Anthocyanin Pigmentation in Rice *(Oryza sativa L.)*: Towards Genetic Engineering of the Pathway for improved Stress Resistance

A thesis submitted for the degree of
Doctor of Philosophy

By

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CERTIFICATE

This is to certify that Gandikota Madhuri has carried out the research work embodied in the present thesis entitled *"Anthocyanin Pigmentation in Rice (Oryza sativa L.): Towards Genetic Engineering of the Pathway for Improved Stress Resistance"* for the degree of **Doctor of Philosophy** under my supervision in the Department of Plant Sciences, School of Life Science, University of Hyderabad.

This work has not been submitted for the award of any degree or diploma of any other University or Institute.

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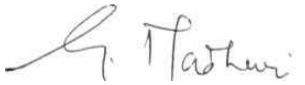
DECLARATION

I hereby declare that the work presented in this thesis entitled ***"Anthocyanin Pigmentation in Rice (Oryza sativa** L.): **Towards Genetic Engineering of the Pathway for Improved Stress Resistance"*** has been carried out by me under the supervision of **Prof. Arjula Ramachandra Reddy** in the Dept of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad - 500 046, and that this work has not been submitted for any degree or diploma of any other University or Institute.



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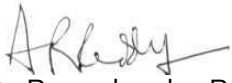

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Dedication

This work is dedicated to my parents,

Lokapavani** and **Narayana

with profound gratitude for sustaining, nurturing , and sharing dreams with me, and finally allowing me the risk of going after those cherished dreams. Their constant support made me believe that dreams need not die, that faith and hope

can still live in

the present age of anxiety. A note of appreciation is extended to my sisters

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for their belief in me and above all bearing with my untimely lifestyle. To my supervisor

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Gandikota Madhuri

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Abbreviations

ABA	Abscisic Acid
Amp	Ampicillin
APS	Ammonium Persulphate
BCIP	5-bromo 4-Chloro-3-Indolyl phosphate
Bz2	*Bronze-2*
Chs	*Chalcone synthase*
EDTA	Ethylenediamine tetra acetic acid
GSH	Glutathione
GST	Glutathione-S-Transferase
Hyg	Hygromycin
kb	Kilobase
kDa	Kilodaltons
MOPS	4-Morpholinepropanesulfonic acid
Mw	Molecular Weight
NBT	Nitroblue tetrazolium
nm	nano meters
PAGE	Poly acrylamide gel electrophoresis
PAL	Phenyl alanine ammonia lyase
PEG	polyethylene glycol
PVP	Polyvinyl pyrrolidine
SDS	Sodium dodecyl Sulphate
TEMED	N,N,N,N (Tetramethyl) Aminomethyl ethylene Diamine
Ubi	*Ubiquitin*
Hph	*Hygromycin phosphotransferase*

1. INTRODUCTION

1. Introduction

Nature has bequeathed upon plants many valuable life assets such as flower color, fragrance and capacity to survive under unfavorable conditions such as diseases, drought, cold and heat, to name a few. Plants, with their sedentary life style, have developed various means of survival through a long history of evolution. These strategies include development of various shades of color for pollination, synthesis of a milieu of compounds involved in many biological phenomenon such as signal transduction, symbiotic associations, biotic and abiotic stress responses and as modulators of various plant metabolic processes. Accordingly, plants invest huge amounts of energy expenditure for the synthesis of secondary metabolites in a species-specific manner. Such adaptive metabolites include flavones, flavanones, dihydroflavonols, isoflavonoids, aurones, leucoanthocyanins, proanthocyanins and anthocyanins synthesized by a complex pathway generally termed as flavonoid pathway. The pathway that leads to the production of vividly colored anthocyanins though various intermediates and by products (mentioned above) is broadly referred to as the anthocyanin pathway.

Anthocyanin pathway has been rigorously investigated in many plants, with maize serving as the model cereal plant. Other plants include *Antirrhinnum* and *Petunia,* both dicots. The genetic analysis of this pathway in these plants revealed a regulatory hierarchy that governs the temporal and spatial expression of the pathway. Structural and functional elucidation of the individual genes governing the pathway-has become possible mainly due to the developments in transposon tagging and new strategies of gene cloning. The functional analysis was accelerated mainly because of the availability of wealth of mutants in maize, both spontaneous and transposon induced. The pathway was finally dissected by a combination of both genetics and molecular biology and transposon tagging.

With the availability of well characterized genes, gene products and reproducible transformation procedures in several plant species, it is now possible to isolate corresponding genes from target plants and investigate the structure/function

and regulation both in heterologous and homologous systems. Most importantly, it is now possible to utilize these regulatory genes of one species to drive the expression of the pathway in other plants. In essence, these developments opened the new era of metabolic engineering in plants. This approach becomes even more relevant here as flavonoids have been increasingly implicated in survival functions of plants.

The functional role of flavonoids and their derivatives in plants including rice is still to be established unequivocally. In fact, we have had no prior information on molecular biology of flavonoid pathway in rice- Asia's most important crop plant that supplies the bulk of dietary calories to more than a billion people. Recently, our laboratory has begun the molecular genetic analysis of the anthocyanin pathway in rice with the objective of genetic engineering of rice for improved stress resistance. Several cDNA clones encoding enzymes and regulatory proteins have been isolated and sequence characterized. An experimental system of UV-B inducible anthocyanin production has been established (Reddy *et al.*, 1994; 1995; 1998: Reddy 1996).

Our earlier studies show an increase in PAL (Phenylalanine Ammonia lysase) activity in response to UV-B stress (Reddy *et al,* 1994). The elevated levels of the transcripts of *Pal, Dfr* and *Ans* (Reddy 1995) were accompanied by an increase in the purple anthocyanin pigments. Accumulation of anthocyanins as a consequence of UV-B stress is direct evidence that these compounds have prophylactic role in protecting the plants from UV-B mediated free radical damage. The present work further extends the UVB studies in rice particularly its influence on the expression of CHS (Chalcone synthase) and GST (Glutathione S transferase).

Owing to the non-destructible nature of flavonoids, they are used extensively as visible markers in transformation experiments. Such examples include. use of maize *C1* and *R* in *Arabidopsis* and *Nicotiana* (Lloyd *et al.*, 1992), maize *Lc* in *Arabidopsis*, tomato and *Petunia* (Quattrochio *et al,* 1993; Goldsbrough *et al.*, 1996; Moonney *et al,* 1995). In additon, in view of the proposed role of the involvement of flavonoids under stress, the pathway genes were employed in metabolic engineering strategies. Such an example of pathway engineering, as a consequence of the

introgression of the novel steps of pathway in plants is shown by the transfer of ***Sts*** gene from grape to tobacco, exhibiting protection against the bacterial pathogen *Botrytis cinerea* (Hain *el al.*, 1993). Similar *Sts* trasngenic were shown to offer protection against *Phytophthera infestans* in tomato, and potato (Thomzik 1995; Stahl 1994) and also in rice against *Pyricularia oryzae* (Lorenzen, 1997).

Metabolic pathway engineering has emerged as an important tool in improving the plants' internal machinery to combat adverse stress conditions. Flavonoid/ anthocyanin biosynthetic pathway controlled by well-characterized structural and regulatory genes with their allelic series governing specific biochemical steps offers unique advantages for such genetic engineering exercises. The goal of such exercises would be to enhance the accumulation of the beneficial intermediates of the pathway, by sense over expression or antisense suppression, which can potentially offer stress resistance in plants.

The major objective of the dissertation is to analyse the anthocyanin pathway in rice and to develop transgenic rice plants with the over-expression of flavonoid genes to test the functional role of the flavonoids in stress response.

The specific objectives are as follows:

1. Identification and analysis of GST in rice.
2. Functional characterization of a cDNA sequence. *CT24* of maize (a partial homologue of *Bz2* encoding GST) by expressing in *E.coli.*
3. UV-B induced enhancement in the activity of target enzymes GST and CHS and its relation to anthocyanin production.
4. Transformation of rice with target genes of maize using constitutive promoters.

- Development of plant expression vectors for structural and regulatory genes using different promoter sequences, from both plant and viral origin.
- Development of a transient expression system for transformation experiments.
- Development of stable transgenic lines carrying over-expression constructs in order to test the premise of the role of preformed intermediates in conferring disease resistance.
- Molecular analysis and evaluation of the developed transgenic plants.

2. LITERATURE REVIEW

2. Literature Review

There are three major routes for the production of secondary metabolites in plants: the shikimate pathway through which flavonoids and anthocyanins are produced, isoprenoid pathway leading to the production of alkaloids, steroids, terpenoids, carotenoids etc., and the polyketide pathway for the synthesis of aromatic compounds. Of the three pathways, shikimate pathway is the major defense pathway in plants by which the phenyl propanoids and flavonoids are synthesized forming the bulk of metabolites. The genetic/chemical attributes of the flavonoid gene pigment system, particularly in maize offered unique advantages to study gene expression, regulation, and the resulting phenotypic diversity observed in higher plants. Flavonoid biotechnology, today, is one of the active areas of research, contributed to the spectacular progress in advancing the knowledge of secondary biosynthetic pathways in general.

The present literature survey deals with the general features of the flavonoid/anthocyanin pathway which includes the structural aspects of the compounds, genetics, molecular biology and genetic engineering of this pathway in plants. In addition, the physiological relevance of the products of this pathway is described with emphasis on the role of these compounds in protecting plants under adverse conditions, such as UV-B stress, and pathogen attack. Further, recent advances in manipulation and genetic engineering of flavonoid pathway with reference to floral color modification, reporters in plant transformation, and in development of new strategies for improved defense response in plants are mentioned.

2.1 Structure and Function of Flavonoids

2.1.1. Structural diversity

Higher plants exhibit remarkable variation in flower, fruit and foliage color largely due to the underlying diversity of flavonoids. The chemical identity of flavonoids/anthocyanins and the consequent phenotypic expression has been worked out initially using standard organic chemical procedures, later followed by the high resolution physical and analytical procedures such as various forms of chromatography,

spectroscopy, including proton NMR and mass spectroscopy. Of the thousands of structurally different flavonoids produced in higher plants a given plant species normally accumulates a definite combination of a few of these molecules.

Anthocyanins are the brightly colored compounds belonging to the general class of flavonoids. Chemically, anthocyanins are classified as water-soluble glycosides, which arc the derivatives of polyhydroxyl and polymethoxyl compounds of 2-phenylbenzopyrylium (flavylium cation). They are derived from a flavonoid molecule consisting of a typical A-ring benzoyl and B-ring hydroxycinnamoyl system composed of three planar rings A. C and B as shown in (Fig 2.1.1). While the B ring of the flavonoid skeleton (Fig. 2.1.1) originates from the phenylpropanoid pathway, the A ring is derived from acetyl-malonyl pathway. The precursors of the flavonoid pathway are Malonyl-Co A (obtained by the condensation of acetyl-Co A and carbondioxide catalysed by acetyl Co A carboxylase), and 4-coumaroyl CoA (obtained from Shikimate/ phenylpropanoid pathway). Most classes of flavonoids derived from this *C15* precursor show similar A ring hydroxylation pattern at 5 and 7 position but differ widely in the B and C ring substitutions. Hydroxylation, methylation, methoxylation, glycosylation, acylation and oxidation / reductions in the C and B ring produce a bewildering array of flavonoids The occurrence and characterization of such structurally diverse flavonoids among wide range of plant genera have been recorded and reviewed extensively (Harborne, 1993).

On the basis of their structural diversity, and the oxidation level of the central pyran nucleus, flavonoids are broadly classified into 12 groups Chalcones, aurones, flavones, flavonols, flavanones, dihydrochalcones. catechins, flavan-3-4-diols, bi-flavonoids, iso-flavonoids, proanthocyanidins and anthocyanins (red/purple and blue pigments).

Different classes of the above group of compounds are specialised to perform distinct chemical, physical and biological properties. For instance, anthocyanins are brightly colored and relatively stable but not physiologically very active, whereas flavonoids, are not so bright in appearance but are involved in several physiologically

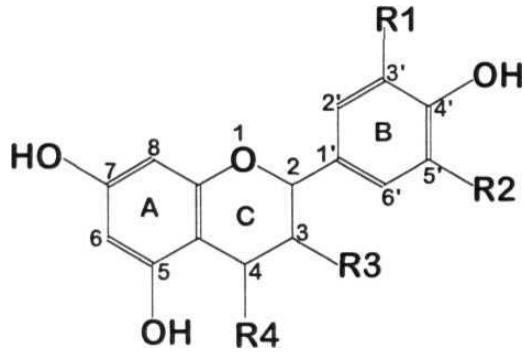


Fig 2.1.1. The basic flavonoid molecule. Modification of the **'C'** and **'B'** give rise to an array of flavonoids.

Flavanone, R3-H, R-4-=O
Dihydroflavonol, R3-OH, R4—O
Leucoanthocyanidin, R3-OH, R4-OH
Anthocyanidin, R3-OH, 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-=O, R4-=O, C2=C3
Monohydroxyl flavonoid (e.g., pelargonin), R1-H, R2-H
Dihydroxyl flavonoid (e.g., cyanin), R1-OH, R2-H
Trihydroxyl flavonoid (e.g., delphinin), R1-OH, R2-OH

significant functions. In addition, plants are known to synthesize a range of natural products, from phenylpropanoid/flavonoid pathway precursors and intermediates that are structurally related and share common biosynthetic enzymes. Such compounds include complex lignin's - structural polymers predominantly found in xylem cell walls, anti-microbial phytoalexins including isoflavonoids and furanocoumarins, organic acids and their esters (Harborne 1993).

The extensive structural diversity of flavonoids found in plants suggests that they have definite functions in plants. Originally, the main function of flavonoids in plants was hypothesized to be their role in protection from UV-B damage Later, a range of functions have been attributed to flavonoids - mostly on the basis of experimental evidence from several plant genera In few cases, however, circumstantial evidences were the basis for assigning new functions. Such attributed functions include, fundamental biological processes, such as, attracting birds and insects for pollination, (the color factor), modulation of hormone responses and free radical scavenging. Most importantly flavonoids are reported to have a role in plants defense mechanism against diseases (viruses, bacteria, fungi) and pests. Further, flavonoids were also implicated in pollen viability and fertility and as signal molecules in various transduction pathways including those associated with abiotic stress responses.

