggest the role of multiple promoters in the regulation of R gene expression, the possibility of multiple regulators influencing tissue specificity of various R alleles is still not ruled out. Sequence comparisons of promoters from different members of the R gene family, like R(Lc), R(Sn) and B, together with deletion experiments, are being pursued in order to further elucidate the molecular basis for the differential expression of these genes.

Diversity of the promoter and 5'-untranslated leader sequences among the R genes provides an opportunity to study the coevolution of transcriptional and translational mechanisms of gene regulation. The structural and functional relationship of various members of the R gene family can be possibly explained by a recent postulate about the common evolutionary origin of these genes from a single ancestral gene [28]. While R and B, which are located on two different chromosomes have possibly resulted from a genome (or chromosome) duplication event, Sn and Lc, the displaced repeats of R that are not ubiquitous in their distribution (apparently restricted to a closely related group of maize

races) may represent a more recent intrachromosomal duplication of the R-containing region, a result of unequal exchange or translocation. Sequence divergence during the course of evolution may then have contributed to the establishment of functionally distinct genes, each one with its own tissue specificity; this hypothesis was confirmed by cDNA sequence analyses, indicating that Lc and Sn are more closely related to R than R to B [28, 34].

ANTHOCYANIN BIOSYNTHESIS IN PLANTS—REGULATORY GENES ARE HIGHLY CONSERVED

Molecular analyses of anthocyanin synthesis in several plants, cloning of the key pigmentation genes and gene swapping experiments in recent years have revealed another interesting feature: a high degree of conservation of the regulatory genes controlling anthocyanin synthesis (Table 1) in diverse plant species. Schematic illustration of anthocyanin pathway in maize is presented in Fig. 1; the pathway is similar in snapdragon and petunia for all major steps.

In most maize tissues, the entire pathway from CHS to UF3GT is regulated by R and C1, but in seedlings CHS expression can occur independently of R [36]. In snapdragon the pathway is regulated except for the first two steps, whereas in petunia the regulated part starts with DFR [35].

Table 1. Regulatory genes controlling anthocyanin pigmentation in selected plant species

Plant	Regulatory genes	References
Maize	R, C1, B, Vp1, Pl, P	7, 36
Antirrhinum majus	delila, eluta, rosea	37–39
Petunia	an1, an2, an4, an10, an11	40, 41
Arabidopsis	ttg	42

The R gene family of maize is highly homologous to the delila from snapdragon [31]. Similarly, R(Lc) can substitute for ttg (transparent testa glabrous) and an2 of *Arabidopsis* and *Petunia* respectively and the combination of C1 and Lc can induce expression of structural pigmentation genes in tissues where they are normally silent [41, 43]. These observations clearly indicate that regulation of pigmentation pattern in diverse plant species may be orchestrated by functionally homologous regulatory genes that appear to be derived from common ancestor [39].

In this brief review of the structure and function of R genes, we have outlined the recent studies at the molecular level in the context of elegant genetic experiments carried out by maize researchers since 1911. The significant developments in the genetic, biochemical and molecular analyses are presented step-wise in Table 2.

