Anthocyanin Biosynthetic Pathway in Rice *(Oryza sativa* L.): UV-B-Response, Molecular Cloning and Characterization of *Dihydroflavonol reductase* and *Anthocyanidin synthase* cDN As

A Thesis
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This is to certify that I, Vaka Subba Reddy, have carried out the research work embodied in the present thesis in the Department of Plant Sciences under the supervision of **Professor Dr. Arjula Ramachandra Reddy,** for the full period prescribed under the Ph.D., ordinance of the University.

I declare that no part of this thesis was earlier submitted for the award **of** research degree of any University.

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Date: March 27, 1996 **Vaka Subba Reddy**

Contents

1. Introduction

Anthocyanins, a class of flavonoid compounds, are ubiquitous flower and fruit pigments of flowering plants. Anthocyanins together with other flavonoid pigments impart a wide range of visible colors to almost any plant part. These pigments are synthesized through a multistep biosynthetic pathway, generally referred as anthocyanin pathway. The biosynthesis of anthocyanins in plants is an ideal experimental material for the genetic and molecular analysis because this pathway is an example of an integrated gene interaction system among higher plants. Some of the major attributes of the pathway are; co-ordinate regulation in a spatial and temporal fashion, phenotypes are readily observable and therefore can be used as genetic marker and mutations in the pathway are not lethal and by and large lead to visible phenotypic changes which can easily be identified and isolated. Thus it turned out to be the most intensively studied secondary metabolite pathway in higher plants at the genetic, biochemical and molecular level.

The anthocyanin pathway has been extensively exploited in the study of a variety of genetic and molecular phenomena in plants: gene interaction, markers for linkage and mapping, gene isolation, gene organization, expression and regulation, characterization and isolation of transposable elements and transformation for many useful purposes. Genetics and molecular biology of anthocyanin pathway is rigorously investigated in a few plants, notably maize, snapdragon and *Petunia*. Extensive studies in maize lead to the establishment of genetic and molecular basis of anthocyanin production, distribution and regulation. More than 20 loci are known to be associated with anthocyanin biogenesis in maize (Coe et al., 1988). The role of many of these loci in the biosynthesis of flavonoid pigments is elucidated. The trans-regulation by *rnyb* family of anthocyanin genes viz., *C*\ and *R* and the role of environmental factors, for instance light, have been clearly demonstrated. On the contrary, almost nothing is known about the molecular and biochemical basis of anthocyanin biosynthesis in rice.

Genetics of anthocyanin pigmentation has been studied in rice mostly by early plant breeders. These studies described the identification of specific loci and their linkage relationship, mostly in japonica lines. Indica-japonica comparisons were also reported. Three classes of genes were mainly described: i) basic or fundamental genes, *C* (chromogen), *A* (activator), *P* (purple), ii) distributive or localizer genes, *Pl* (purple

leaf), *Pn* (purple node), *Prp* (purple pericarp) and iii) inhibitory genes, *IPl*-1 to *IPl*-6 which inhibit anthocyanin pigmentation in a range of tissues. However, a systematic approach to establish a relationship between the identified genes and their specific role in the anthocyanin biosynthetic pathway has been lacking. Biochemistry, photoregulation (particularly in response to UV-radiation) and molecular biology of the anthocyanin pathway in rice plant have not been studied at all. Recently, studies on molecular characterization of the pathway have been initiated and molecular cloning of the chalcone synthase *(CHS)* cDNA has been reported. This cDNA clone encoding chalcone synthase, the key enzyme of anthocyanin pathway, was isolated from a leaf specific cDNA library of an indica rice line Purpleputtu (Reddy et al., 1994) and sequence characterized (Scheffler et al., 1995). These studies opened up the anthocyanin gene-pigment system in rice for further analysis.

Anthocyanin and flavonoids are implicated in diverse plant functions particularly under stress situations. Having a wealth of information on the basic aspects of flavonoids and availability of easily adaptable analytical methods, the role of the pathway in stress response could be tested. Particularly interesting is the possibility of anthocyanins and flavonoids having a protective role against the UV-B light damage in plants.

It has been reported earlier that UV-B radiation is harmful to plants in many ways including reduction in plant growth, reduced photosynthetic efficiency, biomass production and also the diminished ability of crop plants to compete with weeds. It has been predicted that every 1% decline in the ozone layer would lead to 1% decrease in crop yield. Studies on the deleterious effects of UV radiation have been largely confined to temperate plant species. Not much is known about its effects on tropical plant species. Among tropical crop species, rice *(Oryza sativa* L.) is the foremost food crop of the developing world, providing food to more than three billion people and therefore any reduction in its yield by UV-B radiation can have adverse consequences in developing countries. In fact, there are only a few studies on the effects of UV-B radiation on rice crop. These studies mainly focused on parameters like leaf area, biomass, tiller number and photosynthetic capacity, all agronomic traits of rice plant.

Information on the presence of UV-absorbing pigments and their role in ameliorating the harmful effect of UV light in rice is scanty. Accumulation of the UV-B absorbing pigments is one of the ways by which plants alleviate harmful effect of UV-B

light. A few investigations have reported that UV-B treatment increases the amount of UV-absorbing pigments in some rice cultivars. Since an increased levels of UV-absorbing compounds are thought to offer protection against the harmful UV-B damage, information about the presence and regulation of their biosynthesis is valuable in selecting rice cultivars with increased resistance to UV-B light.

In view of the above information, it emerges that a powerful tool in rice genetics and molecular biology of UV-B regulation can be developed if the anthocyanin pathway and its genes are characterized in detail and put in proper perspective. This objective becomes more significant in view of the importance of rice as a major world food crop.

The aim of the present study is to understand the genetics, biochemistry and molecular biology of anthocyanin pathway in rice.

Specific objectives are:

- Identification of rice genotypes for distinct anthocyanin phenotypes.
- Structural determination of anthocyanidins in rice.
- Determination of genetic blocks that accumulate precursors and intermediate metabolites in the pathway.
- Detection and elucidation of UV-B induction of anthocyanin pathway in rice.
- Isolation of cDNA clones encoding enzymes and regulatory proteins of anthocyanin pathway.
- Sequence characterization of specific cDNA clones, dihydroflavonol reductase *(DFR)* and anthocyanidin synthase (*ANS*).
- Molecular manipulation of phenypropanoid and anthocyanin biosynthetic pathway genes.

2. Literature Review

Plant cells are inherently capable of performing numerous and definite functions during differentiation and growth. In addition, they respond to a complex set of environmental signals through a series of metabolic corrections. Elucidation of such mechanisms require a thorough investigation on the expression and regulation of specific genes of relevant metabolic pathways. This, understandably, involves complex phenomena that result from the interaction of a chain of molecular (beginning with transcription of genes and the final production of a functional protein) and developmental events. Specialized cells synthesize numerous structurally diverse compounds with a range of functions. Such compounds, originating from the secondary metabolite pathways, including phenylpropanoids and flavonoids (anthocyanins) are reported to be involved in plants' response mechanisms to biotic and abiotic stress factors.

Studies on the control of multistep metabolic pathways are of great significance because of synthesis and accumulation of novel and functionally important secondary metabolites in plants. Also, regulation of secondary metabolism is of immense importance as the level and distribution of these compounds is strictly governed by a complex set of regulatory genes. These regulatory mechanisms determine co-ordinate expression of the structural genes encoding enzymes of secondary metabolic pathways. This is mainly achieved through interaction between *cis*-elements and *trans-factors* in a spatial and temporal fashion.

The phenylpropanoid and anthocyanin pathways in higher plants emerged as excellent model systems to study the molecular basis of tissue-specific, co-ordinate and developmental regulation of gene expression. Particularly, the anthocyanin pathway has been studied in depth at the biochemical, genetic and molecular levels in several species, notably *Zea mays, Petunia hybrida* and *Antirrhinum majus* (reviewed by Coe et al., 1988; Valming et al., 1984; Martin et al., 1991).

2.1 General phenylpropanoid and flavonoid biosynthetic pathways

Phenylpropanoid pathway leads to the synthesis of the basic precursor of flavonoids, 4-coumaryl CoA. This is formed by three sequential enzyme mediated

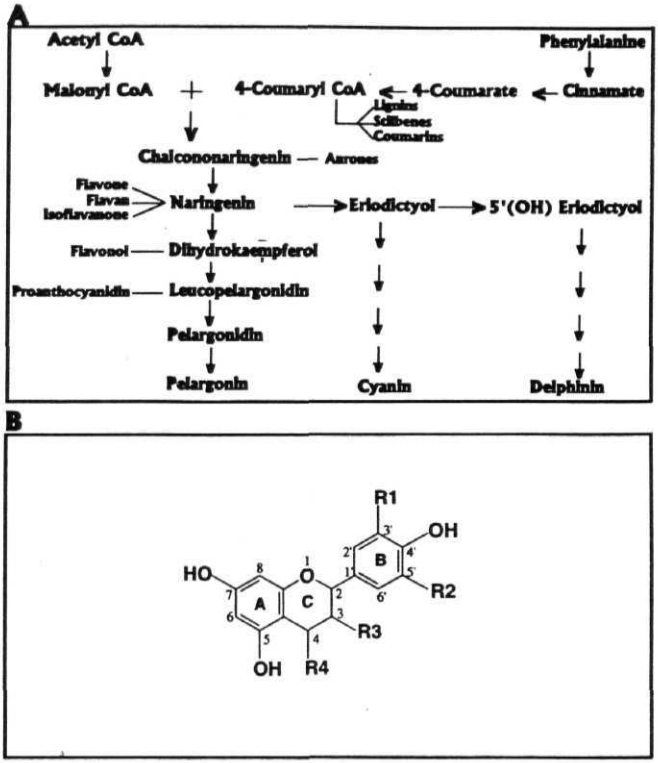

Figure 1 A) General phenylpropanoid and flavonoid biogenesis in plants.
→, indicates the main channel of the pathway; —, indicates branches of the pathway.

B) The basic flavonoid molecule: modifications of V and 'b' rings give rise to an array of flavonoids.
Flavanone, R3-H, R4->=O
Dihydroflavonol, R3-0H, R4->=O
Leucoanthocyanidin, R3-OH, R4-OH
Anthocyanidin, R3-0H, R4-H, O1=C2, C3=C4
Anthocyanin, R3-OGlc, R4-H, O1=C2, C3=C4
Flavone, R3-H, R4->=O, C2=C3
Flavan, R3-H, R4-H, O1=C2, C3=C4
Isoflavanone, shift of aryl group (B ring) from C2 to C3 position
Flavonol, R3->=0, R4->=0, C2=C3
Monohydroxyl flavonoid (eg. pelargonin), R1-H, R2-H
Dihydroxyl flavonoid (eg. cyanin), Rl-OH, R2-H
Trihydroxyl flavonoid (eg. delphinin), Rl-OH, R2-OH

reactions beginning with the conversion of phenylalanine to trans-cinnamic acid by phenylalanine ammonia lyase (PAL, EC-4.3.1.5). The cinnamic acid is then hydroxylated by cinnamate 4-hydroxylase (C4H, EC-1.14.13.11) to form 4-coumaric acid which is activated by the addition of coenzyme A by 4-coumarate: CoA ligase (4CL, EC-6.2.1.12). Malonyl CoA is synthesized from acetyl CoA and CO_2 catalyzed by acetyl CoA-carboxylase (EC-6.4.1.2).

Flavonoid pathway (Figure 1A) originates from the phenylpropanoid pathway by the stepwise condensation of three units of malonyl CoA and one unit of 4-coumaryl CoA catalyzed by the enzyme, chalcone synthase (EC-2.3.1.74) leading to the formation of the basic C-15 flavonoid skeleton, the chalcononaringenin. This is followed by isomerization of the chalcone, either non-enzymatically or by chalcone isomerase, to form naringenin (flavanone), the central intermediate of the flavonoid pathway. From this step onwards, the pathway diverges into several branches each yielding a different class of flavonoids, the end products of the main pathway being anthocyanins. Therefore, this pathway is also referred generally as the anthocyanin pathway.

The degree of oxidation of the C-ring (Figure 1B) determines the subclass of flavonoids formed such as isoflavonoids, dihydroflavonols, flavones, flavonols, catechins, proanthocynidins and anthocyanins (Figure 1B). Further, various substitutions like hydroxylation, methylation, glycosylation or acylation account for the overwhelming diversity of flavonoids observed in nature (Heller and Forkmann, 1988; Harborne, 1988). More than four thousand different kinds of flavonoids including anthocyanins from all kinds of plant species have been reported

Anthocyanins

Anthocyanins (Greek *anthos*, flower and greek *kyanos*, blue), the term used originally to describe the blue pigment of cornflower, *Centaurea cyanus* (Marquart, 1835), are the most important sub group of visible flavonoid pigments. More than two hundred different naturally occurring anthocyanins are reported (Harborne, 1988) and the number is growing. Anthocyanins are almost universal plant colorants and largely responsible for the brilliant orange, pink, scarlet, red, mauve, violet and blue colors of flowers, fruits, roots, leaves and other plant organs. They accumulate in vacuoles (Wagner, 1982; Joanny and Reddy, 1992) of epidermal or sub epidermal cells as anthocyanoplasts (Pecket and Small, 1980).

The anthocyanin pathway begins with the conversion of naringenin (flavanone), by hydroxylation at the C3 position by flavonone-3-hydroxylase (F3H) activity to dihydroflavonol, a pale yellow pigment. That next step is the reduction of dihydroflavonol by dihydroflavonol reductase (DFR) to form a colorless leucoanthocyanidin (Reddy et al., 1987). This compound is converted into a colored anthocyanidin in a two step fashion, catalyzed by anthocyanidin synthase (ANS), an NADPH dependent oxidoreductase (Menssen et al., 1990). One of the last steps of the pathway, the glyocosylation of anthocyanidin molecule, leading to the formation of anthocyanin is catalyzed by the enzyme UDP-glucose-flavonoid-3-oxy-glycosyl transferase (FGT). Further, acylation of anthocyanin is catalyzed by the glutathione s-transferase (GST) which is associated with transport of anthocyanins into vacuoles (Marrs et al., 1995).

2.2 Physiological functions of phenylpropanoids and flavonoids

Phenylpropanoids and their flavonoid derivatives are reported to perform several active physiological functions in plants. There are reports that flavonoids including anthocyanins have a role in a) protecting the plants from harmful radiation particularly UV-B radiation, b) plant defence responses (phytopathogenes), c) auxin transport regulation, d) plant-microbe symbiosis, e) male gametogenesis and f) stress response mechanisms.

2.2.1 Protection from harmful UV-radiation

Plants undergo a variety of physical and metabolic alterations when exposed to biologically harmful UV radiation. These include inhibition of growth, leading to reduced height, leaf area and dry weight (Caldwell et al., 1980; Teramura, 1983; Beggs et al., 1986; Murali et al., 1988; Tevini and Teramura, 1989). These changes are accompanied by an increased accumulation of UV-B absorbing compounds including phenylpropanoids, flavonoids and anthocyanins (Caldwell et al., 1980; Wellmann, 1983; Teveni et al., 1991). Epidermal cells of parsley leaves respond to UV-radiation with an increased vacuolar deposition of UV-absorbing flavonoid glycosides which may prevent UV-damage of the underlying tissue (Schmelzer et al., 1988). This was confirmed by *in situ* hybridization of *CHS* mRNA and flavonoid end products in epidermal cells (Schmelzer et al., 1988).

A direct evidence that flavonoid molecules play an important role in UV-protection has been obtained from the studies of *Arabidopsis sin* mutants. These mutants do not accumulate flavonoids and other phenolic compounds, hence are more susceptible to UV-radiation than that of wild type plants (Li et al., 1993). In *Arabidopsis*, a recessive mutant line showing sensitivity to UV radiation was isolated and characterized. These seedlings, under UV light, suffer severe developmental aberrations eventually leading to death (Lois and Buchanan, 1994). This single gene defect was shown to be a block in the synthesis of kaempferol, a flavonol. On the other hand, the wild type seedlings accumulate high concentrations of this flavonol under UV-B light and grow normally. These results suggested that the flavonols, whose production had been interrupted in UV-sensitive mutants, were essential for the protection of *Arabidopsis* against the UV-damage.

Most of the flavonoids have a high absorbance (log E, 4 to 4.5) in 250 to 270 nm range where proteins and nucleic acids also absorb (Harborne, 1988). Kootstra (1994) demonstrated the protection of plasmid DNA from UV-induced DNA damage by naringenin, a flavanone and rutin, a flavone using plasmid DNA. In an elegant set of experiments the formation of cyclobutane pyrimidine and pyrimidine (6,4) pyrimidone dimers by UV-radiation was monitored in *Zea mays* plants using individual monoclonal antibodies (Stapleton and Walbot, 1994). The data from these experiments revealed that anthocyanin/flavonoid containing maize lines could protect the DNA more efficiently than non-flavonoid producing genetic strains.

2.2.2 Role in defence response (phytoalexins)

Plants synthesize an array of specific flavonoids like isoflavonoids and flavones which act as phytoalexins in response to fungal or bacterial phytopathogens or elicitor treatments. Pisatin, an isoflavonoid, has been shown to function as phytoalexin in epicotyls of pea seedlings (Noborous et al., 1981). Isoflavonoids and flavones were reported to act as phytoalexins in legume species. For instance, daidzein and tricin were produced in high amount in plants infected with *Ascochyta imperfecti* in *Medicago sativa* (Olah and Sherwood, 1973). Glyceollin, an isoflavonoid involved in soybean - microbe interactions as phtoalexins (Ebel, 1979) was induced by infection with pathogens or treatment of plant tissues with pathogen derived elicitors (Ebel, 1986). Furanocoumarins were produced in response to a fungal pathogen in parsley

cell cultures (Dangl et al., 1987). The role of flavonoids in disease resistance is yet to be unequivocally established.

2.2.3 Role in auxin transport regulation

The plant growth regulator, indole-3-acetic acid (IAA, an auxin), is a phytohormone that is transported from the site of synthesis in shoot apices and young leaves to subapical target tissues in which it exerts its many developmental effects. This transport is facilitated by a proton gradient driven efflux of IAA anions through plasma membrane receptors. Quercetin, a flavonol, and apigenin, a flavone, can inhibit IAA transport by blocking the polar efflux step and causing a net IAA accumulation in transporting cells as it was tested in *Cucurbito pepo* L. hypocotyls and other plant tissues (Jacobs and Rubery, 1988). Flavonols are widely distributed across the plant kingdom and exert their effects at micromolar concentration (almost same as endogenous levels). These results suggest that flavonols play a role as natural auxin transport regulators (Jacobs and Rubery, 1988).

2.2.4 Role in plant-microbe interactions

The soil micro-organisms such as *Rhizobium* species establish nutritionally beneficial symbiotic relationship by fixing the atmospheric nitrogen in root nodules on host plant cells of the family *Leguminosae*. The symbiosis begins with the formation of nodules on roots by the activity *of nod* genes including *nod D,A,B* and *C*. These genes are induced by compounds in exudates of plant hosts (Peters et al., 1986; Rossen et al., 1985). Flavonoids are the first known signal molecules involved in the establishment of *Rhizobium-legume* symbiosis (Redmond et al., 1986; Kosslack et al., 1987). The symbiotic interaction of *R.meliloti* and alfalfa results in the formation of nitrogen-fixing root nodules; Luteolin, a plant flavone acts as an inducer molecule for *nod A, B, C* expression and was isolated from alfalfa plant exudate (Peters et al., 1986). The isoflavones daidzein and genistein have been identified in soybean root extracts as the major components that are responsible for the induction of *nod* genes in *Bradyrhizobium japonicum* (Kosslack et al., 1987). Free flavones and flavonols released by pea and clover roots (Firmin et al., 1986; Redmond et al., 1986) act as signal molecules in nodulation process by *Rhizobium*.

Another example of microbial response to flavonoids is the induction of virulence (*vir*) genes of the opportunistic pathogen *Agrobacterium tumefaciens*. The transformation of plant cells by *A. tumefaciens* is mediated by the *vir* genes of Ti plasmid. This *vir* region of Ti plasmid contains six complementation groups of which *vir A* and *vir G* loci are constitutively expressed. The other *vir* loci *(vir B, C, D* and *E)* are only expressed if *Agrobacterium* are cultivated with plant cells. The expression of *vir E* locus was monitored with a gene fusion between *vir E* and *lac Z* gene to determine the *vir* inducing activity of extracts of pollen and stigma of *Petunia hybrida*. These compounds were identified as flavonol glycosides namely kaempferol-3-glycosides and quercetin-3-glycosides. Thus, flavonoids induce the *vir E* gene expression which is a crucial event in agroinfection and *Agrobacterium* mediated plant transformation (Zerback et al., 1989).

2.2.5 Role in male gametogenesis

Coe et **al.,** (1981) first isolated and characterized a maize mutant deficient in producing functional yellow pollen. The recessive *whp* mutant sheds normal amount of non-pigmented pollen, which germinates on the silk, but no seed is set after most pollinations. The condition is sporophytically determined by the expression of stable recessive mutation at two chalcone synthase *(CHS)* loci in maize, *C2* (colored-2) and *Whp* (white pollen). Later, it was identified that flavonols play a definite role in the growth of the pollen tube. A high abundance of flavonols and ***CHS*** mRNA were demonstrated in male reproductive organs of *Petunia hybrida* (Taylor and Jorgensen, 1992). Transgenic plants carrying antisense *CHS* gene activity completely lack the flavonol in male reproductive organs. This phenomenon renders the plants self-sterile. Such antisense transgenic plants could be made fertile by supplying exogenous flavonol. Hence such phenomenon is called as reversible or conditional male sterility. This finding clearly specifies the role of flavonol in pollen maturation and fertility in these plants (Ylstra et al., 1994; Mo et al., 1992).

2.2.6 Other functions of flavonoids

Flavonoids, particularly quercetin and myrcetin can act as antioxidants to ascorbic acid cofactor in various enzymatic reactions. The antioxidant activity of flavonoids depends primarily due to 3-hydroxyl, 4-carbonyl and 3',4'- dihydroxyl

substitutes (Figure 1B) of flavonoid molecule (Pratt, 1965). The anthocyanidins, a major class of flavonoids, are known to be excellent scavengers of oxygen radicals (Saskia et al., 1995). Certain flavonoids are reported to act as insecticides (Scalbert, 1991) and also inhibit viral growth (Mucsi and Pragai, 1985; Kaul et al., 1985). It is likely that many more functions of flavonoids remain to be uncovered.

2.3 Stress-induced phenylpropanoid and anthocyanin biosynthetic pathways in plants

Plants posses specific defence mechanisms against different forms of environmental stress conditions, both biotic and abiotic. The peripheral epidermal cell layers alter their metabolic channels to produce numerous compounds required for the protection of internal cell layers from harmful UV-radiation, high light, temperature, wound, fungal elicitor and phytopathogens. In this context, general phenylpropanoid and flavonoid pathways are of great functional significance (Dixon and Lamb, 1990). As a result of these multi-step multibranch pathways several structurally diverse low molecular weight compounds with a wide range of functions are synthesized in physiologically altered cells and are accumulated in specific regions to protect the internal sensitive photosynthetic and other cell machinery of plants. As can be anticipated from the rapid production and increased accumulation of these secondary metabolites, the transcription and translation of the genes responsible for these pathways are co-ordinately regulated. The key enzymes phenylalanine ammonia lyase and 4-coumarate:Co A ligase of phenylpropanoid pathway and chalcone synthase of the flavonoid pathway are of great significance since they determine the specific end products to be synthesized toward alleviating the deleterious effect of stress. The stress mediated regulation of expression of the genes encoding these enzymes was extensively studied.

Phenylpropanoid derivatives have been implicated in disease resistance (Halbrock and Scheel, 1989) and UV-light resistance (Caldwell et al., 1983) in plants. They have high extinction coefficients in the biologically damaging UV-B range and primarily accumulate in the epidermal layers of plant organs. Phenylpropanoids are also required for the biosynthesis of polymeric lignin which is an important structural component in disease resistance mechanisms (Moerschbacher et al., 1990). Upon UV-radiation or elicitor treatment, transcription rates of *PAL* and *4CL* genes were increased

several folds in cultured parsley cells and protoplasts, with a concomitant accumulation of flavonoids and coumarins in epidermal cells of plants (Dangl et al., 1987, 1991; Chappel and Hahlbrock, 1984; Hahlbrock, 1981). Tissue-specific increases in *PAL* mRNA level were also observed in bean hypocotyls infected with *Colletotrichum lindemuthianum* (Bell et al., 1986). In *Arabidopsis*, the *PAL*-1 gene is induced by wounding and pathogen attack (Davis and Ausubel, 1989; Davis et al., 1991; Dong et al., 1991) in a tissue specific manner (Oh! et al., 1990). Immunohistochemical analysis of young parsley buds localized *PAL* to oil duct epithelial cells (a site for constitutive furanocoumarin synthesis), developing xylem (a site for lignin synthesis) and epidermal cells (a site for flavonoid biosynthesis) (Jahnen and Hahlbrock, 1988; Schmelzer et al., 1988). Three different *PAL* genes, *gPAL-*, *gPAL-2* and g*PAL*-3 from bean have been isolated and characterized (Cramer et al., 1989). RNase protection assays revealed they are differentially expressed: *gPAL-* is expressed in nearly all tissues (roots, stems, flowers and leaves); *gPAL-2* is expressed, at very high levels, in flower petals and to a lesser extent in roots and shoots and gPAL-3 is expressed in roots only. However, various stress factors alter their distribution in hypocotyls. While all three genes are activated by wounding, g*PAL*-1 and *gPAL-2* are expressed by high intensity illumination of etiolated hypocotyls and fungal infection stimulates g*PAL*-1 and 3 gene transcription (Liang et al., 1989a, 1989b).

In parsley, atleast three of the four *PAL* genes are activated either by UV light or fungal elicitor in cultured cells and in wounded roots. Interestingly, only the parsley *gPAL-3* gene is active in wounded leaves (Lois et al., 1989). *Arabidopsis PAL*-1 is inducible by wound and pathogen attack (Davis and Ausubel, 1989 and Dong et al., 1991).

The induction of *CHS* expression in response to internal and external stimuli has been studied intensively. By *in situ* hybridization analysis in parsley, it was shown that the expression of *CHS* and accumulation of flavonoids were restricted to epidermal cells of UV-light treated etiolated seedlings (Jahnen and Hahlbrock, 1988; Schmelzer et al., 1988; Ohl et al., 1989). This general scheme for light regulation of the *CHS* expression holds true for *Antirrhinum* (Lipphardt et al., 1988), *Petunia* (Koes et al., 1989) and *Arabidopsis* (Feinbaum and Ausubel, 1988). The Induction of isoflavonoid synthesis in primary leaves of bean is another example of U V induced *CHS* mRNA and flavonoid pathway (Beggs et al., 1986). The etiolated suspension cultured parsley cells,

when treated with UV containing white light, trigger a series of transcriptional and biosynthetic reactions culminating in vacuolar deposition of flavonoids (Kreuzaler and Hahlbrock, 1973; Schmelzer et al., 1988). In *Arabidopsis*, the expression of *CHS* gene and flavonoids is induced by blue light in seedlings and by high intensity white light stress in mature plants (Feinbaum and Ausubel, 1988; Feinbaum et al., 1991). In legumes CHS turned out be the key enzyme in the biosynthesis of isoflavonoid phytoalexins (Lamb et al., 1989). There are many more reports of flavonoid derived compounds playing a critical role in plant-microbe interactions of either pathogenic or symbiotic nature (Long, 1989).

In French bean, soybean, alfalfa, pea, and *Petunia*, the CHS is encoded by multigene families (Ryder et al., 1987; Wingender et al., 1989; Dalkin et al., 1990; Harker et al., 1990 respectively). Differential transcription of specific members of the ***CHS*** gene families was observed in French bean (Kreuzuler et al., 1983) and soybean cell suspension cultures (Wingender et al., 1989) in response to UV light. In *Petunia*, the *CHS* multigene family are spatially (flower specific) and temporally expressed and in addition they can be induced in young seedlings and in cell suspension cultures by illumination with UV-light (Koes et al., 1989). Kubasek et al., (1992) analyzed the steady-state expression levels of phenylpropanoid and anthocyanin biosynthetic pathway genes in light stressed *Arabidopsis* seedlings. The expression of the genes (*PAL*-1, *CHS, CHI* and *DFR)* was more in light stressed seedlings than that of etiolated seedlings. Further, the expression was prominent in UV-B and blue light treated seedlings (Kubasek et al., 1992).

Parsley protoplasts and cell cultures respond differentially to UV-B radiation (flavonoid glycosides) and fungal elicitors (furanocoumarins) (Dangl et al., 1987). The expression of the phenylpropanoid pathway genes *PAL, BMT* and *XMT* involved in the synthesis of furanocoumarins are inducible by both the stress factors and flavonoid pathway genes are stimulated by UV radiation only (Dangl et al., 1987). In another study, simultaneous treatments of parsley cells with UV light and fungal elicitor resulted in quantitative changes in both responses (flavonoid and furanocoumarin synthesis). While UV radiation reduced elicitor-induced furanocoumarin synthesis, the elicitor treatment completely blocked the UV induced accumulation of flavonoids by repressing the transcription of the chalcone synthase gene (Lozoya et al., 1991).

Low temperature (15 C) has also been demonstrated to increase anthocyanin production in *Sorghum* (Shichijo et al., 1993). Accumulation of anthocyanin pigments and expression levels of phenylpropanoid and anthocyanin biosynthetic genes were monitored in maize seedlings during the short term low temperature treatment (Christie et al., 1994). These maize lines are genotypically well defined in terms of anthocyanin production. A significant increase in the transcription rates of the genes *PAL, ACL, C2, CHI, Al, A2, Bz* and *Bz2* (structural genes of the phenylpropanoid and anthocyanin biosynthetic pathways) with a corresponding increased-levels of end products, was observed in 7-day-old maize (B 37N) seedlings upon exposure to cold stress at 15 C (Christie et al., 1994). This has lead to the conclusion that the anthocyanin biosynthetic pathway genes can be considered as *Cor* (cold-regulation) genes. Plant hormones like gibberelic acid are also involved in regulation of flavonoid/anthocyanin biosynthesis (Weiss et al., 1990).