2.1.2 Flavonoid biosynthesis

The biosynthetic pathway leading to the production of flavonoids and anthocyanins have been described in detail in many plants. The biochemical steps and the responsible enzymes have been identified, characterized and reviewed (For. e.g. Heller and Forkmann. 1988). To date, the biochemistry of flavonoids in rice has not been rigorously analyzed. Among cereals, unlike rice only maize flavonoid biochemistry is known. The flavonoid biosynthetic route has two component pathways, the phenylpropanoid pathway and the flavonoid pathway. A simplistic version of the pathway is shown in Fig.2.1.2. The first step in the flavonoid pathway is the de-amination of phenylalanine to transcinnamic acid by phenylalanine ammonia lyase (PAL). PAL activity links primary metabolism with the phenylpropanoid pathway, the beginning of secondary metabolic pathway. Cinnamate, thus formed is hydroxylated

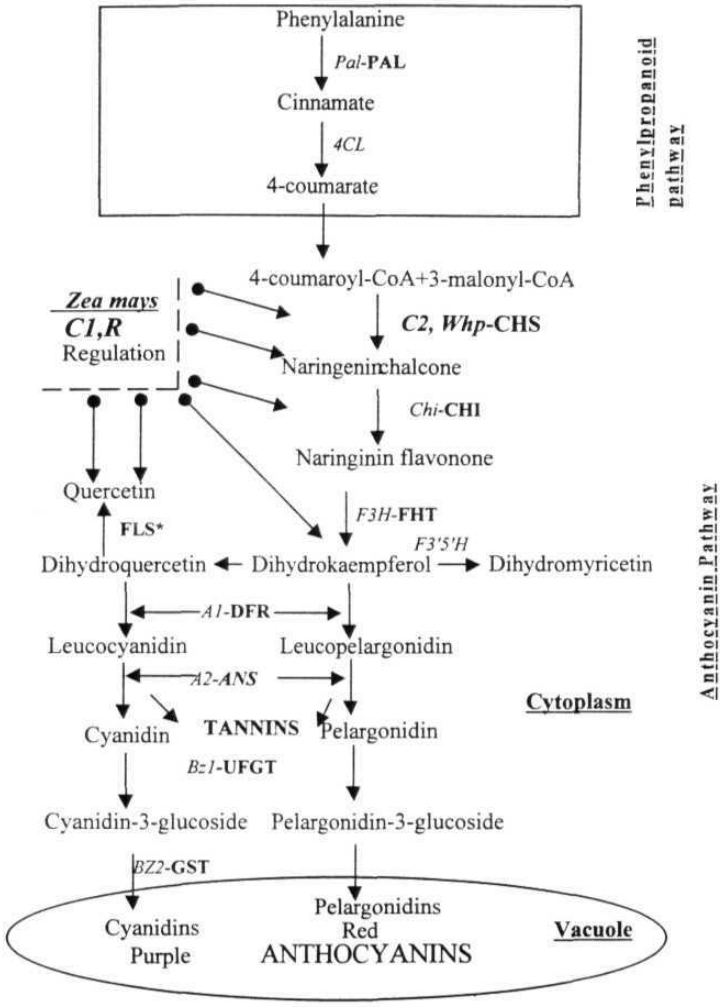


Fig. 2.1.2. Schematic representation of the flavonoid biosynthetic pathway

Genes are represented in italics; Enzymes in Capitals; dotted lines and T represent regulation.

by Cinnamate-4-hydroxylase to form 4-coumarate which is further transformed to 4-coumaroyl Co-A by 4-coumarate Co- A ligase. The above steps constitute the phenylpropanoid pathway , and all the subsequent steps belong to the flavonoid pathway.

Chemico-genetic analysis followed by molecular investigations in various plant species lead to the elucidation of the individual biosynthetic steps of the flavonoid pathway, as shown in Fig.2.1.2. Evidences came predominantly form maize, *Petunia, Antirrhinnum* and *Matthiola*. However, only the maize pathway is explained in some detail in the present context.

The first step in the flavonoid pathway is the condensation of the three molecules of malonyl-Co A and 4-coumarate Co-A to form the first C/5 chalcone intermediate (4,2', 4`, and 6' -tetrahydroxy chalcone) catalyzed by chalcone syntheses (CHS) (Fig.2.1.1.1). Isomerization of this product would lead to the formation of naringenin (2S - flavonone) catalysed by chalcone isomerase There are two types of chalcone isomerases found in nature, one catalysing the cyclization of 6'-hydroxychalcone to 5-hydroxyflavonone. and the other isomerising both 6'-hydroxy and 6'-deoxychalcone to 5-hydroxy and 5-deoxyflavanones. the precursors for iso-flavonoids. Iso-flavonoid formation are catalysed by 2-hydroxy-isoflavone synthase, a mixed cytochrome P450 monooxygenase that is involved in the oxidative rearrangement of the flavanone. with an aryl shifts from the position 2 to 3. Although leguminaceae members are specialized in producing these group of compounds this iso-flavonoid branch pathway is completely missing in cereals.

The hydroxyflavonols are formed from flavanones via hydroxylation at the 3 position catalyzed by the flavanone 3-hydroxylase. These dihydroflavonols are the biosynthetic intermediates in the formation of flavonols, catechins, leucoanthocyanidins, proanthocyanidins and anthocyanidins. Dihydroflavonols are converted to flavan 2,3-trans-3, 4- cis-diols generally called as leucoanthocyanidins, by the enzyme dihydroflavonol reductas (DFR). Leucoanthocyanidns are the colorless active precursors for the synthesis of catechins, proanthocyanidins and anthocyanins. While catechins

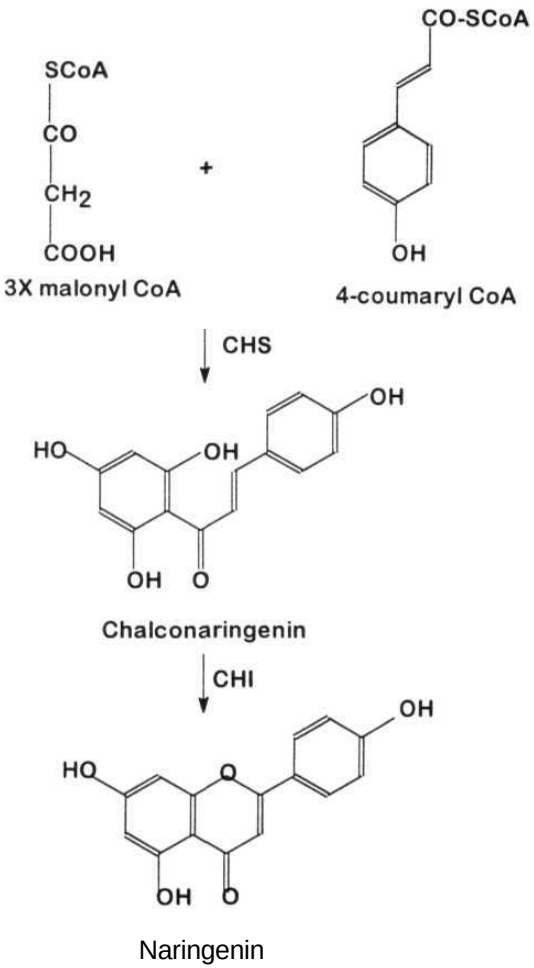


Fig 2.1.1.1. Formation of Naringenin catalysed by chalcone synthase (CHS)

are synthesized from leucoanthocyanidins by the action of flavan 3,4-cis-diol reductase, the proanthocyanidins are formed by the condensation of catechins and leucoanthocyanidins. Polymers of leucoanthocyanidins are also found in many plants.

The exact chemical steps from leucoanthocyanins to anthocyanidins are unknown. Genetic analysis revealed the conversion of leucoanthocyanidin to the corresponding colored anthocyanidin catalysed by the enzyme anthocyanidin synthase, which belongs to a class of plant dioxygenases. This enzyme has been characterized from only a few plant species. The next obligatory step is the glycosylation at the 3' position of anthocyanidin aglycone to form the anthocyanin glucoside, namely, cyanidin-3-glucoside. This 3-O-glycosylation of anthocyanins and flavanols is catalyzed by flanonol-3-glucosyl transferase (FGT). The function of GST in anthocyanin pathway was recently uncovered by Marrs *et al,* (1995). Cyanidin-3-glucoside is the substrate for Glutathione-S-Transferase (GST) which tags the anthocyanins with glutathione and thus mediates its movement into vacuoles. The glutathione conjugates of anthocyanins transiently serve as transport intermediates. The glutathionation of anthocyanins is depicted in Fig. 2.1.1.2. Malonylated anthocyanins are found as the final products in vacuole, which might have been formed as a part of the pathway that removes the glutathione tag in the vacuole

There is not much information about the intracellular site(s) of enzyme assembly and synthesis of various intermediates of flavonoid biosynthetic pathway. Ultra-structural details of the process of synthesis accumulation and mobilization into organelles are lacking. The enzymes of flavonoid and phenylpropanod metabolism seem to be associated with the endoplasmic reticulum (Wagner *et al,* 1984). The flavonoid intermediates are proposed to be channeled through multienzyme complexes, and are organized in such a way that the compound produced by the preceding enzyme in the sequence becomes the substrate for the subsequent enzyme reaction (Hrazdina and Wagner 1985). The PAL activity was located on the lumen of the ER, where it has an easy access to the substrate pool of phenylalanine The next enzyme in the complex, cinnamate-4-hydroxylase, remains embedded in the ER membrane but channels its product, p-coumarate to the cytoplasmic face of ER. The remaining

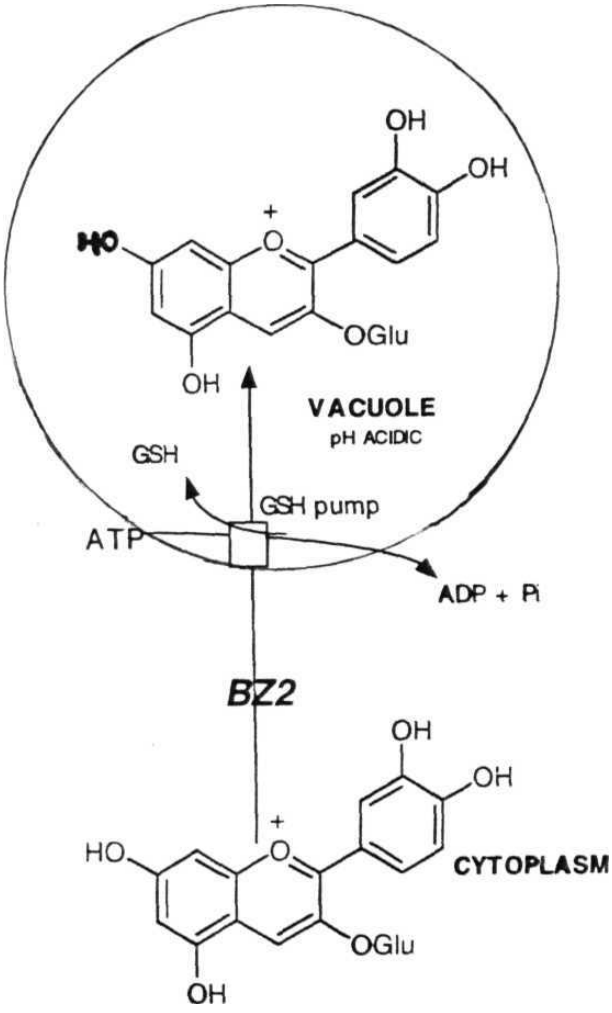


Fig. 2.1.1.2. Formation of glutathione intermediate catalyzed by *Bz2* encoded Glutathione-S-transferase (GST)

enzymes of the pathway like p- coumarate Co-A ligase, CHS, CHI, DFR, are on the cytoplasmic face of the ER and are held by week forces. The phenyl propanoid and flavonoid pathway products are then sequestered in the ER lumen after glycosylation by specific glycosyl transferases present on the lumen face or in the lumen. From here the products are then transported to the plasma membrane or to the central vacuole mediated by GST where the pigments are ultimately localized (Hrazdina and Jenssen 1990).

Since CHS and glutathione-S-transferase are the subject of this dissertation work, a detailed account about these enzymes is reviewed below.

2.2 Glutathione S-transferases in higher plants

GSTs (E.C: 2.5.1.18) have been reported in several plant species GSTs in plants are associated with the detoxification of the potential alkylating agents, including pharmacologically active compounds (Habig *et al.*, 1974). GSTs catalyse the transfer of -SH group of glutathione (γ-Glu-cys-gly; GSH) to the electrophilic sites of a variety of compounds, and thus neutralise and render the product more water soluble. These glutathione conjugates are hypothesized to be metabolized further by cleavage to glutamate and glycine residues, followed by acetylation of the resultant free amino group of cysteinyl residue and the final product mercapturic acid (Boyland and Chasseaud 1969; Wood. 1970).

The natural substrate of GST enzymes in plants, however, remains unclear. One of the well-worked out natural substrate is trans-cinnamic acid (Dean *et ai,* 1991; Diesperger and Sanderman 1979; Edwards and Dixon 1991), an important intermediate of the phenylpropanoid pathway. Phytoalexins are also reported to be natural substrates for GST's (Li *et al*., 1997).

In plants, GSTs are primarily involved in herbicide detoxification and most of the research is focussed on the ability of certain herbicides to serve as substrates for GST enzymes. However, many of the recently discovered GSTs are found to be involved in response processes to abiotic stress conditions.

2.2.1 Classification of Plant GST's

Todate, the recognized sixteen plant GSTs are classified into three major types based on an evolutionary tree and the gene organization, (Droog *et al,* 1995). Type 1 *Gsts* include all maize *Gsts,* characterized by the presence of two introns and three exons. These *Gst's* are responsive to environmental perturbations such as dehydration (Kiyosue, *et al.,* 1993), wounding (Kim, *et al.,* 1994), pathogen attack (Dudler *et al,* 1991), auxin responsive genes such as tobacco *Par-B.* Type II *Gst* have nine introns, and to date there is only one representative example from carnation (Meyer *et al.,* 1991, Itzhaki, and Woodson 1993). Type III *Gst* are characterized with a single intron. Majority of the cDNAs/genes belonging to the type III category are originated from diverse sources, involved in various other functions with significant sequence similarities with known GSTs. Characteristically, the type III GSTs are responsive to numerous environmental stress conditions such as pathogen attack (Taylor *et al,* 1990), oxidative stress (Wingate *et al,* 1988; Levine *et al,* 1994; Tenhaken *et al,* 1995) and heavy metal stress (Czarnecka *et al,* 1988; Hagen *et al,* 1988; Wingate *et al,* 1988; Mauch and Dudler 1993). Examples of this class are heat shock inducible genes such as *Gmhsp26-A* or *GmGST26-A* from soybean (Czarnecka, *et al.,* 1988 Hagen *et al,* 1988), also responsive to a variety of chemicals including 2,4-D. ABA, cadmium and other heavy metals(Droog *et al,* 1995) and a number of other genes belonging to auxin-regulated tobacco genes (Takahashi *et al.,* 1990; 1991; 1995).

The maize *Bz2* belongs to the type III GST, and is responsive to cold stress (Droog *et al,* 1995; Christie *et al,* 1994). In addition, *Nicotiana plumbaginifolia LS 216,* the tobacco *C7,* potato *Prp1* or GST1 and soybean *GmGX1* also belong to this category of *Gsts.* Though the above classification of *Gsts* into different classes is based broadly on the substrate specificity and gene organization, nevertheless, closely related *Gsts* have distinct substrate preferences and conversely, the highly divergent enzymes recognize the same substrates (Alfenito *et al,* 1998). For instance, *An9* and GST III belonging to type I GST and soybean *GmGST26A* (type III) could complement *Bz2* mutation (type III) (Alfenito *et al,* 1998). On the other hand, other type I *Gsts,* including the *Arabidopsis Gst EST H36860* (cloned as most closely related to *Petunia An9),* maize *Gst1* and *GstIV* failed to complement the *Bz2* mutation and restore

pigmentation in transient expression studies, thus indicating the differences in substrate preferences. The proof for the involvement of GST in abiotic stress resistance in shown in transgenic tobacco seedlings over-expressing both glutathione S-transferase and glutathione peroxidase (GPX) cDNAs resulting in an increase in the GST- and GPX-specific enzyme activities showing an improved tolerance to chilling or salt stress (Roxas *et al.*, 1997).

GSTs from pumpkin sarcocarp are identified and biochemically characterized (Fujita *et al,* 1994), however, these GST's could not be included in the above classification due to the lack of information about gene organization

2.2.2 GST's and their role in anthocyanin sequestration

Anthocyanins synthesized in the cytoplasm are ultimately sequestered into the vacuole with the aid of *Bz2* encoded GST which catalyses the formation of anthocyanin - GSH conjugates forming a transfer intermediate allowing the transport into the vacuole and thus impart a deep red or purple color. The glutathionation of anthocyanins is presumed to transport the bioreactive molecules from cytoplasm and actively transport the conjugates through a Mg-ATP requiring GS-X pump (Marrs *et al* 1995).

An9 locus, is involved in the last step of *Petunia* anthocyanin pathway, (Gerats *et al.,* 1982) similar to *Bz2*. *An9* and *Bz2* exhibit extensive sequence divergence. On the other hand *An9* cDNA shows significant sequence similarities with type I GST, which encodes a protein of 26kDa protein showing GST activity. Interestingly, *An9* cDNA was shown to functionally complement maize *Bz2* and produce anthocyanins in aleurone. Other *Gsts* that could complement *Bz2*, though less efficiently are *GmGST26A* and maize GST III (Alfenito *et al,* 1998).