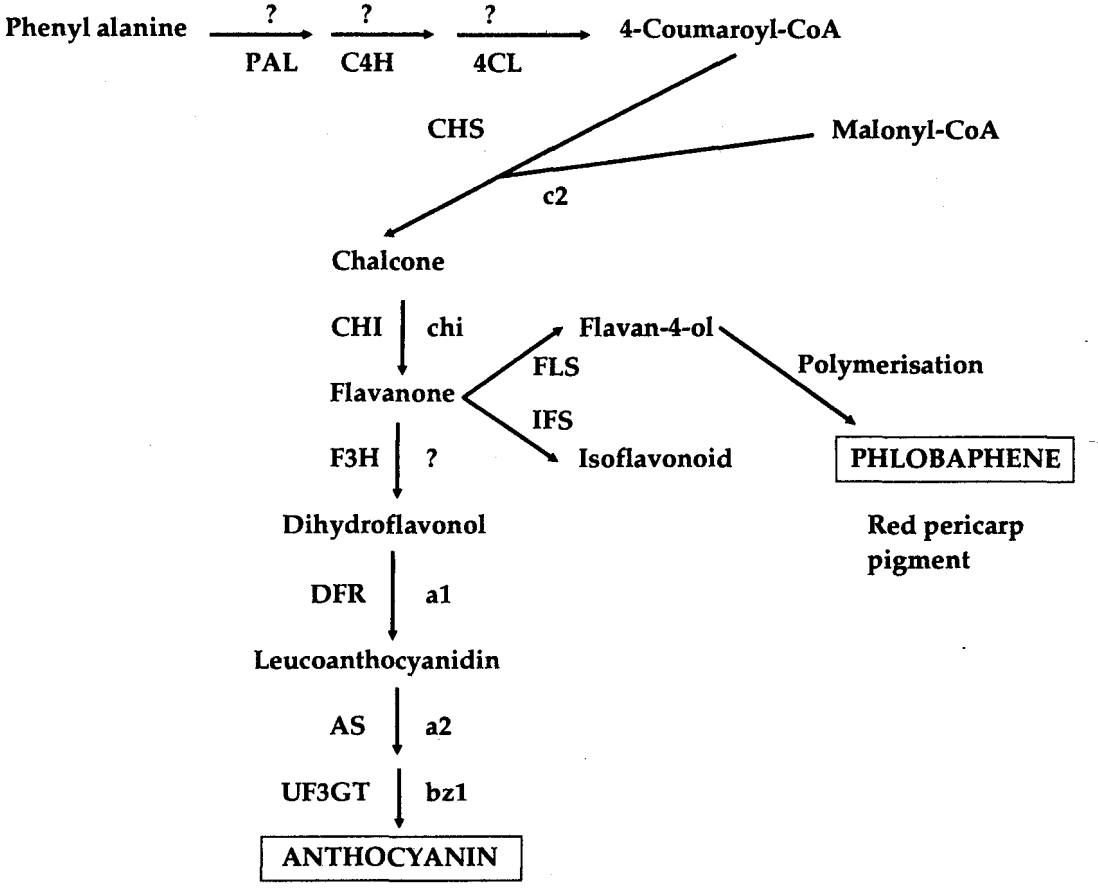

Fig. 1. **Schematic presentation of the anthocyanin biosynthetic pathway in maize. Each reaction is catalysed by a specific enzyme: PAL—phenylalanine ammonia-lyase; C4H—cinnamate 4- hydroxylase; 4CL—4-coumaroyl-coA ligase; CHS—chalcone synthase; CHI—chalcone isomerase; F3H—flavone 3-hydroxylase; DFR—dihydroflavonol reductase; FLS—flavonol synthase; IFS—isoflavonoid synthase; AS—anthocyanin synthase; and UF3GT—UDP-glucose: flavonol 3-O-glucosyl transferase. Gene coding for the enzyme, wherever known, is indicated opposite each enzyme on the other side of the arrow. Genes not yet discovered in maize for specific enzymes are indicated by marks of interrogation(?). Figure adopted and modified from Kroes et al. [35].**

HIERARCHY IN GENETIC REGULATION—REGULATION OF THE REGULATOR

The anthocyanin biosynthesis in maize is an illustration of the genetic regulation. There are structural genes like C2, Bz1 and A1 which code for specific enzymes in the biosynthetic pathway; there are regulatory genes like R and Cl that coordinately regulate the expression of these structural genes, and the regulatory genes in turn, can be regulated by diverse

Table 2. Significant developments in the understanding of structure and function of the R gene family

Year	Key finding	Reference(s)
A. Genetic:		
1911	R gene identified as a dominant factor for red or purple colour in aleurone	8
1921	Identification of multiple alleles at R influencing aleurone and plant tissue pigmentation; B, a functionally duplicate gene of R, discovered	9
1946	R alleles control organ, tissue and cell-type specificity of pigmentation	10
1948–1958	Compound structure of the R locus, having separate determiners for seed (S) and plant colour (P); step-wise mutation events discovered	5, 44, 45
1956	Paramutation discovered	6
1970–1975	Presence of multiple gene copies governing tissue-specific pigmentation in R-ch	16, 17
1976	Tissue-specific units of R occur as differentiated components of a tandem duplication	12
1978	Gametic imprinting phenomenon discovered in certain R alleles	60
B. Biochemical:		
1962	Linear sequence of gene action in anthocyanin biosynthesis proposed	46
1964	R is required for leucoanthocyanidin production	47
1979–1987	R gene product regulates enzymatic activities encoded by A1, C2 and Bz1	20, 48, 49
C. Molecular:		
1988	R-Navajo cloned by transposon tagging with Ac	24
1989	R(Lc) cloned using R-nj probe and gene product characterized; cDNA sequencing of R(S) and R(Lc); molecular evidence for transcriptional activator role of R	21, 26
1989–1990	Isolation of B utilizing R genomic sequences; molecular homology of B-Peru and B-I genes with R(Lc) discoverd	15, 50
1990	Cell-autonomous pigmentation induced by particle bombardment of R(Lc) and B-I chimeric gene constructs in various plant tissues	25, 50
	Molecular analysis of paramutation at R locus	58
1991	Molecular analysis of R(Sn)	74
1992	Molecular analysis of allelic diversity at B	32
	Homology of products of R gene family with delila gene of *Antirrhinum majus*	39
	Activation of anthocyanin production in other species using R gene	43
1993	Molecular analysis of regulation of R(Lc) gene expression	33
	Molecular analysis of paramutation at B locus	59