In summary, the co-ordinate regulation of phenylpropanoid and anthocyanin pathway genes in response to developmental cues and environmental signals emerges out as an excellent model system to study the underlying molecular mechanisms of these pathways.

2.4 Expression of *PAL, 4-CL* and *CHS* genes in response to **UV-light** and elicitor is regulated through specific *cis* elements

Phenylalanine ammonia lyase, 4-coumarate:CoA ligase and chalcone synthase are crucial enzymes which regulate the phenylpropanoid and anthocyanin pathways. They have been analyzed extensively for the regulatory elements that control their expression in response to various abiotic and biotic stresses in plants.

PAL is encoded by three to four genes in *Phaseolus vulgaris* (Cramer et al., 1989), parsley (Lois et al., 1989) and four to five genes in *Arabidopsis* (Ohl et al., 1990). Analysis of bean *gPAL-2* and *Arabidopsis PAL-1* promoter deletions and *in vivo* footprinting of parsley *PAL-1* promoter revealed some clues regarding the nature and organization of *cis* element network involved in the regulation of *PAL* genes (Dangl, 1991; Ohl et al., 1990; Lois et al., 1989). Differential expression, spatial and developmental regulation of these *PAL* genes were observed under light and elicitor treatments. Unidirectional and internal deletion analyses of bean *gPAL2* promoter-*GUS* fusion in transgenic tobacco revealed that the presence of *cis* element sequences

between -280 bp and -150 bp are required and sufficient for root apex, xylem and to wound specific expression. Internal deletion of 100 bp between -250 and -150 bp abolishes xylem expression. Analysis of parsley *PAL*-1 *cis* elements using *in vivo* dimethyl sulpahate (DMS) footprinting method revealed the presence of different *cis* elements involved in response to UV light and elicitor treatments (Lois et al., 1989). Several sequence segments for functional ***cis*** active elements located between -280 and -70 bp of the bean g*PAL*-2 promoter were observed based on their homology to putative *cis* elements of the parsley *PAL-]* promoter (Lois et al., 1989) and *Arabidopsis PAL-l* promoter (Ohl et al., 1990).

Analysis of the 1.3 Kb 4*CL*-1 promoter of parsley with *GUS* fusion gene construct in transgenic tobacco revealed a significant role of the 36 bp (deletion from bp -210 to -174) sequence for normal activity (Dangl et al., 1987,1991; Hauffe et al., 1991) and a 597 bp sequence for complete activity but not in response to wound or elicitor or UV-light treatment. This suggests that the light inducible expression is determined by the sequences located outside -597 region in 4*CL*-1 promoter. The high expression levels of a 597 bp promoter in conjunction with 4*CL*-1 cDNA in response to elicitor or UV-light treatment suggests the necessity of the coding region. These ***cis***-acting sequences in the coding region may function either as binding sites for transcription factors or they may function to stabilize nascent 4*CL*-1 transcripts (Dangl, 1991; Douglass et al., 1991).

Regulation of ***CHS*** expression have received a great deal of attention in characterizing the role of the regulatory elements of *CHS* genes (Dangl et al., 1989: Lamb et al., 1989; Hahlbrock and Sheel, 1989; Dixon and Harrison, 1990). Analysis of ***cis*** acting elements for the *CHS* promoter in parsley using *in vivo* foot printing method revealed four light-dependent foot-prints (Schulze-Lefert et al., 1989a; 1989b and Block et al., 1990). They are co-ordinately expressed and each of them has the ability to respond to either UV-light, blue light or mixed wavelengths (Dangl, 1991). Further, two separate light responsive units were identified (Schulze-Lefert et al., 1989a; Donald and Cashmore, 1990). In each unit, one of the two foot prints showed homology to "G-box", a sequence highly conserved in a number of light and stress related plant gene promoters (Giuliano et al., 1988; Schulze-Lefert et al., 1989b) and the other foot prints in both the units are unrelated to each other. The first foot print I in the TATA proximal light response unit (Schulze-Lefert et al., 1989b) shows strong homology with

the conserved sequence element (box I) in the phenylpropanoid genes of several plants. This unit was shown to be sufficient for directing light dependent gene expression in transient parsley protoplasts (Weisshaar et al., 1991). Three cDNA clones encoding proteins that bind specifically to the sequence of foot-print II were isolated and characterized. All these proteins contain a conserved basic and leucine zipper domain, but their participation in light mediated activation of the *CHS* gene in parsley is not clear.

Cis-controlling elements *of Antirrhinum CHS* gene were confirmed using UV-inducible expression of a chimeric *CHS* promoter (Lipphardt et al., 1988). The TATA proximal sequence -197 to -39, independent of its orientation, is demonstrated to be sufficient to confer UV induction, the sequence between -357 and -197 enhances the UV responsive expression and a strong enhancer is located at -661 to -564.

Cis acting elements were determined in *Petunia* using ***CHS*** promoter-*CA T* construct on a -220 to +1 light responsive fragment (Meer et al., 1990). *Cis*-elements regulating cell-type specificity of *CHS* expression was also observed in *Petunia. A* dominant negative *cis*-element involved in directing flower specific expression of *CHS-A* gene was observed and the 5' TACPyAT 3' sequence located within the -142 to +81 (Meer et al., 1990). The involvement of "TACCAT" *cis*-element regulating flower specific expression of CHS in *Antirrhinum* was also identified (Sommer et al., 1988).

Another important feature of phenylpropanoid and anthocyanin biosynthetic pathway genes is that they exhibit a strong sequence homology in their promoter regions. These regions were referred to as "box I" and "box II". The conserved sequences of box I and box II are T(A)CTCACCTACC(A)C and CCAACA(T)AAC(C)TCC respectively (Stuitje and Mol, 1991). This could explain their co-ordinate expression in specific cell types in response to external stimuli or in developmental regulation.

2.5 **Photoreceptor-mediated** anthocyanin biosynthesis in plants

One of the most extensively studied regulatory effects of light on specific metabolic pathways was the induction of anthocyanin biosynthesis (Hahlbrock and Grisebach, 1979). Three types of photoreceptors mediate the biosynthesis of anthocyanins in plants. These are phytochrome, blue/UV-A photoreceptor (cryptochrome), or specific UV-B photoreceptors (not characterized yet) through which

plants perceive light signals of specific wavebands (Mohr and Schafer, 1983; reviewed by Mohr, 1986). Often, these photoreceptors exert cumulative effect on the induction of anthocyanin pigments. For example, all three photoreceptors were able to mediate the induction of anthocyanin pigments in cultured parsley cells (Bruns et al., 1986; Ohl et al., 1989). In *Arabidopsis*, the transient accumulation of anthocyanins was induced independently by three photoreceptors viz., phytochrome, blue/UV-A photoreceptor and UV-B photoreceptor (Kubasek et al., 1992).

The exclusive role of UV-B light in the induction of anthocyanins has been observed in cell suspension cultures and seedlings of parsley as well as in seedlings of *Sorghum*. In these plants, anthocyanin production was induced by UV-B light alone whereas other photoreceptors, phytochrome and cryptochrome, could modulate the photoresponse triggered by UV-B light (Wellmann, 1974; Yatsuhashi et al., 1982). Both phytochrome and blue/UV-A photoreceptor were involved in stimulation of anthocyanins in turnip seedlings whereas only phytochrome was shown to be responsible for the production of anthocyanins in red cabbage seedlings (Siegelman and Hendricks, 1957).

In *Triticum aestivum* coleoptiles, only UV-B radiation is effective alone but the magnitude of its response (anthocyanin formation) can be modulated with red and far-red light reversible mechanism (Mohr and Drumm-Herrel, 1983). Takeda and Abe (1992) observed that a specific UV-B photoreceptor was involved in the induction of anthocyanins using carrot cell suspension culture system in 2,4-dichloro-phenoxyacetic acid (2,4-D) depleted culture medium while phytochrome modulates UV-B response. Phytochrome controls the *de novo* synthesis of phenylalanine ammonia lyase activity in cell suspension cultures of parsley after pre-irradiation with UV-light (Wellmann and Schopfer, 1975).

Photoregulation of ***CHS*** mRNA accumulation was analyzed in parsley and mustard plants. In both species *CHS* mRNA accumulation in young etiolated seedlings was primarily under phytochrome control while UV-B photoreceptor regulates the *CHS* mRNA levels in leaves of adult reetiolated plants. Thus both photoreceptors were involved in the regulation of flavonoid biosynthesis in these plants at different developmental stages (Frohnmeyer et al., 1992).

2.6 Genetic and molecular basis of phenylpropanoid and anthocyanin pathways

2.6.1 Phenylpropanoid Pathway

The genetic basis of phenylpropanoid pathway is not well understood. However, the enzymology and molecular biology of one of its constituent genes, the *PAL,* encoding phenylalanine ammonia lyase has been rigorously studied. The *PAL* is the key gene of the pathway and its activity regulates the entry of L-Phe pools into the secondary metabolic pathways. The 4-CL activity determines the removal of CoA esters into end products of specific metabolic branches. The genes encoding PAL and 4-CL have been cloned and analyzed from various plant species.

Phenylalanine ammonia lyase (PAL)

In plants, multigene families are known to encode PAL: 3 to 4 genes in *Phaseolus vulgaris* (Cramer et al., 1989), *Oryza saliva* (Minami et al., 1989) and 4-5 genes in *Petroselinum crispum* (Lois et al., 1989), and *Arabiodopsis thaliana* (Ohl et al., 1990). The principal regulatory mechanism of *PAL* gene expression is the *de nova* mRNA synthesis from various *PAL* genes which are differentially expressed in different target tissues. Further, the coding regions of various *PAL* genes in bean share extensive homology, however, the structurally diverse *PAL* promoters differ from one another (Bevan et al., 1989; Liang et al., 1989a, 1989b).

Cinnamate 4-hydroxylase (C4H)

This is a cytochrome P-450 dependent microsomal mixed-function oxygenase which uses NADPH as a cofactor and catalyzes the formation of coumaric acid from cinnamic acid. Cloning of *C4H* genes has not been reported so far.

Coumarate:Co A ligase (4CL)

Two highly homologous genes (extending over promoters as well as coding regions) encoding 4CL in parsley and potato were isolated. Sequence conservation between parsley and potato *ACL* genes (200 bp) proximal to "TATA" region was reported (Douglas et al., 1987; Dangl, 1991). Several constitutive sites of protein-DNA interaction between -195 bp and -78 bp were found in the promoter region in parsley (Lois et al., 1989) and in potato (Becker-Andre et al., 1991) suggesting the regulation of *ACL* expression through trans-elements.

2.6.2 Anthocyanin pathway

Although, the genetic regulation of anthocyanin pathway has been extensively studied in maize, *Petunia* and *Antirrhinum,* the maize system is described here in some detail because of its close evolutionary relationship with rice. An excellent and extensive review on anthocyanin-gene pigment system in maize has been provided by Coe et al., (1988). There are three classes of genes responsible for the synthesis, modification and regulation of anthocyanin pigments in maize: Class I: structural genes which encode enzymes catalyzing various enzymatic conversions in the pathway, namely *C2* (colored-2), *A* (anthocyanin-1), *Al* (anthocyanin-2), *Bz* (bronze-1) and *Bz2* (bronze-2). Class II: These are enhancers and modifiers of pigment like *In* (intensifier) and *Pr* (purple-red) which control the quantity and quality of anthocyanin pigments respectively. The presence of the dominant *Pr* determines the synthesis of cyanidin based anthocyanins while the intensifier *(in)* allele in homozygous condition increases the formation of anthocyanin pigments in the aleurone tissue of maize (Coe, 1957; Reddy and Peterson, 1978). Class III include the regulatory genes like Cl (colored-1), *Pl* (purple plant), *R* (red) and *B* (booster) which regulate the expression of the structural genes and *Vp* (viviparous-1) regulates the expression of *C1* regulatory gene (McCarthy et al., 1989). Most of these genes were isolated using transposon tagging strategy and are listed in Table 1 along with the encoded enzymes and protein factors. The four regulatory loci C1, *Pl*, *R* and *B* were subjected to a rigorous investigation **in** maize. They co-ordinately regulate the expression of the structural genes of the anthocyanin biosynthetic pathway in a spatial and temporal fashion. C1, which is required for the production of anthocyanins in aleurone and embryo (Coe, 1985), was cloned (Paz-Ares et al., 1986, 1987; Cone et al., 1986) and functionally characterized (Paz-Ares et al., 1990). *Pl* regulates the anthocyanin production in most of the plant body (Gerats et al 1984; Cone and Burr, 1989) although strong light can induce pigmentation in recessive *pl* mutant plants (Coe, et al., 1988). The regulatory loci Cl and *Pl* that encode protein factors containing two major domains; a basic amino terminus (amino acids 1-20) and an acidic carboxy terminus (amino acids 230-273). The basic domain shows a 40% homology to c-*myb* related protooncogene products from human and animals which interacts with DNA (Katzen et al., 1985; Majello et al., 1986) whereas the acidic domain shows similar functions as transcriptional activator (Goff et al., 1991). The regulatory loci *(Cl* and *Pl)* regulate the expression of C2, *A*

Table 1 Molecular analysis of anthocyanin pathway genes and gene products in maize (reviewed by Coe et al., 1988)

Gene	Gene product	Reference
Structural		
C2 (colored-2)	CHS (chalcone synthase)	Wienand et al.,1986
Zm CHI	CHI (chalcone isomerase)	Grotewald and Peterson, 1994
A (anthocyanin-1)	DFR (dihydroflavonol reductase)	O'Reilly et al., 1985; Reddy et al., 1987
A2 (anthocyanin-2)	ANS (anthocyanidin synthase)	Mennssen et al., 1990
Bz (bronze-1)	FGT (flavonol glycosyl transferase)	Fedoroff et al., 1984
Bz2 (bronze-2)	GST (glutathione s-transferase)	Theres et al., 1987; Marrs et al., 1995.
Modifier / Enhancer		
Pr (purple)	F3H (flavonoid-3-hydroxylase)	Forkmann et al., 1980
In (intensifier)	not known	Coe et al., 1957; Reddy and Peterson, 1978
Regulatory		
Cl (colored-1)	encodes *c-myb* protooncogene family protein, (transcriptional factor)	Paz-Ares et al., 1986,1987, 1990; Cone et al., 1986
Pl (purple plant)	encodes *myb* protooncogene family protein (transcriptional factor)	Coe, 1985; Cone and Burr, 1989
R (red)	encode *myc* protooncogene family protein	Dellaporta et al., 1988
B (booster)	-do-	Chandler et al., 1989
Vp 1 (viviparous-1)	regulates the expression of Cl and *R*	McCarty et al., 1989

and *Bz* structural genes. A different allele of the *C* locus, namely C1-/ has been isolated and characterized from maize which is devoid of acidic domain compared to the wild type protein (Cone et al., 1986; Paz-Ares et al., 1987; Cone and Burr, 1989). Other alleles of the *C* locus like *C-S*, *c-p* and *c-n* were also characterized molecularly (Scheffler et al., 1994). Using maize C1 cDNA as the probe, C1 related cDNAs were isolated from kernel and leaf specific cDN A libraries of *Hordeum vulgare* (Marocco et al., 1989) and from the floral library of *Antirrihnum majus* (Jackson et al, 1991). Also, seven *myh* related cDNAs from floral derived cDNA library from *Petunia* were isolated and characterized (Meer, 1991).

Other regulatory loci, *R* and *B* super family in maize exhibit extensive allelic diversity with respect to the tissue specificity and developmental timing of anthocyanin production. The *R* locus regulates the expression of C2, *A* and *Bz* structural genes (Dellaporta et al., 1988) and thereby synthesis and distribution of anthocyanin pigments in certain tissues of plant and seed while the *B* locus controls expression in plant tissues independent of the *R* locus (Styles et al., 1973; Chandler et al., 1989; Gerats et al., 1984). They encode basic helix-loop-helix transcription activators related to L-*myc* gene products (Cansonni et al., 1992). These regulatory loci (C1, *R* in kernel and *Pl*, *B* in leaf tissue) regulate the expression of atleast three structural genes such as C2, *A1* and *Bz* in the anthocyanin pathway (Cone et al., 1986; Paz-Ares et al., 1987; Ludwig et al., 1989; Dellaporta et al., 1988; Cone and Burr, 1989; Chandler et al., 1989; Wienand et al., 1990).

In summary, the available information suggests that there is a highly structured hierarchy of genes for regulation of anthocyanin pathway in maize and most likely other plants as well. The hierachy consists of structural genes at the bottom and regulatory genes and also the pleotropic genes like *Vp* at the top. It appears that anthocyanin pathway is not only regulated by the regulatory genes dedicated to the pathway but also the other genes belonging to signal transduction pathways. Superimposed on this is the interaction between the regulatory circuits that govern the pathway and environmental and developmental cues. Thus this pathway is amenable for exploitation in both fundamental and applied research. All these aspects are to be studied in details in rice as much as it is an important food crop for the most of the humanity.

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals

Flavonoid pigment markers were purchased from Carl Roth GMBH, Germany. Anthocyanin markers were a gift from Prof. Dr. G. Forkmann, MPI, Koln, Germany. The following chemicals were obtained from Sigma company, USA: Agar, Agarose, BSA, Calf-thymus DNA, Cellulose-avicell TLC plates, EDTA, EGTA, Ficoll, Glucose, Glycerol, IPTG, LiCl, Maltose, 2-mercaptoethanol, MOPS, MS ready mix, NaCl, Oligo-(dT)-cellulose, Peptone, Potassium acetate, PVP, Phenylalanine, SDS, Sodium acetate, Tris, Triton X-100, Vannilin, Yeast extract, X-gal. The following salts, acids and solvents were obtained from local companies: Acetic acid, n-Amyl alcohol, n-Butanol, Chloroform, Ethanol, HC1, Isopropanol, Magnesium, Methanol, Sodium borohydride, Sodium hypochlorite, Sodium tetra borate, Tris-buffer saturated phenol and Zink. X-ray films and intensifying screens were purchased from Kodack, USA. Restriction enzymes, Klenow enzyme, T4 DNA ligase, CIAP, Exonuclease III, S1-nuclease, 1 Kb ladder, λ DNA (digested with EcoRI and Hind III), Lysozyme and RNase were purchased from New England Biolabs, England, Boehringer Mannehim, Germany and Sigma, USA. Random primer labelling kit, λ-ZAP cDNA synthesis kit, ***in*** *vitro* packaging kit and *E.coli* strains (XL1 cells, XL1-Blue MRF' cells, SOLR cells) were purchased from Stratagene. Ampicillin, Tetracycline, Kanamycin were purchased from sigma, USA. Hybond N membrane and α-dCTP^{32} were purchased from Amresham UK. Sequencing kit was purchased from Perkin Elmer, USA.

3.1.2 Rice genetic stocks

The identity and source of rice lines were described in Table 2. Most of the lines are from DRR and a few are from TNAU. All japonica lines are from the Hokkaido University, Japan (gift from Prof. T. Kinoshita). The Japonica lines are of defined genotypes with clear phenotypic descriptions of anthocyanin accumulation in various plant parts.

Table 2 Source of rice lines

Line	Subspecies	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
Blackputtu	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
R27 (P)	indica	DRR, Hyderabad, India
N22W	indica	DRR, Hyderabad, India
N22B	indica	DRR, Hyderabad, India
Hamsa	indica	DRR, Hyderabad, India

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

3.2 Methods

3.2.1 Screening of rice germplasm for anthocyanin pigmentation phenotype

The rice lines used in this study (Table 2) include land races and cultivars of indica and a few genetically defined japonica types. They were selfed for at least three generations and were made true breeding lines. The indica lines were selected from a rice germplasm collection at the Directorate of Rice Research, Hyderabad and Tamilanadu Agricultural University, Coimbatore, India after an extensive visual screening for pigment phenotypes of various plant parts in the monsoon crop season (July-November) of 1990.

The experimental plants were grown either in the field or in the net-house in pots. The plants experienced uniform environmental conditions during scoring for anthocyanin pigmentation phenotypes as described below. At Hyderabad, India (longitude 78°4' E; latitude $17^0$3' N; altitude 600 meter above mean sea level) the plants were experienced to an average day/night temperature of 30.1/20.7 C and RH ranging between 76 and 60% respectively. At midday, sunlight intensity was about 2800 μmole m" s . All the experimental plants were grown in a 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 plant: 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 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 before further analysis. The visual pigment phenotypes of various plant parts were photographed using a Wild Photomakroskop M400 without any filters .

3.2.2 Crosses and embryo rescue

The F1 hybrid plants were obtained from reciprocal crosses of three different rice lines, Purpleputtu (Class I), N 22W (Class II) and N 22B (Class IV). Emasculation (5-6 PM) and pollination (9-11 AM) were done using the standard bagging method. The pollinated panicles were harvested 15 days after pollination (DAP) and the Fl seeds were embryo-rescued by culturing on 1/2 MS medium. The cultured embryos

were incubated in darkness at 25 ± 1^0C until germination and subsequently transferred to light. The young seedlings at the three leaf stage were transferred to 1/2 MS liquid solution (Murashige and Skoog, 1962) and were grown under lab conditions. After seven days, the seedlings were transplanted into soil along with the parents. The F1 plant phenotype was scored for purple pigmentation in various tissues/organs.

3.2.3 Biochemical analysis

3.2.3.1 Characterization of anthocyanidin pigments

The plant organs/tissues namely leaf blade, leaf sheath, ligule, auricle, collar, node, internode, stigma, apiculus, husk and pericarp were frozen in liquid N_2 and stored at -20 C for further analysis. The stored or freshly harvested tissues were extracted with (25 mg/mL) acidified methanol (1% v/v) for 24 h at 4 C in dark. To one mL of the extract, 0.75 mL of water and 2 mL of chloroform were added which resulted in 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 hydrolyzed with 2N HC1 for 40 min in a boiling water bath. The hydrolyzates, after washing with ethyl acetate, were extracted into iso-amyl alcohol and evaporated to dryness. The final residue was redissolved in methanol-HCl and separated by Thin Layer Chromatography (TLC) on cellulose plates using four 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) 4. BAW (n-butanol:acetic acid:water, 4:1:5, v/v/v) After drying, the chromatogram was scanned on a densitometre. The separated compounds were identified on the basis of RF values, fluorescence under visible and UV light with or without ammonia and the characteristic absorption maxima (Harborne, 1965, 1967, 1988; Reddy, 1974). Anthocyanidin pigments from pericarp tissue of Purpleputtu were purified by preparative TLC with AHW as the developing solvent 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.

3.2.3.2 Characterization of proanthocyanidin and other flavonoid pigments

Mature dehulled seeds and other rice tissues were extracted with 10% aqueous methanol (50 mg/mL) for 24 h at room temperature (RT) with occasional shaking. To

one 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 the following standard analytical tests.

a). Methanol-HCl test: To 0.1 mL of methanolic plant extract, 2N HC1 (final concentration) was added and boiled for 10 min. Presence of leucoanthocyanidins and proanthocyanidins are revealed by this test as they produce red color in this reaction (Harborne, 1967).

b). Butanol-HCl test: Assay mixtures contain 0.1 mL of aqueous or acidified methanolic extracts and 1 mL of butanol-HCl reagent (95:5, v/v). After thorough mixing, they were incubated for 1 hour at boiling temperature in unstoppered tubes and cooled. The red color of the residue was a positive indication for the presence of proanthocyanidis. This residue was re-dissolved in methanolic-HCl and tested for its absorbance maxima using a Shimadzu UV-160A Spectrophotometer (Bate-Smith, 1975; Waterson and Butler, 1983).

c). Vanillin test: Catechin and proanthocyanidins were also detected by either spraying the developed chromatograms or treating the crude methanolic extracts in a test tube with a mixture of 5% (w/v) vanillin in ethanol and concentrated HC1 (4:1, v/v). Both compounds produce a red color (Price et al., 1978).

d). Sodium borohydrate/HCl test: This reagent is specific for the detection of flavanones (Eigen et al., 1957). Preparation of $NaBH_4$ reagent: 0.1 g of $NaBH_4$ was dissolved in a minimum distilled water and adjusting the volume to 10 mL with isopropanol. Only freshly prepared solution was used either as a spraying reagent on developed chromatograms (which were then exposed to concentrated hydrochloric acid vapors for 30 seconds) or used as a test tube reaction with acidic or aqueous methanolic tissue extracts. Flavanones were revealed as purple to magenta spots.

e). **Zinc/HCl test (Pew's test):** Zinc powder and a drop of 5N HC1 were added to the crude plant extract or used as a spraying reagent on developed chromatograms. Only dihydroflavonols react to give a deep purple red or cherry red colors (Pew, 1948).

f). Magnesium/HCl test (Shinoda's test): This test was applied in the same way as Zinc/HCl, but magnesium wire was used instead of Zink powder. The development of a deep-red or magenta color was an indication for the presence of flavonols (Harborne, 1965).

3.2.3.3 Screening for UV-B induction of anthocyanin pigments in rice

Rice cultivars of both indica and japonica types were grown in nursery beds under a continuous irrigation for 20 days during kharif (July-Nov.) season in 1992 at Hyderabad in black loam soil. Twenty day-old seedlings were transferred from the seed-bed to field and grown till maturity. The plants experienced uniform environmental conditions as described above. Both seedlings (4-day-old etiolated seedlings after exposing to sunlight) and field grown plants (40-day-old) were examined visually for the presence of anthocyanin pigmentation and were classified into three groups. The first group was acyanic with nine cultivars, the second group was moderately cyanic with four cultivars and the third group was cyanic with three cultivars. The cultivars belonging to cyanic group possessed abundant amounts of anthocyanin in the shoots, but not in the root. Out of the three cyanic cultivars, `Purpleputtu' was chosen for further experimental studies, and an acyanic cultivar Blackputtu' was used for comparison. Both cultivars obtained in homozygous state in 1990 were further selfed for six generations at Hyderabad. Mature dry seeds of the above mentioned cultivars were harvested and used for further experiments.

3.2.3.4 Culture conditions for screening

The seeds were sterilized in Na-hypochlorite 5% (v/v) for 5 min, then extensively washed and soaked for overnight in sterile distilled water. The seeds were sown on 25 mL of solid 0.4% (w/v) agar medium in 4" diameter petridishes and were kept in black cardboard boxes at 28±1 C in complete darkness. Etiolated seedlings at indicated time intervals were exposed to light of various wavebands for a short period and were returned to darkness. Unless otherwise mentioned, all experiments were carried out with 4-day-old etiolated seedlings exposed to 30 min of sunlight (SL).

3.2.3.5 Light sources

Seedlings were exposed to mid-day (12-3 PM) sun light (2800-3300 umol m" sec) or colored light by opening the boxes in SL. The different colored lights were obtained by filtering the SL through a red light filter (λmax 650 nm, 1600-1900 μmol m^{-2} sec^{-1}), far-red light cut-off filter (λmax 750 nm, 1300-1500 umol m^{-2} sec^{-1}) and a

blue light filter (λmax 457±40 nm, 650-750 umol m sec) using filters obtained from Carolina Biological Supply Co., Burlington, NC, USA. The SL free of UV-B (>320 nm, Klien, 1979) was obtained by filtering SL through a 4 mm thick window glass plate (2200-2600 umol m^{-2} sec^{-1}). The seedlings were exposed to various colored lights in a black plastic box covered with the respective light filter. The temperature of the box was maintained at 28±1 C by circulating water around the box. The seedlings were also exposed to brief red and far-red light pulses in a darkroom using the artificial light sources (Manga and Sharma, 1988). Red light (λmax 650+40 nm, 20 umol m~ sec") was obtained after filtering the output of two white fluorescent tube-lights through two layers of red Plexiglas sheet. Far-red light (λmax >750 nm, 16 umol m^{2} sec^{1}) was obtained using a 150 Watt projector lamp whose output was filtered through a far-red filter (Manga and Sharma, 1988).

3.2.3.6 Quantitative estimation of anthocyanins and other UV-B absorbing compounds

A pair of seedlings of uniform height were harvested and anthocyanins were extracted in 2 mL of acidified (1% w/v HC1) methanol for 24 h at 4 C with occasional shaking. Anthocyanins were quantitatively estimated by measuring A535 (Harborne, 1967). For qualitative analysis, aglycones were obtained by hydrolyzing one mL of the above extract with an equal volume of 2 N HC1 at 100 C for 40 min. The hydrolyzate was extracted with isoamyl-alcohol, evaporated to dryness and the residue was dissolved in 30 uL of acidified methanol. Equal volumes of aglycone extracts (5 uL) were spotted on to a cellulose TLC plates and chromatogram was run using different solvent systems as described above. UV-B absorbing compounds were extracted by shaking a pair of seedlings in 2 mL of 80% (v/v) methanol at 4 C in darkness. The UV-B absorbing compounds was quantitatively determined by measuring absorbance at 300 nm (Dangl et al., 1987).

3.2.3.7 Phenylalanine ammonia lyase enzyme activity

The PAL extraction and activity were essentially similar to the one described by Goud et al., (1992). Five shoots of uniform height were homogenized at 4 C using a

pre-cooled mortar and pestle, with 200 mg of sea sand and 150 mg of polyvinylpolypyrrolidone (PVP) in 3 mL of 0.1 M borate buffer (pH 8.8) containing 50 mM 2-mercaptoethanol. The homogenate was centrifuged at 18,200 g for 30 min at 4 C and the supernatant was applied to a Sephadex G-25 column (2 cm x 10 cm) equilibrated with 0.1 M borate buffer (pH 8.8). The fractions constituting void volume were pooled together and were used for assay. The PAL assay was performed at 25 C in an assay mixture consisting of 1 mL of enzyme extract and 0.5 mL of 50 mM L-phenylalanine (Saunders and McClure, 1975). The PAL activity was assayed by monitoring the increase in the absorbance at 290 nm against the control without phenylalanine over a period of 4 h at 1 h intervals. The rate of appearance of trans-cinnamic acid was taken as a measure of enzyme activity using an increase of 0.01 A at 290 nm equal to 3.09 nanomoles of trans-cinnamic acid formed (Saunders and McClure, 1975). The PAL activity is expressed in pkat (picomol trans-cinnamic acid formed per second) per shoot.

3.2.3.8 Statistical analysis

All data points in figures and in relevant tables represent the mean of values obtained from atleast three independent replicates and standard errors calculated thereof.