2.3 Genetics and Molecular Biology of the flavonoid pathway

2.3.1. Genetics of the flavonoid pathway

Much of the advances in understanding this pathway in plants is mainly because of brightly colored, non-lethal and visually scorable flavonoid /anthocyanin mutant phenotypes and several transposon induced mutants in maize, which served as "ready

made" genetic variability. In addition, simple and reproducible chemico-physical techniques, coupled with well-established genetic/molecular methodologies have contributed to the identification of genetic determinants governing the synthesis of specific flavonoid molecules.

Genetic-biochemical and molecular analysis of *Zea mays* flavonoid biosynthesis revealed functionally distinct classes of genes dispersed in the genome, acting sequentially in a temporal and spatial manner (Coe *et al,* 1988; Neuffer *et al,* 1997). Broadly, the maize genes involved in the anthocyanin pathway can be grouped into three classes. Class I genes are structural genes encoding enzymes that control the single steps in the biosynthesis and subsequent modification Class II genes are the regulatory genes encoding transcription activator proteins that interact among themselves and with the promoter elements that switch on the whole pathway or parts of the pathway, and determine the tissue specific distribution of the pigments. Lastly, class III genes are largely known as modifying genes that influence the flavonoid concentration. intensity and distribution of pigments Most of the known flavonoid genes are thus classified into one or the other class and exhibit clear phenotypes. Gene-enzyme relationships of the individual step reactions in the pathway have been well characterized in maize, *Petunia,* and *Antirrhinum.* Almost all the genes involved in the pathway in these plants are isolated and characterized enabling one to understand the molecular basis of expression and regulation.

In maize, the well characterized structural genes include, the *Pal* (phenylalanine ammonia lyase), the *C2* (chalcone synthase), the *CHI* (chalcone isomers), the *A1* (dihydroflavonol reductase), the *A2* (anthocyanidin synthase). the *Bz1* (flavonoid 3-O-glycosyltransferase) and the *Bz2* (glutathione -S-transferase). Activity of all the enzymes are obligatory for full expression of color phenotype and absence of any one of the enzyme leads to non-color phenotypes. For instance, maize tissue devoid of F3GT, and GST is brown, where as the wild type is purple. Dominance-recessive relationship between alleles at individual loci, complementation pattern between non-allelic gene loci and the regulatory interaction all leading to predictable phenotypes have been elegantly demonstrated in maize (Coe *et al,* 1981; Neuffer 1997). For instance, in

maize, the regulatory loci, *B* (booster), *C1* (colored-1), *P* (pericarp), *Pl* (plant color), *R (red)* and *Vp1* (viviparous) encode trans-activating factors governing the expression of the pathway genes (Forkmann 1993; Neuffer *et al,* 1997) Well-characterized regulatory genes in *Antirrihinum* include *Delila, Eluta* and *Rosea* (Martin *et al,* 1991) and *An], An2, An3, and An4 in Petunia (Gerats et al.,* 1982b; Beld *et al,* 1989).

In addition, a range of mutants belonging to individual steps of the flavonoid pathway are characterized enzymatically in several ornamental plants which lack clear cut genetic information. For example in *Gerbera, Dahlia, Helichrysum, Sinningia cardinalis, Saintpaulia* and *Zinnia* a deficiency or reduction of CHS enzymatic activity has been demonstrated (Forkmann *et al,* 1989). Similarly, *Petunia* RL101 line is a mutant for *Dfr* gene (Meyer *et al,* 1987).

The structural genes, enzymes, biosynthetic steps, and the regulatory features have been clearly elucidated in several other plant species such as barley (Jende strid *et al,* 1993; Kristianten 1984), *Arabidopsis* (Shirley *et al,* 1993; 1995; Martin *et al,* 1991; Feinbaum and Ausubel 1988; Feinbaum *et al,* 1991; Kubasek *et al,* 1992). *Antirrihinum* (Martin *et al,* 1991; Jackson *et al,* 1992) and *Petunia* (Wiering 1974; de Vlaming *et al,* 1984; Cornu *et al,* 1990; Van Tunen and Mol 1991, Gerats *et al,* 1992). In maize, *Petunia* and *Antihirrinnum,* the regulatory genes, their gene families have been isolated, and the molecular phenomenon of regulation has been extensively reviewed in maize (Coe *et al,* 1988; Dooner *et al,* 1991; Neuffer *et al,* 1997) *Petunia* (de Vlaming *et al,* 1984) *Antirrihinum* (Gerats *et al.,* 1992; Martin and Gerats 1993) and an overview of all the above plants can be found in (Holton *et al,* 1995). Table 2.1 summarizes the available information on the structural genes in representative plants.

In rice, the information on purple/red pigmentation has been restricted largely to the phenotypic descriptions, the inheritance of specific loci governing the pigmentation pattern and their classical map positions (Ramaih and Rao 1953; Kinoshita and Maekawa 1986; Kinoshita and Takahashi 1991; Reddy *et al,* 1994; 1995; 1996a; 1998: Reddy 1996). The anthocyanin gene pigment system in rice is not clearly elucidated Based on the limited information, the rice anthocyanin pathway is explained

to consist of structural genes, the C (Chromogen), *A* (Activator) *Rc* and *Rd* (determining the brown pericarp), and the regulatory genes *P* (purple) and *Pl* (purple leaf), with a number of alleles, determining the distribution of purple pigments in various plant organs. Apart from the above-mentioned genes, rice genome has a class of dominant inhibitor loci that eliminate color expression in diverse tissues. In the presence of these inhibitor genes, a typical wild type dominant anthocyanin gene behaves as a recessive. The presence of such multiple inhibitor alleles is an interesting feature in rice, though the significance of this observation is obscure at present Detailed phenotypic effects of the inhibitor alleles in rice have been described (Reddy *et al*, 1995) though the mechanism of action is not yet clarified. Such a dominant inhibitor allele, the *C1-I* which eliminates color in heterozygous state, in maize tissues, has been extensively characterized (Coe *et al*, 1962; Paz -Ares *et al*, 1987;1990). The *Cl-I* protein is a truncated one arising out of an insertion of a 8bp sequence in the third exon coding the C-terminus of the *C1-I*protein The truncated version is short by 21 aminoacids (Paz-Ares *et al*., 1990).

Recent studies on biochemical and molecular aspects of the pathway in rice are beginning to add much needed information to this pathway. The rice pathway has been demonstrated to be UV-B responsive and this property has been exploited by the molecular analysis of the pathway (Reddy *et al*, 1994). Several structural cDNA sequences have been cloned and sequenced (Reddy *et al*, 1996; 1998). Table 2.1 includes the status of rice structural genes isolated and characterized so far. Such studies have naturally paved the way for genetic engineering of rice for altered pathway (Madhuri *et al*, 1998).

2.3.2 Molecular biology of the flavonoid biosynthesis

Maize is a model system to understand the molecular basis of anthocyanin synthesis, accumulation and metabolism in plants. Anthocyanin pigments owing to their brightly colored visible phenotype enabled one to decipher the physiological/biological meaning of this pathway in plants, to dissect various molecular genetic phenomenon such as transposon activity, breakage-fusion bridge cycle, genetic and cytologic crossing over, and also served as markers in molecular mapping and

Table 2.1. Structural genes encoding anthocyanin biosynthetic enzymes

Enzyme	Maize		Rice		*Petunia*		Barley	
	Locus	Clone	Locus	Clone	Locus	Clone	Locus	Clone
Chalcone synthase (CHS)	*c2* *Whp*	+ +	*chs*	+	NA	+	-	
Chalcone isomearse	NA	+	NA		*Po*	+		
Flavonone 3-hydroxylase	NA	+	NA		*Ani*	+	*ant* 17? *ant* 22?	+
Flavonoid 3'-hydroxylase	*pr*		NA		*Ht1/Ht2*	+[c]	-	
Dihydroflavonol reductase (DFR)	*al*	+	*Dfr*	+	*An6*	+	*ant* 18	+
Anthocyanidin synthase	*a2*	+	*ans*	+	NA	+	*ant 1?* *ant 2? ant 5?*	
Flavonoid 3-glucosyl transferase	*Bzl*	+	NA		NA	+		+
UDP rhamnose: anthocyanidin -3-glucoside	NA		NA		*Rt*	+	NA	
Anthocyanin acyl transferase (AAT)	NA		NA		*Gf*		NA	
Anthocyanin methyl transferase (AMT)	NA		NA		*Mf1/Mf2*	+[c]	NA	
Glutathione S-transferase(GST)	*Bz2*	+	NA		*An1 3*	+	NA	+

transformation Investigations into the molecular organization of the genes governing the anthocyanin pathway revealed that the structural genes of anthocyanin pathway are under the control of regulatory genes that determine the intensity, and tissue specific expression of the purple color. The protein products of the regulatory genes of *Zea mays,* such as those belonging to the *C/Pl* and *RIB* family encode transcription activators with functional domains interacting among themselves and also the promoters of the structural genes leading to the regulation of gene expression. Such regulatory protein products are related to the *myb* and *myc* class of transcription activators having an acidic domain and a basic bHLH domain The bHLH domain is conserved among various species.

The *Cl* gene product having an amino terminal basic *myb*-homologous domain binds to the DNA of promoters of structural genes while the C-terminus containing the amphipathic acidic alpha helix is thought to be responsible for transcription activating function (Paz-Ares *et al,* 1987). Thus, anthocyanin biosynthesis requires atleast one bHLH protein *{RIB)* and one *Myb* homologous protein (Goff *et al.,* 1992). Another regulatory locus, *P* gene encodes a *myb*-homologous protein that binds and activates a set of structural genes in floral tissues of maize. A different class of regulatory genes such as ***Vpl*** has pleiotropic effects which facilitates seed germination but blocks anthocyanin biosynthesis (Robertson, 1955), *Vpl* regulates anthocyanin biosynthesis in seed by regulating the *Cl* promoter (Hattori *et al.,* 1992). Other regulatory/modify ing genes include the *In,* (Intensifier) which determine the intensity of purple color in aleurone. In maize, the timing, the distribution and amount of anthocyanins in a given tissue are determined by a complex regulatory hierarchy comprising mainly two gene families are the *Cl,* and *R /B* and *In. R* genes constitute a small gene family which includes *R(S)* (Perrot and Cone 1989), *R (Sn)* (Tonelli *et al,* 1991), *R(Lc)* (Ludwig *et al,* 1989) determining the temporal and spatial pattern of anthocyanin accumulation (Coe 1962). Members of the *B* family include *B1-Peru* (Chandler *et al,* 1989), *B1-I* (Radicella *et al.,* 1992). Both *R* and *B* act as duplicate genes (Goff *et al,* 1990). Similarly, *Cl* regulates the expression in kernel, that is scutellum and aleurone of maize while its homologue *Pl* regulates the expression in plant body (Paz-Ares *et al.,* **1986,1987;** Cone ***et al,* 1989;** Scheffler 1994). Also, different regulatory genes in

maize have specific target genes. The transcription of the structural genes *C2, Chi, F3H, Al, A2, Bz1* and *Bz2* is regulated by *RIB* and *C1* genes in various plant tissues (Dooner and Robbins 1991; Bodeau and Walbot 1992; Deboo *et al,* 1995). The *P* locus governs the expression of *C2, Chi* and *Al* in pericarp tissue (Grotewald *et al,* 1994) and in cultured maize cells (Grotewald *et al,* 1998).

In snapdragon, the regulatory mutants of *delila* and *eluta* show distinct patterns of *Chs* and *Chi* mRNA accumulation from those of *F3H* and *Dfr* (Martin *et al,* 1991; Jackson *et al,* 1992) while the regulatory mutants *an1*, *an2* and *an11* affect the anthocyanin levels in *Petunia* floral color (Quattrocchio *et al,* 1993). Moreover, the regulatory loci also govern the type of pigments accumulated. For instance, *C1/R* family of genes control the production of anthocyanins. 3-hydroxyflavonoids, proanthocyanidins and flavonol glycosides, and whereas the *P* locus controls 3-deoxyflavonoids and 3-deoxyanthocyanidins, C-glycosylflavones, and phlobaphanes. Thus, there appears to be specific regulatory elements governing the sets of genes in different parts of maize plant leading to the tissue specific phenotypes. Most of such genes involved in the synthesis and regulation of anthocyanins/flavonoids have been identified, cloned and characterized from a number of plant species [reviewed in Holton and Cornish 1995).

With the elucidation of regulatory mechanisms, the genes and the corresponding enzymes, the genetic engineering of plants for modified pathway has become possible. Again rice lags behind in this area of gene regulation. So far not a single regulatory gene in rice has been shown to specifically influence the anthocyanin expression. The efforts of this lab in that direction yielded the isolation of several rice homologues of maize anthocyanin genes (Reddy *et al,* 1998). Particularly, the prospects of using well defined regulatory genes of model plants such as maize across many other plant species has become indeed a reality. The present work further tested this possibility in rice with success.

2.4 Biological functions of flavonoids

2.4.1 Flavonoids as UV protectants

One of the strategies developed by plants to over come the increased UV-B radiation is the production of UV-B absorbing compounds such as flavonoids and anthocyanins. The production of such flavonoid compounds as a protective measure against damaging UV-B radiation is also seen lower organisms such as ferns and bryophytes (Markham *et al.* 1998;Veit *et al,* 1996). Flavonoids with their light absorbing, light scattering. UV filtration and photo-repair properties protect plants from the damaging UV-B radiation (Rozenna *et al,* 1997; Lois 1994; Tevini *et al,* 1991; Reuber *et* ***al,*** 1993; Li *et al,* 1993). Flavonoids localized in the upper epidermal cells absorb most of the active UV-B radiation and effectively protect the sensitive inner cells from the damaging effects of UV-B radiation (Caldwell *et al,* 1983; Beggs *et al,* 1994). An important physiological consequence in response to UV-B light in plants is thought to be the differential induction of the flavonoid genes, resulting in the production of flavonoids, flavones, iso-flavonoids and anthocyanins (Taylor and Briggs 1990; Jordan 1996; Fiscus and Booker 1995; Reuber *et al.,* 1996; Klapper *et al,* 1996). There seems to be a differential accumulation of classes of flavonoids among UV susceptible and tolerant varieties of rice indicating their role as UV-B protectants rather than simple UV-B screens (Ken Markham *et al,* 1998). There are exceptions to the rule of the involvement of flavonoids in affording plant protection as presence of *Lc* in transgenic *Petunia* did not afford any protection against UV-B. even though increased ratio of quercetin to kaempferol was observed in all the transgenic lines (Ryan *et al,* 1998).

2.4.2 Flavonoids and their role in male sterility and pollen development

One of the important function of flavonoids is the association of flavonoids with pollen fertility. The involvement of flavonoids in pollination was first uncovered in a maize mutant namely *Whp {White pollen)* at the duplicate locus, of the *C2* also encoding the chalcone synthase. While *C2* is expressed in seed and other plant parts, *whp* is expressed in reproductive organs such as anthers, pollen, and stigma Maize plants deficient in *CHS* activity produce white sterile pollen in contrast to wild type yellow pollen (Coe *et al,* **1981).** It is **a** general observation that in maize and *Petunia*

the fertile pollen contain abundant amounts of flavonols (Styles and Ceska 1977; Coe *et al.*, 1981; Koes *et al.*, 1989; Davies *et al*, 1993; Pollak *et al*, 1993; Ylstra *et al.*, 1995)

In maize and *Petunia*, pollen deficient in chalcone synthase and therefore chalcones and flavones are not functional on similar *CHS* deficient stigmas, but are normally functional on wild type stigmas and thus exhibit a phenomenon of **conditional male fertility,** (Coe *et al*, 1981; Pollak *et al*, 1993; Ylstra *et al.*, 1995; Mo *et al.*, 1992.). Similarly, *Petunia* transgenics for *Chs* exhibiting co-suppression or anti-sense suppression are also male sterile (Taylor and Jorgenssen 1992; van der meer *et al.*, 1992). The fertility function in such plants is chemically restored by spraying the infertile pollen with low concentrations of a flavonol, the kaempferol. However, the requirement of flavonoids for pollen fertility/sterility is not universal. Several flavonoid mutants of *Arabidopsis* , including a null mutation with impaired *CHS* enzyme and protein did not affect male fertility (Burbulis *et al.*, 1996; Ylstra *et al.*, 1995; Shirley *et al.*, 1995). Also, a transposon induced mutation in *Antihirinnum* did not effect fertility (Sommer and Saedler 1986).