Note. Work on specific R alleles not included.

mechanisms. A commonly known phenomenon is the influence of transposable genetic elements; alleles of R such as R-st [51] and R-mb [52] provide examples for this. Can transposons act as general regulators of structural genes in normal development? This question is still under debate and no conclusive answers are available. Phenomena that are less understood but equally fascinating from the gene expression point of view are paramutation and imprinting.

Paramutation. The term 'paramutation' was first coined by R. A. Brink [6] for 'a directed, metastable and heritable change at a locus, resulting from an interaction between alleles in the somatic cells of appropriate heterozygotes.' Marked reduction can be observed in the anthocyanin pigmenting effect in aleurone of one class of R alleles (termed paramutable), by meiotic segregation from heterozygotes involving a second allelic class (termed paramutagenic). While the standard R-r and R-nj alleles represent the paramutable alleles, R-mb and R-st are paramutagenic. For instance, the R/R and R/R-st kernels obtained by selfing heterozygous R/R-st plants, will display notable reduction in the aleurone pigmentation intensity in comparison with the parental R kernels. Such paramutated R kernels are designated as R' [53, 54]. A paramutagenic allele like R-st, on the other hand, is not altered with respect to either aleurone phenotype or paramutagenicity in association with R. However, when R' is heterozygous with r or with a deficiency, it regains nearly all of its original level of pigmentation [55]. The mechanism behind such a phenomenon is still obscure.

Paramutation is not restricted only to the R locus; the unlinked member of the R gene family, B, also shows similar behaviour under the influence of a paramutagenic allele, B-I [56]. The paramutation phenomenon observed in the B allele is not entirely similar to that of R; the B' allele, the product of paramutation itself is capable of causing paramutation in another B allele [56].

The mechanism by which paramutation occurs at a high frequency in a given locus evoked interesting hypotheses from several researchers. Brink [53] speculated that the paramutagenic alleles provoked the process but were not the exclusive trigger for it. Schwartz [57] proposed that transposons are involved in paramutational changes. Although there is no experimental support for this hypothesis, it remains a possibility. Recently, Dooner et al. [36], hypothesized that a highly efficient homology sensing machinery and homology-searching process operate in the somatic cells, by which exchange of chromatin-associated proteins and possibly DNA methylation patterns (but not DNA sequences) occur in transinteraction phenomena like paramutation.

The molecular basis of paramutation is beginning to be understood. Based on gel blot analysis using restriction enzyme isoschizomers, Kermicle and Alleman [58] showed that DNA of the paramutant R' allele was hypermethylated relative to that of its progenitor. Are paramutational changes correlated with changes in the degree of methylation of the paramutable allele? Investigations by Patterson et al. [59] revealed that methylation

differences are not correlated with the differential expression of B and B' alleles. With the cloning and characterization of R, the time is now ripe to understand the molecular reasons for paramutation.

Gametic imprinting. The phenomenon of 'gametic imprinting' refers to the functional difference between the male and female gametes carrying a specific gene, which is in discordance with a basic, if tacit, assumption of Mendelian inheritance, that is, equivalent gene expression following passage through male and female gametophytes [60].

The R gene, when present in two or three doses in the endosperm, confers solid colouration in the aleurone in the presence of other complementary anthocyanin genes. However, the pigment producing ability is drastically reduced when a single dose of R transmitted through the pollen combines with two r doses in the egg. After Emerson [61] associated the splashing of colour or mottling in the endosperm with the heterozygosity of the R factor resulting from xenia (rr/R aleurone), Kermicle [60] resolved whether the mottling effect is due to a single dose of R or due to transmission of R through the male gametophyte. Using B10(R) translocation, he demonstrated that the mottled rr/RR and solidly coloured RR/r phenotypes could relate to differential R expression following maternal and paternal transmission to the endosperm.