3.2.4 Molecular biological methods

3.2.4.1 Extraction of genomic DNA

Genomic DNA was isolated from leaf tissue as described by Dellaporta et al., (1983). Freshly harvested one gram leaf tissue was quick frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. The fine powder was thoroughly extracted with 15 mL of extraction buffer (100 mM Tris pH 8, 5 mM EDTA pH 8, 500 mM NaCl, 10 mM 2-mercaptoethanol) containing 1 mL of 1% SDS and incubated at 65 C for 10 min. To this, 5 mL of potassium acetate (5 M) was added, mixed well, incubated on ice for 20 min and centrifuged at 10000 rpm at 4 C for 20 min. The supernatant was passed through a miracloth filter into a clean tube containing 10 mL of isopropanol, mixed well, incubated at -20 C for 30 min and centrifuged at 10,000 rpm at 4 C for 15 min. The pellet was recovered and dissolved in 1 mL of TE. The insoluble debris were removed from this solution by centrifugation for 10 min,

precipitated with 1/10 volume of 3M sodium acetate and 2.5 volumes of ice-cold absolute ethanol, washed in 80% ethanol, dried and dissolved in appropriate volume of TE. The concentration of DNA was tested spectrophotometrically and quality was checked through gel analysis.

3.2.4.2 Southern analysis

Genomic DNA (6 µg) was digested with 12 units of various restriction enzymes at 37 C (or suggested temperatures according to recommendation of manufacturers). The digested DNA was electrophoresed (25 V/overnight) through a 0.7% agarose gel in a running buffer of 1 X TAE (40 mM Tris-acetate, 50 mM EDTA). The DNA was depurinated in 0.25 N HC1 for 10 min, denatured in a solution containing 1.5 M NaCl and 0.5 N NaOH for 30 min, neutralized in 1.5 M NaCl, 0.5 M Tris pH 8, for 30 min, and rinsed in 20 X SSC (1 X SSC = 0.15 M NaCl, 15 mM sodium citrate) for 30 min, and transferred to Hybond N^+ (Southern, 1975). The blots were baked for 2 h at 80°C in an vacuum oven or UV cross linked at 1200 Joules for 2 min. Prehybridization and hybridization were performed (6 X SSC, 10 X Denhardt's, 0.5% SDS, and 100 µg/mL sheared denatured calf thymus DNA) for 3 h and overnight respectively at 60 C. Prior to autoradiography, the blots were washed twice in 2 X SSC, 0.5% SDS for 30 min each and once in 0.2 X SSC, 0.1% SDS for 30 min. The filters were exposed to X-ray film (Kodak, USA) at -70 C with intensifying screens.

3.2.4.3 Extraction of total cellular RNA

Total cellular RNA was isolated from various tissues of rice plant according to the method described by Weisshaar et al., (1991). The harvested tissues were quick frozen in liquid nitrogen and stored at -70 C. Five grams of freshly harvested tissues or frozen tissues were ground in liquid nitrogen to a fine powder, transferred to tubes with extraction buffer (150 mM Tris pH 9, 100 mM NaCl, 20 mM EGTA pH 8, 1% SDS), containing 100 uL of 2-mercaptoethanol and 5 mL of buffer saturated phenol pH 8. The extracts were incubated for 10 min at RT and centrifuged at 3000 rpm at RT for 15 min. The supernatant was extracted atleast three times with phenol-chloroform. Finally the aqueous upper phase was precipitated with 1/10 volume of 3 M sodium

acetate and 2.5 volumes of ice-cold ethanol, kept at -70 C for 30 min and centrifuged at 10000 rpm at 4 C for 15 min. The resultant pellet was dissolved in 5 mL of DEPC-treated water. The total RNA was precipitated with an equal volume of ice-cold 5 M LiCl, incubated on ice-cold water for over night and centrifuged at 4500 rpm at 4 C for 15 min. The pellet was redissolved in one mL of water and precipitated with alcohol. The resultant pellet was washed in 70% ethanol and redissolved in 200 µL of water. The quality of total RNA was checked through 1.2% denatured agarose formaldehyde gel and was estimated spectrophotometrically.

3.2.4.4 Isolation of poly A^+ RNA

Isolation of poly A RNA was performed through Oligo-(dT)-cellulose affinity chromatography (Weisshaar et al., 1991). Oligo (dT)-cellulose(0.5 g) was washed in 40 mL of 0.1 M NaOH and 5 mM EDTA and repeatedly washed in the binding buffer without SDS (0.5 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA pH 8) until the pH reached to 7.5. Total RNA (500 µg) was incubated with Oligo-(dT)-cellulose with 20 mL of binding buffer with 0.5% SDS, washed three times with binding buffer, resuspended in 2 mL of fresh binding buffer and poured onto the column. After washing the column with 25 mL of binding buffer, the poly A RNA was eluted with the buffer (10 mM Tris pH 7.5, 1 mM EDTA pH 8, 0.5% SDS). The fractions were precipitated with alcohol, washed in 70 % ethanol, dried and dissolved in an appropriate volume of water.

3.2.4.5 Northern analysis

Equal concentrations of poly A RNA were separated through 1.2% agarose formaldehyde gel (each 100 mL gel consisting of 1.2 g of agarose, 10 mL of 10 X MOPS (200 mM MOPS, 100 mM sodium acetate, 10 mM EDTA, pH 7), 73 mL of water, boiled and added 17 mL of 37% formaldehyde), transferred to Hybond N^+ solid support, UV-crosslinked and/or baked. Prehybridization and hybridization were performed at 60 C for 3 h and overnight respectively in 1 M NaCl, 10% Denhardt's solution, 2% SDS and 100 µg/mL sheared denatured calf thymus DNA. The

membranes were washed three times (10 min each) at 60 C in 2 X SSC, 0.5% SDS. The filters were exposed using an X-ray film with intensifying screens at -70 C.

3.2.4.6 Random-primer labeling

The cDNA fragments were used as templates for preparation of probes by random primer labeling reaction (Stratagene). 24 uL of template DNA (40 ng) with 10 μL of primers were denatured by boiling for 5 min and snap cooled. To this, 10 uL of dNTPs for α-dCTP^{32} buffer, 50 μCi α-dCTP^{32} (3000 Ci/mM) and 5 units of Klenow were added and incubated at 37 C for 30 min. Sephadex G-25 spin column was run to remove unincorporated a-dCTP . The resultant probe was calculated for specific activity as follows; cpm/μg= cpm X 2.5 X total volume of the probe X 1000/40; where "2.5" is a Czrenkoff factor. The probe was denatured by boiling for 5 min, snap cooled and added to the hybridization solution.

3.2.4.7 cDNA library construction, screening and isolation of cDNA clones

The four-day-old etiolated Purplcputtu rice seedlings were exposed to four tubelights (UV-B containing white light source from SON-T Agro 400 W, Philips) for 30 min and returned to darkness. Shoots were harvested at 0, 4, 6, 8, 12, 16, 20 and 24 hours time intervals, frozen in liquid nitrogen and pooled. Total RNA and poly A RN A (two cycles of Oligo-(dT)-cellulose affinity chromatography) were prepared from these pooled tissue as described above. The poly A RNA was used to direct double stranded cDNA synthesis using λ-ZAP, XL 1-Blue MRF' synthesis kit according to the suppliers' recommendations (Stratagene). The first-strand synthesis was initiated with 70 units of Moloney murine leukemia virus-reverse transcriptase (MMLV-RT), 10 raM dATP, dGTP, dTTP and 5 mM 5-methyl dCTP methyl nucleotide mixture at 37^0C. The second-strand synthesis was performed at 16 C with 3 units of RNase H, 100 units of Pfu DNA polymerase I and 10 mM dATP, dGTP, dTTP and 26 mM dCTP nucleotide mixture. The first and second-strand syntheses were monitored with a-dATP^{32} (800 Ci/mmol). The double-stranded cDNA was filled-in with four dNTPs (2.5 mM each) and 5 units of Pfu DNA polymerase I. After being heat killed, the

cDNA was phenolyzed, alcohol precipitated, EcoRI adapters were ligated and then treated with kinase and Xho 1 restriction enzyme. The size fractionated cDNA through sephacryl S-500 containing >1 Kb cDNA fragments was ligated into λ-ZAP vector (digested with EcoR I-Xho I and treated with CIAP). *In vitro* package of *X* vector was performed with gigapack II packaging extract (Stratagene).

The library was titered on XL 1-Blue MRF' (E.coli) cells on agar plates with top agarose containing IPTG and X-gal. This primary library was amplified on XL1-Blue MRF' cells without IPTG and X-gal. The 1 X 10^6 pfu. of the amplified library (50000 pfu/petri plate) were used for screening. Filter-lifts were performed with Hybond N nylon membranes (denatured, neutralized, rinsed (in 20 X SSC) for 2 min each wash and UV crosslinked) and hybridized with cDNA fragments of the *A* (1.3 Kbp), the *A2* (1.4 Kbp) and the *C* (2.1 Kbp) from maize as the hybridization probes. Prehybridization and hybridization were performed as described for Southern analysis. After four rounds of probing, the purified plaques were separated. *In vivo* excision of pBluescript $SK^{(-)}$ phagemid along with the cloned cDNA fragment from λ-ZAP vector was performed with the help of ExAssist helper phage and SOLR *(E.coli)* strain from the plaques grown on XL 1-Blue MRF' cells (Stratagene).

3.2.4.8 Restriction fragment analysis of cDNA clones

Restriction maps of the isolated cDNA clones and their identities were confirmed by restriction analysis using suitable restriction endonucleases and plasmid Southern analysis using the corresponding cDNA fragments from maize as the hybridization probes.

3.2.4.9 Exonuclease Ill-derived set of sequential deletion clones

The nested Exo-III deletion mutants were developed for complete sequence of the nucleotide strands according to the method described by Henikoff (1987). The rice *DFR* and *ANS* clones in pBluescript $SK^{(-)}$ were digested with two restriction enzymes that digest only once within the polylinker site at one end. The restriction enzyme that cleaves at the site closer to the insert leaves a 5' protruding end while the second enzyme that cuts farther from the insert leaves a 4-base 3' protrusion. Exo III digestion proceeds uniformly from the accessible 3' hydroxyl end and adjacent to the insert since

Exo III does not attack a 4-base 3' protrusion. Kpn I (produces a 4-base 3' protrusion) and Xho I (leaves a 5'protruding end) were used for the *DFR* clone while Apa I and Xho I were used for the *ANS* clone to generate 3' Exo-III deletion clones which were served as templates for universal primer sequencing reactions. After digestion with two restriction enzymes separately, the linearized plasmid (5 μg) was alcohol precipitated and dissolved in 60 uL of Exo-III buffer (66 mM Tris-HCl pH 8 and 0.66 mM MgCl2).

The plasmid DNA was digested with 500 units of Exo-III at 37 C and 5 to 8 aliquots (2.5 μL) were removed for each 30 second interval into a tube containing 7.5 uL of S1 mix (172 uL of water, 27 uL of 1 OX S1 buffer containing 1.1 mL of 3 M KoAc (pH 4.6), 5 mL of 5 M NaCl, 5 mL glycerol, 30 mg $ZnSO_4$ and 60 units of S1 nuclease. The S1 nuclease reaction (to remove undigested single stranded DNA) was performed by incubating the reaction tubes at RT for 30 min. The reaction was stopped by addition of S1 stop buffer (0.3 M Tris base and 50 uM EDTA pH 8) and heat killed at 65 C for 10 min. At this step, 2 uL aliquots were removed and analyzed on agarose gel electrophoresis to test the extent of Exo-III digestion (Figure 14A). These deletion clones were blunt ended with 1 uL Klenow mix (3 uL of 0.1 M Tris-HCl pH 8, 6 uL of 1 M MgCl2, 20 μL water and 3 units of Klenow) and 1 uL of dNTPs (0.125 mM in each of the four dNTPs) at 37 C for five min. The resultant clone was ligated with 40 uL T4 DNA ligase mix (0.8 mL water, 0.2 mL 5 X ligase buffer containing 2.5 mL of 1M Tris-HCl pH 7.6, 0.25 mL of 1 M MgCl2, 0.5 mL of 0.1 M DTT, 0.5 mL of 0.1 M ATP, 2.5 gm PEG 6000 and water to 10 mL) and 5 units of T4 DNA ligase at RT for 30 min and transformed into XL1 cells. The right sized deletion clones were further tested by restriction analysis.

3.2.4.10 Isolation and purification of plasmid DNA for sequencing analysis

The Qiagen (Germany) plasmid purification kit was used for the purification of plasmid DNA. The overnight bacterial culture (3 mL, $1X10^{o}$ cells/mL, A600 = 1.5 to 3) grown in LB medium with an appropriate antibiotic was pelleted at 15000 rpm at 4 C for 5 min. The pellet was resuspended completely without cell clumps in 0.3 mL P1 buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0 and 100 μg/mL RNase). The bacterial cell suspension was alkali lyzed with 0.3 mL P2 buffer (200 mM NaOH and

1 % SDS) at RT for 5 min. The lyzate was neutralized and enhanced the precipitation with 0.3 mL P3 buffer (3M KoAc pH 5.5) and incubated on ice for 10 min. Supernatant was recovered by centrifugation at 10000 rpm at 20 C for 15 min. The resultant supernatant was applied onto a Qiagen-tip 20 (containing a resin) column equilibrated with QBT buffer (750 mM NaCl, 50 mM MOPS pH 7 and 0.15% Triton x 100). The column was washed (4 x 1 mL) with QC buffer (1M NaCl, 50 mM MOPS and 15% ethanol pH 8.5) and the plasmid DNA was eluted with 0.8 mL elution buffer QF (1.25 M NaCl, 50 mM Tris-HCl and 15% ethanol pH 8.5). The plasmid DNA was precipitated with 560 µL of isopropanol and centrifuged immediately at 15000 rpm at 20 C for 30 min. The resultant pellet was washed in 70% ethanol, dried under vacuum and dissolved in 30 uL of 10 mM Tris-HCl (pH 8.5). The concentration of this purified plasmid DNA was estimated by spectrophotometry and agarose gel electrophoresis (Figure 14B) and was used as the template DNA for sequencing reactions.

3.2.4.11 DNA sequencing analysis

Sequences were determined by dideoxy termination method (Sanger et al., 1977) using fluorescence-based dye primer cycle sequencing ready reaction kit (Perkin Elmer corporation, USA). In a total of 5 µL (for A and C reactions) or 10 uL (for G and T reactions) reaction volume containing 1 or 2 µL (225 ng/µL) of purified template plasmid DNA and 4 uL or 8 uL of their corresponding ready reaction premix (containing an appropriate ddNTP, fluorescence-based dye primer, M13 reverse or -21 M13 universal primer, dATP, dCTP, dTTP, 7-deaza-dGTP to minimize band compression, Tris-HCl pH 9 at 25^0C, MgCl2 and AmpliTaq DNA polymerase CS+) with DMSO as denaturing agent. To each reaction about 20 uL of mineral oil was overlayed on aqueous layer to prevent thermal evaporation. The reaction tubes were placed in a DNA thermal cycler using the following program.

1. rapid thermal ramp to 95 C and 95 C for 30 seconds
2. rapid thermal ramp to 55 C and 55 C for 30 seconds
3. rapid thermal ramp to 95 C and 70 C for 1 min

(the above thermal cyclings (1,2 and 3) were performed for 15 cycles in total) and

4. rapid thermal ramp to 95 C and 95 C for 30 seconds
5. rapid thermal ramp to 70 C and 70 C for 1 min

continued for 15 more thermal cycles (4 and 5) and stopped thermal cycler with rapid thermal ramp to 4 C and hold.

After completing thermal cycling, the reaction products were combined from the bottom of each of the four tubes into a tube containing 80 µL of 95% ethanol, kept on ice for 30 min and centrifuged at 15000 rpm at 4 C for 30 min. The pellet was washed, vacuum dried and kept at -20 C. Prior to loading the sample onto an acrylamide gel, the pellet was resuspended in 4 µL of deionized formaide/50 mM EDTA pH 8 (5:1, v/v). The sequences were revealed on the ABI PRISM 373 DNA Sequencing System (Perkin-Elmer, USA).

3.2.4.12 Computer analysis for the sequence comparison

Mac vector program using Macintosh system was employed for the sequence comparison analysis.

3.2.4.13 The cDNA fragments of the anthocyanin and phenylpropanoid pathway genes used in making sense and antisense constructs

The cDNA fragments for the genes involved in anthocyanin biosynthetic pathway were used for making transcriptional fusion constructs. The source and properties of the cDNA fragments are shown in Table 3. Further, small restriction fragments of the coding sequences were also used for making antisense constructs.

3.2.4.14 Restriction map of the *pActin*1 transcriptional fusion cassette

This transcriptional fusion cassette was a gift from Dr. Richard A. Jefferson, CAMBIA (through the Rockefeller Foundation program). The total size of the cassette is 4 Kbp in pSP 72 as background plasmid (2.46 Kbp). The sizes of the *pActin* 1 promoter and *nos* gene 3' region as terminator are 1.37 Kbp and 0.2 Kbp respectively. The *pActin* 1 sequences consist of 840 bp promoter region, 80 bp first exon and 450 bp first intron.

3.2.4.15 Preparation of competent cells

One mL of XL-1 cells from the overnight culture was inoculated into 100 mL of LB medium without antibiotic. The cells were grown till they reached an OD_{600} to

Table 3 List of cDNA fragments of the genes involved in the anthocyanin and phenylpropanoid pathways

Gene	Description	Source/Reference
A. Anthocyanin patthway		
i) Structural genes		
A] (anthocyanin-1)	1.4 Kbp cDNA of maize	O'Reilly et al., (1985)
A 2 (anthocyanin-2)	1.3 Kbp cDNA of maize	Mensson et al., (1984)
Bz1 (bronze-1)	1.7 Kbp cDNA of maize	Fedoroff et al., (1984)
C2 (colored-2)	1.48Kbp cDN A of maize	Wienand et al., (1986)
Chs (chalcone synthase)	1.2 Kbp cDNA of rice	Reddy et al., (1994); Scheffler et al., (1995)
ii) Regulatory genes		
C1 (colored-1)	2.1 Kbp cDN A of maize	Paz-Ares et al., (1986)
P (purple)	1.2Kbp cDN A of maize	Lechelt et al., (1989)
R (red)	2.5 Kbp cDN A of maize	Ludwig et al., (1989) Dellaporta et al., (1988)
B. Phenylpropanoid pathway		
PAL (phenylalanine ammonia lyase)	2.5 Kbp cDNA of rice	Minami et al., (1990)

0.4. Cells were harvested in a 50 mL Falcon tube at 3000 rpm for 10 min at 4 C. From then onwards every step was performed in cold room. The harvested cells were resuspended in 15 mL of TFB I buffer (30 mM potassium acetate pH 5.8, 50 mM MnCl2, 100 mM RbCl, 10 mM CaCl2 and 15% glycerin, autoclaved) and incubated for 10 min on ice. This suspension was centrifuged at 4 C for 10 min at 3000 rpm. To the resultant pellet, 4 mL of TFB II buffer (10 mM MOPS pH 7, 75 mM CaCl2, 10 mM NaCl and 15% glycerin, autoclaved) was added and mixed gently with a pipette. Aliquots (50 µL) were frozen in liquid nitrogen and stored at -80 C for further use (Sambrook et al., 1989).

3.2.4.16 Transformation of plasmid DNA

One nanogram of plasmid DNA was added to 50 uL of XL1 competent cells and given a heat shock at 42 C for 2 minutes. The cells were recovered by incubating them at 37 C for one hour in one mL of LB medium without antibiotic. 100 uL of the transformed cells were plated on LB-agar plates with an appropriate concentration of antibiotic and with 40 uL of IPTG (100 mM) and 16 uL of X-gal (50 mg/mL) for the selection of recombinant plasmids (Sambrook et al., 1989).

3.2.4.17 Miniprep of plasmid DNA

The overnight grown bacterial culture (1.5 mL) was harvested by centrifugation. To the bacterial pellet, 200 uL of TELT buffer (50 mM Tris pH 8, 2.5 M LiCl, 62.5 mM EDTA pH 8, autoclaved and 0.4% Triton X-100 added later) and 20 µL of freshly prepared lysozyme (10 mg/mL) were added. After thorough mixing, the bacterial suspension was lyzed at 95 C for three min. The bacterial debris was removed by centrifugation at 15000 rpm at 4 C for 20 min. The pellet was discarded with a wooden tooth-pick. The plasmid DNA was recovered by adding 100 uL of ice-cold isopropanol to the supernatant, incubated on ice for 15 min and centrifuged at 12000 rpm at 4 C for 20 min. The resultant pellet was washed with 70% ethanol (v/v), dried and dissolved in appropriate volumes of TE (10 mM Tris pH 8 and 1 mM EDTA pH 8).

The isolated plasmid DNA was used for restriction analysis for its identity (Sambrook et al., 1989).

3.2.4.18 Maxiprep of plasmid DNA

The overnight grown 500 mL bacterial culture was centrifuged at 4000 rpm at 4 C for 10 min. The pellet was resuspended thoroughly in 15 mL of solution I (50 mM glucose, 25 mM Tris pH 8 and 10 mM EDTA pH 8). This suspension was alkali lyzed with freshly prepared 20 mL of solution II (0.2 N NaOH and 1% SDS) and incubated on ice for 10 min. This was neutralized with 15 mL of solution III (5 M potassium acetate pH 4.8), mixed gently and incubated on ice for 10 min. The supernatant was recovered from this bacterial lyzate by centrifugation at 10000 rpm at 4 C for 30 min. To the supernatant, 0.6 volumes of isopropanol was added, incubated at RT for 20 min and centrifuged at 10000 rpm at 4 C for 30 min. The pellet was recovered, dried and dissolved in 5 mL of TE. The RNA contamination was removed from this preparation by adding equal volumes of 5M LiCl, incubated on ice for 15 min and centrifuged at 10000 rpm at 4 C for 10 min. To the supernatant one volume of isopropanol was added and incubated on ice for 10 min. Plasmid DNA was recovered by centrifugation at 10,000 rpm at 4 C for 10 min. The pellet was dried and dissolved in 5 mL of TE. After RNase (10 μg/mL) treatment at 37 C for 1 h, the total protein contamination was removed with phenol-chloroform extractions. The final aqueous phase was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of ice-cold ethanol. Plasmid DNA was recovered by centrifugation at 10,000 rpm at 4 C for 20 min, washed in 70 % ethanol, dried and dissolved in appropriate volume of TE (Sambrook et al., 1989).

3.2.4.19 Restriction digestion of plasmid DNA and isolation of fragments

The reaction was carried out in a total volume of 20 μL (or scaled upto 50 μL where ever needed) containing 2 uL (0.5 μg) of plasmid DNA, 2 uL appropriate restriction enzyme (10 X buffer), 1 uL BSA (1 μg/μL, if necessary), 15 (or 16) μL double distilled water and 1 unit of restriction enzyme. The reaction was incubated at 37 C (or at appropriate temperature according to the restriction enzyme) for 2 hours

and the resultant restriction fragments were isolated after 1% agarose gel (running buffer 1 X TAE).

3.2.4.20 Calf Intestinal Alkaline Phosphotase (CIAP) reaction

This reaction was performed to remove the 5' phosphate groups to prevent self-ligation of plasmid vector. The reaction was carried out in a total volume of 25 µL containing, 20 uL of restriction digested plasmid DNA in 10 mM Tris pH 8 (10 pmol ends), 2.5 µL 10 X CIAP buffer (0.5 mM Tris pH 9, 10 mM MgCl2, 1 mM ZnCl2, 10 mM spermidine) and 2.5 uL CIAP enzyme (0.1 U/uL). The reaction was incubated at 37 C for 30 min. The total volume was increased to 300 uL with the stop buffer (10 mM Tris pH 7.5, 1 mM EDTA, pH 7.5, 200 mM NaCl, 0.5% SDS), phenolyzed, alcohol precipitated, washed in 70% ethanol, dried, dissolved in TE and used in further manipulations.

3.2.4.21 Fill-in reaction

The reaction was performed in a total volume of 100 uL reaction containing 10 uL of digested plasmid DNA (1 µg), 10 uL of 10 X Klenow buffer (50 mM Tris pH 7.5, 7 mM MgCl2 and 1 mM DTT), 1 uL of dNTPs (125 µM of each), 1 µL Klenow enzyme (1 U/uL) and 78 uL of double distilled water. The reaction was incubated at 37 C for 1 h, heat inactivated (65 C for 10 min), phenolyzed, unincorporated nucleotides were removed by spin column (sephadex G-25), alcohol precipitated, washed in 70% ethanol, dried and dissolved in an appropriate volume of TE.

3.2.4.22 Ligation reaction

The following principle was used to calculate the concentration of fragment and plasmid DNAs needed (3:1, ratio of fragment to vector) for ligation reaction (Sambrook et al., 1989). Fragment size/vector size X 100 X 3 where "100" denotes the amount (ng) of plasmid DNA and "3" denotes the number of times of fragment DNA (ng) required for ligation.

The reaction was carried out in a total volume of 20 µL containing 1 uL (100 ng) of restriction digested plasmid DNA (4 Kbp), 3 uL (300 ng) of fragment DNA (1.5 Kbp),2 uL 10 X ligase buffer (50 mM Tris pH 7.5, 5 mM $MgCl_2$, 1 mM DTT and 0.5

mM ATP), 1 μL of hexammine cobalt chloride (10 μM), 12 μL of sterile DD water and 1 μL of T4 DNA ligase (5 U/μL). The reaction was incubated at 16 C for 2h or overnight where necessary. After completion of the reaction, an aliquot of 10 μL was used for transformation analysis.

4. Results

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

Initially, about 600 rice lines from the DRR (Directorate of Rice Research) germplasm collection were screened for pigment distribution in different plant parts. Sixteen rice lines were selected after such a screening and made true breeding for color phenotypes. Figure 2 displays purple and green phenotypes exhibited by the field grown rice genotypes at vegetative and reproductive stages. A representative sample of pigmentation phenotypes of specific organs of different rice lines, including F1 plants, is shown in Figure 3. The purple/red phenotype is vivid and shows a sharp localization with an even distribution in the given tissue. The intensity of color of a given tissue/organ varies between lines, pericarp being the strongest. A visual observation revealed the existence of variation in distribution of pigments in different tissues among different genotypes. The combination of pigmented plant organs in a given genotype constituted the basis of classification.

Although the selected rice lines exhibit the purple/red color in a variety of organs, some of the prominent organs like leaf sheath, leaf blade, midrib, leaf margins, leaf tip, ligule, auricle, collar, node, internode, sterile glumes, husk, apiculus, stigma and pericarp were observed critically for pigmentation pattern. None of these lines showed purple anthocyanin pigmentation in roots and anthers. In the process of screening, three distinct pigmentation phenotypes were observed, viz., purple, red and brown in addition to colorless. It is clear from Table 4 that there is variation among lines in accumulation of pigments in a given tissue or combinations of tissues.

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

Based on the observations from Table 4, the selected lines were classified into four distinct classes. Class I lines, Purpleputtu and G2237 exhibit purple color in all arial parts except the node. Class II includes a majority of the tested lines H-126, A58, H113, R 27(P), G-962, TN1013, Crossa and N 22W and exhibits purple color only in certain plant organs. Class III lines, A 136, Hamsa and Whiteputtu, do not exhibit

Figure 2

Field grown rice plants.

1) vegetative 2) reproductive stages of Purpleputu (purple color)

3) vegetative stage of N 22B (green color).

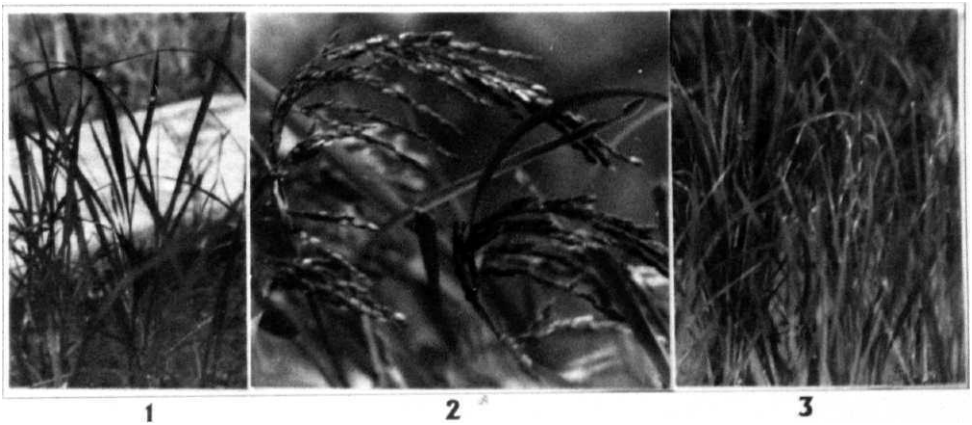

Fig. 2

Table 4 Classification of rice lines by screeinig for purple pigmentation distribution

Subspecies	Class	Genotype	Phenotype[a]									
			Lb	Ls	Co	Au	Lg	No	In	Ap	Hu	Pe
Japonica		**Known**										
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^B *A Rc Rd*	G	G	G	G	G	G	G	G	C	B
Indica		**Predicted**										
Purpleputtu	I	*C A P* Pl^w	P	P	P	P	RP	G	P	R	P	BP
G 2237	I	*C A P* Pl^w	P	P	P	P	RP	G	P	R	P	BP
R 27 (P)	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^+ *Ilb*	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^+ Pl^+ *Rc Rd Ilb*	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

purple color in any of the plant parts. Class IV lines, A5 and N 22B, exhibit brown color in the pericarp tissue and thus are distinct from the rest.

4.3 Detection of a dominant leaf color inhibitor gene

Variability among the classified rice lines with respect to the pigmentation pattern was established. Since these lines are already verified as true breeding, genetic difference is the 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, reciprocal crosses among appropriate lines were made. To start with this aspect of the problem, three rice lines, viz., Purpleputtu (class I), N 22 W (class II) and N 22B (class IV) were selected. The parental (Purpleputtu and N 22B) phenotypes along with the phenotypes of the corresponding F1s are shown in Figure 3. A summary of phenotypic comparison of three parents and the respective F1s in ten different tissues is presented in Table 5.