2.4.3 Flavonoids **and their role in plant** defense

Flavonoids have a key role in stress response mechanisms in plants. There is an impressive body of information on the anti-bacterial, anti-fungal and anti-viral properties of flavonoids indicating the role of these compounds in defense response (Bloor, 1995; Kramer *et al*, 1984; Martin 1995; Beretz *et al*, 1978; Musci and Pragai 1985; Weidborner and Jha 1993; French and Towers 1992; Kodama *et al* 1991). While anthocyanins by themselves are toxic in some plants, the aglycone moieties or other intermediates of the pathway are toxic in others. The accumulation of stress induced prenylated isoflavonoids, isoflavans, furanocoumarins, 3-deoxyanthocyanidins and flavonols was described in detail (Treutter and Feucht, 1990). Deoxyanthocyanidins from *Sorghum* have been shown to be toxic to the pathogen *Colletotrichum graminicola* (Snyder and Nicholson 1990; Tenkuano et al 1993). Sakuranetin, a methylether derivative of naringenin was also implicated in the resistance of rice plants against blast infection (Kodama *et al.*, 1992; Dillon *et al*, 1997). Maysin, a C-glycosylflavone was demonstrated to confer resistance of maize plants to corn earworm *Helicoverpa zea*

(Byrne *et al.*, 1996; Mc Mullen *et al.*, 1995)

Polymerised anthocyanins - proanthocyanidins are also reported to exhibit anti-microbial activity (reviewed in Scalbert 1991). Further, anti-fungal properties of flavonoids have been demonstrated ***in*** *vitro* against many fungal pathogens. Anti-bacterial nature of naringenin was demonstrated *in vitro* against a major rice pathogen, *Xanthomonas oryzae* (Padmavathi 1997). The role of iso-flavonoids, as a class of phytoalexins (low molecular weight anti-microbial compounds) produced due to attempted infections, is shown in Leguminaceae members including *Medicago sativa* (Dalkin *et al,* 1990), soybean (Zacharius *et al,* 1989) and bean (Gnanamanickyam 1977). The rice phytoalexins oryzaalexins and momilactones are shown to inhibit *in vitro* the growth of the fungal pathogen *Magnoporthe griesae* (Cartwright *et al.*, 1981).

2.5 Transgenic approaches to modify secondary metabolic pathways in plants

Metabolic pathway-engineering is aimed at enhancing the production of target compounds in a multi-step biosynthetic pathway. In order to use such a powerful tool, knowledge about the genetic and molecular basis of the entire pathway is absolutely essential. Secondary metabolic pathways in plants are receiving increasing attention as their study offers innovations in agriculture and horticulture besides being important in chemical and pharmaceutical industries. In this context, one of the most thoroughly analysed secondary metabolic pathway in plants is the anthocyanin biosynthetic pathway. Recent evidences indicate that pathway engineering is successfully applied in several plant species to alter the genetic makeup and produce novel flavonoids by introducing the missing gene/function or suppressing the activity of the endogenous gene. Such examples of flavonoid pathway engineering are discussed in the following paragraphs.

2.5.1. Flavonoid pathway and its role in biotic stress

The best example of metabolic pathway engineering is the introduction of grapevine stilbene synthase *(Sts)* gene into tobacco which produced resveratrol in transgenics showing an increased resistance to the bacterial pathogen *Botrytis cinerea* (Hain *et al,* 1993). Further, tomato and potato *sts* transgenics are also reported to

exhibit an improved resistance against the fungal pathogen, *Phytophthora infestans* (Thomzik 1995; Stahl *et al.*, 1994). In addition, preliminary results from the analysis of the *sts*-rice transgenics also showed an improved resistance against *P. oryzae* (Lorenzen *et al.*, 1997).

In addition, the strategy of altering the metabolic flux by sense suppression is demonstrated in transgenic tobacco plants exhibiting *Pa!* sense suppression, showing a decreased levels of phenylpropanoids with a concomitant increase in the disease susceptibility towards *Cercospora nicotianae* (Maher *et al.*, 1994) Different strategies of altering the metabolic flux in plants through genetic engineering of secondary metabolic pathways are under progress in many labs including ours. Such transgenics with engineered pathways would prove to be extremely useful in generating crop plants with improved defense.

Our approach in rice is to alter the flow of metabolic flux of the anthocyanin biosynthetic pathway by directing the metabolism through over-expression or anti-sense suppression to regulate the products of interest In effect, we aim at driving this important secondary metabolic pathway for the desired result- the improved defense response.

2.5.2 Flavonoids and flower color

Structurally diverse flavonoids and anthocyanins contribute to vivid floral colors observed in nature. Often, compounds with defined structures impart the expected color to the organ in which they are localized. For instance, anthocyanins such as delphinidins with three hydroxyl groups in the B-ring impart a bluish or mauve color, while cyanidins with two hydroxyl groups are purple/red, and pelargonidins with one hydroxyl group are pink/scarlet/orange. In addition, such differences in the floral color depends on the chemical nature of anthocyanins, their acylation and methylation status, the pH of the vacuole (in which the pigments are normally located), presence of metal ions, and most importantly, the extent of co-occurrence of other flavonoids.

Flavonoid pathway genes are extensively used to modify floral color of several plant species. For instance, *Petunia* line *RL101* transgenic line carrying the maize *Al*

gene results into a new bright red floral phenotype. This is due to the fact that *RL101* line is deficient in the DFR but has the substrate accumulated in reasonable amounts in the petals upon which the maize enzyme can act (Meyer *et al,* 1987). Other factors like co-pigments influence the final color of these flowers. Further, different phenotypes, ranging from uniformly red to sectored and variegated phenotypes are produced by the *Al* transgene in the subsequent generations due to the hyper-methylation of the introduced gene (Meyer and Heidmann 1994). Interestingly, *Petunia* flowers with the maize *A]* transgene are paler red while flowers carrying the *Gerbera Dfr* are bright, although, both sequences encode dihydroflavonol-4-reductase (Elomaa *et al.,* 1996). Thus, the expression of a single anthocyanin gene, namely *Dfr,* produces a range of flower color phenotypes in heterologous plants.

The *Chs* gene encoding the chalcone synthase, has been used extensively in the modification flower color in ornamentals. The floral color in such transgenics carrying the active chalcone synthase gene was altered through a number of genetic engineering excersises including antisense inhibition, sense suppression, and over-expression to produce a range of variegated *Petunias* (Napoli *et al,* 1990; Jorgensen 1994;1995) or sectored phenotype (Van der Krol *et al.,* 1988). In addition, modification of flower color in *Chs* transgenics has been demonstrated in several ornamental plants such as *Chrysanthemum, Cyclamen, Pelargonium, Lisanthus.* and *Gerbera* (Elomma *et al.,* 1993; Courtney-Gutterson *et al.,* 1994; Elomaa *et al.,* 1996; Davies *et al.,* 1997; 1998).

Other flavonoid genes such as chalcone reductase (*CHR*) are also used in the genetic engineering of floral color. Introduction of the *alfalfa* CHR cDNA under the control of 35S *CaMv* promoter into *Petunia* resulted in the production of 6-deoxychalcones (which are otherwise absent in *Petunia)* thus, changing the flower color from white to pale yellow, and deep purple to pale purple (Davies *et al.,* 1997). Another example of such a diversion of the flavonoid pathway is transgenic *lisianthus,* carrying the *Anntirhinnum FLS* transgene (encoding flavone synthase) that exhibits a novel flower phenotype (Elomma *et al.,* 1993).

Many ornamental flowers, including roses, exhibit varying degrees of red

purple shades largely due to the accumulation of the cyanidin and pelargonidin derivatives. On the other hand, blue roses including other blue ornamental flowers are not normally found in nature. Production of blue roses might be possible by the development of transgenics carrying flavonoid 3',5'- hydroxylase (F3'5'H) gene to produce delphinidin glucosides that may impart blue color, given the appropriate pH and co-pigmentation (Griesbach 1996). Blue colored pigments in *Petunia* have been generated with trade names Surfinia ™ and Blue Moon ™ which are in the process of commercialization. Infact, with the availability of rapidly developing transformation methodologies for rose, the commercial production of blue roses and other such novel flowers would soon be in the market place.

2.5.3 Anthocyanin genes as *reporters* in transformation

Genes encoding the structural or regulatory protein/enzymes of anthocyanin pathway have been used as reporters in transformation experiments in many cell types of maize and a few other plants owing to the visible, benign, dispensable and cell autonomous pigmentation property. Use of the anthocyanin genes as novel visible markers in transformation in maize aleurone was first shown with *Lc* (Leaf color) a member *of R* gene family (Ludwig *et al,* 1990). *Lc,* along with *Cl* from maize under the control of 35S promoter activates the anthocyanin pathway in heterologous plants such as *Arabidopsis* and *Nicotiana* (Lloyd *et al,* 1992). The *R* and *Cl* genes have been extensively used as reporters in maize transformation experiments as their activity results in the production of purple color (Bowen 1992). The maize *Lc* activates the anthocyanin pathway in tomato (Goldsbrough *et al,* 1996) and *Petunia* (Quattrocchio *et al,* 1993; Bradley *et al,* 1998) can complement *ttg* mutation in *Arabidopsis* (Quattrocchio *et al,* 1993). Further, the *R* and *B* genes were used to generate stable sugarcane transgenic lines where the production of purple color was used as a non-destructible marker (Bower *et al,* 1996). Ectopic expression of *Del* (from *Antirrhinum)* also lead to the increased anthocyanin expression in tomato and tobacco using (Mooney *et al,* **1995).** In addition, the ectopic expression of *C1/R* or *P* producing anthocyanins or 3-deoxy flavonoids in Black Mexican Sweet corn cell lines producing anthocyanins (Grotewald *et al.,* 1998).

2.6. Rice as a choice plant for genetic engineering

Rice constitutes the staple food for more than 50% of the world's population Owing to its importance as the primary food source, rice breeding is an important fanning enterprise of the world particularly in Asia Conventional rice breeding strategies have led to the development of many rice cultivars with high yield and other important agronomic traits. However, traditional breeding methods are fraught with several limitations, such as, sexual incompatibility between species and long drawn genetic processes. Advent of genetic engineering and biotechnology provided immense opportunities and scope to overcome these limitations.

Rice plant is emerging as the favorite target for molecular biologists and genetic engineers owing to its smallest cereal genome (0.4x10 bases) with l/10th of size compared to the human genome, and is thrice as complex as *Arabidopsis* genome (Shimamoto, 1995, Terada and Shimamoto 1993) In addition, recent advances in rice molecular biology, such as development of highly saturated molecular maps extensive analysis of expressed sequence tags, cDNA sequences and numerous regulatory genes, coupled with the availability of well established transformation protocols makes it as an ideal cereal for experimental research. Also, rice is endowed with rich germplasm containing considerable genetic variation that can be used in genetic engineering excercises. Further, identification and isolation of several genes coding for resistance to diseases, pests, and abiotic stresses across many crop plants provided ample scope to transfer these traits into rice by genetic transformation. Among the cereals, rice occupies a pivotal position with respect to the application of modern biology tools for improving plant productivity and production.

2.6.1 Genetic transformation of Rice

There is an extensive work in the development of efficient, reliable and reproducible transformation system since the first report of plant regeneration from rice protoplast for *Japonica* by Fujimura (1985) and in ***Indica*** rices by **Lee** ***et al.***, (1989). Significant progress has been made on gene transfer of monocots using direct DNA delivery-methods such as electroporation and PEG mediated transformation of protoplasts (Potrykus, 1991). Several transgenic plants were obtained by the direct DNA uptake of

protoplasts in rice resulting in the successful transformation of *Japonica* rice lines (Toriyama *et al,* 1988, Shimamoto *et al,* 1989). In the meantime, several groups regenerated *Indica* and *Indica*-type varieties and thus developed transformation protocols for *Indica* rices (Christou *et al.,* 1991, Datta *et al,* 1992; Ghosh Biswas *et al,* 1994a, , Peng *et al,* 1992, Xu and Li 1994, Peng *et al,* 1992). There is a varying degrees of success among these labs with the transformation system being labour intensive and also having poor regeneration and/or low fertility mainly arising due to long tissue culture period. Ghosh Biswas *et al,* (1994b) attempted to reduce the long tissue culture period by using the scutellar callus as the source of protoplasts. Paul Christou's group, have developed reasonably reproducible and efficient transformation system using immature embryos.

The advent of the microprojectile bombardment offered a much easier way of transformation of regenerable tissues (Sanford *et al,* 1987). Thus, using microprojectile bombardment a number of *Japonica* and *lndica* rice cultivars have been successfully transformed (Christou *et al,* 1991, Cao *et al,* 1990, Li *et al,* 1993, Sivamani *et al,* 1996). Although, the *lndica* rices turned out to be relatively recalcitrant, alternative transformation methods such as *Agrobacterium*-mediated gene transfer (Chan *et al,* 1993, Hiei *et al,* 1994, Dong *et al,* 1996) using embryogenic callus have also been developed.

Rice *in vitro* culture has strong genotype and culture dependence (Christou 1993). Sivamani *et al,* (1996) have reported the genotype independent production of transgenic rice plants using the biolistic method, while Zhang *et al,* (1996) have reported a genotype and environment independent transformation system for *lndica* rice using embryogenic suspensions. With the development of genotype independent, straight forward regeneration and transformation techniques, it is now possible to introduce genes of interest into rice- a first step for the development of rice via genetic engineering. In the recent past *Tp309* has extensively used as a transformation workhorse to genetically engineer the p-carotene biosynthesis in rice endosperm Burkhardt (1996) demonstrated the genetic engineering of rice towards β-carotene by transforming *Tp309* with phytoene-synthase and phytoene-desaturase gene constructs.

Further, the biochemical investigations upto Rl generation of these transgenic plants revealed the expected enzyme products. Kumpatla *et al.,* (1997), demonstrated the phenomenon of chimerism, gene silencing monocots using *Tp309* as a model plant

3. MATERIALS AND METHODS

3. Materials and Methods

3.1 Chemicals and Reagents

Agar, Agarose, acrylamide, ammonium persulphate, ampicillin, anti-AP antibodies, BSA, CDNB, calf-thymus DNA, EDTA, ethidium bromide, ficoll, glucose, glycerol, IPTG, LiCl, LB medium, maltose, 2-mercaptoethanol, MOPS, NaCl, NZCYM medium, protein molecular weight standards, potassium acetate, PVP, SDS, sodium acetate, sucrose, TEMED, Triton-X-100, Tris, X-gal and all the chemicals used for tissue culture media preparation are obtained from Sigma, USA. Acetone, bromphenol blue, Coomassie Brilliant blue, ethanol, glacial acetic acid, methanol, ninhydrin, ortho-phosphoric acid, polyethylene glycol, sulphosalicyclic acid, trichloroacetic acid were of analytical grade purchased from local companies. DNA labeling kits are from Stratagene, USA or BARC, India. All the restriction enzymes and modifying enzymes such as T4 DNA ligase, CIAP, 1kb ladder, *X* ladder *(Hind III-EcoR1* digest) were obtained from Gibco-BRL Gaitheresburg. MD. Gene clean kit, DNA markers (λ ladder) are obtained from promega, Membranes for nucliec acid transfer (Hybond N+ and Hybond C for Western transfers) were obtained from Amersham, Arlington Heights, IL. Hygromycin is obtained from Calbiochem, La Jolla, CA. Gold and micro-carriers are obtained from BIORAD.