R imprinting is an allele-specific response. For instance, R- r:standard, R-d:catspaw and R-mt are paramutable in nature. Nonmottling alleles such as R-st and R-mb show dosage effect but not imprinting response, although they are paramutagenic as mentioned earlier [60].

The functional difference between the male and female gametes carrying the R gene is expected to have a molecular basis. These gametes are probably differentially imprinted prior to fertilization, most likely during gametogenesis. The differences in the methylation pattern appear to have some role in the imprinting mechanism in the case of animal systems [62], but the picture is far from clear. Information on the molecular mechanisms of initiation, maintenance and erasure of imprinting is still lacking.

Phasic constitution of the aleurone — the R-Navajo case. 'Phasic constitution' is a term coined by Coe [63] to describe the phenotypic differences between the upper and the lower portions of the endosperm or between the cell layers flanking the embryo and those more distant. This property of the endosperm, first revealed by the behaviour of the R-nj allele, exemplifies intra-tissue differentiation based on the diffusion (or on the restriction of diffusion) of soluble metabolites.

Our recent analysis of the onset and progression of anthocyanin pigments in various R alleles [64], such as R-st, R-mb, R- nj:Illionois, R-r:standard, R-scm (ex. R-mb) and R-mb/R-nj

heterozygote revealed the following features: (i) there is a conspicuous delay in the onset of pigmentation in R-nj (about seven days under Delhi conditions) in comparison with other R alleles; (ii) while R-st and R-mb anthocyanin pattern formations reflected systematic (clonal) development of the aleurone, in R- nj, the anthocyanins diffused gradually from the silk-attachment region in a typical sun ray-like manner towards the periphery of the crown; and (iii) in both standard R-r and R-scm, pigmentation occurred first in the cells surrounding the silk- attachment region, but progressed in a wave-like manner.

On the basis of genetic analysis, Kumar and Sarkar [65] proposed that R-nj represents a complex with two discrete components: the self-colour (Sc) component is responsible for anthocyanin production while the Navajo (Nj) component regulates the time of onset and termination of pigment synthesis restricting the pigmentation to the crown region of the kernel. The clear-cut differences in the manner of pigment progression in R-nj and other R alleles [64] indicated that the Navajo pattern might not be solely due to delayed onset. It appears that the silk- attachment site has some significance in the anthocyanin pigmentation of certain R alleles, as in some important cellular processes in the developing endosperm, such as starch synthesis, protein body formation and transport [66]. At present, we know little about the molecular nature of the 'regulators' in R-nj, R- mb and R-st. Genetic analyses of R-st [51] and R-mb [52] indicated the presence of specific controlling elements influencing the variegation patterns. Molecular cloningof the R- nj allele [24] opens up the possibilities for utilizing R-nj as a 'reporter' in the study of phasic constitution of the aleurone. Some of the questions that can be addressed are (i) how are only the cells around the silk- attachment site endowed with pigment-producing potential in the Navajo aleurone, although all the aleurone cells have the same genetic constitution, and (ii) why does diffusion-based pigmentation occur only in R-nj and not in standard R-r?

R gene expression—influence of extrinsic conditions. Anthocyanin pigmentation is known to be influenced by several factors, such as position, time, place, conditions, stage and history, collectively termed as 'ambience' [67]. A variety of environmental factors such as light, photoperiod, temperature and stress conditions also influence the expression of some anthocyanin genes.

An R or B allele is, by itself, not sufficient to produce pigmentation in a particular tissue without the appropriate allele of either C1 or Pl. While C1 acts as a transcription activator (and also as a cofactor along with R), the Pl gene displays a light dependence of pigment accumulation in a variety of mature plant tissues. The recessive allele pl has a requirement of direct sunlight for pigmentation, whereas the dominant allele Pl renders R/B less light dependent and has considerable intensifying effect. Transient assays also revealed that the R gene expression was influenced by the action of Pl gene, another regulatory gene in anthocyanin pathway, or light, suggesting that expression of an R/B type protein alone is not enough for pigmentation [36].

Effect of light on R(Sn) gene expression is well-established [14, 36, 68]. However, it is not possible yet to define, with reasonable certainty, the regulatory mechanisms behind the photocontrol of Sn expression. It would be necessary to determine whether influence of light on the R gene expression was being exerted at the transcriptional or translational level.