The phenotype of the various organs of F1s (NB X PP and NW X 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, leaf sheath showed patchy pigmentation to start with and became uniform at maturity. Similarly, hulls show a uniformly lighter pigmentation to start with but faded as it matured. All other tissues were uniformly pigmented as described in Table 5. Reciprocal crosses did not show any differences in phenotype. 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 F1 plants can be ascribed to a dominant inhibitor contributed from the other parents, N 22B or N 22W in the respective crosses. Further, segregation of leaf blade color phenotype in F2 generation of the crosses between N 22B X Purpleputtu and N 22W X Purpleputtu was observed. They segregated green to purple in the ratio of 13:3, with a x of 0.27 (NB X PP) and 0.54 (NW X PP) (Table 6). With regard to the pericarp phenotype, the F1 seeds invariably exhibited the phenotype of the maternal parent, i.e. the F1 seeds from the cross of female Purpleputtu X male N 22B or male N 22W were purple whereas the seeds from reciprocal crosses are brown (NB X PP) or white (NW X PP). This suggests that purple or brown or colorless phenotype of seeds is restricted to the pericarp tissue but not aleurone; pericarp being maternally derived. This is further substantiated by the observation that the pericarp of F2 seeds of

Figure 3

Tissue specific distribution of anthocyanin pigments in rice.

a) N 22B (1), Purpleputtu (2) and F1 (NB X PP) (3) plants.

b) eaf blade (lb), collar (co), leaf sheath (ls), auricles (au), ligule (lg) of
N 22B (1), Purpleputtu (2), Fl plant (NB X PP) (3) and H 113 (4).

c) Node (no), internode (in) of

N 22B (1), Purpleputtu (2), F1 plant (NB X PP) (3) and A 58 (4).

d) Panicle of N 22B (1), Purpleputtu (2), Fl plant (NB X PP) (3) and R 27 (P) (4).

e) Apiculus (ap), hull (hu) and sterile lemma (sl) of

N 22B (1), Purpleputtu (2), Fl plant (NB X PP) (3) and R27 (P) (4).

f) Stigma of N 22B (1), Purpleputtu (2) and Fl plant (NB X PP) (3).

g) Pericarp (pe) of

Purpleputtu (1), N 22B (2), Fl seed (Purpleputtu X N 22B) (3)
F1 seed (NB X PP) (4), F2 seed (NB X PP) (5) and N 22W (6).

h) TLC separation [Each lane, 1-10, contains acid hydrolyzed methanolic extract from 50 mg tissue of PP, note the variation in Cy/Pn ratio]. Arrow indicates the direction of chromatogram (chromatogram was developed in AHW solvent).

1) Leaf blade
2) Leaf sheath
3) Collar
4) Auricles
5) Ligule
6) Internode
7) Sterile glumes
8) Apiculus
9) Hull
10) Pericarp
11) Prericarp of N 22B
12) pericarp of F1 seed (NB X PP)
13) Leaf blade of NB
14) Leaf blade of Fl plant (NB X PP)
15) Pericarp of Fl seed (PP X NB)
16) Pericarp of F2 seed (NB X PP)
17) Pericarp of NW
S) Standard pigment markers
Cy, Cyanidin; Pn, Peonidin

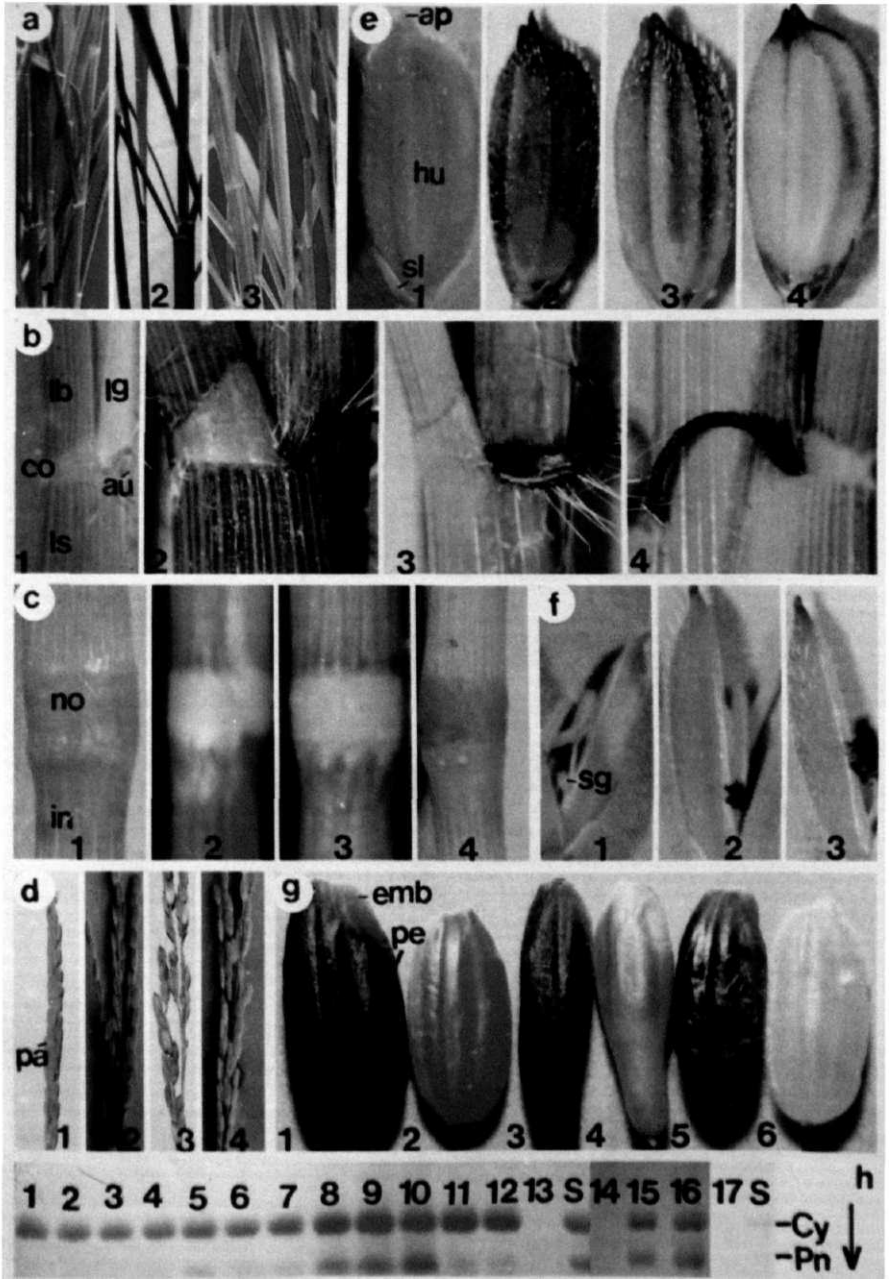
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Fig. 3

Table 5 Phenotype comparison of parents and Fl plants

Parents / **F1**[a]	**Phenotype**[b]									
	Lb	Ls	Co	Au	Lg	No	In	Ap	Hu	Pe
Purpleputtu (PP)	P	P	P	P	RP	G	P	R	P	BP
N 22W (NW)	G	G	G	G	G	G	G	G	C	W
N22B(NB)	G	G	G	G	G	G	G	G	C	B
F1 (NW X PP)	G	P[c]	P	P	RP	G	P	R	P[d]	BP[c]
F1(NBXPP)	G	P[c]	P	P	RP	G	P	R	P[d]	BP[c]

[a] The phenotypic outcome of reciprocal crosses is identical

Abbreviations are same as described in Table 4

[c] Pericarp of F2 seeds; they are of Fl genotype as pericarp is of maternal origin

Young tissue is uniformly light purpled and the pigmentation becomes irregular at maturity

[e] At younger stage patchy; but becomes uniformly pigmented later

Table 6 F2 segregation of the leaf blade phenotype

Cross	F2 segregation for leaf blade colour		Total	Goodness of fit (13 : 3)	
	Green	Purple		X^1	P
N 22B X PP	208	44	252	0.27	0.25-0.50
N 22 W X PP	132	26	158	0.54	0.50-0.75

both crosses are found to be purple. These plants are heterozygous for the dominant allele from Purpleputtu and the recessive allele from N 22B or N 22W in the respective crosses (Table 7). This would also mean that the dominant inhibitor allele has no effect on pericarp color.

4.4 Identification of anthocyanidin pigments accumulating in purple tissue

As elaborated in the previous section, three basic pigmentation phenotypes were observed in rice lines; purple, red and brown. Further, at the visual level, variation in the intensity and tissue specific accumulation of the pigments was noticed. These pigments were isolated from extracts of various tissues in order to identify and quantify each of them. The objective of the experiment is to identify the role of the anthocyanin pathway genes that are operative in rice and determine the genetic block in the pathway in various lines. Toward this goal, the qualitative and quantitative analysis of the pigmented tissue were done using a combination of spectrophotometry, TLC, characteristic chemical tests and NMR spectroscopy. The results from these studies are given in Figure 3h, 4A, 5 and in Table 7 and 8. Spectral analysis revealed that 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 corresponded to λ_{max} 525 for the unhydrolyzed samples and λ_{max} 540 for the hydrolyzed samples. In contrast, all green colored tissue extracts failed to show this peak. Extracts from brown pericarp tissue always showed a peak at λ_{max} 457 for the unhydrolyzed samples and two peaks, one at λ_{max} 447 and the other at λ_{max} 540 for the hydrolyzed samples (Figure 4A).

Since the anthocyanin pigments correspond to λ_{max} 525 and anthocyanidins to the 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 the characteristic absorption maxima, they do not contain any of the anthocyanin class of pigments. The spectral characteristics of the unhydrolyzed extract from the brown tissue correspond to the proanthocyanidin class of compounds (λ_{max} 457) which, upon acid hydrolysis, are converted to anthocyanidin with the characteristic peak at λ_{max} 540 in addition to λ_{max} 447 (Figure 4A). In conclusion, the spectral

Table 7 Tissue specific accumulation of proanthocyanidins and anthocyanidins

Parents/F1 and other rice lines	Proanthocyanidins		**Anthocyanidins**[a]	
	Leaf blade	Pericarp	Leaf blade	Pericarp
Purpleputtu (PP)		-	+	+
N 22B (NB)		+		
N22W(NW)		.	. .	.
NB X PP (F1 seed)	NA	+	NA	
PP X NB (F1 seed)	NA	-	NA	+
NB X PP (F1 plant)				+
NW X PP (F1 seed)	NA	-	NA	-
PP X NW (F1 seed)	NA	-	NA	+
NW X PP (F1 plant)				+
A5		+		
G 962 [b]				
H 126			+	
H 113				+
TN 1013			+	
Crossa	-	-	+	-
Hamsa[c]				

[a] Products of acid hydrolysis; yields both cyanidin and peonidin

Accumulation (transient) of leucoanthocyanidins in pericarp

[c] Acyanic control

NA, not applicable; +, presence and -, absence of pigments.

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 blade and pericarp tissues is shown in Table 7 as the representative data. The unhydrolyzed samples of the brown pigmented tissue lack both cyanidin and peonidin. However, the hydrolyzed samples showed a red band corresponding to cyanidin, as expected. The results of TLC analysis completely matched (Figure 3h) with that of spectral analysis. In order to test the presence of other pigments like flavonoids, several diagnostic tests were performed on the tissue extracts. These included NaBH4 for flavanones, Zn^{+2}/HCl heat test for dihydroflavonols and Mg'VHCl heat test for flavonols. None of the samples tested positive.

4.5 Confirmation of cyanidin and peonidin-based anthocyanins as major and minor pigments respectively

Using NMR analysis of purified anthocyanin preparation from the pericarp tissue of Purpleputtu material, it was confirmed that 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') and 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-CH3 group (Figure 4B). The delta values were comparable to those of the marker pigments. These data lead to the conclusion that the major pigment of pericarp of Purpleputtu extract is cyanidin and the minor pigment is peonidin, a methyl derivative of cyanidin (Figure 25, step 12).

4.6 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 (Figure 3). Also, visual assay would fail to detect any difference, if present, in the composition of the pigments. This is important since two different anthocyanidin pigments, viz.,

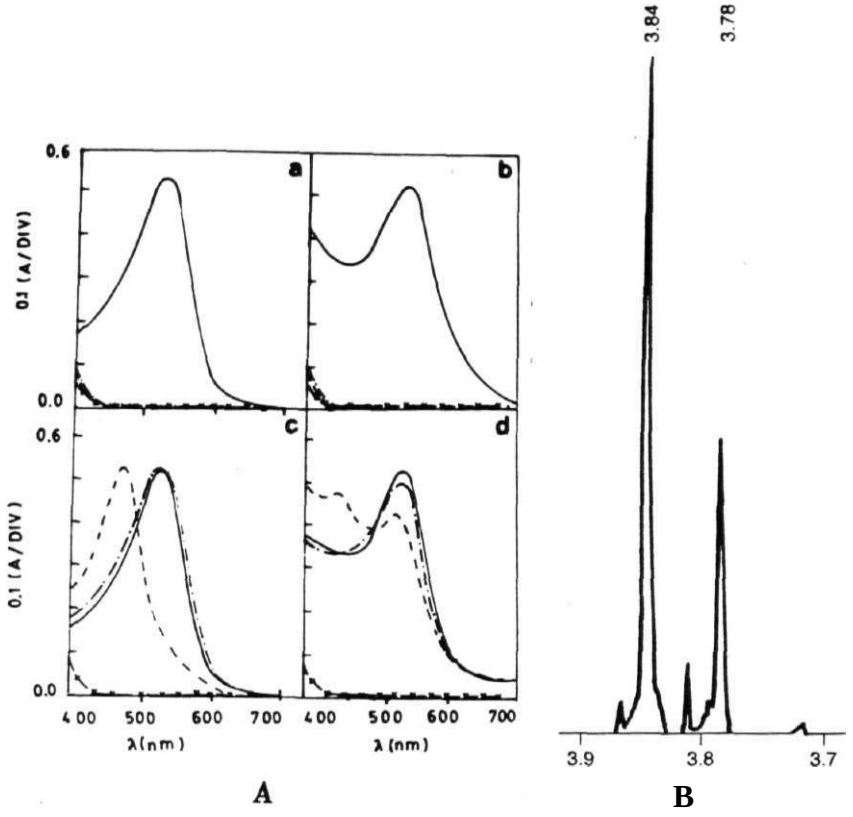


Figure 4

A) Spectral analysis of anthocyanins, proanthocyanidins and anthocyanidins (experimental approach is described under the Materials and Methods section, 3.2.3).
a) unhydrolyzed and b) acid hydrolyzed leaf blade methanolic extracts.
c) unhydrolyzed and d) acid hydrolyzed pericarp methanolic extracts.
N 22B (—), Purpleputtu (—), F1 plant (NB X PP) (——) and N22W (-x-x).

B) Proton NMR spectroscopy of peonidin. The delta ppm values are 3.78 (CD3OD solvent) and 3.84 for $O\text{-}CH_3$ group of peonidin present at the 3' position. This peak is absent in cyanidin proton NMR spectroscopy (experimental approach is described under the Materials and Methods section, 3.2.3.1).

Table 8 Qualitative and quantitative analyses of anthocyanidin pigments of different plant parts of Purpleputtu

Plant organ	Phenotype	Anthocyanidin (in nano 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 millimolar extinction coefficient of 31.6

Ratio of peonidin to cyanidin

cyanidin and peonidin were found to accumulate in the purple/red tissues. Purpleputtu, which accumulates pigments in most plant 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 (Figure 3h, lanes 1 to 10). The extent of anthocyanidin pigments accumulating in different plant parts of Purpleputtu is given in Table 8 and can be compared with the corresponding visual phenotypes (Figure 3). Pigment accumulation is the highest in the pericarp tissue (693.9 nano moles/mg f.wt); the vegetative tissues of plant body accumulate less than 300 nano moles/mg f wt. It is interesting to note that peonidin, the minor pigment of leaf and other vegetative parts (~3 nano moles/mg f.wt.) except ligule (~28 nano moles/mg f.wt.), occurs in relatively high amounts in pericarp (~293 nano moles/mg f.wt.), a floral derived organ. Further, there is an apparent correlation between the increased levels of cyanidin with that of peonidin.

4.7 Accumulation of leucocyanidins in pericarp tissue of G 962

In the process of characterization of anthocyanin pigments, it was observed that the unhydrolyzed methaonolic extract of pericarp tissue of G 962 showed λ_{max} 280 (a characteristic absorption for leucoanthocyanidin) and the corresponding hydrolyzed extract showed λ_{max}540 (Figure 5). It is well known that the colorless leucoanthocyanidins are converted to their corresponding anthocyanidins upon heating with HC1. The dehulled, white colored pericarp when exposed to hydrochloric acid fumes turned purple and the aqueous colorless methanolic extract, when heated with 2N HC1 for 5 minutes, turned red (an important color test). TLC analysis of the hydrolyzed extract and subsequent spectral analysis confirmed cyanidin as the colored pigment product (Figure 5). These results revealed that the accumulating flavonoid in G 962 is a leucocyanidin.

4.8 Chemical nature of the brown pigment

During the phenotypic survey, it was observed that two lines, N 22B (indica) and A 5 (japonica), showed a brown pigmentation in their pericarp tissue; no other tissue accumulated the brown pigments. Qualitative analysis revealed the accumulation of proanthocyanidin compounds in this tissue. Moreover, leucoanthocyanidins were not accumulated in the brown pericarp because this tissue is not colorless and an

Figure 5

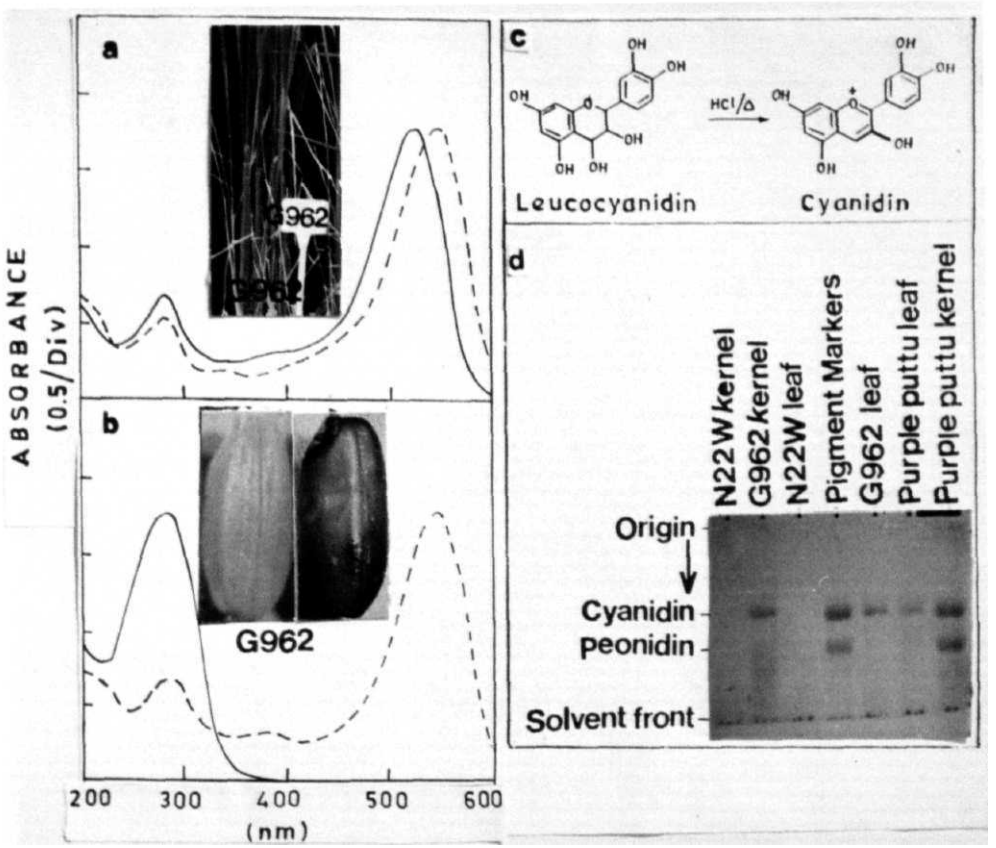
Accumulation of leucocyanidins in G 962 rice line

a) Spectrum of (—) unhydrolyzed and (—) acid hydrolyzed methanolic extracts of leaf blade. Inset shows phenotype of leaf blade.

b) Spectrum of (—) unhydrolyzed and (—) acid hydrolyzed methanolic extracts of pericarp. Insct shows untreated (left) and dehulled acid fumes treated (right) pericarp tissue.

c) Conversion of leucocyanidin to cyanidin by acid fumes (a reduction reaction)

d) Thin layer chromatographic analysis of acid hydrolyzed methanolic extracts of pericarp and leaf blade tissues ((acid hydrolyzed methanolic extracts of leaf blade and pericarp tissues of N 22 W and Purpleputtu were used as controls in TLC analysis; chromatogram was developed in AHW; arrow indicates the direction of chromatogram).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G962
b
G962
ABSORBANCE
(0.5/Div)
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300
400
500
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(nm)
c
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Leucocyanidin
Cyanidin
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N22W kernel
G962 kernel
N22W leaf
Pigment Markers
G962 leaf
Purple puttu leaf
Purple puttu kernel
Origin
Cyanidin
peonidin
Solvent front

Fig. 5

unhydrolyzed extract of this showed λ_{max} 457 (Figure 4A) instead of λ_{max} 280, characteristic of the leucoanthocyanidin pigment as observed in white pericarp tissue of G 962 line (Figure 5). Although this supports the fact that the brown pigment is proanthocyanidin, not anthocyanidin or anthocyanin, it needed confirmation by further tests. The unhydrolyzed (aqueous or acidified) methanol extract of brown pericarp showed a positive response to butanol-HCl heat test (red), 1% vanillin in HCl test (red) and also with 0.5% vanillin in ethanol, used as a spraying solution on TLC plates (the brown color bands turn red). The unhydrolyzed 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 hydrolyzed methanolic (aqueous or acidified) extract from the brown pericarp showed a peak at λ_{max} 540 (Figure 4A). This compound in the hydrolyzed extract is found to be cyanidin after TLC (Figure 3h, lane 11) because it comigrated with the cyanidin marker pigment with an RF value 0.49 (AHW). In addition, a minor pigment with an RF 0.69 has also been observed (Figure 3h, lane 11). Thus the red band represents cyanidin pigment derived from proanthocyanidins after hydrolysis. The compound(s) in the brown streak at the front is likely the cause for the peak at λ_{max} 447 observed in the spectral curve for this sample (this may be the oxidized and polymerized compounds of catechin, the initiating unit for the synthesis of proanthocyanidins) as shown in Figure 4A. The compound that comigrated 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, brown color of the pericarp of the tested class IV rice lines (Table 4) is predominantly due to the accumulation of procyanidins and a residual amount of propeonidins, the proanthocyanidin class of compounds (Table 7).

4.9 Effect of sunlight on anthocyanin synthesis

The 4-day-old etiolated rice seedlings of about 15 cultivars including indica and japonica were exposed to direct sunlight (SL). Based on the visual observation of the intensity of anthocyanin pigments, these rice lines were classified into three distinct classes viz., cyanic, moderately cyanic and acyanic (Table 9). Seedlings of cyanic group were deep purple in color, possessing copious amounts of anthocyanins in shoots while acyanic group do not accumulate any anthocyanin pigments in shoots. Moderate

Table 9 Response of rice lines to UV-B irradiation

Group*	Cultivar
Cyanic	Purpleputtu, G 2237 and R 27 (P)
Moderately cyanic	H 126, G 962, Crossa and TN1013
Acyanic	Blackputtu, A 5, A 58, H 136, H 113, Whiteputtu, N 22W, N 22B and Hamsa

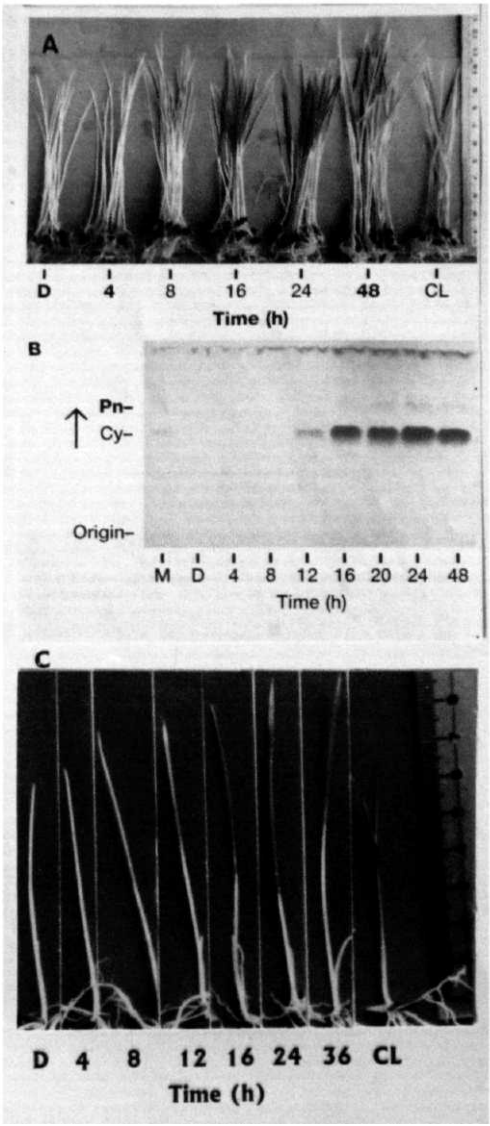
*Classification was made on the basis of visiual observation of the intensity of purple pigments of the 4-day-old seedlings exposed to UV-B radiation

Figure 6

A) Anthocyanin accumulation in Purpleputtu seedlings after a saturating SL pulse. Four-day old etiolated seedlings were exposed to 30 min SL, transferred back into dark and were photographed after the indicated dark intervals.

B) Separation of acid hydrolyzed anthocyanin extracts by TLC. Anthocyanins were extracted from the above mentioned seedlings at indicated time points. Cyanidin (Cy) and Peonidin (Pn) were clearly identified. Arrow indicates the direction of chromatogram. The chromatogram was developed AHW solvent. (D-Dark control; M-Standard markers).

C) Anthocyanin accumulation in a single PP seedling treated with SL (as described above).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Cy–
Origin–
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24
48
Time (h)
C
D
4
8
12
16
24
36
CL
Time (h)

Fig. 6

levels of anthocyanin pigments were accumulated in some of the tested rice lines and hence grouped as moderately cyanic (Table 9).

The etiolated Purpleputtu seedlings, upon exposure to sunlight, under defined conditions at defined time intervals, accumulate greatly increased amounts of anthocyanin pigments (Figure 6A) where as the dark-grown control seedlings totally lack anthocyanins. The induced pigments are localized primarily in the apical portions of the growing shoot while the leaf sheath is almost colorless. On the other hand, roots do not show any anthocyanin pigment upon exposure to sunlight. The etiolated acyanic Blackputtu seedlings do not show any visible anthocyanin upon exposure to sunlight. Similarly, none of the tested acyanic lines exhibit SL-induced anthocyanin synthesis in seedlings. The induction of anthocyanin was exclusively mediated sunlight as evident by the fact that the Purpleputtu seedlings grown for 5 days under the cool white fluorescent tube lights (26 μmol m" sec") accumulated greatly reduced amounts of anthocyanins (Figure 6A and 6C, CL). The induction of anthocyanins upon exposure to sunlight can also be analyzed with a single seedling picked up randomly from a bunch of etiolated seedlings. The 4-day-old seedling was exposed to sunlight for 30 minutes, returned to darkness and photographed at the indicated time points (Figure 6C) with a control seedling grown under cool white fluorescent tube lights (26 μmol m" sec").

The extent of photoinduction of anthocyanin synthesis appears to depend upon the age of seedlings, younger seedlings being the most responsive to sunlight. The results (Figure 7A) reveal that photoinduction is the maximum in 4-d-old etiolated seedlings and thereafter it gradually declined with age. It was observed that a 5 min exposure to SL is sufficient to induce detectable levels of anthocyanin pigments in Purpleputtu. The magnitude of photoinduction increased with an exposure to SL (Figure 7B) and in fact, a 30 min exposure saturated the response (Figure 7B). The time course of photoinduction of anthocyanin pigments in Purpleputtu shoots shows (Figure 8) that a 30 min SL exposure leads to a massive accumulation of anthocyanins after a lag of about 4 h, attaining a peak at 24 h after which there is a gradual decrease in next 24 h (40% reduction). The anthocyanin pigments in SL-exposed Purpleputtu seedlings were separated on cellulose TLC plates into two distinct bands of Rf value 0.49 and 0.68 (acetic acid/HCl/water, solvent mixture) at all tested time points of

Figure 7

A) Age dependent anthocyanin accumulation in Purpleputtu seedlings. Seedlings were grown in darkness from sowing. At the time points indicated on abscissa, seedlings were removed from darkness, irradiated with 30 min SL and transferred back to darkness. Anthocyanin content was estimated at 24 h from the end of light treatment.

B) Effect of SL on anthocyanin accumulation as function of duration of treatment in Purpleputtu seedlings. 4-day-old dark grown seedlings were irradiated with SL for different durations indicated on abscissa and transferred back to darkness. Anthocyanin content was estimated at 24 h from the end of light treatment.

Figure 8

Time course of anthocyanin accumulation in Purpleputtu seedlings. Seedlings were grown in darkness and the 4-day-old seedlings were irradiated with 30 min SL and transferred back to darkness. Anthocyanin content was estimated at time points indicated on abscissa.