3.2 Plasmids used: The plasmids and bacterial strains used in this work and their source is described in Table 3.2. In additon to the plasmids listed, expression vector *pET CT24* carrying *CT24* cDNA under the control of *T7* RNA ploymerase in *BL21 (DE3)* cells, *pCT24* cDNA were obtained as a gift from Klaus Theres and Gregor Schmitz, Max Planck Institute, Koln.

The plasmids pUOH *Myb C1*, pUOH *Myb* C7(as), pUOH *C2,* pUOH *Myb R(as),* and pUOH *Chs* (as), are the plasmids with anthocyanin genes are obtained from our lab. The plasmid pUOH *A2,,* and plasmid pUOH *R* are from Udo Wienand (Univ of Hamburg's Plasmid collection). pUOH *Bz2* is a gift from Virginia Walbot (Stanford University). Plasmids pMON 999, pAHC17, and p35H are from ILTAB gene bank. pBlueScript KS+ is from Stratagene, La Jolla, CA.

Table 3.2 Description of plasmids carrying specific cDNA sequences

Plasmid	cDNA of interest	Source	Insert size
pUOH Myb *C1*	*C-1* of *Zea mays*	UH	2.5kb
pUOH *C2*	*C2* of *Zea mays*	UH	1.4kb
pUOH Myc *R*	*R* of *Zea mays*	UH	2.5 kb
pUOH *A2*	*A2* of Zea mays	UH	1.2kb
pUOH *Bz2*	*Bz2 of Zea mays*	SU	1.1kb
pUOH*CT24*	*CT24 of Zea mays*	MPI	1.0kb
pAHC17	*Ubiquitin* promoter	ILTAB	2.0kb
pMON 999	E-35S promoter	ILTAB	1kb
p35H	Hygromycin	ILTAB	850bp

UH: All these plasmids are in Actin constructs available at Univ.of Hyderabad, Hyderabad, India;
ILTAB: Internationa] Laboratory for tropical Agricultural Biotechnology, La Jolla, CA..
MPI: Max Planck Institute, Koln, Germany; SU: Stanford University.

3.2.1 Bacterial Strains: *E.coli* strain DH5 α (Gibco BRL, Gaithersburg, MD) was used for transformation experiments. BL21 (*DE3*) cells were used for *CT24* over-expression.

3.2.2 Antibodies: Anti-CHS antibodies *Zea mays* are obtained from Dr. Loverine Taylor, Univ. of Georgia, USA. Anti-*Pug b* a GST antibodies were obtained from Dr. Masayuki Fujita, Kagawa, Japan.

3.2.3 Rice lines: *Indica* and *Japonica* cultivars of rice differing in their anthocyanin pigment intensity were used in the present investigation. The experimental plants were grown in the field or in the net house. All plants are grown in clay soil with continous irrigation. At Hyderabad, India, (longitude 78 ° 4" E; latitude 17 °3' N; altitude 600 meter above sea level) the plants experienced an average day/night temperature of 30.1 °C/20.7 °C and RH ranging between 76 to 60%. The sunlight intensity was about 2800

u mol /m^{-2}/sec. These varieties were procured from various sources as shown in the table and were repeatedly selfed before being and used as experimental material.

Table 3.2.3: Rice lines **and** the anthocyanin phenotypes

Line	Type	Source	Phenotype
Purpleputtu	*Indica*	TNAU, Coimbatore	All parts of the plant are purple
TN1013	*Indica*	TNAU, Coimbatore	Lb+,Ls+,ln+,Lg+ are purple
G962	*Indica*	DRR, Hyderabad	Lb+,Ls+,Lg+, In+, Ap+ are purple
TN1013	*Indica*	TNAU, Coimbatore	Lb+,Ls+,Lg+,In+,Ap+ are purple
N22B	*Indica*	DRR, Hyderabad	All parts are green except pericarp is brown
N22W	*Indica*	DRR, Hyderabad	All parts are green except except apiculus is red
R27W	*Indica*	DRR, Hyderabad	Lb+,Ls+,Co+,Au+,Lg+,In+, Ap+ are purple
Hamsa	*Indica*	DRR, Hyderabad	All parts are green
H113	*Japonica*	HU, Sapporo.	Co+, Au+, Lg+, In+, Ap+ are purple
Tp309	*Japonica*	ILTAB	Genetic composition not known. All parts of the plant are green
IR 8	*Indica*	DRR, Hyderabad	Genetic composition not known. All parts of the plant are green
HRI2	*Indica*	DRR. Hyderabad	Genetic composition not known. All parts are green. Susceptible to *P oryzac*
1R64	*Indica*	DRR, Hyderabad	Genetic composition not known. All parts are green. Resistant to *P.oryzae*

Ls: Leafsheath, Lb: Leafblade, Co: Corolla, Au: Auricle, Lg: Ligule, In: Internode, Ap: Apiculus
+ denotes the presence of red/purple color, UH: Univ. of Hyderabad, Hyderabad, India DRR: Directorate of Rice Research, Hyderabad, India. TNAU: Tamilnadu Agricultural University, Coimbatore, ILTAB: International Laboratory for tropical Agricultural Biotechnology, La Jolla, CA.

Table: 3.2.4 Stocks used for Tp309 transformation:

N6 Macroelements 20X stock

KNO_3	56.6g/L
$(NH_4)_2SO_4$	9.26g/L
KH_2PO_4	8.0 g/L
Mg $SO_4 7 H_20$	1.8g/L
$CaCl_2.2H_20$	3.3g/L

B5 Microelements

Mn $SO_4 .H_20$ or	758g/L
Mn $SO_4 .4H_20$	1000mg/L
$ZnSO_4$ or	112mg/L
$ZnSO_4.7 H_20$	200mg/L
KI	75mg/L
$Na_2MOO_4.2H_2O$	25mg/L
H_3BO3	300mg/L
$CuSo_4.5H_2O$	2.5mg/L
$CoCl_2.6H_2O$	2.5mg/L

B5 vitamins
Gamborg's vitamin powder(G2519) 2.8g/250ml

MS medium Fe-EDTA 100X
EDTA Ferric-Sodium salt 4.15g/L

BAP, NAA, and ABA are dissolved in 1M KOH initially then made up with water.
2,4-D is dissolved in 100% EtOH, quicky dissolved in water.

3.2.5a Culture, transformation and regeneration *of Japonica*

Medium	N6 macro 20X	B5 micro 100X	B5 Vit I00X	MS-FF.DA 100X	Suc g/L	Pro mg/L	Gln mg/L	CEH mg/L	Man g/L	Sor g/L.	NAA mg/L	BAP mg/L	IAA mg/L	ABA	Hyg •ll
NB	50ml	10ml	10ml	10ml	30	500	500	300	…	---	…	…	2	…	…
NBO	50ml	10ml	10ml	10ml	30	500	500	300	47	47	…	—	2	…	…
NH50	50ml	10ml	10ml	10ml	30	500	500	300	—	…	…	—	2	…	130
PRNH50	50ml	10ml	10ml	10ml	30	500	500	300	…	…	1	2	…	5	130
RNH50	50ml	10ml	10ml	10ml	30	500	500	300	…	…	0.5	3	—	…	130
I/2MS	½ MS salts	—	5ml	—	10										130

3.2.5b Culture, transformation and regeneration of *Indica*

Media	KNO_3 mg/l	NH_4NO_3 mg/l	$CaCl_2$ $2H_2O$ mg/l	$MgSO_4$ mg/l	KH_2PO_4 mg/l	Myo-Inositol mg/l	CEH mg/l	Suc g/l	Fe-EDTA ml/l	CC-micro ml/1	Nicotinic acid ml/l	Thiamine-HCl ml/1	Glycine ml/1	Pvro. HCl ml/l	Mal g/l	Hyg	NAA ml/l	KT ml/l	ABA ml/l	IAA ml/l
CC	1212	640	558.8	120	1364	90	300	30	10	10	6	8.5	2	2	30	--	…	…	…	8
CC40	1212	640	558.8	120	1364	90	300	30	10	10	6	8.5	2	2	30	40	…	…	…	8
CC50	1212	640	558.8	120	1364	90	300	30	10	10	6	8.5	2			50	…	…	—	8
PRNI	1212	640	558.8	120	1364	90	300	30	10	10	6	8.5	2			50	1	2	5	—
RNI	1212	640	558.8	120	1364	90	300	30	10	10	6	8.5	2			50	0.1	2.5	5	—

pH 5.8; phyta-gel 2.6gms for all the media.

3.2.6 RBS -Liquid

Sucrose for Japonica (RBS)
Maltose for Indica (RBM)

NaH_2PO_4	240
KNO_3	4040
$(NH_4)_2SO_4$	330
$MgSO_4$	120
$CaCl_2 2H_2O$	187
FeEDTA	4.5ml
B5 Micro	10ml
B5 Vita	10ml
Proline	500mg
Glutamine	500mg
CEH	300mg
Sucrose	30g
2,4-D	2mg/l
pH	5.8

3.3 Microbiological methods

3.3.1 Bacterial Transformation

E.coli strain DH5 α were grown at 37 °C either on solid (1.2 % agar) or in liquid medium. Liquid cultures were grown initially in 2ml of LB medium in a test tube, and later in 1 litre flasks for plasmid isolations.

Competent cells of *E.coli* were prepared as follows. One ml of DH5 α cells from an overnight grown culture were inoculated in 100 ml of LB medium without the antibiotic. The cells were grown till they reached an OD_{600} of 0.4-0.6. Cells were then harvested into precooled 50 ml falcon tubes at 3000 rpm for 10 min at 4 C. All the operations were performed under sterile conditions at 4° C. After the centrifugation, the cells were re-suspended in 15 ml of TFB 1 buffer (30 mM potassium acetate pH 5.8, 50 mM $MnCl_2$, 10 mM $CaCl_2$ and 15% glycerol, 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 $CaCl_2$, 10mM NaCl and 15% glycerol, autoclaved) was added and mixed gently with a pipette. Aliquots (50 ul) were frozen in liquid nitrogen and stored

at -80 ^{0} C for further use.

Transformation of the competent cells was done as follows: Frozen *E.coli* cells (50 *u*l of competent cells) were thawed on ice to which 1 ng of plasmid DNA or 100ng of ligation mix were added. The suspension was carefully mixed with a pipette tip and incubated on ice for 30 min. A heat shock of 42 C for 45 s was applied, followed by an incubation on ice for another 5 min. 900 *u*\ of LB supplemented with 20 mM glucose were added and the bacterial suspension was incubated at 37° C and 100 rpm for 1h. Aliquots of the suspension were spread evenly on solid LB supplemented with the antibiotic. The bacteria were incubated at 37° C overnight.

For long-term storage, single colonies of bacteria were picked and inoculated in 2 ml of LB supplemented with ampicillin as the antibiotic. After overnight growth. 625 *u*l of the suspension were added to 375 *u*\ of 40% glycerol to bring the final concentration to 15%. The bacteria were quick-frozen in liquid nitrogen and stored at -70 °C.

3.3.2 Growth of *E.coli* and Induction of *CT24*

E.coli strain *DE3* containing the expression vector *CT24* cDNA sequence under the control of T7 RNA polymerase was grown in NZCYM medium at 37 C in a rotary shaker until the O.D $_{600}$ of the growing culture reached 0.6-0.9. At this stage, the *E.coli* cells were induced with 0.4mM IPTG and the cultures were allowed to grow for an additional three hours. After three hours, the cells were harvested by centrifugation at 5000 rpm, 15 mins at 4 C suspended in a sonication buffer (100mM Tris, pH 8.8, 200mM DTT, 0.5mM sucrose/glycerol, 5mM EDTA. 0.2ml of 20% SDS, 2mM PMSF) and boiled for 5 mins in a water bath. After boiling. the cells were sonicated for 120 seconds at 12000 Hz using an ultra-sonicator. The sonicated cells were then centrifuged and aliquots of the supernatant was dissolved in 50mM Tris pH 8.8 and 5mM EDTA and used for protein estimation. About 75*u*g of protein was used for 12% SDS-PAGE.

For the purpose of enzyme assay(s) the cells were suspended in the sonication buffer (without DTT) and sonicated for 120 seconds at 12000 Hz. The extracts are

then dissolved in a buffer containing 50mM Tris pH 8.8 and 5mM EDTA and used for protein estimations. The GST activity of the crude bacterial extract was determined by assaying the production of S-2-,4-dinitrophenyl glutathione whose absorbance was read at λ340. The reaction was initiated with the addition of 1mM CDNB. The enzyme activity was carried out at 25°C for 120 seconds and the activity was determined spectro- photometrically as the increase in absorbance at λ340. The non-enzymatic reaction consists of enzyme and GSH. Enzyme activity was expressed as activity units where 1 unit of activity is the amount of the enzyme that catalysis the formation of 1μmol of S-2, 4-dinitrophenylglutathone/min at 28°C using lmM concentration of GSH and lmM of CDNB. Specific activity was defined as units of activity per mg of protein.

For Western blot analysis , about 30μg of the induced and the control cell extracts were used for 12% SDS-PAGE, transferred to Hybond C or ECL membrane and probed with Anti-GST antibodies from pumpkin.

Purification of the induced *CT24* polypeptide was done by electro-elution of the seperated band. The band of interest was cut from 12-15 preparative SDS-PAGE gels, and electroeluted using the BIORAD electro eluting apparatus. The eluted protein was concentrated by acetone precipitation and the purity band was checked by SDS-PAGE. This eluted protein was mixed with incomplete adjuvant and was used to raise the poly clonal antibodies in rabbit. For the primary dose, the antigen was prepared as follows: 150μg of the purified protein was made upto 1ml with PBS solution, and was emulsified with an equal volume of Complete Freund's adjuvant to make up to a total volume of 2ml of emulsion. (The emulsification of the antigen with the adjuvant is made by mixing the antigen, in and out of the Lud-lock glass hypodermic syringe to forma thick emulsion. This emulsion was injected sub-cutaneously into the rabbit on either sides of its thighs. Booster doses of the antigen was administered after four weeks of the primary injection. For booster doses about 100 μg of protein mixed in the incomplete Freund's adjuvant was injected. Subsequently the second booster is given exactly after a week of the first booster. With in a week of the second booster the blood of the rabbit was collected from the

ear-veins of the rabbit, and the serum seperated by low speed centrifugation. Aliquots of the serum was stored at -20 C.

3.4 Molecular Biological methods

3.4.1 Miniprep of plasmid DNA

A rapid and simple method was used to isolate very small amounts of plasmid DNA from single bacterial colonies. Overnight-grown colonies were picked with inoculation loops, and incubated in 12 *u* lysis buffer (25 mM Tris-HCI, pH 7.5, 25 mM Na_2 EDTA, 0.5 mg/ml lysozyme, 0.1 mg/ml RNase , 10% glycerol, v/v, bromophenol blue). The bacteria were lysed for 5 min 2 *ul* phenol/chloroform (1:1, v/v) and vortexed for 30 s and centrifuged at 12,000rprm in a tabletop centrifuge. The supernatant was examined by gel electrophoresis in the presence of ethidium bromide and compared with the original plasmid. Masterplate was incubated overnight at 37^0C

Small amounts of DNA suitable for restriction analysis and molecular cloning were isolated by a small-scale preparation based on alkali lysis as described in Sambrook *et al* (1989).

3.4.2 Maxiprep of plasmid DNA

Overnight grown 500 ml bacterial culture was centrifuged at 4000 rpm at 4 °C for 10 min. Large volumes of bacterial suspensions were lysed by alkali treatment, and the nucleic acids were purified by column chromatography (Qiagen, Hilde, FRG). For the lysis and purification, the plasmid Maxi Protocol of Qiagen was followed. The pellet was suspended 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 lysed 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 lysate 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 treating with 2wl of 20mg/ml of RNA-ase for 1 hour at 37°C. The RNA-ase was removed by phenol :Chloroform and the DNA was finally precipitated with $1/10^{th}$ volume of sodium acetate and 2.5 volumes of ethanol.