CONCLUSIONS

Anthocyanin biosynthesis in maize is one of the well-understood metabolic pathways. Cloning and characterization of several structural genes and important regulatory genes like R and C1 provide possibilities for further analyses of their interaction and the influence of extrinsic factors on anthocyanin pigmentation.

The alleles at R show more phenotypic variation than any other locus in higher plants. Several members of the R gene family have been recently analysed at the molecular level. The first demonstration of complementation, using molecular tools, of a regulatory gene involved the R(Lc) coding sequence under the control of the constitutive CaMV promoter. Substantial progress has been made on the structural and functional dissection of the R genes. One important conclusion is that differences in the tissue-specific pigmentation governed by the R gene family members such as R(S), R(Lc), R(Sn) and B are mostly due to promoter differences and/or tissue-specific regulators rather than variations in the coding frame. The R gene system is highly amenable for further probing the molecular mechanisms behind the differential gene expression.

The maize system offers the advantage of studying genetically defined transactivators such as R. The high degree of sequence conservatism in the anthocyanin regulatory genes in model systems such as maize, snapdragon and petunia and their functional homology as revealed by gene swapping experiments are interesting from the evolutionary viewpoint.

The mechanisms by which a regulatory gene like R is regulated in phenomena such as paramutation, phasic constitution and imprinting are still not clear, although there are several speculations.

SOME AREAS OF INTEREST FOR FUTURE RESEARCH

Selection of stable transformants using the R marker system. The R gene as a versatile visible marker for selecting stably transformed cells lineages that can give rise to transgenic plants is rapidly gaining prominence. The most novel feature of this technique is that cell-autonomous expression can be visualized in almost all tissues of maize without disturbing the plant integrity. Transfer of this marker into meristematic cells may permit the identification of stably transformed somatic cell lineages, which can be observed as pigmented sectors in the developing plant. Stably transformed lineages that give rise to germinal tissues should yield transformed maize plants in the next generation [25].

Both GUS, a commonly used reporter system, and R can be visually monitored in the same tissue; however, the R reporter system allows quantification of gene expression in a living tissue by counting unambiguously the number of pigmented cells, unlike the GUS system. Transformation technique using chimeric R gene constructs shall be helpful in the following ways:

(i) introduction of R constructs mutagenised in vitro shall allow addressing questions regarding contributions of different sequence motifs to R function;

(ii) fusion with R(Lc) DNA shall also help analysing tissue-specific promoters from maize and possibly other monocots for their cis- acting control regions; and

(iii) questions related to influence of light on gene expression, such as, whether induction of pigmentation is influenced by genetic constitution of a specific locus or by light in different tissues, can be addressed.

Altered gene expression—paramutation and cosuppression. Plant transformation experiments, involving anthocyanin genes, recently revealed an intriguing phenomenon, where introduction of a transgene results in the suppression of the expression of the homologous, endogenous gene [69]. Since the introduced transgene was also suppressed in plants in which the endogenous gene was suppressed, the phenomenon was referred to as 'cosuppression' or 'repeat-induced gene silencing (RIGS)'. RIGS, which appears to be commonly occurring, can be a problem of considerable significance in genetic engineering experiments. Workers are now drawing parallels between the two transinteraction phenomena, paramutation and cosuppression. While paramutation is based on allelic interactions, cosuppression is due to interactions between copies of the same sequence placed at nonallelic (ectopic) locations [69]. Anthocyanin biosynthetic genes such as R can be exploited as 'reporters' for study of trans-interaction phenomena in plants.

Analysis of DNA–protein and protein–protein interactions. Preliminary molecular characterization of the R gene family and several of the anthocyanin biosynthetic genes along with the availability of a transient assay will be valuable in understanding the protein–protein interactions (between the regulatory gene products of R and C1) as well as the structural gene promoter–regulatory protein interactions.

Exploiting the regulatory gene homology in different plant species. With the advent of sophisticated molecular techniques, cloning and sequencing of anthocyanin regulatory genes in diverse plant species have become feasible. Gene swapping experiments in recent years [39, 43] indicated that these regulators are highly conserved, both structurally and functionally, and interchangeable between plant species and that their expression patterns determine the mode of pigmentation in different species. These findings may have two

immediate implications: (i) plants with novel colouration patterns may be obtained by simultaneous introduction of C1 and R-type genes under the cell type-specific promoters; and (ii) from the evolutionary point of view, further studies may provide clues regarding the plausible mechanisms by which different regulatory mechanisms might have developed from a common origin.

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