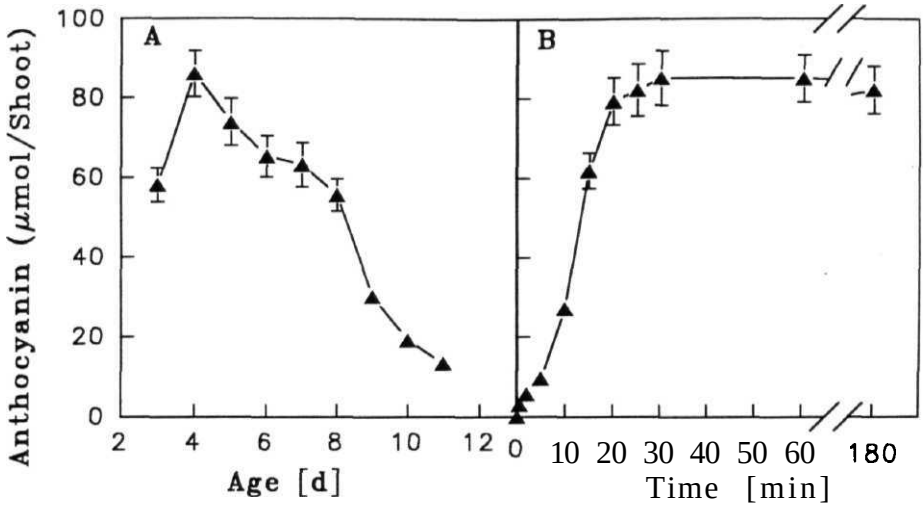
A
B
Anthocyanin (μmol/Shoot)
100
80
60
40
20
0
2
4
6
8
10
12
Age [d]
0
10
20
30
40
50
60
180
Time [min]

Fig. 7

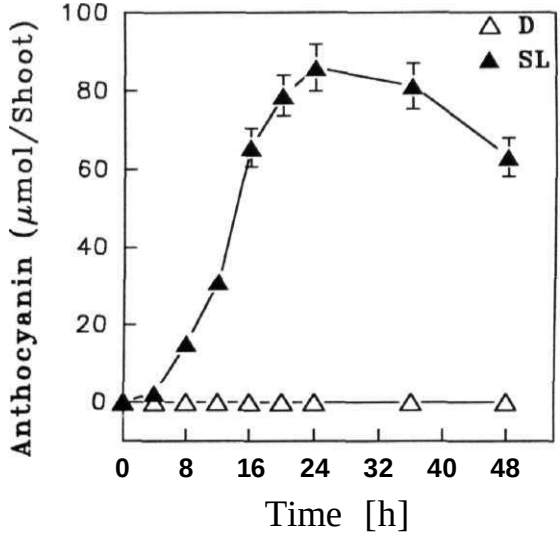
D
SL
Anthocyanin (μmol/Shoot)
100
80
60
40
20
0
0
8
16
24
32
40
48
Time [h]

Fig. 8

Table 10 Effect of various light treatments on anthocyanin level in Purpleputtu seedlings

Irradiation	Anthocyanins	
	µmol/shoot	(%)
Dark	0.00	0.00
SL	85.9+1.2	100
SL + FR*	58.9+1.8	68.56
SL + FR* + RL*	92.4±2.8	107.56
SL + FR* + RL* + FR*	59.8±2.5	69.61
RL* + SL	84.4±1.9	98.25

Obtained using an artificial light source (Manga and Sharma, 1988)

Seedlings exposed to WG, RL, BL or FR lights did not accumulate any anthocyanin pigments

Table 11 Differential accumulation of anthocyanins and UV-B-absorbing compounds in light treated seedlings

Irradiation*	Purpleputtu		Blackputtu	
	Anth	UV-B AC	**Anth**	UV-B AC
Dark	0.0	1.06 ±0.05	0.0	1.01 ±0.01
SL	85.9 ± 1.2	2.55+0.02	2.5 ± 0.02	2.20 ±0.21
WG	0.0	1.54 ±0.01	0.0	1.38 ±0.20

Seedlings were grown in darkness for 4 days and were exposed to either SL or WG for 30 min and transferred to darkness. Anthocyanins and UV-B-absorbing compounds were estimated after 24 h incubation in darkness

Anth, anthocyanins; UV-B AC, UV-B-absorbing compounds

accumulation (Figure 6B). The bands were identified as cyanidin and peonidin by comparing their Rf values and absorbance maxima with that of standards.

4.10 Effect of UV-B light on anthocyanin accumulation

The foregoing analysis clearly indicates the role of sunlight in anthocyanin synthesis in Purpleputtu seedlings. However, the specific light fraction that is responsible for such an induction is not clear. Therefore, the photoinduction of anthocyanin in seedlings was characterized with respect to the effectiveness of different wave bands of light. Table 10 shows that in Purpleputtu seedlings, only sunlight can induce anthocyanin accumulation and this accumulation is triggered by the UV-B component of SL. This is based on the fact that the seedlings exposed to SL-filtered through window glass, (WG), which cuts off UV-B radiation (Klein, 1979) completely lack anthocyanin pigments. Also, there was no anthocyanin accumulation in seedlings exposed to RL, FR or BL. However, the SL-mediated anthocyanin accumulation decreased by about 30%, if SL exposure was followed by a 10 min FR pulse. Further, this effect of FR pulse is nullified if followed by a brief RL pulse. SL also induced the accumulation of UV-B-absorbing compounds in both cyanic Purpleputtu and acyanic Blackputtu seedlings (Table 11). The photo-induced accumulation of UV-B-absorbing compounds was independent of UV-B, as evidenced by the fact that when seedlings (both Purpleputtu and Blackputtu) exposed to either sunlight or window glass accumulated nearly the same level of UV-B-absorbing compounds. By contrast, the acyanic cultivar Blackputtu accumulated only a small amount of anthocyanin which was less than 1/30 th of anthocyanin amount accumulated by Purpleputtu seedlings.

4.11 Photostimulation of PAL activity

The possible relationship between photo-induced anthocyanin accumulation and PAL activity was investigated by following the kinetics of PAL enzyme activity in Purpleputtu seedlings after a brief exposure to SL. Data in Figure 9A show that SL irradiation of seedlings leads to a rapid induction of PAL activity resulting in more than a two-fold increase with a peak at 4h (peak-I). After a minor decline, PAL activity showed again a 4 fold increase with a peak at 12 h (Peak-II). Subsequently, by 20 h, PAL activity declined to the level of dark control and thereafter dipped below that of

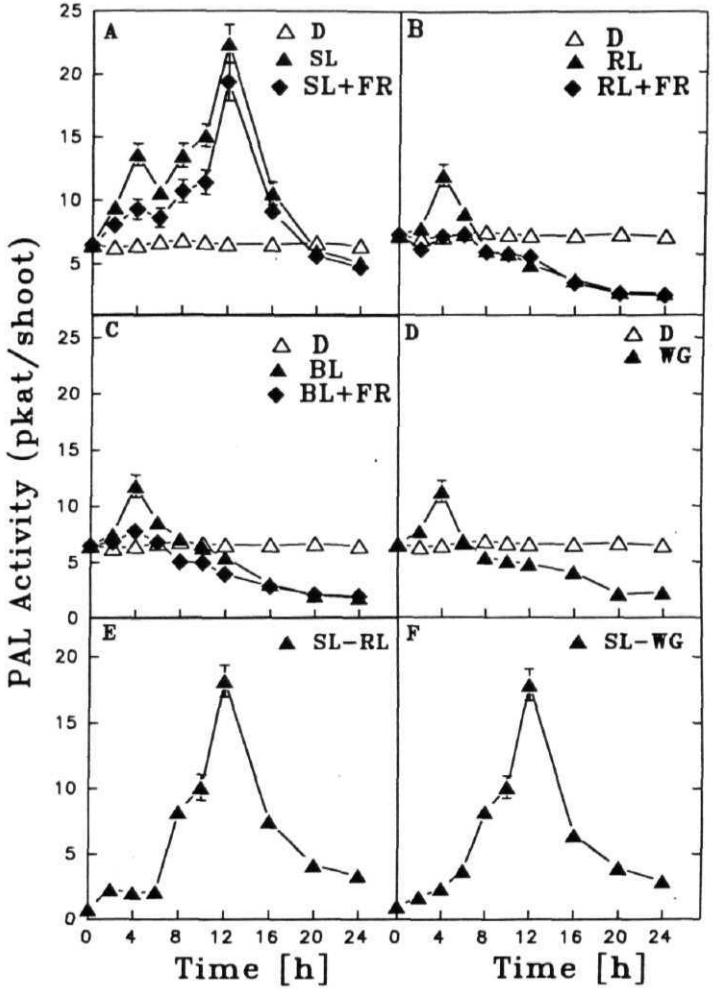

Figure 9 Time course of phenyalanine ammonia lyase (PAL) activity in Purpleputtu seedlings in response to various light treatments. Seedlings grown in darkness upto 4-days from sowing were exposed to 30 min of various light treatments and transferred back to darkness. Incase of SL, BL or RL followed by FR (A, B and C respectively), seedlings at the end of light treatment were irradiated with a 10 min FR light pulses using an artificial light source. The data for E and F were calculated after subtracting values obtained for time course of PAL under SL from the values obtained with RL (B) or WG (D) respectively.

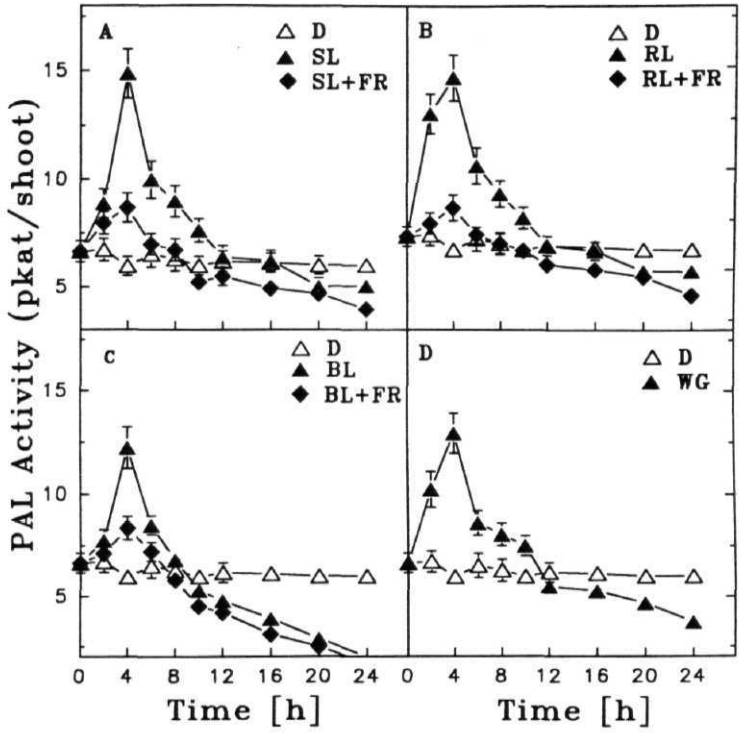

Figure 10 Time course of PAL activity in Blackputtu seedlings in response to various light treatments. Seedlings grown in darkness upto 4 days from sowing were exposed to 30 min of various light treatments and transferred back to darkness. In case of SL, BL or RL followed by FR (A, B and C respectively), seedlings were irradiated with a 10 min FR light pulse using an artificial light source at the end of light treatments. The kinetics of PAL with WG were shown in D.

the dark grown seedlings. However, when SL treatment was immediately followed by a 10 min FR pulse, the PAL induction was subdued during an initial 4 h period and the induction profile of PAL showed only peak-II at 12 h (Figure 9A). In contrast, either a RL or a BL exposure which does not induce anthocyanin accumulation in the Purpleputtu seedlings, induced PAL activity and the induction profile showed only peak-I at 4 h. Thereafter, the PAL activity declined below that of dark level after 8 h. The RL as well as BL-mediated PAL induction was blocked by a 10 min FR pulse (Figure 9B and C) indicating that peak-I is phytochrome-dependent and is also RL/FR reversible. Purpleputtu seedlings, irradiated with SL, filtered through a window glass, showed only the peak-I of PAL similar to RL and conspicuously lacked the peak-11 of PAL induction (Figure 9D). The photoinduction of PAL activity in Purpleputtu seedlings was also plotted by subtracting the curve obtained after RL or WG treatment from the one obtained after SL treatment. Figure 9E and 9F clearly reveal that upon such a subtraction, only peak-I I of PAL is discernible, substantiating the conclusion that, the PAL peak-11 is specifically UV-B inducible in Purpleputtu seedlings.

The time course of photoregulation of PAL in acyanic Blackputtu seedlings is presented in Figure 10. It is evident that in Blackputtu seedlings, SL triggers an induction of PAL activity with a peak level at 4 h from the end of irradiation (Figure 10A), which is similar to the peak-I observed in Purpleputtu seedlings with WG, BL and RL (Figure 9). However, Blackputtu seedlings do not show the second peak of PAL photoinduction i.e. peak-II at 12 h. In as much as the kinetics of photoinduction of PAL in Blackputtu seedlings irradiated with SL, WG, BL and RL are essentially similar and, moreover, induction of peak-I and the effect of SL, BL or RL on PAL induction can be nullified by a terminal FR pulse indicating the photoinduction of peak - I of PAL in Blackputtu seedlings is presumably mediated by phytochrome (Figure 10B, C and D).

4.12 Construction of a shoot specific cDNA library from UV-B exposed Purpleputtu seedlings

In pursuance of our objective of elucidating the UV-B induction mechanism vis-a-vis anthocyanin gene-pigment system in Purpleputtu, a cDNA library was made using a poly A^+ RNA prepared from shoots of UV-B treated 4-day-old Purpleputtu seedlings. The abundance of transcripts of specific genes of the pathway at this stage of seedling

Construction of cDNA library

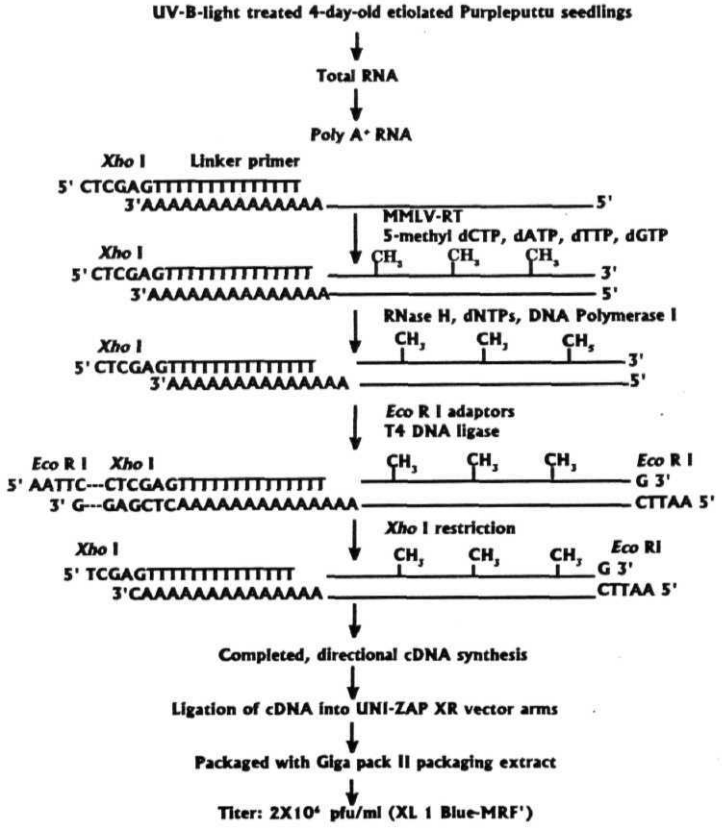

Fig.11

growth was the main criteria. Figure 11 summarizes the important features and steps of cDNA library using the *X-ZAP* vector and XL1-Blue MRF' host system (Stratagene). Five µg of poly A^+ RNA (prepared from the pooled tissues harvested at different time points after UV-B treatment as described in section 3.2.4.7 under Material and Methods) was used for cDNA synthesis. The purity and quantity of poly A^+ RNA was tested by hybridizing poly A^+ containing blot with the *GAPDH* (1.2 Kbp cDNA sequence of maize) in addition to spectrophotometric estimation. The titer of this library was estimated as 2.5 X 10^6 pfu/mL/µg. The resultant primary library was amplified to 1 X 10^{10} pfu/mL. The 1 X 10^6 pfu of this amplified library was used for screening to isolate cDNA clones of the anthocyanin biosynthetic pathway genes.

4.13 Isolation and molecular characterization of cDNA sequences of anthocyanin biosynthetic pathway genes

As a first step towards the characterization and manipulation of anthocyanin pathway genes in rice, the library was screened using full length cDNA sequences of corresponding genes from maize including the *A1* (encodes dihydroflavonol reductase), the *A2* (encodes anthocyanidin synthase) and the *C* (encodes a *myb* related transcriptional activator protein) as the hybridization probes. Several positive plaques from the library, were isolated some of them were subjected to *in vivo* excision (Stratagene) which yield pBluescript $SK^{(-)}$ along with the cloned cDNA sequence. The results of the screening process and number of clones are given in Table 12. In total, 10 *Os-DFR* (encodes dihydroflavonol reductase), 40 *Os-ANS* (encodes anthocyanidin synthase) and 5 *Os-cMyb* (encodes a *myb* related transcriptional activator protein) cDNA clones were analyzed for their restriction maps and identity. All these tested cDNA clones were identical in their restriction maps but differ from one another in length at the 5' end sequences. The largest cDNA clones for each of these genes from the tested cDNA clones were isolated and their restriction maps (Figure 12) were analyzed. The restriction maps, derived from this analyses are displayed in Figure 13. EcoR I-Xho I digestion releases the entire cloned sequence from the pBluescript plasmid vector.

The size of the largest cDNA clone for *Os-ANS* was estimated as 1.6 Kbp (Figure 12A, lane 3) and its internal restriction sites were analyzed by various restriction enzymes including Pst I (Figure 12A, lanes 1 and 2). The corresponding

Table 12 Summary of cDNA library screening and cDNA clones

cDNA clone[1]	Hybridization probe[2]	Number of clones analysed[3]	Size of the largest cDNA clone isolated
Os-DFR	*A1* (1.3 Kbp)	10	1.5 Kbp
Os-ANS	*A2* (1.4 Kbp)	40	1.6 KbP
Os-cMyb	*C1* (2.1 Kbp)	5	1.4 Kbp

cDNA clones isolated from UV-B induced shoot specific cDNA library by screening $1X10^6$ pfu (50000 pfu/petri plate)

Maize cDNA sequences

All these clones were analyzed for their restriction maps and selected the largest clone for sequence characterization

Figure 12

Restriction enzyme analysis of *Os-ANS*,*Os-DFR* and *Os-cMyb* cDNA clones.

A) *ANS* cDNA clone: The bottom part of the gel is shown at high contrast in the small photograph on right side. (Note the 1.6 Kb cDNA insert and its restriction sites).
1 Kb ladder (M), Pst I (1), Pst I-Xho I (2) and Eco RI-Xho I (3).

B) *DFR* cDNA clone: (Note the 1.5 Kb cDNA insert and its restriction sites).
i) 1 Kb ladder (M), Eco Rl-Xho I (1)
ii) 1 Kb ladder (M), Apa I (1), Bam H I (2), Eco R I (3), Kpn I (4), Pst I (5), Sac I (6), Spe I (7), Xba I (8) and Xho I (9)

C) *Os-cMyb* cDNA clone homologue of maize *C1*: (Note the 1.4 Kb cDNA insert and its restriction sites)
i) 1 Kb ladder (M), Eco R I-Xho I (1).
ii) 1 Kb ladder (M), Apa 1(1), Bam H I (2), Eco R I (3), Spe I (4), Xho I (5), Pst I (6), Xba I (7), Sac I (8) and Kpn I (9).

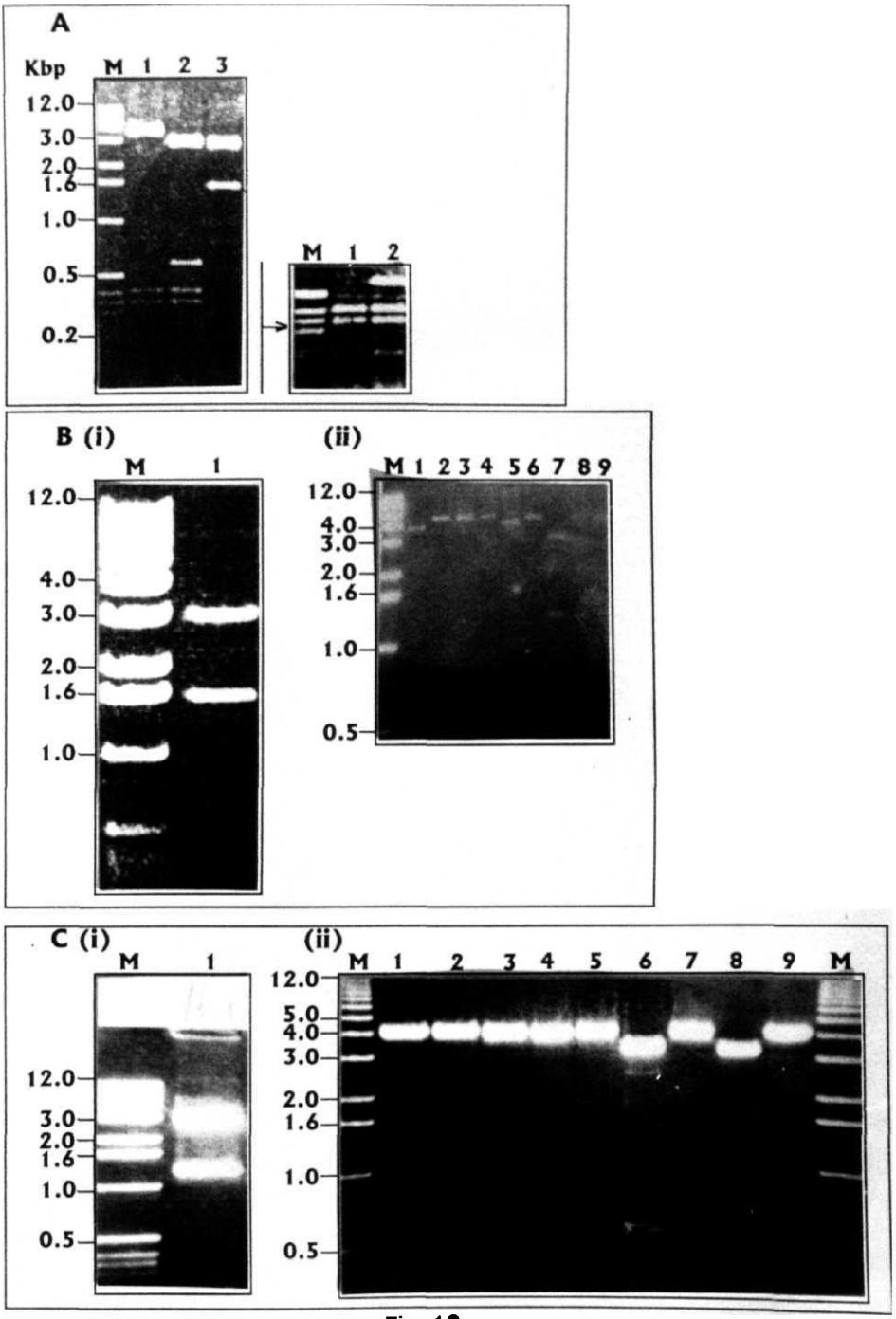
A
Kbp
M 1 2 3
12.0
3.0
2.0
1.6
1.0
0.5
0.2
M 1 2
B (i)
M 1
12.0
4.0
3.0
2.0
1.6
1.0
(ii)
M 1 2 3 4 5 6 7 8 9
12.0
4.0
3.0
2.0
1.6
1.0
0.5
C (i)
M 1
12.0
3.0
2.0
1.6
1.0
0.5
(ii)
M 1 2 3 4 5 6 7 8 9 M
12.0
5.0
4.0
3.0
2.0
1.6
1.0
0.5

Fig. 12

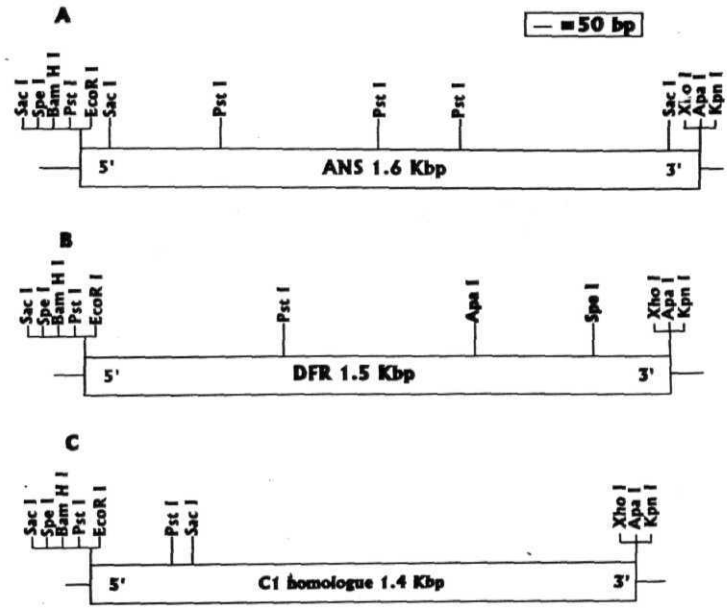

Figure 13
Restriction maps of *Os-ANS, Os-DFR* and *Os-cMyb* cDNA clones in pBluescript $SK^{(-)}$ from rice (maps are constructed from the data in Figure 12).
A) *ANS* encodes anthocyanidin synthase
B) *DFR* encodes dihydroflavonol reductase
C) *Os-cMyb* homologue of maize *C* encodes a regulatory protein factor

Figure 14

A representative agarose gel analysis of Exonuclease III derived deletion clones of the *Os-DFR* cDNA clone.

A) 1 Kb ladder (M), Exo III not treated control (1) and the lanes (2-13) show the sequential deletion clones of the *Os-DFR* removed at 25 seconds interval from Exo-III reaction (Exo III reaction was conducted at 37°C for the period of 5 min, details are described under the Materials and Methods section, 3.2.4.9).

B) 1 Kb ladder (M), the purity and size of the selected deletion clones (from above) through agarose gel electrophoresis after Qiagen miniprep and the size of the clones are:
4.2 Kb (1), 3.9 Kb (2), 3.6 Kb (3), 3.3 Kb (4) and 3.1 Kb (5).

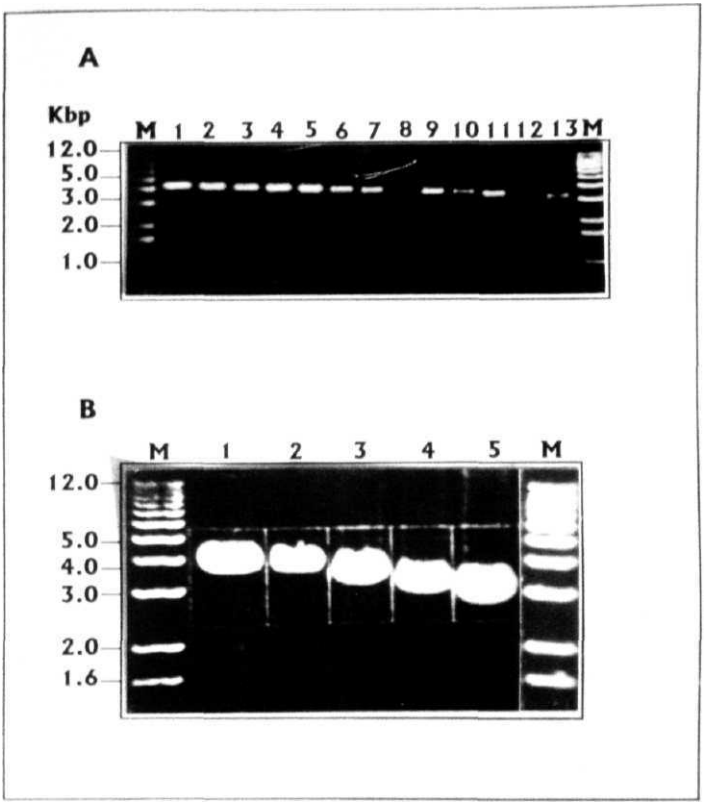
A
Kbp
M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
12.0
5.0
3.0
2.0
1.0
B
M 1 2 3 4 5 M
12.0
5.0
4.0
3.0
2.0
1.6

Fig. 14

restriction maps derived from this analysis was shown in Figure 13A. The 1.5 Kbp largest *Os-DFR* cDNA clone (Figure 12B (i), lane 1) was isolated and analyzed. Out of the nine tested restriction enzymes, Pst I, Apa I and Spe I recognized the internal sequences of the *Os-DFR* cDNA clone (Figure 12B (ii) lanes 1-9). The *Os-DFR* restriction map was derived from this analysis and shown in Figure 13B. Like wise, the *Os-cMyb* cDNA clone, which encodes a regulatory protein, was analyzed for its size (1.4 Kbp, shown in Figure 12C (i), lane 1) and restriction map, derived from the figure 12C (ii), lanes 1-9) and shown in Figure 13C. Further, the *Os-Myb* clone was characterized by sequencing both the 5' and 3' ends and showed extensive sequence homology with the maize *C* cDNA sequence (sequence data not shown).

4.14 Partial characterization of *dihydroflavonol reductase (DFR)* and *anthocyanidin synthase* (*ANS*) cDNAs

The *Os-DFR* and *Os-ANS* cDNA clones were treated with Exo **III** and the resulted deletion clones were monitored for their size through an agarose gel. The Exo III deletion clones (derived from the 3' end) of the *Os-DFR* and *Os-ANS* are further characterized. As a representative data, the Exo **III** clones for the *Os-DFR* clone are shown in Figure 14A. The extent of digestion of Exo III was controlled in such a way to get about 100 bp for a 20 seconds interval at 37 C. Clones of the desired size were used to transform and the plasmid DNA was purified using Qiagen plasmid purification kit. The size and quality of these deletion plasmid DNA clones were shown in Figure 14B. The clones differ from one another in length by about 300 bp. This purified plasmid DNA was used for sequencing using the fluorescence-based dideoxy termination method (described in 3.2.4.11 under Materials and Methods section).

Comparison of the *Os-DFR* single strand nucleotide sequence with that of maize *A* cDNA showed an extensive homology (78%) (Figure 15). Several additional single nucleotide insertions at various sites and a three nucleotide deletion at the 1106 site were also observed. The sequence reveals the untranslated 3' and 5' regions and also **poly** A^+ tail. Further, the deduced protein sequence from *Os-DFR* showed high stretches of homology between the two species.