3.4.3 Restriction digestion of plasmid DNA and isolation of fragments

Restriction digestions of the plasmid DNA was carried out in a total volume of 20 ul containing (0.5 wg) of plasmid DNA, 2 wl of appropriate restriction enzyme (10 X buffer), 1 wl BSA (1 ug/ul, if necessary), 15 (or 16) wl double distilled water and 1 unit of restriction enzyme. The reaction was incubated at 37^0C (or at an appropriate temperature according to the restriction enzyme) for 1 hour and the digestion pattern was analysed on 1% agarose gel.

Agarose slabs containing the desired DNA fragment were excised with a scalpel blade under UV light. DNA fragments were isolated by melting the agarose in a high salt buffer and subsequently binding the DNA to glass particles with Gene clean kits. Alternately, high molecular weight plasmid DNA (larger than 5kb) was purified by dialysis method or low melting agarose method. During the purification using the dialysis tubing the band of interest was cut and placed in the dialysis tube and electrophoresed in 0.5 X TAE at 60 volts for 1hr. At the end of the run the polarity was reversed for short time after which the DNA was moved out into the solution was extracted with butanol. The lower phase is extracted with phenol chloroform and then re-precipitated with acetate and alchohol and stored for further use at -20°C.

Calf Intenstinal Alkaline Phosphatase (CIAP) reaction was carried out in a total volume of 25 wl containing, 20 ul of restriction digested plasmid DNA in 10 mM Tris pH 8 (10 pmol ends), 2.5 wl 10X CIAP buffer 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 wl with the stop buffer , phenolyzed, alcohol precipitated, washed in 70% ethanol, dried, dissolved in TE and used in further manipulations. Alternately, the CIAP reaction was carried out along with restriction digestion of the plasmid molecule in order to avoid the steps of phenol CHC13. The digested plasmid was

electrophorosed and the band of interest was identified, cut and purified by Gene clean and further used in cloning.

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 wl containing 1 wl (100 ng) of restriction digested plasmid DNA (4 kbp), 3wl (300 ng) of fragment DNA (1.5kbp), *2* wl 10 X ligase buffer ,12 wl of sterile DD water and 1 *u*\ of T4 DNA ligase (5 U/*u*l). The reaction was incubated at 16° C for 2 h or overnight where necessary. After completion of the reaction, a aliquot of 10 wl were used transformation analysis.

3.5 Construction of expression vectors

Plasmid DNA extraction, restriction reactions and pattern analysis are carried out according to the standard procedures as described above.

pMAC2: The 1.4kb *C2* cDNA fragment was excised from pUOH *C2* using *Bam H1* and ligated at the same *Bam HI* site of pAHC17 to from pMAC2. The restriction pattern of pMAC2 was checked using of enzymes and the sequence confirmed. The primer used for sequencing was *Ubi 1* primer with the sequence (5'GTCGATGCTCACCCTGTT 3').

pKS17: For the construction of the plasmids described below, the intermediate vector pKS17 was used. pKS17 was constructed by excising the 2kb *Pstl* fragment carrying the *ubiquitin* promoter and the intron from pAHC17 and cloned into the *Pstl* site of pBSKS to form pKS17. The direction of the plasmid was confirmed restriction analysis.

pMA*C1*: Plasmid pMA*C1* was constructed by excising a 2.8kb fragment containing the *Cl* cDNA and the *Nos* terminator from pUOH *Myb Cl* using *Hind 111-Bgl 11* to form p99C1S. From p99C1s the Hind III-ClaI fragment was excised and

ligated into *Hind III-Cla I* site of pKS17 to form 7.46 kb pMA*C1*.

PMAC7(as), pMA*Chs*(as), pMA*R* (as): Other plasmids, namely pMAC7(as), pMA*Chs*(as), pMA*R*(as) were essentially cloned along the same lines as those of pMA*C1*, i.e the fragment of interest was excised from the actin construct using *Hind III-Bgl II* sites to form the intermediary plasmids p99*C1*s, p99*Chs* and p99*R* respectively. From each of the above plasmids the *Hind III-Cla I* fragment was excised and ligated to the corresponding sites of pKS17 to form pMACl(as), pMAChs (as) and pMAR (as) respectively. The identity of all these plasmids was checked using restriction analysis and confirmed by sequencing. The primer used for sequencing was that of *Ubi 1*.

pMA*R*, pMA*Bz2*,pMA*A2*: The *R* and the two structural gene cDNAs the *A2* and *Bz2* were cloned under the control of 35S promoter. Construction of the plasmid pMA*R*1 was achieved by excising 2.5kb *EcoR1* fragment from pUOH *R1* and ligating it into the same site of pMON 999. The identity ofpMA*R1* was checked by restriction analysis and confirmed by sequencing using *R* forward primer. 5'ACTGGATCCCTACGCAAGCTGCCCGG 3'.

Construct pMA*B2* was made by excising 1kb *Bz2* cDNA from the plasmid *pBz2* (kind gift of Virginia Walbot, Stanford) using *EcoR1*. This fragment was cloned into the *EcoRI* site of pMON999. The construct pMA*A2* was made by excising the 1.4kb *A2* cDNA from pUOH *A2* and was cloned into the same site of pMON 999. The orientation of all the inserted fragments was done by restriction analysis and sequenced using the *Nos* primer. The sequence of the primer is 5'GTAACATAGATGACACCGCG 3'. The sequencing is done by Applied Biosystems model 373A apparatus.

The identity of the sequenced cDNA's were compared with the original cDNAs from the available data base by sequence alignment using DNASTAR package for Macintosh (DNASTAR Inc. Madison WI, USA).

3.6.1 Extraction of genomic DNA

Genomic DNA was isolated from leaf tissue as described by Dellaporta. 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, 50 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 sol III was added (60ml of 5 M Pot. Acetate, 115ml of acetic acid in 200ml of distilled water) 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 into a clean tube containing 10 ml of isopropanol, mixed well, incubated at -20° C for 30 min and then 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 low speed centrifugation for 5 min, and was subjected to phenol-chloroform. The aqueous phase was then precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of ice-cold absolute ethanol for 12h. and later centrifuged at 12000 rpm. The pellet thus formed was washed in 80% ethanol, dried and suspended in appropriate volume of TE. The concentration of DNA was tested spectro-photometrically and its quality through gel analysis. This method was used when the amount of plant tissue was reasonably high.

In order to extract the rice DNA with high yields with low amounts of tissue the following procedure was followed: The tissue was harvested and ground to a fine powder in liquid N2. The powdered tissue was transferred to a conical flask and 2.5ml/gm of LETS buffer was added (100mM LiCl, 10mM EDTA, 10mM Tris (pH 7.5) , 1% SDS, 14mM mercapto ethanol and heated for 10 mins in hot water bath until the clumps of the tissue disappeared. To this 10ml of phenol:CHC13 was added and mixed well to form a milky suspension. The suspension was then transferred to a falcon tube and centrifuged at 5000 rpm for 5 mins at room temperature. The aqueous layer was taken and extracted with phenol:CHC13 followed by one round of CHC13. To the aqueous phase half the volume of 7.5M ammonium acetate was added and incubated overnight at 4°C. The solution was then centrifuged at 10000rpm at 4°C for 10 mins. To the supernatant equal vol. of iso-propanol was added and incubated

overnight a t 4°C. The following day the solution was centrifuged at 4°C for 10min at 10000 rpm to obtain the DNA pellet. The DNA pellet was dissolved in 2ml of TE. RNA contamination from the DNA samples is removed by treating the DNA with 1 ul of 20mg/ml of RNA-ase for 1h at 37°C. The phenol:CHC13 step was repeated and the DNA was finally precipitated in 1/10 vol. of 3M sodium acetate and 2.5 vol. ethanol.

3.6.2 Extraction of total RNA

Total RNA was isolated from various tissues of rice plant as follows: Rice seedlings were harvested quick frozen in liquid nitrogen and stored at -70° C. Five grams of the harvested tissues (preferably fresh) were ground in liquid nitrogen to a fine powder, transferred to tubes with extraction buffer (150 mM Tris pH 9.0, 100mM NaCl, 20mM EGTA pH 8, 1% SDS), containing 100 wl of 2-mercapto ethanol and 5 ml of buffer saturated phenol pH 8. The extracts were properly mixed and incubated at 55 C for 10 mins. The extracts were then centrifuged at 5000 rpm at RT for 10 min. The upper supernatant was extracted at least twice with phenol-chloroform and finally with chloroform. The upper aqueous phase was then precipitated with 1/10 volume of 3M sodium acetate and 2.5 volumes of ice-cold ethanol, kept at -70° C for 45 min and centrifwged at 10,000 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 5M LiCl and incubated for overnight at 4 C and then centrifwged at 4500 rpm at 4 C for 10 min. The pellet was re-dissolved in one ml of DEPC water and precipitated with alcohol. The resultant pellet was washed in 70% ethanol and finally dissolved in 200 wl of water. The quality of total RNA was checked spectrophotometrically and also through 1.2% formaldehyde gel.

3.6.3 Northern analysis

Equal concentrations of total RNA were separated on 1.2% agarose formaldehyde gel (each 100 ml gel consisting of 1.2 g of agarose, 10 ml of 1 Ox MOPS (200 mM MOPS, 100 mM sodium acetate, 10mM 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 3h and overnight respectively in 1M Nacl, 10% Denhardt's

solution, 2% SDS and 100 ug/ml sheared denatured calf thymus DNA. The membrane were washed three times at 60° C in 2 X SSC, 0.5% SDS for 10 min each. The filters were then exposed using an X-ray film with intensifying sreens at -70 C.

3.6.4 DNA detection by Southern blot analysis

Genomic DNA sequences were detected by preparation of Southern blots and hybridisations (Southern 1975). For this purpose, approximately 10 ug of DNA was digested with an appropriate restriction endonuclease for a specific time and loaded on an 0.8% agarose gel. After electrophoretic seperation, the DNA in the gel was depurinated for 10 mins in 0.25M HC1 and subsequently denatured in 0.5M NaOH, 1.5M NaCl, for 45 mins. Finally, the gel was washed three times in neutralisation buffer (0.5M Tris HC1, pH 7.2, 1.5M NaCl, 1mM Sod. EDTA) for 10 mins each and blotted using 20X SSC (3M NaCl and 0.3M Sod. Citrate) onto Hybond N+ membrane by capillary transfer for overnight. The blots were then baked at 80°

3.6.5 DNA slot blot analysis

DNA slot blot analysis was performed on the transgenic plant as a preliminary step first to look for the presence of gene of interest. For DNA slot blot analysis, about 3ug of plant DNA was added to 10ul of sterile double distilled water and denatured for 3-5 mins in a boiling water bath, snap cooled followed by the addition of 2ul of 2XSSC. The Hybond N + membrane was pre-wetted in 2XSSC and placed on top of the 2 layers of 3mm whatman paper on a slot blot manifold apparatus. The manifold was then clamped unto which the DNA samples were applied to the slots and a slight vaccum was applied for 45s to 1min. The membrane was then air dried and vaccum baked followed by pre-hybridization and hybridization.

3.6.6 **Random-primer** labeling

The cDNA fragments were used as templates for preparation of probes by random primer labeling reaction (Stratagene) or BARC 24 u\ of template DNA (40 ng) was denatured by boiling and snap cooled. To this, 10 u\ of primers, 15 u\ of dNTPS (excluding the radio-active dNTP), 15ul of the respective dNTP buffer, 50 uCi of α-dNTP 32 (3000 Ci/mM) and 5 units of Klenoow were added and incubated at 37°

C for 1 hour. After the incubation period, the radioactive probe was purified using Sephadex G-25 spin column to remove any un-incorporated dNTP. The specific activity of the resultant probe was calculated as follows: cpm/ug • 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 then added to the hybridization solution.

3.6.7 Hybridizations

Prehybridisations were performed with 0.5M potassium phosphate buffer, 7% SDS and 100ug/ml of salmon sperm DNA for 3-5h. Hybridisations were also performed in 0.5M Pot. phosphate buffer pH 7, 7% SDS, and 100 ug/ml sheared denatured calf thymus DNA including the radioactive probe for 12-16h. The blots were washed twice (2X SSC +1% SDS) for 30 mins, then (2X SSC+0.5% SDS) for 30 mins at 62°C. Finally the blots were placed in 2X SSC at room temperature for one hour. The filters were exposed to X ray film (Kodak, USA) or Indu film at -70°C with intensifying screen.

3.6.8 Polymerase Chain Reaction

Genomic DNA were amplified by PCR in a Perkin thermal cycler. 1 OOng of the DNA was added to the 50ul PCR mix containing (10ul PCR buffer, 5ul of MgC12, l-2ul of 10uM primers, 1ul (10mM) of each dNTP's, and 1U of Taq. Polymerase (GIBCO-BRL) covered with mineral oil. DNA was denatured at 94°C for 5mins, followed by 40 amplification cycles of 50°C for 1min annealing, extension at 72° C for 2mins, and denaturation at 94°C for 45s. The terminal delay was set at 72 C for 5mins. The PCR reaction was carried out in thermocycler Biometra trio thermoblock. Amplified DNA fragments were electrophoresed on 1% agarose gel and detected by ethidium bromide staining.

The primer sequences for *H1* are 5' CGTCTGTCGAGAAGTTTC 3'

H3 are TACTTCTACACAGCCATC

Ubi GTCGATGCTCACCCTGTT

NOS GTAACATAGATGACACCGCG

3.7 Protein and immunological methods

3.7.1 Protein extractions

For the extraction of total rice proteins, a modified procedure of Goday et al., 1988 was used. The tissue was finely ground in liquid nitrogen and extracted with extraction buffer (gm/2ml) containing 0.25M Tris-HCl (pH 8.0) , 0.4% SDS, 20mM EDTA, 2mM PMSF, and 5% mercaptoethanol by mixing and boiling for 5mins. The extract was centrifuged at 12,000g for 10mins, and proteins in the supernatant were precipitated with 15% TCA at 4°C. The pellet was washed with 80% acetone thrice. The resulting pellet was dissolved in Tris buffer and used for protein estimations. The amount of protein was calculated using Lowry *et al.*, (1951) or Bradford methods (1976). About 30 ug of protein were loaded on to SDS- PAGE (12%) and electorphoresed according to the Laemmeli (1970). Proteins were visualized by gel staining with Coomassee Brilliant Blue.

A rapid isolation protocol for the immediate use of proteins was done as follows: The tissue was harvested and ground to fine powder in a mortar and pestle under liquid nitrogen. The fine powder was extracted (500 ul /gm) into the extraction buffer containing 62.5 mM Tris (pH 6.8) and 5mM EDTA was added, mixed well and the centrifuged at 12,000 rpm. The supernatant was taken and used for protein estimation. Bradford's method of protein estimation involves the addition of 5ml of Bradfords reagent to 1 00ul of the protein extract and incubating for 5 mins at room temperature. The O.D of the samples were taken at 595nm within 5mins after the addition of Bradford's Reagent.

3.7.2 **SDS-PAGE**

SDS-PAGE was performed according to Laemmeli et al., (1970). For mini gels about 30ug of protein was loaded, while the maxi gels required 75-100ug of protein. The seperation and stacking gel composition is as follows: Seperating gel solution (30ml) contains 16ml of 28% acrylamide solution, 4.5ml of 3M Tris buffer (pH 8.9), 3ml of 1%SDS, 6.45 ml of distilled water, 100ul of ammonium persulphate

(10% APS), and 30ul of TEMED. Stacking gel solution (5ml) contains 0.9% acrylamide (28%), 2ml of 0.5M Tris (pH 6.7), 0.5ml of 1%SDS , 0.6ml of water, 40 ul of 10% APS, and 10ul of TEMED. Electrophoresis was carried at 150V after which the gels were stained with Coomassie blue. Gels were treated with a solution containing 7.5% methanol and 7% glacial acetic acid. From these gels the induced *CT24* protein was identified by comparison with that of the control sample, and the band was cut and then electro-eluted using the BIORAD electro elution apparatus following manufacturers instructions. The purity of the electroeluted band was checked on SDS-PAGE. The 22kDa polypeptide was used to raise polyclonal antibodies in rabbit.