A partial cDNA sequence for the *Os-ANS* was characterized (Figure 16). **The** comparison of available single strand nucleotide sequence with that of the *A2* of maize showed a high percentage of (76%) homology at the nucleotide level. The deduced

Figure 15 Comparison of nucleotide sequence of the *Os-DFR* and *Zm-A1* cDNA clones. Nucleotides in small case denote maize sequences not homologue to the corresponding position in rice sequence. -, indicates insertion of a single nucelotide in rice and not found in maize.

```
rica     GCCGCTCTAG AACCANTGGA TCCCCGGGCT GCAGGAANTC GGCACANTAG   50
rica     CTATCATATA TNTCTKRCGC AATCCAACAC AAGCACCGMG GMGTAGTACT  100
rica     ACTACTTGCO CCGCGCGTGT TAGATTCGCG TGCGAATCCA ACACAAGCAG  150

rica     ATCGATCACG CACGGTACGC CATGGGCGAO GCGGTOAAGG GGCCAGTGGT  200
maize_____atgg agagaggt  -g t  - - a a  a g  c  40

rica     GGTGACGGCG CSCGTCGGGC TTCGTCGGCT CATOGCTCGT CATGAAGCTC  250
maize      c  -  gg                    c                        90

rica     CTCCAGGCCG GCTACACCGT CCGCGCCACA GTGCGCGACC CCTCTAACGT  300
maize                            g  g  c       t   g g          140

rica     TGGGAAGACG AAGCCGTTGC TGGAGCTGGC GGGGTCGAAG GAGAGGCTGA  350
maize                  a  a    c  tc  c  ag a c   cc   t  190

rica     CGCTGTGGAA GGCCGACCTK GGCGAGGAAG GCAGCTTCGA CGSCSBGCGA  400
maize     ca a     a       g  cg              c   a gc- -  240

rica     TGCAGGGGTT GCACGGGACG TGTTCCACGT CGCGACGCCC ATGGACTTCG  450
maize     -     c     c  -   c          c              c  290

rica     AGTCCGAGGA CCCGGNAGAA CGAGGTGATC AAGCCCACCG TGGAARGGGW  500
maize    t a a      t-     t     a        g g     - a a  340

rica     TGCTGAGATC ATCATGCGGG GCCTGCAGGG ACGCCGGCAC CGTCAAGCGC  550
maize      a - - g         -     a  a  g           gcg      390

rica     ATCGTCTTCA CCTCCTCCGC CGGGACCGTC AACATCGAGG AGCGGCAGCG  600
maiz             t              g      c g     a      a   440

rica     CCCCTCCTAC GACCACGACG ACTGGAGCGA CATCGACTTC KGTCGCCGCG  650
maize    g  gt       g g  aa g    C    g         t c t      490

ri'ca    TCAAGATGGA CCGGATGGAT GTACTTCGTG TCCAAGTCAT TGGCGGAGAA  700
maize         -                          t  aa cc            540

rica     GGCCGCCATG GAATACGCGA GGGAGCACGG GCTGGACCTC ATCAGCGTCA  750
maize      g  c   cg     g c         C       g g  c a     590

rica     TCCCCACGCT CGTCGTCGGO CCCTTCATCA GCAACGGGAT GCCGCCGAGC  800
maize       g        g    c  g        gcgtcc         c      640

rica     CACOTCACCG CGCTGGCGCT GCTCACGGGG AACGAGGCCC ACTACTCGAT  850
maize     t a                ca            c c g              690
```

```
rice    CCTGAAGCAG GTGCAGTTCG TCCACCTCGA CGACCTCTGC GATGCCGAGA  900
maize       c              c  a                          c      740

rice    TCTTCCTCTT CGAGAGCCCC GAGGCGCGCG GCCGCTACGT CTGCTCCTCC  950
maize                      a  g  cc  gc  g                  g  790

rice    CACGACGCCA CCATCCACGG CCTCGCGACG ATGCTCGCGG ACATGTTCCC 1000
maize          t                     cg c      ag   t g  a     640

rice    GGAGTACGAC GTGCCGCGGA GCTTTCCCGG GATCGACGCC GACCACCTCC 1050
maize    c             c   a   g  c       c - - - g          890

rice    AGCCGGTGCA CTTCTCGTCG TGGAAGCTCC TCGCCCACGG GTTCAGGTTC 1100
maize             g          c aa         ag a  t         cc   940

rice    AGGTACACGC TGGAGGACAT GTTCGAGGCC GCCGTCCGGA CGTGCAGGGA 1150
          aag
maize     C                     c      a       t   ca     990

rice     GAAGGGGCTT CTCCCGCCCT GCCGCCACCG CCGACGACGG CCGTGGCCGG 1200
maize         c  c a    --    --     t     -        -a g  a    1040

rice     AGGAGACGGC TCGGCGGGTG TGGCCGGCGA GAAGGAACCG ATACTNGGGA 1250
maize    cttt - - -    t c c  cac        c   gg   cgat  cg 1090

rice    NGGGGAACCG GGACGNCGGT TGGTGCTGAA ACAGAAACGT TGGTCAAATG 1300
maize   cttaa                                                  1095
rice    AGTGTTGACT AGTGAGTCCA GAGAACGGTA TTGAAATTGA TCGTGTNTTC 1350
rice    NTGCGCCTTG CCTCGTTGGC TTCGTCTATT TCACAATGCG AGATTTGGAA 1400
rice    TAAATCAGAG CGGTTAATCC TGTAAGTTCA TATGTAACGT ACCCATTTGA 1450
rice    TTTTTTATTG GTTACATATG GTTACTCCCA AAAAAAAAAA AAAAAAAAA  1499
```

Fig. 16 ***Anthocyanidin synthase*** **- a partial cDNA sequence**

CNTGNGTHGA TGGGCAGCGC GAAGAACGCC TCGCTCCGCG GCGCGCAGGC 50

GGCCGAGGAC NTCGCCCCGG GAGGCCGTGA TCCGGCGATG TGCATGACGC 100

CCCACTCCTC GGCCCGCCGC GCGCACCSSC NTCCACGCAC GCGTGCCCTG 150

CCGTCGCCGT CCGTTGTCCG AACGCGGAGA TGTCGACGAC GGGGATGCGG 200

GCGGTRGACR TACGYYSGTC GKACGACGGC GCCGTTGGYG AGSMTCTCCA 250

GGGCGTCGTC CGACGTGKAC GRCGMTGSTS CYCKSCTCSS GGCGSRCGGT 300

GACSYAGCTC CCGGCGNCTS GTGCACCTGM AKCCCCGGCA CGCCGTTGTG 350

GAGGATGAAG GAGAGCGCGC TGACGTCGGT GTGTGCCTCG ACGCCGACGG 400

GAGGTCAGGC CGCGGGCACC TCGGGTAGTA GTTGATCTYG AGCTGGAGGA 450

GCAGGTCGTC GTCGACGCCG GCCAGCTCAT GTCCTCTCAG GCGGCGTTCC 500

AGCGTCTCTT CCAGGCAGGC CGAGGCCGAG GGAGAGGATG GCGAGCAGCT 550

TGGACGCGAG GGTCCGGACG SGGCCGCCGA AGTCGCGCGA CACGGGGACG 600

TACTTCGGCG GGTTCGCCGG CCAGAGCGAG TGGTCGGCGA GGTGGTCCGG 650

GTGGACGAGG TGGAACAGGT AGTYCTYCCA CTCCCTCTTC CCGCTGGCGT 700

TGGCGGCGAG CTTGCTGCCG TACCCCTGCA GCCGCCCCGC CGCCGGGTCG 750

TTGGCGTACG CCTCCTTCTC CGCGATGGGC AGCGCGAAGA ACGCCTCGCC 800

CGCCTCGTGC CGAATTCCTG CAGCCCGGGG GATCCACTAG TTCTAGAGCG 850

GCCGCCACCG CGGTGGAGCT CCAGCTTTTG TTCCCTTTAG TGAGGGTTAA 900

TTTCGAGCTT GGCGTAATCA TGGTCATAGC TGTTTCCTGT GTGAAATTGT 950

TATCCGNTCA CAATTCCACA CAACATACGA GCCGGAAGCA TAAAGTGTAA 1000

AGCCTGGGGT GCCTAATGAG TGAGTAACTT ACATTAATTG CGTTGCGTNA 1050

ATGNCCGTTT TCAGTTGGGA AAACTTTTGT GNCAGTGATT AATGAATTGG 1100

GCAA

aminoacid sequence comparison of the *Os-ANS* with the deduced protein of the maize *A2* cDNA revealed that it encodes an NADPH dependent oxidoreductase in rice.

4.15 Molecular analysis of *anthocyanidin synthase* (*Ans*) gene in rice

In order to get an estimation about the number of *A2* related gene sequences present in the rice genome, Southern blot analysis of restriction enzyme digested genomic DNA from Purpleputtu was performed using the full length *Zm-A2* cDNA sequence as the hybridization probe. Hybridization at high stringency detects at least two high molecular weight fragments in the range of 18 Kb - 7 Kb (Bcl I, Hind III, BamH I, EcoR V, Xba I and EcoR I) and four fragments in the range of 8 Kb - 1 Kb (Sac I and Bgl II) (Figure 17). This suggests that at least two sequences with high homology are present in the rice genome.

The expression pattern of *ANS* gene in leaf blade tissue of 45 DAG purple and green cultivars were performed using Northern blot analysis with *Zm-A2* cDNA sequence as the hybridization probe (Figure 18A). The Purpleputtu seedlings showed at least a two folds higher expression than that of other lines including acyanic (Blackputtu, N 22W, N 22B) and moderately cyanic lines (G 962, Crossa,TN 1013). The size of the transcript was estimated as 1.25 Kb. Further, *ANS* gene expression was also carried out in various tissues of Purpleputtu which were harvested at different stages of plant development (Figure 18B). The same size of the transcript was observed in all the tissues independent of developmental regulation (leaf blade, 45 DAG; leaf sheath, 60 DAG; ligule and auricles, 80 DAG; and pericarp at maturation stage).

UV-B light induces the expression *of anthocyanidin synthase* gene in Purpleputtu: The 4-day-old etiolated Purpleputtu seedlings were exposed to SL, RL and WG (as described in Materials and Methods section, 3.2.3.5) and transferred back to darkness. The poly A^+ RNA was prepared from these samples harvested after 4 h of incubation in dark after treatment. Equal amounts of RNA were separated on Formaldeyde-agarose gel and transferred to Hybond N^+. The blot was hybridized using 1.3 Kbp *Zm-A2* cDNA sequence as the hybridization probe. The expression of *ANS* gene transcript showed 5 folds more in SL treated seedlings comparaed to dark grown seedlings and about 2-3 folds higher levels compared to RL and WG treated seedlings (Figure 20B,

Figure 17

Genomic Southern blot analysis of *Ans* locus in Purpleputtu. Each slot contains 10 µg of geneomic DNA digests. Hybridization probe was the 1.3 Kb Zm-*A2* cDNA sequence.

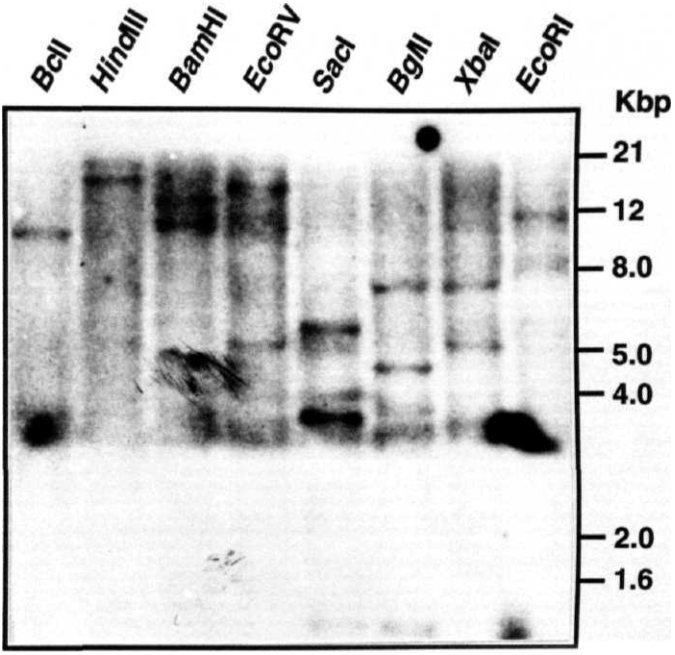
BclI
HindIII
BamHI
EcoRV
SacI
BglII
XbaI
EcoRI
Kbp
21
12
8.0
5.0
4.0
2.0
1.6

Fig.17

upper panel). The same blot was hybridized using the 1.2 Kbp *GAPDH* cDNA sequence as the hybridization probe to check for equal amounts of RNA loaded in each lane (Figure 20B, lower panel).

4.16 Genotype dependent *DFR* gene expression in parents and Fl hybrid

Expression of the *DFR* gene in leaf blade tissue of 45 DAG Purpleputtu, N 22B parents and Fl hybrid (NB X PP) was analyzed through Northern blots (Figure 19, upper panel). Purpleputtu and N 22B showed almost equal levels of expression whereas the Fl plants showed highly reduced levels of (about 50%) the *DFR* transcript. The same blot was also hybridized with the 1.2 Kb of *GAPDH* cDNA sequence of maize as the hybridization control to equalize the poly A^+RNA content in each lane (Figure 19, lower panel). Drastic reduction in *DFR* specific transcript in Fl may be due to several reasons. It can be speculated that there is an interaction between different inhibitors. It is not possible to explain this phenomenon as the genetic background of these lines are different.

4.17 Differential expression pattern of the *PAL* and *DFR* genes

The purpose of this experiment was to analyze the differences in the expression of anthocyanin pathway genes between two genetically diverse lines, purple (Purpleputtu) and green (N 22B) genotypes. The 4-day-old etiolated seedlings of Purpleputtu and N 22B were exposed to SL for 30 min and transferred back to darkness. The shoots of these seedlings were harvested at different time intervals after treatment (0, 4, 8, 12 and 16 h) along with a dark control. The poly A^+ RNA was prepared from these samples and separated on a 1.2% denaturating Formaldehyde-agarose gel and transferred to Hybond N^+. The blot was probed using the 1.9 Kbp EcoR I- Sac I fragment of rice *PAL* cDNA and 1.5 Kb EcoR I-Xho I fragment of rice *DFR* cDNA as the hybridization probes. Figure 20A shows the expression pattern of the *PAL* gene (upper panel) and the *DFR* gene (lower panel) in Purpleputtu and N 22B.

PAL gene expression pattern: The *PAL* gene transcript is inducible in Purpleputtu by UV-B light at 0, 4, 8, 12 and 16h (Figure 20A, a-f, upper panel) with a basal level of expression in dark control. Further, the expression is gradually increased from 4 hours to 12 h with the maximum at 16 h. The expression level of *PAL* gene in N 22B is restricted to dark control and 0 time point (considered as the basal level of expression)

Figure 18

Northern blot analysis of *Am* gene in rice.

A) The expression of *Am* gene in leaf tissue (45 DAG) of various rice lines. 10 µg of poly A^+ RNA (each lane) were fractionated on an 1.2% formaldehyde agarose denaturation gel. Hybridization probe was the 1.3 Kb Zm-*A2* cDNA.
Purpleputtu (1), Blackputtu (2), N 22W (3), N 22B (4), G 962 (5), Crossa (6) and TN 1013 (7). The size of the transcript is 1.25 Kb.

B) The expression of *Ans* gene in various tissues of Purpleputtu. 10 µg of poly A^+ RNA (each lane) were fractionated on an 1.2% formaldehyde agarose denaturation gel. Hybridization probe was the 1.3 Kb *A2* cDNA of maize.
leaf blade 1), leaf sheath 2), ligule 3), auricles 4) and pericarp 5). The size of the transcript is 1.25 Kb.

Figure 19

Northern blot analysis of the *DFR* gene in rice.

Upper panel) The expression pattern of the *DFR* gene in leaf tissue (45 DAG). 10 µg of poly A^+ RNA (each lane) were fractionated on an 1.2% formaldehyde agarose denaturation gel. Hybridization probe was the 1.5 Kb *DFR* cDNA of rice. The transcript size is 1.4 Kb.
F1 (NB X PP) (1), N 22B (2) and Purpleputtu (3).

Lower panel) The same blot was hybridized with the 1.2 Kb *GAPDH* cDNA sequence of maize.

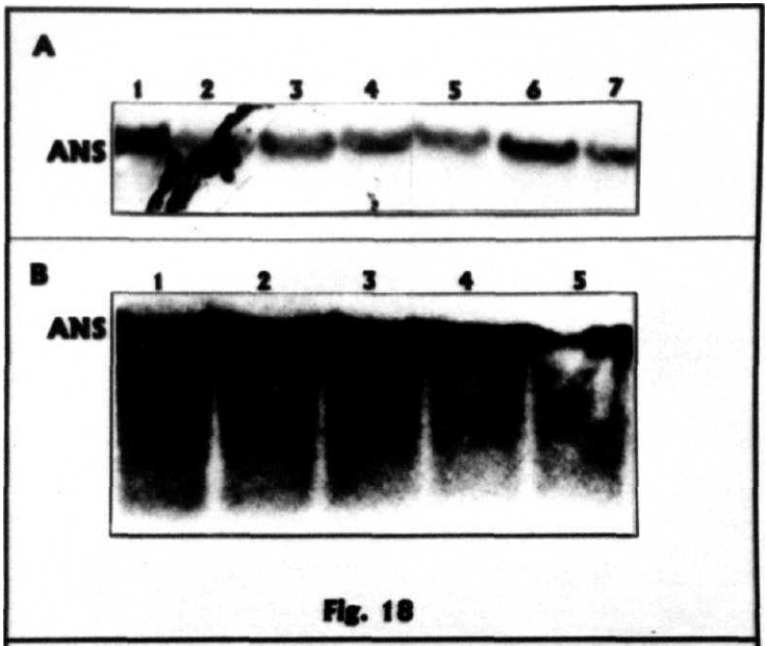

Fig. 18

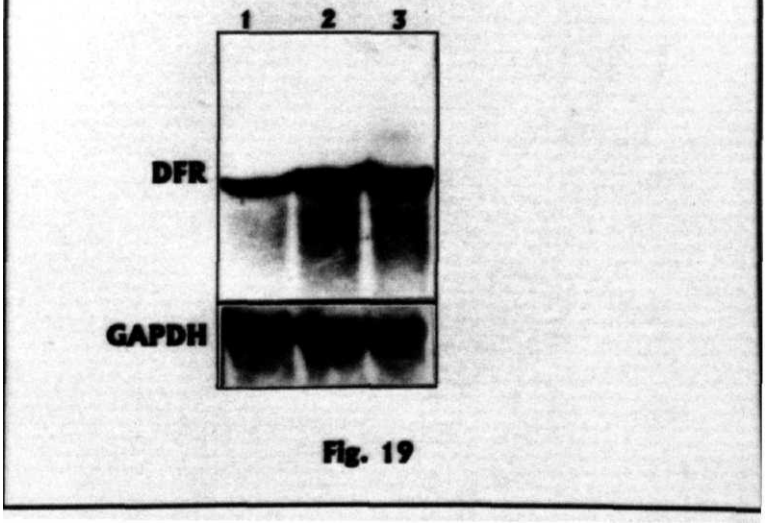

Fig. 19

and again increased at 16 h time point (Figure 20A, g-l, upper panel) with no expression at 4, 8 and 12 h. These results reveal the differential transcript levels of the *PAL* gene in Purpleputtu and N 22B rice genotypes. Further, these results correlate well with the production of anthocyanins in these lines.

***DFR* gene expression pattern:**

The expression of the *DFR* gene is inducible in both the genotypes by UV-B but they differ in the magnitude of induction responses (Figure 20A, lower panel). In Purpleputtu, the transcript is gradually increased from 0 to 16 h time point (Figure 20A, a-f, lower panel) while in N 22B the transcript rate is high at 4 h and showed a gradually decreased appearance from 8 h to 16 hours. The rate of transcript at 12h and 16h is dipped below the dark levels in N 22B (Figure 20A, g-1, lower panel) which is not the case in Purpleputtu where the level of transcript showed higher than that of dark control. Since *DFR* is one of the structural genes of the anthocyanin pathway, its activity explains the difference in the production of anthocyanin end products in these two lines in addition to the activity of the earlier genes in the anthocyanin pathway including the *PAL* and *CHS*.

4.18 Molecular manipulation of the anthocyanin biosynthetic pathway genes by making transcriptional fusion constructs

Different classes of flavonoids originating from diverse branches of the parental phenylpropanoid pathway are inducible in response to biotic and abiotic environmental stress factors. Flavonoids are reported to serve a multitude of functions in plants (as described in 2.2 in Literature Review section). In order to test the relationship between specific pigment levels and magnitude of disease resistance, a series of sense and antisense constructs for each of these genes in the pathway became obligatory. This is because, each reaction is controlled by a specific structural gene in addition to an overall regulation of the pathways by different regulatory genes. Further, the gene expression is influenced by environmental signals and developmental cues. Towards this end, several transcriptional fusion constructs were made for the genes involved in the phenylpropanoid and anthocyanin pathways. Since the rice anthocyanin genes were not yet well characterized, the corresponding maize genes were used for preparing constructs for testing the above hypothesis.

Figure 20

Northern blot analysis of the *PAL* and *DFR* genes in UV-B treated Purpleputtu and NB seedlings. 4-day old etiolated seedlings were exposed for 30 min SL and shoots were harvested at various time intervals. Each lane contains 10 µg of poly A^+ RNA (prepared from these tissues).

A) Upper panel) Differential expression pattern of the *PAL* transcript. Hybridization probe was the 1.9 Kb EcoR I-Sac I fragment of *Os-PAL* cDNA. The size of the transcript is 2.5 Kb.

Dark (a), 0 h (b), 4 **h** (c), 8 h (d), 12 h (e) and 16 h (f) of PP. **Dark** (g), 0 h (h), **4** h (i), 8 h (j), 12 h (k) and 16 h (1) of N 22B.

Lower panel) Differential expression pattern of the *DFR* transcript. Hybridization probe was the 1.5 Kb Os-*DFR* cDNA. The size of the transcript is 1.4 Kb.

Dark (a), 0 h (b), 4 h (c), 8 h (d), 12 h (e) and 16 h (f) of PP. Dark (g), 0 h (h), 4 h (i), 8 h (j), 12 h (k) and 16 h (1) of N 22B.

B) Upper panel) Effect of UV-B light on the expression of *Ans* gene in 4-d-old Purpleputtu seedlings exposed to various lights for 30 min. 10 µg of poly A^+ RNA (each lane) were fractionated on an 1.2% formaldehyde agarose denaturation gel. Hybridization probe was the 1.3 Kb *Zm-A2* cDNA.

Dark (1), SL (2), RL (3) and WG (4).

Lower panel) The same blot was hybridized with the *Zm-GAPDH* cDNA.

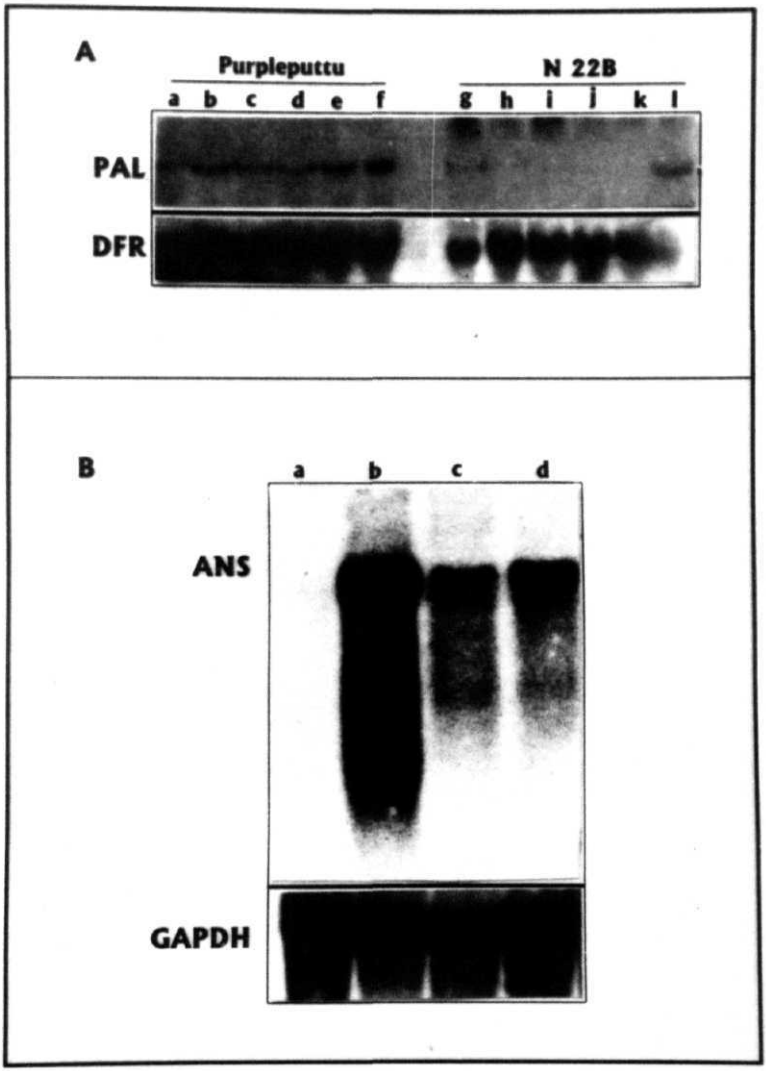

FIG. 20

The details of specific cDNA sequences, their sizes and cloning details were shown in Table 13. Sense constructs were made using full length coding sequences of cDNA fragments. Antisense constructs were made using small restriction fragments of coding sequences, mostly from the 5' end from rice and maize genes (Table 13). All these constructs were made in a transcriptional fusion cassette between the rice *Actin*-gene 1 promoter region and *NOS* terminator in the pSP 72 based plasmid vector. The restriction digestion of *pAclin* 1 cassette was analyzed using relevant enzymes including Sac I (Figure 21 A, lane m), Pst I (Figure 21A, lane n), Bgl I (Figure 21B, lane p) and EcoR I (Figure 21B, lane q). The restriction map of this cassette derived from this analysis is shown in Figure 22.

The cDNA sequences of the anthocyanin biosynthetic pathway genes (Table 13) were deinserted from the respective plasmid vectors by digesting with relevant restriction enzymes as described (Table 13), isolated through agarose gel electrophoresis and, in some cases, the ends of cDNA sequences were filled with dNTPs for cloning purpose. The selected cDNA sequences were then ligated in between *pActin* 1-gene promoter and *NOS* terminator region of *pActin* 1 cassette (details of isolation of cDNA fragments from plasmid vectors, filling-in and ligation reactions were mentioned in Materials and Methods section). The XL1 *(E.coli)* host cells transformed with the ligation product using ampicillin resistance as a bacterial selectable marker. The recombinant plasmids with cloned cDNA sequence were isolated using colony hybridization with the corresponding cDNA sequence as the hybridization probe. The plasmid DNA (prepared from the selected transformed colonies) was digested with appropriate restriction enzymes and the fragments were detected (data not shown). The resultant constructs were verified for desired orientation and expected sizes of the cloned cDNA sequences using restriction analysis through agarose gel electrophoresis and partial sequencing of the junction regions of the promoter-cDNA sequence-terminator. This was achieved by two different primers corresponding to the poly linker sites of the *pActin* 1 cassette (one for the 5' end, TGCAGGTCGACTCTAGAG; and the other for the 3' end, GAGCTCGGTACCC).

Restriction enzyme analysis of sense and antisense constructs:

The sense constructs of the cDNA sequences of the *A\, A2, Bzl,* C1, *PAL* and *R* genes were subjected to restriction analysis and the results are shown in Figure 21 A. Similarly the antisense constructs of cDNA sequences of the *A\, A2, Bzl,* C1, *Cl, CHS,*

Table 13 Summary of molecular constructs for the genes involved in the phenylpropanoid and anthocyanin biosynthetic pathways

cDNA	Sense construct	Antisense construct
A1	1.4 Kbp EcoR I fragment, filled-in and cloned into Sma I	950 bp BspH I-Sma I fragment, filled-in and cloned into Sma I
A2	1.3 Kbp EcoR I fragment, filled-in and cloned into Sma I	650 bp Pvu II fragment and cloned into Sma I
Bz1	1.7 Kbp BamH I-Hind III fragment, filled-in and cloned into Sma I	1.2 Kbp Pml I fragment and cloned into Sma I
C2	1.4 Kbp BamH I fragment, filled-in and cloned into Sma I	1.1 Kbp Nae I-Sma I fragment and cloned into Sma I
Cl	2.1 Kbp EcoR I fragment, filled-in and cloned into Sma I filled-in	2.1 Kbp EcoR I fragment, and cloned into Sma I
P	1.2 Kbp Sal I fragment and cloned into Sal I	1.2 Kbp Sal I fragment and cloned into Sal I
R	2.5 Kbp EcoR I fragment, filled-in and cloned into Sma I	845 bp Pst I fragment and cloned into Pst I
CHS[1]		920 bp Pvu I-Pml I fragment, filled-in and cloned into Sma I
PAL[1]	2.5 Kbp EcoR I fragment, filled-in and cloned into Sma I	

All cDNA sequences were cloned in between *Actin* 1 promoter and *NOS* terminator in a transcriptional fussion cassette

[1]cDNA fragment from rice

P and *R* genes were also restriction digested and the results are shown in Figure 21B. The sense constructs of the regulatory loci *C* and *R* were analyzed with a wide range of restriction enzymes (Figure 21C). Thus restriction maps were derived for all constructs. As a representative data the restriction maps of only the *A*1, *A2, C* and *C*2, both sense and antisense constructs, were shown in Figure 23 and 24. Confirmation of the *A*, *A2* and *C* constructs for their orientation and sizes of sequences cloned was outlined below as representative cases.