3.7.3 Western blot anlaysis.

The SDS-PAGE gels were electrophoretically blotted onto nitrocellulose filters with an electroblotting buffer (25mM Tris-HCl (pH 8.3), 100mM glycine and 20% methanol) using a Biorad transfer apparatus. After the transfer, the proteins were visualised by Ponceau-S solution (0.2% Ponceau-S in HC1 (pH 7.6) and 150mM NaCl). The membrane was then blocked with TBST (5% low fat milk in TBS solution, (20mM Tris pH 7.5;150mM NaCl) . The membrane was then blocked with TBS+ 5% non fat dry milk for an hour and transferred to a solution containing the primary antibody with an appropriate dilution. A dilution of 1:500 was used for CHS antibody where as 1:200 was used for pumpkin antibodies. The blot is then incubated with alkaline phosphapase conjugated secondary antibody at a dilution of 1:20000. The blot was washed three times with TBS, followed by a wash with TBS containing Tween 20. The bands were visualized using BCIP and NBT in AP buffer (1 OOmMmM Tris (pH 9.5), lOOmM NaCl and 5mM MgC12. NBT : 50mg of NBT dissolved in 700ul of diethyl formamide and 300ul of AP buffer. BCIP: 50mg of 5-Bromo 4-Chloro-3-Indolyl phosphate (BCIP) dissolved in diethyl formamide). Both NBT and BCIP are made and store at -20° in dark until use.

3.7.4 Rice seedling **growth and culture** conditions

Rice seeds were sterilized in Na-hypochlorite 5% (v/v) for 5 min, then washed extensively and soaked overnight in distilled water. This seeds were sown on

solid 0.4% (w/v) agar in petri dishes and kept in dark for germination. Etiolated seedlings at regular intervals were exposed to sunlight for a short period and were returned to darkness. Seedlings were exposed to mid-day sun light (2800-3300 umol m 2 sec 1) or by keeping the seedlings outside. After the light pulse the seedlings are returned to darkness and harvested at regular intervals for pigment extraction and GST enzymatic activity of the rice. To study the spatial distribution of the GST activity in single seedlings an *Indica*line R27 showing variation in the amount of anthocyanins in base, tip and middle regions, was harvested, pooled and used for enzymatic analysis. Stress treatments to the seedlings is given to the 8 day old seedlings by treating them with either 20% of poly ethylene glycol (PEG) or 100*u*M ABA or keeping the seedlings at 4° C in a refrigerator. Control seedlings received water at room temperature. Seedlings were harvested at regular intervals in liquid nitrogen and quick frozen.

3.7.5 Biochemical methods

Estimation of anthocyanins was done according to the protocols described in (Harborne, 1967). UV-B induced seedlings of uniform height, were extracted with 1% v/v of acidified methanol (25mg/ml) for 24 hours at 4 ° C in dark with occasional shaking. For every ml of the extract, 0.75 mL of water and 2ml of chloroform were added resulting in Folch partition. Anthocyanin concentration in the upper phase of the partition was calculated from A_{525} using a millimolar extinction coefficient of 31.6.

The protocols for GST extraction and activity are essentially as described in Mozer *et al*., (1983) with slight modifications. The samples for GST activity were harvested and homogenised in liquid nitrogen in a pre-cooled mortar and pestle with 200mg polyvinylpolypyrrolidine in the 50mg/ml of the homogenising buffer containing 25mM Tris pH 7.8, 1mM EDTA. The homogenate was centrifuged at 20,000 g for 20 min at 4°C and the supernatant was used for protein estimations and subsequent enzyme assay. The GST assay was performed at 25° C in an assay mixture consisting of 1mMGSH, 1mM CDNB and enzyme in an 1ml assay mix. GST activity is measured as S-2,4, dinitrpphenyl glutathione formed.

For Western Blot analysis, the samples are harvested at regular intervals an aliquot of the protein samples used for enzymes activity, were also used for Western blot analysis by dissolving the samples in 2X sample buffer boiled for 3-5 mins and finally loading onto 12% SDS-PAGE to be used for Western Transfer.

All the data points in the figures and tables representing GST activity are the mean values from atleast three independent replicates and standard errors calculated thereof.

3.8 Tissue Culture Methods

3.8.1 Transformation of Tp 309

Mature dehulled seeds were surface sterilised in 70% (V/V) ethanol for 1 minute followed by 50-100% (V/V) commercial bleach for 50 minutes with gentle stirring . Seeds were then rinsed with sterile water 3-5 times and placed on NB medium for callus induction at 25°C in dark. After four weeks of culturing on NB medium the calli were ready to be used for bombardment with in two weeks of the subculture.

3.8.2 Bombardment Conditions

Co-transformation was the general rule. For the generation of chalcone synthase transgenics two plasmids, one containing the selection marker (*hph*: hygromycin phosphotransferase) *p35H* and the plasmid containing the gene of interest i.e pMAC2 were used . For the purpose of transformation gold suspension was prepared as follows: The gold particles are 1 .0um (BIORAD). gold micro-carriers are 1.8 um. : 6 (1.0um) mg of gold was weighed in an siliconized eppendorf tube and suspended well in 100 *u*\ of ethanol. The tube was then centrifuged and the gold pellet was re-suspended in sterile water and vortexed. This procedure was repeated twice and finally the gold was suspended in 1 00*u*l of sterile water. From the above gold suspension about 50ul of the gold suspension was taken and was used for bombarding 5 plates. To this suspension 5 Mg (the two plasmids mixed in the ratio of 1:7) was precipitated in the presence of 20*u*l of 100mM spermidine and 50*u*l of 2.5M $CaCl_2$ and allowed to settle at room temperature for 10 min. The gold/DNA

suspension was then centrifuged and after discarding the supernatant , it was resuspended in 50«l of ethanol. About 10ul aliquot of this DNA coated onto gold particles was placed onto the centre of the microcarrier, alllowed to dry and used for each bombardment with Bio-Rad PDS 1000/He biolistic particle delivery system. A pressure of 1100 psi was applied to accelerate the delivery of the particles. After bombardment the calli were incubated in the same medium (NBO) for 16-20h and then transferred to NH50 medium. The detailed steps involved in the transient expression and stable transgenics is clearly mentioned below. The following formulations is used for co-bombardment in stable transgenics is clearly mentioned below. After the bombardment the steps involved in *Indica* transformation are schematically represented below.

3.8.3 *Indica* transformations

In case of *indica,* seeds were placed on MS medium for callus induction for four weeks. After the four week period, the small compact embryogenic calli were placed on RB medium in a conical flask on a gyratory shaker at 110-130 rpm. Medium was replaced every five days. It takes about four weeks to establish the suspension cultures after which they are ready for bombardment. Medium was replaced three days before the bombardment. Steps involved in the regeneration of *Indica* and *Japonica* in the following paragraphs.

3.8.4 Transient expression of anthocyanin

Two regulatory genes, namely pMAC1 and pMAR and the selection marker gene (p35H) were bombarded in the ratio of (3:4:1) (4:3:1) (2:5:1) (5:2:1) (3:3:1) (4:4:1) ratio. A transient expression system for the anthocyanin production in *Tp309* callus was established as follows: The callus was initiated for four weeks in NB medium and sub-cultured every fifteen days. The calli, within two weeks of the second subculture were used for bombardment.

The plasmids used for bombardment are the two maize regulatory genes *C1,R* and a structural gene *C2* along with the selection marker. These gene constructs (hph:pMACV: pMA*R*: pMAC2) were mixed in the ratio of 1:3:3:4 w/w on 1um (o.d)

particles (5«g of plasmid/2.5mg particles). The bombardment was done on NBO medium and the calli were kept in NBO medium overnight and then transferred to RNH50 medium supplemented with auxins. These plates were then placed in the growth chamber with the photo period 16h at 110-130 «M/m2 PAR. Photographs of the calli were taken using a Photographic system (Nikon N6000) connected to stereo microscope with Kodak tungsten slide film. The steps involved in the transient expression of anthocyanins are schematically represented below:

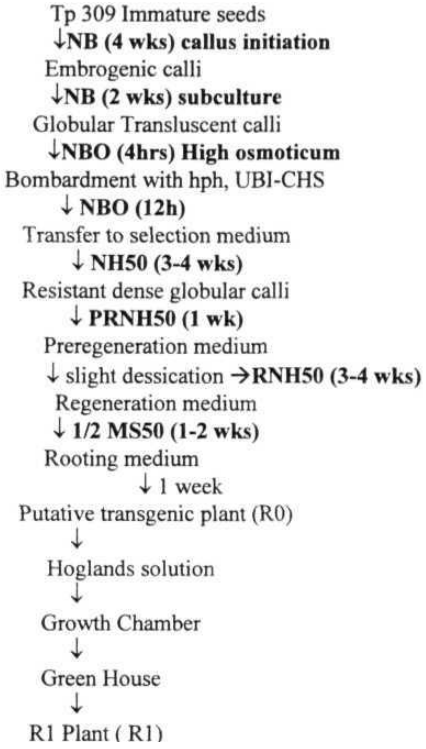
Flow diagram for the transient production of anthocyanins in Tp309

Tp 309 Immature seeds
↓ **B (4 wks) callus initiation**
Embrogenic calli
↓**NB (2 wks) subculture**
Globular Transluscent calli
↓**NBO (4hrs) High osmoticum**
Bombardment with *hph, Ubi-C2, Ubi-C1. E35S*-R
i **NBO (12h)**
Transfer to selection medium
↓ **RNHSO (48hrs-3days)+Light+2mg/L IAA**
Purple calli

3.8.5 Generation of Transgenic Rice Plants

Transformation of rice (*Oryza saliva* .L, sp. *Japonica,* cv *Tp309)* was done according to the protocols described in Chen *et al.,* (1998). A plasmid containing the gene of interest (pMAC2) and a plasmid harboring the selection marker gene (p35H) were used for transformation. For the generation of *C2* transgenics *hph* and pMAC2 were mixed in the ratio of 1:7 and bombarded onto the calli of *Tp309* with PDS 1000 He biolistics system , BIORAD on 1*u*m (o.d) particles. The regeneration steps are as described in Chen *et al.,* (1998). Essentially after the bombardment the calli were placed in various media as follows:RH50 (4wks-dark), PRNH50 (1wk-dark), RH50 (2-3 wks-light) at which stage 2-3 cms long regenerated plants were transferred into magenta boxes with ½ MS medium with hygromycin (2wks-light) for rooting. Any albionos or hygromycin escapes cannot survive after this step. The plantlets from magenta boxes were then transferred to growth chamber (2-3 wks) and then to green house.

Flow diagram for the generation of Tp309 transgenic plants



3.8.6 Nomenclature of the transgenic plants

The transgenic plants were grown in glass house conditions. The primary transgenic plants are designated as RO plants. The numbering of the transformed plants is given as *C2-#*. # indicates 1, 2,3,----so on representing different chalcone synthase transgenic plants, generated as a result of independent integration events. In some cases *C2-#*a,b,or C are denoted which says they arose form the same calli probably may be siblings.

The plants obtained from the RO seed is represented as R1 and the numbering is given as *C2-1-#*, where C2-1 represents additional generation, while # denotes the number of the transgenic plant. The R2 plants are represented as C2-1 -1 -#.

3. 8.7 Crosses and embryo rescue

The genetic crosses of rice lines *Tp309* (Japonica) and *Purple puttu* (indica) was done as follows: Emasculation was done during the sunset time around 5-6 PM and pollination was done around 9-11 using the standard bagging method. The mature seed were harvested 3 to 4 weeks after po. categorized llination and the F1 seeds were embryo rescued by culturing in 1/2 MS medium. The cultured embryos are incubated in dark and subsequently transferred to light. The Fl plant phenotype was scored in various tissues.

3.9 Southern Analysis of the transgenic plants

Molecular analysis of the regenerated plants was performed as follows: Genomic DNA was isolated from the primary transgenic RO plants using the procedure of Dellaporta. These plants were tested for the independence of individual integration events as well as for the structural integrity of the chimeric gene cassette using appropriate restriction enzymes. *Hind III* was used to check the independence of the integration events, while *Bam H1* is used to check for the presence of intact cassette. In some case a double digest of *Hind III / Sma I* was used to check for the place of intact casette i.e 2kb *Ubi.* and 1.2kb cDNA fragment.

3.10 Screening for *Pyricularia oryzae*

Scoring for disease resistance of the transgenic plants was conducted at Directorate of Rice Research. Rajendranager, Hyderabad during the March 1997, and July 1998. R1 and R2 seeds were germinated in small pots containing clay soil and maintained under the same conditions as the un-transformed *Tp309* (control) plants. Other controls, the susceptible control *HR12* and a resistant control *1R64* were also germinated under similar conditions. 40 day old seedlings were subjected to *Pyricularia oryzae* infection either by spraying the spores. Alternatively the infection of transgenic plants is given as follows: *IC9* strain of *Pyricularia oryzae* was first used to infect the *HR12* leaves. The leaves of *HR12* after full blown infection were used to infect the transgenic plants by placing the infected *HR12* leaves on the transgenic plants. The temperature during the infection season is around 28 C at 95%

relative humidity. After 6-8 days (based on plant development and the fungus spread) these plants were scored. Based on the disease symptoms and spread of infection disease response to *P.oryzae* is classified as following, resistant variety has non-spreading lesions with restricted disease symptoms of pin head size with a score of 1, moderately resistant variety where the rate of spreading is higher than resistant *IR64* with lesions slightly larger than; pin head (2-5), Moderately suseptible variety with large necrotic lesions (Score 6-7), highly sensitive variety like , *HR12* showing severe infection lesion the score of 8-10.

4. RESULTS

4. Results

4.1 Detection of GST activity in rice

GST activity in the seedling extracts of various rice lines differing in purple pigmentation levels was assayed and the results are given in Table 4.1.1. Maximal GST activity was found in six day old dark grown seedlings compared to the younger or older seedlings. GST levels showed significant decrease with age of the seedlings until 10 days after germination. The highest GST activity was observed when 50mM potassium phosphate buffer, pH 7.5 with 1mM GSH and 1mM CDNB was used. Detectable activities were also observed at pH 7.0, and 8.0 at concentration of GSH and CDNB.

GST activity was assayed in *Indica* (*Hamsa, TN1013, G962)* and *Japonica* (*H1 13)* rice lines accumulating varying amounts of anthocyanins or even totally lacking them (Fig. 4.1.1). These rice seedlings were given a brief exposure to sunlight (sunlight and UV-B light are used synonymously in the present text) transferred back to dark. harvested after 24h and then assayed for GST activity. There is a considerable variation in GST activity levels among the tested rice lines. Earlier experiments from our lab established that under such conditions (a brief exposure to sunlight) anthocyanin pathway is induced with a concomitant increase in the enzymes of the pathway such as phenylalanine ammonia lyase (Reddy *et al*., 1994).