A gene **constructs:** The Pst I-Sac I digestion released the entire cDNA sequence of size 1.4 Kbp from the *A* sense construct (Figure 21 A, lane a) and *A* antisense construct of size 0.95 Kbp (Figure 21B, lane a) cloned in between *Actin* 1 promoter and *NOS* terminator. The second band (0.5 Kbp) in both the lanes belongs to the promoter sequence restricted by Sac I (Figure 22). The restriction maps of the *A* sense and antisense constructs are shown in Figure 23A and B respectively.

***A2* gene constructs:** Restriction digestion of the *A*2 sense construct with Sal I released, as expected, a fragment of size 200 bp (Figure 21 A, lane b) and with Xba I-Kpn I releases the entire cloned cDNA sequence from the cassette (Figure 21 A, lane c). The corresponding restriction map derived from this analysis is shown in Figure 23C. Restriction analysis of the *A2* antisense construct with Sal I revealed a fragment of size 550 bp (Figure 21B, lane b) and with Hind III-Kpn I released the entire cloned sequence of size 650 bp (Figure 21B, lane c) from the *pActin* cassette. The restriction map of this clone is shown in Figure 23D.

C **gene constructs:** A rigorous restriction enzyme analysis was performed on the regulatory locous *C* constructs. Restriction digestion (both sense and antisense) with Pst I clearly identified the orientation of the cloned fragment. The Pst I cleaves the *C* cDNA at the 650 th bp site from the 5' end of the sequence. Based on this recognition site, digestion of sense construct with Pst I released a fragment of size 650 bp (Figure 21 A, lane f) and antisense construct releases a fragment of size 1.45 Kb (Figure 21B, lane f) from the cassette. The corresponding restriction maps derived from this analysis are shown in Figure 24A and B respectively. Further, the *C* and *R* sense constructs were subjected to restriction analysis with a wide range of restriction enzymes which recognize the sequences both within the cDNA and the cassette sequences (Figure 21C).

Figure 21

Restriction enzyme analysis of transcription fusion constructs for the anthocyanin pathway genes. In all cases, 1 Kb ladder is the molecular weight marker (M).

A) Analysis of sense constructs:

a), *A1* (Pst I-Sac I)
b), *A2* (Sal I)
c), *A2* (Xba I-Kpn I)
d), *Bz* (Sac I)
e), *Bz* (Hind III-Kpn I)
f), *C* (Pst I)
g), *P* (Pst I)
h), *P* (Hind III-Kpn I)
i), *PAL* (Sal I)
j), *PAL* (Hind III-Kpn I)
k), *R* (Pst I)
1), *R* (Hind III-Kpn I)
m), *pActin* 1 cassette (Sac I)
n), *pActin* 1 cassette (Pst 1).

B) Analysis of antisenseconstructs:

a), *A1* (Pst I-Sac I)
b), *A2* (Sal I)
c), *A2* (Hind III-Kpn I)
d), *Bz* (Sac I)
e), *Bz* (Un digested)
f), Cl (Pst I)
g), Cl (Hind III-Kpn I)
h), *C2* (Pst I-Pvu I)
i), *C2* (Hind III-Kpn I)
j), *CHS* (Pst I)
k), *CHS* (Hind III-Kpn I)
1), *P* (Pst I)
m), *P* (Hind III-Kpn I)
n), *R* (Bgl I)
o), *R* (Hind III-Kpn I)
p), pActin 1 cassette (Bgl I)
q), pActin 1 cassette (EcoR **1)**

C) Analysis of sense constructs of regulatory loci Cl and *R*

***C1* construct**	***R* construct**
a), Nco I	g), Pst I
b), Pst I	h), Bgl I
c), Taq **I**	i), Sst I
d), Xho **I**	j), Nco I
e), Sma **I**	k), Acc I
f) Hind III	

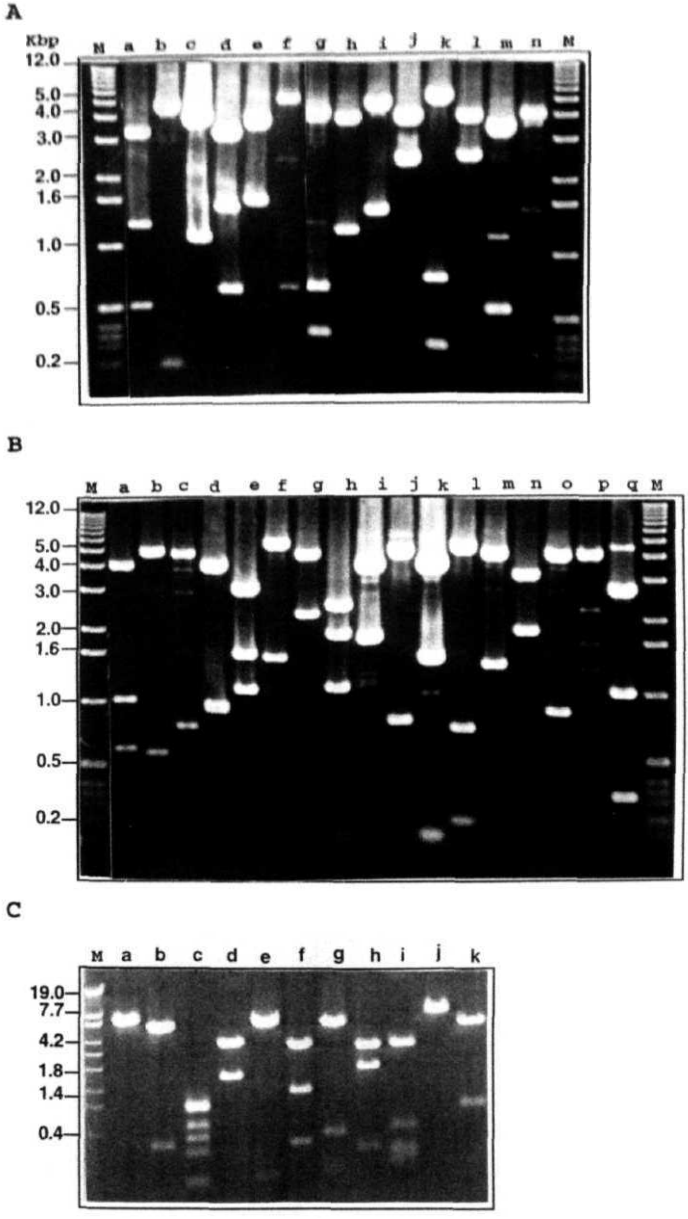
A
Kbp
M a b c d e f g h i j k l m n M
12.0
5.0
4.0
3.0
2.0
1.6
1.0
0.5
0.2
B
M a b c d e f g h i j k l m n o p q M
12.0
5.0
4.0
3.0
2.0
1.6
1.0
0.5
0.2
C
M a b c d e f g h i j k
19.0
7.7
4.2
1.8
1.4
0.4

Fig. 21

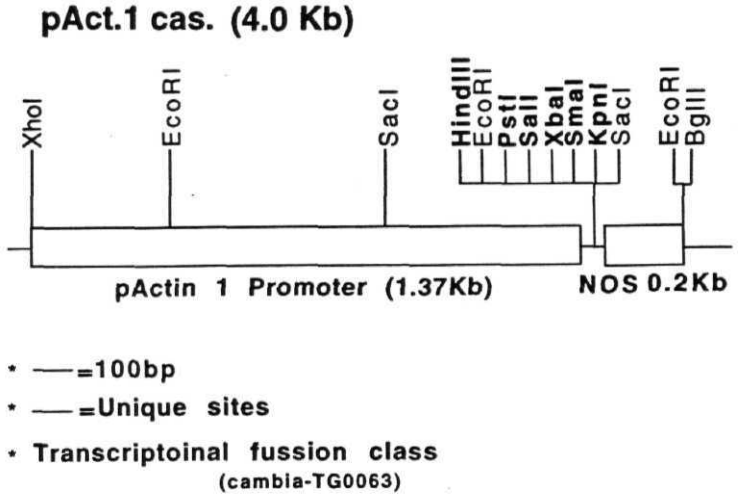

Figure 22 Restriction map *of pActin* 1 transcriptional fusion cassette which is a gift from CAMBIA (Dr. Richard A. Jefferson) through the Rockefeller Foundation.

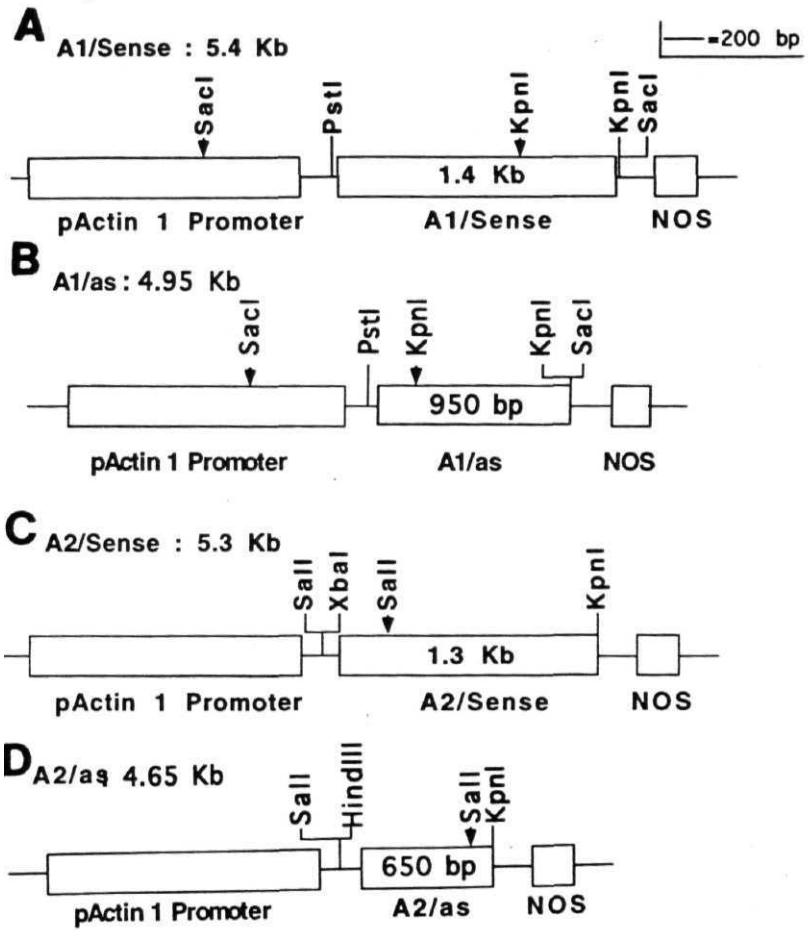

Figure 23 Restriction maps of sense and antisense constructs of the *A1* and *A2* (encode dihydroflavonol reductase and an NADPH dependent oxidoreductase respectively).
A) sense construct of *A1* B) antisense construct of *A1*
C) sense construct of *A2* and D) antisense construct of *A2*

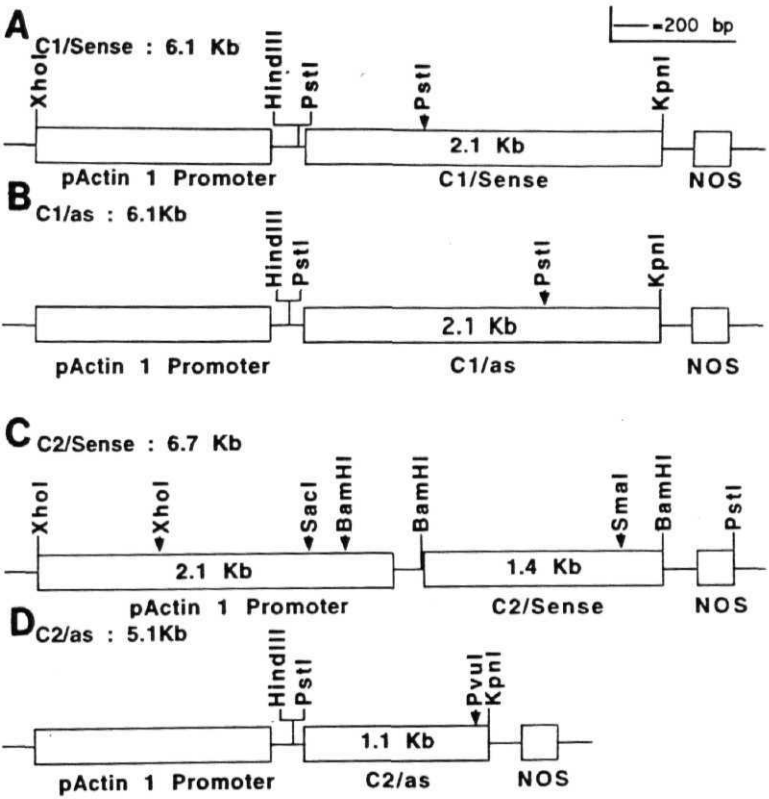

Figure 24 Restriction maps of sense and antisense constructs of the *C1* and *C2* (encode myb related transcriptional protein factor and chalcone synthase respectively).
A) sense construct of *C1* B) antisense construct of *C1*
C) sense construct of *C1* and D) antisense construct of *C2*

Nucleotide sequence of the 5' and 3' ends of the cDNA fragments cloned in between the *Actin* promoter and *NOS* terminator:

The nucleotide sequence of the Sma I digested *pActin* 1 cassette is displayed below.

-Promoter-poly linker site-GATCCC cDNA sequence GGGTACC-poly linker site-*nos*-

The 5' and the 3' end sequences for the *A*, *A2* and *C* constructs were showed as follows.

A gene constructs:

sense-5' AATTC-98 bp-ATAATGGAGAGA-1062 bp-TAA-118 bp-GAATT 3'

antisense-3' CCCCTTGGAG-930 bp-CCTCCTCGAA 5'

A2 gene constructs:

sense-5' AATTC-52 bp-GACATGGAGTCG-1176 bp-TGA-50 bp-GAATT 3'

antisense-3' GACGACAGGA-630 bp-GGCGGCCGTC 5'

C gene constructs:

sense-5' AATTC-6 bp-ATGGGGAGGG-2031 bp-TGA-42 bp-GAATT 3'

antisense-3' TTAAG-42 bp-AGT-2031 bp-GGGAGGGGTA-6 bp-CTTAA 5'

The start and stop codons in the sense constructs were underlined. The numerical numbers (number of base pairs) located in between and outside the start and stop codons belong to the coding sequences and the untranslated leader sequences respectively. Similarly, in antisense constructs, the numerical number (number of base pairs) located in between the 3' and 5' end sequence belong to the coding sequence.

5. Discussions

Anthocyanin biosynthetic pathway in rice is investigated via genetic, biochemical and molecular analysis. The following strategy was adopted to achieve the above goal. Firstly, the variation in pigment phenotypes of rice lines was recorded and accordingly they were grouped into defined classes. Secondly, the stress response of anthocyanin pathway genes was investigated using a variety of experimental tools. Thirdly, certain cDNA sequences, encoding enzymes of the pathway, were cloned from a shoot specific cDNA library. Finally, transcriptional fusion expression vectors of specific genes of the anthocyanin pathway were constructed for eventual manipulation of the anthocyanin/flavonoid pathway in rice.

5.1 Extensive variation in tissue specific anthocyanin pigment distribution is observed among indica rice lines

The existing variation in tissue specific expression of anthocyanin pigmentation was documented in 10 rice lines of the indica sub-species (Figure 2 and 3; Table 4). The difference between the lines were mainly due to the combination of tissues expressing the pigments and the type of pigment. The accumulation and distribution of anthocyanin in rice plant is controlled by coordinated expression of the basic *'C A F* genes and the distributive *'Pl Pn Prp'* genes in addition to the inhibitory genes, *'IPl*-1 to *IPl*-6' (Table 14).

Anthocyanin pigmentation has been studied earlier in rice and many loci have already been reported. These studies have largely focused on the identification and genetic mapping of loci contributing to pigment synthesis and distribution in various plant organs. A summary of the known genes is presented in Table 14. Particularly noteworthy are the alleles at the *Pl* locus besides the *P, Pn* and *Prp* loci because they specify appearance of pigmentation in various tissues; the combination of alleles determine the pattern of pigment distribution. They have been appropriately termed as distributive or localizer genes (regulatory genes of the pathway conditioning tissue specificity). These are in addition to basic genes (structural genes), the *C* and the *A,* that determine pigment synthesis in the plant. Several inhibitory genes which inhibit the function of different *Pl* alleles have also been described (Takahashi, 1982): *IPl*-1,

IPl-2 and *IPl*-3 inhibit the action of both Pl^{w} and Pl^{1} 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^{1} allele. However, the role of the structural and regulatory genes in controlling the individual biosynthetic reactions of anthocyanin pathway and also the molecular basis of inhibition of anthocyanin biosynthesis in specific tissues remains to be established.

5.2 Genotypes of rice lines are predicted from their pigmentation phenotypes

This study being the first systematic attempt to decipher genetic and biochemical basis of the anthocyanin pathway in the indica sub-species, classification of the various lines became necessary to predict their genotypes. This prediction is mainly based on the known genes and their alleles in the japonica subspecies. The four classes are defined on the basis of pigment distribution in ten different plant organs and the phenotypic manifestations of the accumulating pigments. Thus, these rice lines differ mainly in the allelic composition at the C, *A*, *P* and *Pl* loci (Table 4).

A simplified key to predicting genotypes from pigmentation phenotypes is as follows: The presence of the *C* and *A* loci are mandatory for pigment production; presence of the *P* locus is confirmed if apiculus is pigmented; and pigmentation of other aerial tissues determine the status of the specific *Pl* allele present (Table 14). The Pl^{w} allele, in addition to *C, A* and *P* genes contributes pigmentation in pericarp as well as in most other plant organs. Thus, for example, class I lines are all expected to be homozygous for the Pl^{w} allele and therefore 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^{1} alleles in contrast to the Pl^{w} allele condition non-pigmented pericarp; *Pl* contributes to pigmentation in all shoot tissues and Pl^{1} to all except collar and auricle. In the class II R27(P) plants, tissue specific distribution is likely specified by the *Pl* allele, since it shows pigmentation in most aerial tissues. One subgroup of the class II, viz., G 962, TN 1013 and Crossa, are similar except that the pigment distribution is likely determined by the Pl^{1} allele as these lines fail to produce pigmented collars and auricles. The genotype of N 22W is not easily explained merely by the presence of the *C, A, P* and *Pl* loci. It is therefore considered below, separately.

Table 14 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 AA^+ (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:** /'/"'-leaf blade, leaf sheath, auricles, ligule and pericarp *Pl*-leaf blade, leaf sheath, collar, auricles, ligule, node and internode Pl^t-leaf blade, leaf sheath, ligule and inter node /'/"'"-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^t alleles
I-Pl-4, *I-Pl*-5	Inhibit the action of the *Prp* locus and
I-Pl-6	Inhibits the action of Pl^t allele.

Class III, being non-pigmented lines, 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.

5.3 The *Ilb (inhibitor of leaf blade pigmentation)* allele specifically inhibits the synthesis of anthocyanins in leaf blade

The N 22W line (class II) is considered 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 likely that it carries one of the alleles at the *Pl* locus (difficult to predict the specific *PI* allele from the known information) in addition to an inhibitor allele which suppresses pigmentation of the tissues specified by the corresponding *Pl* allele.

If the N 22 W line truly contains the predicted inhibitor gene of pigmentation, it should be detectable in a cross between appropriate genotypes. The ideal tester parent is Purpleputtu since it exhibits purple pigmentation through out the plant and the presence of tissue specific inhibitors present (described above, and in Table 14) has more chances of being detected with this genotype than with any other genotypes in our collection. And indeed, in the Fl of the cross between N 22W and Purpleputtu, the inhibition of pigment production in leaf blade was detected (Figure 3; Table 5). Thus, the failure of pigment production in the leaf blade of F1 plants even in the presence of the Pl^W allele (contributed from the Purpleputtu parent) clearly suggests the involvement of a leaf blade color inhibitor contributed from the N 22W parent. Maternal effect and cytoplasmic inheritance of such an inhibition were ruled out as reciprocal crosses gave rise to same Fl and F2 phenotypic ratios.

Class IV is a special case (brown pigmentation of pericarp) and is represented by a single indica line, N 22B. The phenotype of N 22B is similar to that of N 22W in being not accumulating anthocyanin pigments in any of the aerial tissues, but differs from N 22W as it shows brown pigmentation in pericarp and a colorless apiculus. Incidentally, both have a common origin. It is therefore not a coincidence of detection of the leaf blade specific inhibitor of pigmentation in this line too. The phenotype of various tissues of parents (PP and N 22B) and the Fl plant are shown in Figure 3. Their corresponding pigmentation pattern is presented in Table 5. Spectrophotometry

(Figure 4A and Table 7) and TLC (Figure 3h, lanes 11-17) data confirmed the lack of anthocyanin pigments in leaf blade. All other tissues of the F1 plants and the pericarp of F2 seeds (i.e., Fl genotype, pericarp being a maternal tissue) accumulate the same kind of anthocyanidin pigments (cyanidin and peonidin). In summary, the pattern of anthocyanin pigmentation in Fl plant is identical with that of the Purpleputtu parent except in the leaf blade. This result is identical to that obtained in case of the related N W line with respect to the presence of inhibitor.

Inhibitors of pigmentation have earlier been reported in rice (Table 14; Kadam, 1936; Ramaiah and Rao, 1953; Dhulappanavar, 1973; Kinoshita and Takahashi, 1991; Takahashi, 1982). The inhibitor detected in this study is unique because its inhibitory action is specific to the leaf blade and is unlike the previously reported inhibitors of leaf pigmentation, *IPl*-1, *IPl*-2, *IPl*-3 and *IPl*-6 , whose inhibitory effects extend over a range of tissues. This unique inhibitor is termed as *Ilb (Inhibitor of leaf blade pigmentation).*

Given the presence of *Ilb* in N 22W and N 22B, it is possible to reconstruct their genotypes for the specific *Pl* allele present. The presence of alleles like Pl^{w}, *Pl* or Pl^{i} of the *Pl* locus in N 22B is ruled out as these alleles are known to contribute to pigmentation in tissues that *Ilb* cannot suppress, being a leaf blade specific inhibitor. NW and NB plants likely carry the null allele, Pl^{+} of the *Pl* locus that does not contribute to pigmentation in any of the tissues. The apiculus pigmentation in NW is easily explained by the action of the *P* locus (Table 14; Takahashi, 1957). The F2 progeny of NB X PP and NW X PP segregated for leaf blade color in 13 green : 3 purple ratio suggesting a dominant inhibition phenomenon (Table 6). Their genotypes are *Ilb* Pl^{w} (9, green); *Ilb* Pl^{+} (3, green), *ilb* Pl^{w} (3, purple) and *ilb Pt* (1, green). Based on Fl and F2 progeny analysis of the crosses between the Purpleputtu, N 22B and N 22W, the genotypes of these lines, in terms of anthocyanin production, were confirmed; Purpleputtu - *ilb* Pl^{w}; N 22B - *Ilb Pf* and N 22W - *Ilb Pt.* The allelic status of the *C, A* and *P* loci are common for all the three except the N 22B is homozygous for the null allele P^{+}.

The unresolved part in genotyping the lines is determining the genetic factor(s) responsible for brown pigmentation in the class IV line, N 22B. To resolve this issue,

identification of the brown pigment accumulating in N 22B pericarp became necessary. The brown pigments are distinctly different from the other flavonoids. Therefore, elaborate chemical and physical analyses of these compounds became necessary.

5.4 Cyanidin is the predominant anthocyanidin pigment of rice

The results obtained in this study indicate that the biosynthesis of specific anthocyanin end products contributc red/purple color to various tissues of tested rice plants except the brown pigmented pericarp in N 22B line. There are three common types of anthocyanidin pigments (based on the 'B' ring hydroxylation pattern) encountered among flowering plants: -4' (pelargonidin), -3',4' (cyanidin) and -3',4',5' (delphinidin) hydroxylated forms (Figure 1A). Several modifications like methylation, acylation, glycosylation, malonylation etc., of these three basic moieties give rise to a variety of anthocyanin pigments (Harborne, 1965). In rice, the data from spectrophotometry (Figure 4A), TLC (Figure 3h) and NMR spectroscopy (Figure 4B) confirmed that the major pigment of purple colored tissues is cyanidin and the minor pigment is peonidin, a 3'-methoxy cyanidin derivative (Figure 25). Plants are known to accumulate a combination of anthocyanin pigments; for instance, pelargonidin and cyanidin combination in maize (Coe et al., 1988; Styles and Ceska, 1977), cyanidin, delphinidin and also their methylated forms, peonidin, petunidin and malvinidin in *Petunia* (Gerats et al., 1982, 1984) and cyanidin and delphinidin combination in barley (Meldgard, 1992).

5.5 Cyanidin to peonidin ratios are different in vegetative and floral derived organs

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 tissue specific distribution of pigments. Pigment composition studies have therefore been carried out on various pigment producing tissues.

Purpleputtu (Class 1) accumulates anthocyanin pigments in almost all tissues except node and there exists significant quantitative differences between tissues (Table 8 and Figure 3h, lanes 1 to 10). The total anthocyanidin content is remarkably high (more than 5 folds) in pericarp tissue compared to most vegetative tissues. Interestingly, the peonidin content is higher by several orders of magnitudes 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 anthocyanin biosynthesis in Purpleputtu seedlings. Four-day-old etiolated rice seedlings after exposure to sunlight (UV-B) synthesize 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 synthesized at 16 h (Figure 6B). The *Petunia an* ^{p} mutants at the *An*\ 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 the state of alleles and to some extent by concentration of anthocyanin pigments (Gerats et al., 1984).

The N 22B line is unusual in that it accumulates a brown pigment in the pericarp which was unambiguously identified as proanthocyanidin (its implications with respect to anthocyanin pathway in rice is discussed in a later section).

5.6 G 962 pericarp accumulates leucocyanidin: the colorless precursor of cyanindin

Leucoanthocyanidins are unstable intermediate flavonoid compounds which accumulate in tissues due to the genetic block at anthocyanidin synthase mediated conversion of colorless leucoanthocyanidin to colored anthocyanidin (Figure 25, step 10) in the anthocyanin biosynthetic pathway. Genetic experiments in maize revealed that the *A2* (anthocyanin-2) gene product is associated with this enzymatic step (colored aleurone) and null mutants accumulate leucoanthocyanidins (white aleurone) (Coe, 1955; Reddy, 1964; Reddy and Coe, 1962; Reddy and Reddy, 1971). Identification of leucocyanidins in pericarp tissue of G 962 line (Figure 5) suggested that the impairment in *anthocyanidin synthase.* This was the first report on identification of an *Ans* mutant activity that represents a specific block in the anthocyanin pathway in rice plant.

5.7 N 22B pericarp accumulates proanthocyanidins

The N 22B in our collection and the A5 line from the japonica group have been classified separately solely because they exhibit brown pigmented pericarp due to accumulation of proanthocyanidins (Figure 3g, 2; 3h, lane 11; Figure 4A; Table 7).

Proanthocyanidin accumulation in different tissues of many plant species was well documented. These include vegetative tissues of *Vicia faba* (Crofts et al., 1980), *Phaseolus vulgaris* (Ma and Bliss, 1978), *Lotus corniculalus* (Sakar and Howarth, 1976), in seed coats of *Glycine max* (Todd and Vodkin, 1993), *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 accumulate 3-deoxy proathocyanidins and their oxidized 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 certain japonica rice genotypes identified two genes, the *Rc* and *Rd* whose presence confer a brown pigmentation in 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 color in the pericarp tissue of rice to be a mixture of compounds consisting of catechin, catechol and phlobaphenes. These pigments were considered other than anthocyanins (Takahashi, 1982). The present study unambiguously identified the brown pigment isolated from the pericarps of N 22B and A5 genotypes to be proanthocyanidins. The brown pigment is converted to the corresponding anthocyanidins upon acid hydrolysis (a characteristic test for proanthocyanidins) (Figure 3h, lane 11; Figure 4A). The related flavonoids catechins, catechols and phlobaphenes were absent in N 22B and A5.

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 (Figure 25 summarizes the steps involved in the predicted anthocyanin biosynthetic pathway in rice plant). Proanthocyanidins are polymeric flavonoids synthesized from flavan-3-ols (catechins) and leucoanthocyanidin (Porter et al., 1986; Stafford, 1983). Although two loci, the *Rc* and *Rd* in japonica rice have been implicated in the accumulation of brown pigments in pericarp, their exact role in the pathway is still unknown. The genetics of regulation of proanthocyanidin biosynthetic pathway is known to some extent in seed coats of barley in which procyanidins and prodelphinidins are accumulated (Jende-Strid, 1991). The gene *Ant* 19 encodes dihydroflavononol reductase or leucoanthocyanidin reductase (Kristiansen, 1984) which converts leucoanthocyanidin to catechin (Figure 25, step 13)

Figure 25

Anthocyanin biosynthetic pathway (tentative) in rice. The numbers refer to probable biosynthetic enzymes involved in the pathway. The structural and functional characterization of enzymes, **10, 12**, 13 and 14 are yet to be established.

1) Phenylalanine ammonia lyase
2) Cinnamate-4-hydroxylase
3) 4-Coumarate:CoA ligase
4) Acetyl-CoA-corboxylase
5) Chalcone synthase
6) Chalcone isomerase
7) Flavanone-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
14) Condensing enzyme and/or chain polymerizing enzyme

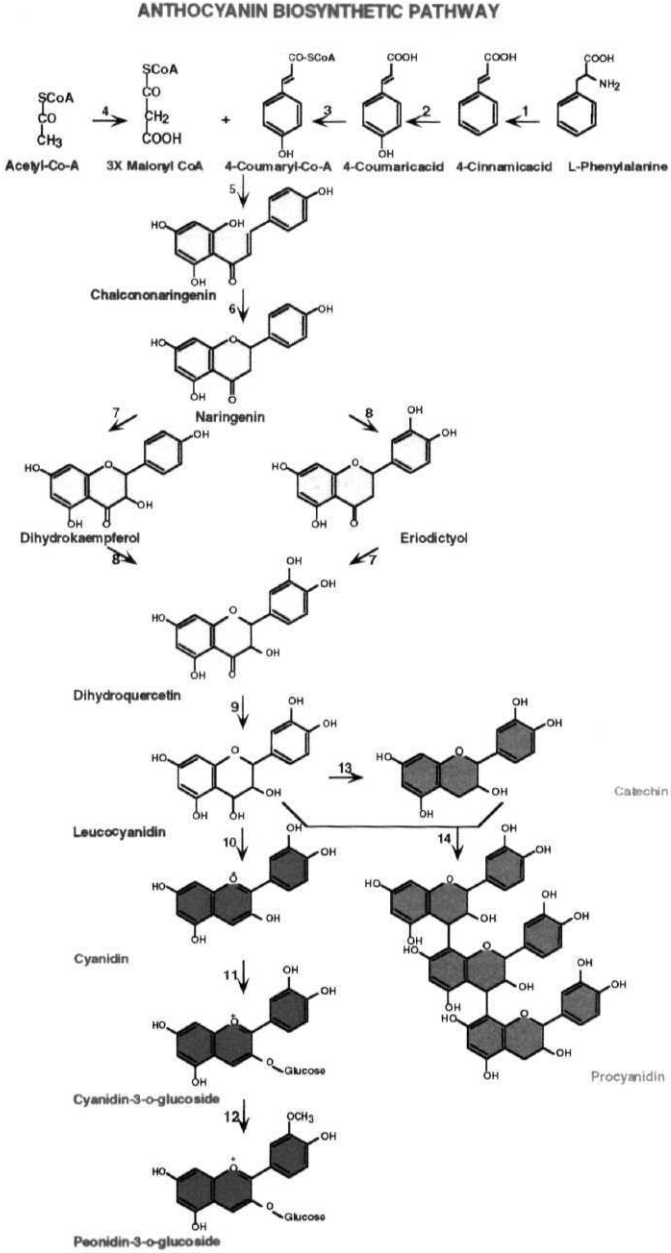
ANTHOCYANIN BIOSYNTHETIC PATHWAY
Acetyl-Co-A
3X Malonyl CoA
4-Coumaryl-Co-A
4-Coumaricacid
4-Cinnamicacid
L-Phenylalanine
Chalcononaringenin
Naringenin
Dihydrokaempferol
Eriodictyol
Dihydroquercetin
Leucocyanidin
Catechin
Cyanidin
Procyanidin
Cyanidin-3-o-glucoside
Peonidin-3-o-glucoside
Glucose

Fig 25

and thereby initiating the polymerization into proanthocyanidins (Figure 25, step 14). The *A2* gene of maize encodes an NADPH-dependent oxidoreductase (Menssen et al., 1990). This enzyme, now referred to as anthocyanidin synthase (ANS) converts leucoanthocyanidin to anthocyanidin (Heller and Forkmann, 1988) as shown in Figure 25, step 10. Thus although these two enzymes are reductases, they probably differ in their oxidative activity. Based on this information (that proanthocyanidins are reduction products), it is concluded that the accumulation of brown pigments (proanthocyanidins) is due to a block at the conversion of leucocyanidin to cyanidin step (Figure 25, step 10). Most likely, the oxidative activity of anthocyanidin synthase is blocked in the brown pericarped rice since the oxidation or oxidoreduction products of this reaction, viz., phlobaphenes or cyanidin, respectively were not accumulated.

Proanthocyanidins and leucoanthocyanidins are important plant defense chemicals since they act as insect 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 programs.

5.8 Anthocyanin biosynthesis in Purpleputtu is induced primarily by UV-B light and modulated by phytochrome

The data from the present study clearly demonstrated that the photoinduction of anthocyanin in shoots of young Purpleputtu seedlings triggered by sunlight (SL) (Figure 6). Since screening of SL by window glass, which cuts off the wavelengths shorter than 320 nm (Klein, 1979) abolishes the photoinduction of anthocyanin in these seedlings, it is proposed that the SL-effect is primarily mediated by the UV-B component of SL (Table 9 and 10). These results are in agreement with that of maize, where it has been shown that while SL stimulates an increase in flavonoid production, the effect of SL is significantly reduced on filtering it through a window glass (Urban, 1959).

The exclusive role of UV-B light in induction of flavonoids has been observed in cell-suspension cultures and seedlings of parsley as well as *Sorghum.* In these plants, flavonoid production was induced only by UV-B light whereas other photoreceptors such as blue/UV-A and phytochrome could only modulate the photoresponse triggered by UV-B light (Wellmann, 1974; Yatsuhashi et al., 1982). In

the present study it is clear that only UV-B induces anthocyanin production in Purpleputtu seedlings and none of the other wavelengths in the visible region of the spectrum (>320 nm) are effective in inducing any detectable levels of anthocyanins (Table 10). Since anthocyanin level induced by UV-B decreased with a FR pulse which inturn could be nullified by a RL pulse, it is evident that phytochrome plays a secondary role in modulating the UV-B response in Purpleputtu seedlings, as in the case of parsley and *Sorghum* seedlings (Wellmann, 1974; Yatsuhashi et al., 1982). In the acyanic Blackputtu cultivar, only a marginal amount of anthocyanin is induced under SL unlike in cyanic Purpleputtu. By contrast, both cultivars accumulate the UV-B-absorbing compounds to the same extent under SL as well as under WG (Table 11). Further, it is evident that the UV-B component of SL (through UV-B photoreceptor) is responsible for the specific induction of anthocyanin pigments.

5.9 UV-B dependent anthocyanin biosynthesis is mediated by through a specific phase of phenylalanine ammonia lyase biosynthesis

It has been shown in many plant species that the photo-induced accumulation of flavonoids is preceded by an induction of several enzymes involved in phenylpropanoid metabolism (Hahlbrock and Scheel, 1989). In the present study, SL clearly triggered photoinduction of PAL in Purpleputtu seedlings with two distinct peaks (Figure 9A). The analysis of the time course of photoinduction of PAL after different light treatments revealed that the occurrence of two PAL peaks was possibly determined by an independent action of two distinct photoreceptors on PAL activity. Our observations lead to the conclusion that the PAL peak-I at 4 h is induced by the phytochrome, as this peak could also be seen in seedlings irradiated with RL or WG (Figure 9B and D) and photoinduction of this peak could be nullified by FR. The second PAL peak was induced specifically by UV-B light, because it was completely missing in seedlings irradiated with WG. Moreover, the FR pulse which abolished peak-I in SL exposed seedlings, had no effect on the appearance of peak-II.

The molecular events leading to induction of the biphasic PAL profile under SL can only be speculated at the moment. It is plausible that these two peaks arise by a stimulation of PAL activity by phytochrome and UV-B photoreceptor on a different temporal scale. Since PAL is encoded by a small multi-gene family in rice (Minami et al., 1989), it is also possible that individual members of the *PAL* gene family may

respond differentially to photoinduction leading to biphasic appearance of PAL. In *Arabidopsis*, transcripts of PAL and other genes of flavonoid biosynthetic pathway are induced independently by three photoreceptors, viz., the phytochrome, the blue/UV-A photoreceptor and the UV-B photoreceptor in a temporally determined fashion (Kubasek et al., 1992). By contrast, in rice, BL induced only peak-I of PAL activity (Figure 9C) which was qualitatively similar to the RL effect; moreover, the BL effect was nullified by a terminal FR pulse. Thus, in rice seedlings, the effect of short exposure of BL (30 min) on PAL activity is evidently mediated by a phytochrome rather than a specific BL/UV-A light photoreceptor.

Since the photoinduction of these two PAL activity peaks precedes the anthocyanin accumulation, it is conceivable that the photoinduction of PAL is a part of the overall induction of the anthocyanin biosynthetic pathway in Purpleputtu seedlings. The above possibility arises from the observation that the photoinduction of both peak-II of PAL and anthocyanin depends exclusively on UV-B light. A correlation between the induction of peak-II of PAL and the anthocyanin formation is also supported by the observation that peak-II is restricted only to the cyanic Purpleputtu line (Figure 10). Although the anthocyanin synthesis is induced by the UV-B and this induction is possibly dependent on peak-II of PAL, it is apparent that in Purpleputtu seedlings, peak-I of PAL also contributes to the anthocyanin production. This becomes evident from the observation that a terminal FR exposure, which blocks the SL-mediated induction of peak-I, also reduces the anthocyanin level by about 30% in Purpleputtu seedlings.

Although, at the moment, only a limited data are available on the possible protection of rice seedlings from UV-B radiation by anthocyanin and other UV-B-absorbing compounds, the available information implies such a role (Robberecht and Caldwell, 1986; Tevini et al., 1991). For instance, seedlings of certain *Arabidopsis* mutants deficient in the production of flavonoids including anthocyanins are hypersensitive to UV-B radiation (Li et al., 1993) and exhibit a lethal response, suggesting that the UV-B-absorbing compounds play a protective role. Similarly, the *Rumex patientia* plants are more sensitive to UV-B damage than *Rumex obtusifolius* which have a higher level of the UV-B-absorbing compounds in the epidermal layers (Robberecht and Caldwell, 1986). It can be argued that the UV-B-induced anthocyanin pigmentation in rice seedlings may have a role in minimizing the UV-B damage. More

direct physiological and molecular genetic data on anthocyanin synthesis and regulation in rice are essential to evolve a strategy to protect this important crop plant from an impending threat of enhanced UV-B radiation in the biosphere.

In summary, these results reveal that there is an apparent correlation between UV-B induction of anthocyanin and induction of peak-II of PAL activity in seedlings of the cyanic cultivar Purpleputtu. At the moment, the genetic determinants in rice associated with anthocyanin synthesis, both UV-B dependent and independent, are absolutely unknown. Therefore, a comparison of rice with that of genetically well-defined maize system becomes necessary here. In maize, at least fourteen non-allelic genes are involved in the synthesis and distribution of anthocyanin pigments in various plant parts and of these, at least four genes, *Pl*, *C*, *R* and *B*, are found to be regulatory (Table 1; Coe et al., 1988; Dooner et al., 1991). Of these four, the recessive *pl* allele conditions a "sun red" phenotype (anthocyanin synthesis) of the portions of the maize plant body that are exposed to light (Briggs, 1966; Coe, 1985).

The light dependent induction of anthocyanin synthesis in the light exposed parts of the plant body was mediated through induced or increased expression of the regulatory gene (Cone and Burr, 1988; Cone et al., 1993). The regulatory locus *R* has been shown to regulate the tissue specific expression of several genes associated with the anthocyanin pathway. The product of the *R* locus was found to be a transcription activator protein of the basic helix-loop-helix class that interacts with the regulatory elements of a number of structural genes of the pathway. Most importantly, the *R* locus was found to be largely responsible for the photoinduction of anthocyanin synthesis in maize seedlings (Taylor and Briggs, 1990). It is reasonable to suggest, therefore, that the UV-B-induced anthocyanin synthesis in rice may be mediated by the UV-B-dependent expression of a regulatory gene analogous to the *R* of maize. In fact, the recent experiments elegantly demonstrate that this maize gene exerts a similar regulatory influence on induction of the anthocyanin pathway in transgenic plants of diverse origin, including dicots such as *Arabidopsis* and *Antirrhinum* (Lloyd et al., 1992). It is speculated that the Purpleputtu seedlings are homozygous for a gene analogous to the *R* gene of maize, mediating the photoinduction of anthocyanins in arial parts of plant body, whereas the recessive acyanic Blackputtu seedlings are defective in this function.

5.10 The rice homologue of the *Zea mays Al* flavonoid gene encoding anthocyanidin synthase

Genomic Southern analysis of Purpleputtu revealed that there are two *A2* homologues (Figure 17). Northern analysis clearly detected one of these two homologues as there is only one detectable *A2* specific transcript, that is 1.25 Kb (Figure 18A). Further, the same 1.25 Kb *A2* gene specific transcript showed up in the extracts of different tissues (Figure 18B) Interestingly, this transcript is showed up in both colored as well as colorless genotypes. It is interpreted that the structural gene *(Al* homologue) *anthocyanidin synthase* is transcriptionally active even in a tissue where anthocyanins are absent. It is suggested that the colorless genotypes are genetically defective in one of the genes (other than *A2* homologue) of the pathway. The expression of *anthocyanidin synthase* gene specific transcript is enhanced significantly in UV-B treated Purpleputtu seedlings (Figure 20B, upper panel) with a very little basal level of expression. In a later section, we show that UV-B treatment enhances other gene transcripts namely *PAL* and *DFR* as well. It is not well understood how flavonoid genes are coordinately switched on in rice. However, it seems likely that different regulatory proteins are produced upon a common signal. In rice, evidence has been obtained that UV-B light induces expression of anthocyanin genes with the concomitant production of anthocyanin end products. The UV-B light may therefore act at an early stage in the signal transduction pathway.

In an attempt to obtain cDNAs of early UV-B induced proteins vis-a-vis enzymes and regulatory protein factors of the anthocyanin pathway, a cDNA library was prepared and screened with the cDNA sequences of anthocyanin pathway genes of maize as the probes. Three different cDNA sequences hybridizable to the cDNA sequences of the *A2*, *A1* and *C* genes of maize were isolated and characterized.

Only one class of cDNAs hybidizable to the *Zm-A2* cDNA sequence (Menssen et al., 1990) of the longest size of 1.6 Kb, named as *Os-ANS*, was isolated and partially characterized from rice (Figure 12A, 13A and 16). It is likely that one of the two *Ans* genes is expressed in response to UV-B light. Alternatively, both genes express in the same way and produce the same kind of transcript. Two homologues of the *Zm-A2*, namely *Ant\7* and *Candi* were molecularly cloned and characterized from *Petunia hybrida* (Weiss et al., 1993) and *Antirrhinum majus* (Martin.C as cited in Weiss et al.,

1993) respectively. The deduced protein sequences of *Ant*17 revealed 48% identity with *A2* sequence and 73% with the *CANDI* sequence (Weiss et al., 1993). Further, deduced protein sequence from *Ant]* 7 showed homology with flavonone 3-hydroxylase (34%) and a gene encoding ethylene-forming enzyme (31%) (Weiss et al., 1993). Based on this information that *A2, Ant\l* and *CANDI* may encode a hydroxylase or an oxidoreductase involved in the conversion of leucoanthocyanidin to anthocyanidin. The *Os-ANS* is partially characterized. The available single strand sequence analysis revealed a 76% homology at the nucleotide level (Figure 16) and high stretches of homology at protein sequence level with the *Zm-A*2 cDNA sequence. This primary evidence indicated that the *Os-ANS* encodes anthocyanidin synthase, an NADPH-dependent oxidoreductase involved in the formation of anthocyanidin from leucoanthocyanidin in rice. The enzymatic step(s) involved in the conversion of leucoanthocyanidin to anthocyanidin, the first colored compound of the pathway is (are) not characterized biochemically. Heller and Forkmann (1988) suggested the possible involvement of dehydration and oxidation steps for this complex biochemical reaction.

5.11 The rice homologue of the *Zea mays Al* flavonoid gene encoding dihydroflavonol reductase

A nearly full size *DFR* cDNA clone (1.5 Kbp), namely as *Os-DFR* (Figure 12B and 13B) was isolated from the shoot (treated with UV-B light) specific cDNA library and compared at the nucleotide (Figure 15) and protein sequence levels with the *Zm-A* cDNA. The data revealed that *Os-DFR* cDNA is the coding sequence for the dihydroflavonol reductase, involved in the conversion of dihydroquercetin to leucoanthocyanidin in the anthocyanin pathway (Figure 25, step 9). Comparison of the deduced protein sequence (not shown) of *Zm-A* (O'Reilly et al., 1985) with that of *Petunia DFR* and *Pallida* cDNA of *Antirrhinum* (Martin et al., 1985) revealed high homology between the three. The deduced protein sequence comparison between the *Zm-A* and *Os-DFR* revealed a 70% homology except a few residues at the N-terminal and C-terminal ends of the sequence. Its activity is associated with the production of pelargonidin and cyanidin in maize plants (Reddy et al., 1987), pelargonidin in *Mathiola incana* (Heller et al., 1985) and cyanidin and delphinidin in *Petunia* (Gerats et al., 1982, 1984). Thus, this enzyme has different substrate specifities in different plant

species. The *Os-DFR* encodes dihydroflavonol reductase, the substrate specificity of which seems to be dihydroquercetin, involved in the production of cyanidin based anthocyanins in rice plant. The dihydroflavonol reductase activity alters the pigmentation pathway in transgenic plants as observed in transgenic *Petunia* plants carrying the *Zm-A* sense sequence. In these plants, a new flower pigmentation pathway (pink to brick red, due to synthesis of pelargonidin) has been established (Meyer et al., 1987). Thus, the wide substrate specificity for DFR plays a crucial role in the manipulation of flower pigment production in flower industry. This requires isolation and characterization *of DFR* genes from diverse plant species.

5.12 *PAL* and *DFR* gene specific transcripts in Purpleputtu seedlings are enhanced by UV-B light

The transcripts patterns of UV-B exposed Purpleputtu seedlings clearly revealed an enhancement of both the *PAL* and *DFR* gene specific transcripts (Figure 20A, a-f, upper and lower panel respectively). It also appears that *PAL* transcript begins to show immediately after 30 min of treatment and showing distinct peak between 12 and 16 h. The PAL enzyme profiles of the induced seedlings also show a similar pattern (Figure 9). Further experiments are needed to correlate these two phenomena. On the contrary, the *DFR* transcript increases from Oh to 16h in induced seedlings. It is interesting and at the same time puzzling that DFR is detected although at the reduced levels, in darkgrown seedlings. It is possible that there is a second *DFR* gene that is associated with the anthocyanin production in a tissue specific manner as in the case of maize. It is speculated that the *DFR* gene is active during germination and early seedling growth. The significance of this observation lies in the fact that flavonoids have multiple properties and are associate with diverse physiological functions during plants' life cycle. The exact role of the *DFR* gene and its products during seedling growth remains to be elucidated.

The UV-B induced *PAL* and *DFR* gene specific expression was also studied in a colorless line namely N 22B and the Northern blot results are shown in Figure 20A, g-l, upper and lower panel respectively. It is clear that while the *PAL* gene specific transcript is not induced in N 22B seedlings. However, it is noticed that the *PAL* transcript detected at dark and 16h which may be due to phytochrome modulation. This is clearly in contrast to the pattern of Purpleputtu. Thus the genotype dependence of

UV-B induced PAL activity is demonstrated. It appears that the *Ilb* influences *PAL* gene transcription since N 22B is homozygous dominant for *Ilb* allele (the leaf blade specific inhibitor).

The PAL is encoded by 3-4 genes in rice (Minami et al., 1989) and it is likely that their promoter regions may respond differentially to photoinduction which results in differential transcript pattern in cyanic (Purpleputtu) and acyanic (N 22B) genotypes. In this context, it is proposed that the promoter of the one of the *PAL* genes (probably the one which responds to UV-B radiation) showed alteration through the action of *Ilb* which resulted in decreased levels of *PAL* transcript and thereby abolished the production of anthocyanins in N 22B. This, however, is purely speculative.

It is interesting that the *DFR* transcript is also inducible by UV-B in N 22B seedlings (Figure 20A, g-l, lower panel). Again the pattern is different from that of the *PAL* profile. Particularly interesting is the observation that the *DFR* transcript is present only at reduced levels in 12h and 16h and this is the stage at which anthocyanin synthesis is rapid in seedlings under UV-B light (Figure 6). Therefore it can be speculated that *Ilb* influences the *DFR* gene transcription that is associated with the biosynthesis of anthocyanins during this stage. It is also possible that the observed differences in *DFR* activity is due to the effect of genetic background (N 22B genetic background is unknown). The reduced levels of the *DFR* transcript in (Figure 19) F1 plants can not be explained simply by assuming the effect of *Ilb* alone. Detailed transcript analysis as well as the structural characterization of regulatory elements of the *DFR* gene is obligatory for elucidating the mechanism of UV-B response and *DFR* gene expression.

5.13 Rice *Actin-1* promoter-driven transcriptional-fusion constructs are made for manipulation of anthocyanin pathway in rice

Plants develop a variety of natural defense strategies to combat pathogen attack and environmental impairments. However, plants often fail to implement these processes due to several reasons including the genotype. The present trend in developing the defense strategies in many plants lies in creating transgenic plants carrying specific engineered genes, which produce novel products to deactivate the causative pathogen and other correction of impaired physiological process. The

secondary metabolites like phenylpropanoids, flavonoids and their derivatives are implicated in plants' resistance by enhancing their endogenous pools. Heritably enhanced accumulation of specific and desired products of these pathways can also be achieved alternatively by using a combination of overexpression and antisense strategies. The approach here is to overexpress or upregulate a gene using sense construct and simultaneously, inhibit or down regulate by antisense sequence of the following gene in the anthocyanin pathway. Such "smart plants" are expected to hyper accumulate specific flavonoid intermediates of interest and thereby resist pathogen attack.

Towards testing the above hypothesis, a series of expression constructs are developed under the *Act* 1 gene promoter for the genes involved in the production of phenylpropanoids and flavonoids including anthocyanins. All these constructs are made in the transcriptional fusion cassette (no ATG codon between the promoter and the *NOS* terminator). The *actin* 1 gene promoter (1.37 Kbp) consists of 840 bp promoter region, 80 bp non-coding first cxon and 450 bp first intron (Figure 22). It is well established that out of eight actin like sequences in rice, the ***actin*** 1 gene *(Act* 1), under the same promoter encodes a transcript that is relatively abundant in all rice cell types and at all developmental stages (Reece, 1988; Reece et al., 1990; McElroy et al., 1990; 1991; Zhang et al., 1991). Actin is a fundamental component of the plant cell cytoskeleton (Seagull, 1989) and hence, it is constitutively expressed in all plant cells. It was observed that the regulatory elements (first exon and intron sequences) are necessary for the maximal *Act* 1 promoter activity in transient assays in rice protoplasts. The *actin* 1 based vectors express the chimeric genes of interest 5-10 and 10-20 times more efficiently than expression vectors containing the maize *Adh* 1 promoter and the CaMV 35S promoter respectively (Zhang and Wu, 1988).

A complete repertoire of transcriptional fusion constructs for anthocyanin producing genes were made (Figure 21, 23 and 24). Sense constructs were made using full length coding sequences. These constructs will be used to over express or to up-regulate the desired genes. The antisense constructs were made using small restriction fragments mostly from the 5' end of the coding sequences. The biosynthesis of anthocyanin pigments in plants involves activity of many genes and is an ideal system to study the inhibition of gene expression by antisense RNA. If one of the enzymatic

steps does not take place because the enzyme is not made (antisense inhibition), the biosynthetic pathway is interrupted resulting in the accumulation of the respective flavonoid intermediate. Van der Krol et al., (1988) were the first to show that the inhibition of the expression of endogenous *CHS* gene (the key enzyme in the pathway) in transgenic *Petunia* plants carrying a small fragment of ***CHS*** cDNA in antisense orientation. Cannon et al., (1989) tested the antisense RNAs complementary to the 5' half of the *GUS* mRNA in transgenic plants and the results showed that the smaller antisense gene segments are more effective in their ability to suppress the corresponding gene expression. Therefore, all antisense constructs were made with the small restriction fragments mostly corresponding to the 5' end of the mRNAs. These gene constructs can possibly be used in altering the shades, texture and intensity of color, tissue specific distribution and the composition of the responsible pigments in the variety of plant species including ornamentals.

Summary

1. Existing variation in anthocyanin-pigment distribution was analyzed in indica rice lines. These are selfed and made true breeding. The tested rice lines were classified into four classes based on the type and distribution of pigment in different tissues. Genotypes of these rice lines were predicted from their phenotypic analysis followed by known genetic nomenclature "C *A P*" system used for japonica subspecies. These genes are tentatively described as structural genes (C , *A)*, regulatory genes *(P, Pl*) and inhibitory genes *(IPl-*\ to *IPl-*6).

2. Specific *Pl* alleles of the *Pl* locus are mainly responsible for anthocyanin pigment distribution in rice. The class 1 lines (Purpleputtu and G 2237) contain Pl^{W} allele as these are pigmented throughout the plant body including pericarp. The class II lines, carry either *Pl* (R 27 P) or Pl^{i} (G 962, TN 1013 and Crossa) or *Pt* (N 22W) allele as they show non-pigmented pericarp and pigmentation one or the other tissues. The class III, Whiteputtu and Hamsa being non-pigmented (acyanic) either carry null alleles of the regulatory genes or more likely recessive alleles of the structural loci. The class IV, N 22B showed brown pigmentation in pericarp (hence classified separately) and carries *Pt* allele.

3. The F1 progeny analyses of the crosses between Purpleputtu (purple), N 22 W (green) and N 22B (green) revealed the presence of the dominant *inhibitor* of leaf blade pigmentation, namely *Ilb* in N 22W and N 22B genotypes.

4. The F2 progeny of NW X PP and NB X PP segregated for leaf blade color in a dominant inhibitory interaction (13 green : 3 purple) with the following genotypes; ***Ilb*** Pl^{W} (9, green), *Ilb* Pl^{+} (3, green), ***ilb*** Pl^{W}(3, purple) and ***ilb*** *Pt* (1, green). The genotypes of Purpleputtu ***(ilb*** Pl^{W}), N 22W (*Ilb Pt)* and N 22B *(Ilb Pt)* in terms of anthocyanin production were confirmed.

5. Based on spectrophotometry, thin layer chromatography and proton NMR spectroscopy of hydrolyzed methanolic extracts of rice tissues, the major and minor anthocyanidin pigments were determined as cyanidin and peonidin respectively. Further, the minor pigment, peonidin accumulates in significantly higher amounts in pericarp tissue.

6. Rice lines, G 962 and N 22B accumulating leucoanthocyanidins and proanthocyanidins respectively in pericarp tissues were identified. In these mutants, the

conversion of leucoanthocyanidin to anthocyanidin, mediated by anthocyanidin synthase, was blocked. It is concluded that the accumulation of proanthocyanidins is also due to a block at the conversion of leucoanthocyanidin to anthocyanidin. Most likely that the oxidative activity of anthocyanidin synthase *(anthocyanidin synthase* encode an NADPH dependent oxidoreductase) is blocked in the brown pericarped rice since the oxidation or oxidoreduction products of this reaction viz., phlobaphenes or cyanidin respectively were absent.

7. Effect of sunlight on anthocyanin biosynthesis in 15 rice genotypes was tested and accordingly classified them into three groups based on the extent of anthocyanin (red color) pigment accumulation in shoots of young seedlings. These are cyanic (accumulates copious amounts of anthocyanins), moderately cyanic (moderate amounts of anthocyanins) and acyanic (no anthocyanin synthesis).

8. The induction of anthocyanins in Purpleputtu was primarily mediated by sunlight as evident by the fact that the Purpleputtu seedlings grown for 5 days under cool fluorescent lights accumulated greatly reduced amounts of anthocyanins.

9. The 4d-old etiolated seedlings are most responsive (production of anthocyanins) to sunlight and a 30 min exposure to sunlight saturated the response which leads to a massive accumulation of anthocyanins with a peak at 24 h. The anthocyanins induced by sunlight are the same as those found in plants grown in continuous light.

10. The induction of anthocyanins in Purpleputtu seedlings is triggered by the UV-B component of SL. This conclusion was derived from the observation that the seedlings exposed to SL-filtered through window glass (WG), which cuts off UV-B component of the SL, RL, FR or BL completely lack anthocyanin pigments. Further, there is no induction of anthocyanins under any of the light treatments in Blackputtu.

11. The SL-mediated anthocyanin accumulation is modulated by RL and FR pulses. In rice, UV-B plays the primary role in the induction of anthocyanins and phytochrome modulates its response.

12. The UV-B responsive anthocyanin biosynthesis in rice is mediated through a specific phase of phenylalanine ammonia lyase activity. The SL mediated induction of PAL showed two peaks, peak-I at 4h and peak-II at 12h in shoots of Purpleputtu and only peak-I at 4h in shoots of Blackputtu seedlings. The other light treatments including WG, RL, BL, and FR indicated that only peak-I at 4h can be inducible in both the cultivars and is phytochrome dependent as evidenced by its reversible nature with

FR pulse. The peak-II at 12h in Purpleputtu is independent of FR pulse (not phytochrome dependent) and hence, UV-B light dependent. The photoinduction of peak-II of PAL and thereby accumulation of anthocyanin pigments depends exclusively on UV-B light. A correlation between the induction of peak-II of PAL activity and anthocyanin formation is also supported by the observation that peak-II is restricted to the cyanic Purpleputtu line.

13. In order to obtain cDNAs of early UV-B induced proteins as well as enzymes and regulatory protein factors involved in the anthocyanin pathway, a cDNA library was prepared from UV-B treated 4-d-old etiolated seedlings using λ-ZAP and XL1-Blue MRF' host system (Stratagene).

14. As a first step towards the characterization of the anthocyanin pathway genes in rice, the library was screened and the rice homologues of the *Zea mays A\, A2* and *C* flavonoid genes were isolated and partially characterized. These are: *Os-DFR* (1.5 Kbp), *Os-ANS* (1.6 Kbp) and *Os-cMyb* (1.4 Kbp) encoding anthocyanidin synthase, dihydroflavonol reductase and a regulatory protein factor respectively. The nucleotide sequence comparison between the rice cDNAs namely *DFR, ANS* and *Os-cMyb* and the corresponding *Zea mays* cDNAs revealed extensive homology (above 70%).

15. Southern and Northern analyses *of anthocyanidin synthase* gene were performed in rice. Genomic Southern analysis revealed that atleast two sequences with extensive homology to *Zm-A2* are present in the rice genome. The *Ans* gene specific transcript is inducible by UV-B light. The expression of *anthocyanidin synthase* gene is abundant in cyanic rice lines compared to acyanic genotypes. The presence of this transcript in various tissues of PP was demonstrated.

16. Differential expression pattern of the *PAL* and the *DFR* genes was observed in PP and N 22B seedlings. The *PAL* and *DFR* gene expressions are enhanced by UV-B in PP. The expression of the *PAL* gene transcript is totally absent in NB seedlings. The *DFR* transcript level was also affected in NB. The results revealed that the induction is genotype dependent and it is speculated that these changes are due to the *Ilb* allele.

17. The rice *Actin* 1 gene promoter-based transcriptional fusion expression constructs were developed for the molecular manipulation of anthocyanin pathway genes towards disease resistance in rice. These include both sense and antisense constructs for the almost entire set of the anthocyanin pathway genes the *A\, A2, Bz1, C\, C2, R, P* and *PAL*.

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