4.1.1 GST activity in response to UV-B light.

Six day old control and UV-B induced roots and shoots of *Hamsa*, *TN1 013*, *G962* and *H1 13* were harvested and used for enzyme analysis as described above. The data on the light induced changes in GST levels reveal that all the tested lines respond with a significant increase after UV-B treatment. Varietal differences were clearly observed. Figure 4.1.1 shows detectable GST activity in both colored as well as colorless lines after an UV-B pulse. Further, there are differences in the GST levels of shoot and root tissues. In general, roots show a higher GST activity than shoots. Though this seems to be the general rule among *indicas*, the *Japonica* lines (*H1 13)* show no such tissue specific differences, that is, the basal GST levels in root and shoot (GST) are similar. Interestingly, the colorless line *Hamsa* shows maximal basal levels

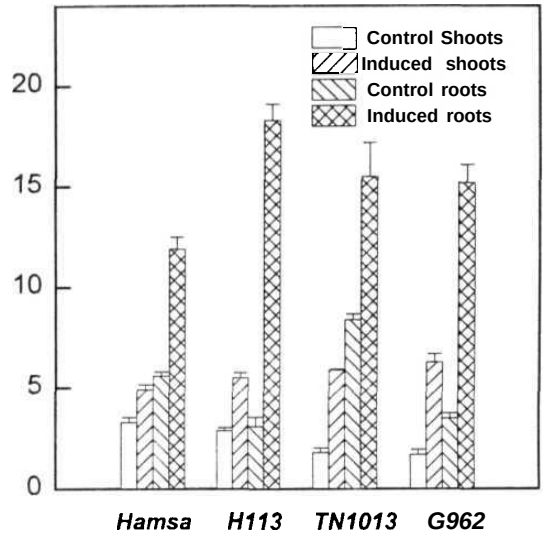


Fig. 4.1.1 Changes in glutathione-S-transferase levels in shoot and roots of different rice seedlings treated with sun light

Rice line	SPECIFIC ACTIVITY					
	Days after germination					
	3davs	4dav	5dav	6day	8day	10dav
N22	2.0	2.3	3.3	4.2	3.5	3
R27	1.8	2.0	2.2	4.5	4.0	3.5
PP	3.5	5	5.5	7	6.5	5
G962	3.0	3	3.5	4	3.8	3
Hamsa	1.4	1.6	2.0	3.3	2.8	2

Specific activity is represented as Units/min/mg of protein

Table 4.1.1. Levels of Glutathione-S-Transferase in various rice seedlings.

compared to the purple colored ones.

Figure 4.1.1 indicates that among the *Indica* lines, *G962* shows a significant increase in both shoot (more than 3 fold) and root (more than 4 fold) GST levels upon induction followed by *TN1013* (3 fold in shoots, 2 fold in roots) and *Hamsa* (2 fold in roots). Interestingly, the *Japonica* line *H1 13* shows a five fold increase in roots but comparatively a small increase in shoots.

4.1.2 Changes in GST activity in UV-B induced seedlings: Time course

Rice seedlings were harvested and assayed for GST levels at regular intervals after UV-B treatment. The time course experiments reveal that all the tested lines show an increase in the GST activity beginning four hours after treatment (Figure 4.1.2). A steady increase in the GST levels was observed in *R27* and *N22* between 4h to 8h with a peak of activity at 20h hour followed by a decline at 24 hours.

In case of *PP*, the increase in GST levels, though not very rapid between 4h to 12h interval was significant increase between 12h to 20h interval, after which the levels fall considerably. In *G962* the activity levels follows the same pattern as *PP*, showing not as much increase in GST levels between 4h to 12h period.

In summary, in all the tested lines the GST activity increased from Oh onwards and reaching a maximum at 20h. after which the activity levels significantly reduced (Fig.4.1.2). Notably, the fully colored *PP* seedlings consistently showed higher levels of GST activities at every time point tested. This is probably due to higher levels of anthocyanins. On the contrary, the colorless *N22* showed least activity, while the activity levels of two lines *G962* and *R27* are found to have the intermediate values.

4.1.3 Western Blot Analysis **of** GST **protein**

In order to test whether UV-B responsive increases in GST activity levels are due to the *de novo* synthesis or due to activation of the enzyme, *PP* and *N22* seedlings were harvested at regular intervals after light pulse and were subjected to irnmunological analysis using anti-Pug *b {Pug b-* GST from pumpkin) antibodies. Western data revealed that the 20h sample exhibited an intense band in both *N22* and

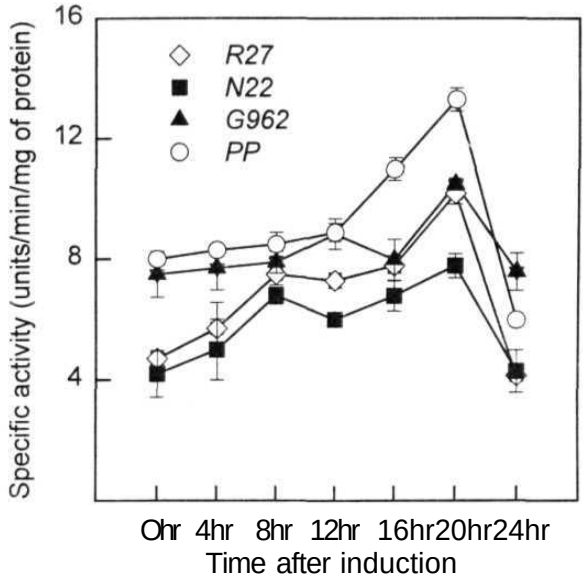


Fig 4.1.2. Time Scan of **glutathione-S-transferase** acitivity in various cyanic and acyanic seedlings in response to sunlight.

Data points represent the mean of three independent experiments

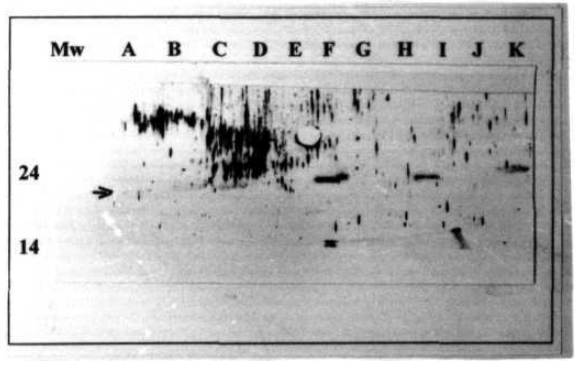


Fig.4.1.3. Immunodetection of 22kDa GST-like protein in sunlight induced purpleputtu and *N22* seedlings.

Mw) Mol.wt A) Oh B) 4h C) 8h D) 12h E) 16h F)20h G) 24h H) Spike I)Seed J) *N22* Oh K) *N22* 20h

PP seedlings (Fig.4.1.3). With in the detection limits of this antibody, the Western data are in agreement with the pattern of GST activities under UV-B treatment. The molecular weight of the GST-like protein in rice was found to be approximately 22kDa.

4.1.4 Spatial distribution of GST in induced seedlings

In our previous experiments, we have observed a peak of GST activity and detectable levels of GST-like protein at 20h after UV-B induction in all the rice lines tested (Fig 4.1.2). Among the tested seedlings, *R27* and *PP* show differential accumulation of the anthocyanin pigment where the base and the tip are more pigmented compared to the middle portion. The enzyme levels in these regions were also analyzed and it was found that the GST levels in the tip and the base are higher than in the middle (Fig.4.1.4). There is about a three fold increase in GST activity in induced seedlings in all the different regions tested. The spatial pattern of pigment accumulation and GST activities in *R27* seedlings thus seem to be correlative.

4.1.5 GST in response to PEG mediated water stress: Enzyme activity and protein levels

In view of the reported increase of GST activity in plants during stress conditions, we have tested for such changes in GST activity and protein levels in rice lines subjected to PEG mediated water stress, cold and exogenous application of ABA. The PEG mediated stress leads to a significant increase in GST activity in all the rice lines tested. However, varietal differences in the GST activity in these lines were clearly observed. Interestingly, all the three lines show almost a similar response pattern, i.e., the increase in GST activity' at 24h after stress treatment is followed by a decrease (Fig.4.l.5A). In case of *R27* and *G962*, this decrease in the GST activity' at 48h is lower than that of control whereas the GST levels in *N22* decline to that of the control. This increase in the GST activity is correlated with the *in vivo* GST levels under PEG mediated water stress (Fig. 4.1.5B). Further, in order to test the effect of the *in vivo* GST levels under different stress regimes, protein extracts of *Hamsa* (a standard rice line that was earlier tested for its stress response) seedlings were used for immunoblotting with anti-*Pugb* antibodies. The results reveal that a 22kDa **GST-**

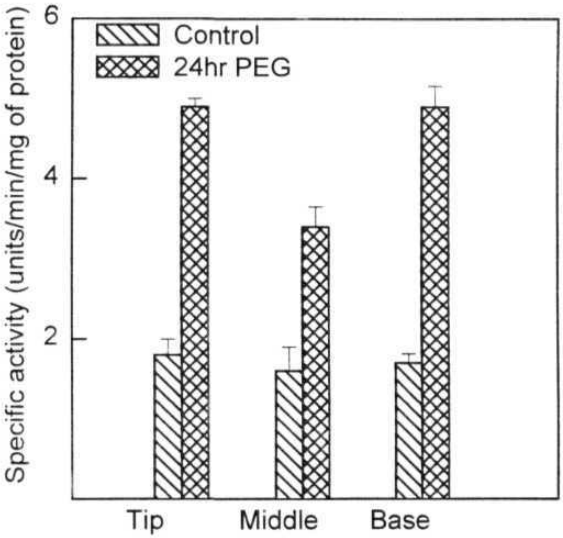


Fig. 4.1.4. **Spatial distribution of GST activity in** *R27* seedlings **20h after exposure to sunlight**

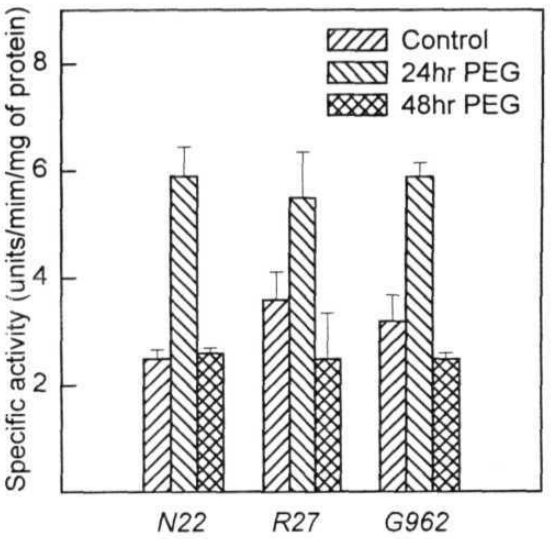


Fig. 4.1.5A. Changes in glutathione- S-transferase activity in different rice seedlings during PEG stress.

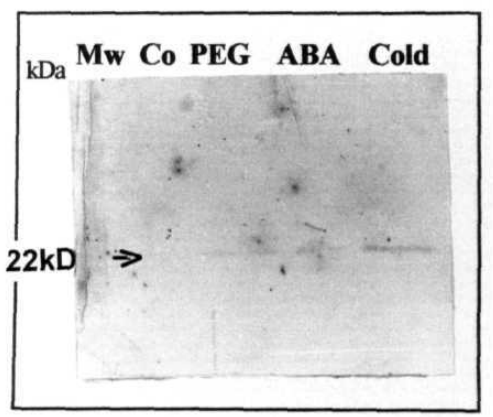


Fig. 4.1.5B. Immunodetection of 22kDa GST-like protein in *Hamsa*ice seedlings. Mw) Mol.Wt.Co) Control PEG) PEG treated ABA) 100 uM ABA treated Cold) Cold treated. Anti-Pug* antibodies are used

like protein is also increased in response to low temperatures and exogenous ABA application (Fig.4.1.5B).

4.2 **The** *CT24* **protein and its functional characterization**

In order to test the function of the *CT24* cDNA (a homologue of the *Bz2* gene encoding GST, showing extensive sequence homology to a number of stress responsive cDNAs of both monocots and dicots) product of maize, we have over-expressed the ***CT24*** cDNA in *E.coli* cells and partially characterized the protein. This was necessary to obtain workable amounts of *CT24* protein to test the premise if *CT24* can be a possible candidate for GST. The *pET* expression vector carrying *CT24* cDNA in *BL2J(DE3)* cells under the control of 77 promoter was used to over express the protein in *E.coli* (Fig. 4.2.1 A) (Courtesy: Schmitz and Theres, MPI, Koln.).

4.2.1 Over expression **of** *CT24* **protein in** *E.coli*

The induced *BL21 (DE3)* cells carrying the *CT24* cDNA were analyzed by SDS-PAGE and the electrophorogram is shown in Fig.4.2.1.B. The presence of the 23-24 kDa polypeptide in Coomasie stained gels was clearly seen in induced cell extracts that was absent in the un-induced cells. Further, the induction was found to be quite substantial.

The sonicated extracts of the control and un-induced *E.coli* cells were assayed for GST activity in order to test whether the *CT24* product can conjugate GSH to the universal substrate, CDNB. A comparison of the GST activity in crude extracts of both induced and control cells revealed about 2 to 4 fold increase in GST activity in induced cells (Fig.4.2.1C). In addition, the **activity** profiles indicate that un-induced cells also exhibit enzyme activity though at reduced levels. The extent of increase in GST activity in induced cells seem to depend upon experimental conditions, such as growth phase of the *E.coli* at which the cells are induced, the culture conditions and the duration of IPTG treatment.

To further examine whether the *CT24* polypeptide is a GST like protein, the Western blot analysis was done with *anti-Pugb* antibodies from pumpkin in un-induced and induced *E.coli* cell extracts. The cell extracts used for GST activity

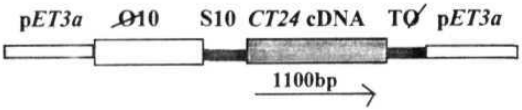


Fig. 4.2.1 A. Plasmid map of *pCT24* expression *(pET)* vector.

Ø 10 is the promoter of *T7* RNA polymerase
S 10 is the translation start site
T Ø is the terminator of the gene 10

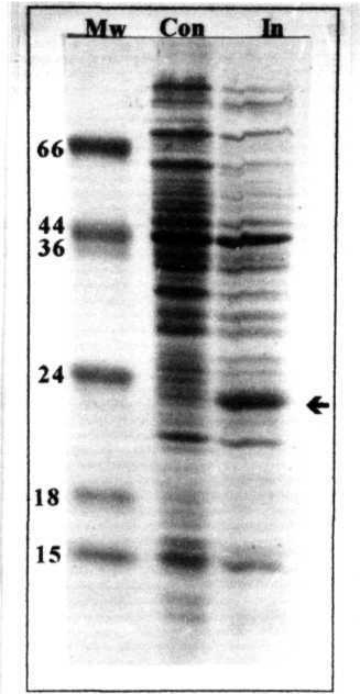


Fig. 4.2.1B. SDS-PAGE Showing over expression of *CT24* protein in *E.coli*

Mw) Mol.Wt Con) Uninduced *CT24* cells In) Induced *CT24* cells
Arrow represents the induced protein

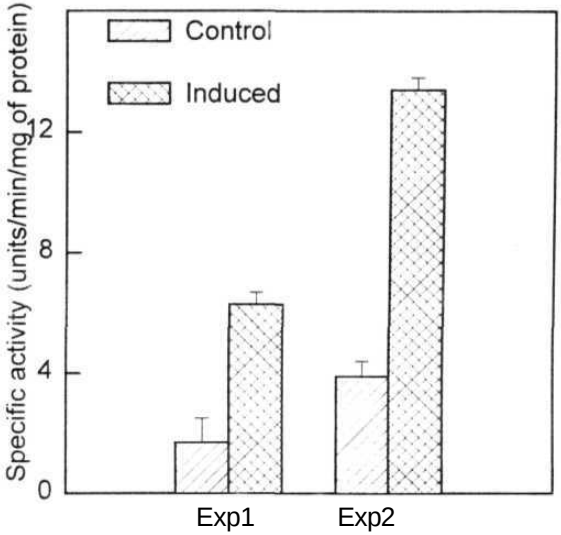


Fig 4.2.1C. Measurement of GST activity in *E.coli* cells expressing *CT24* protein

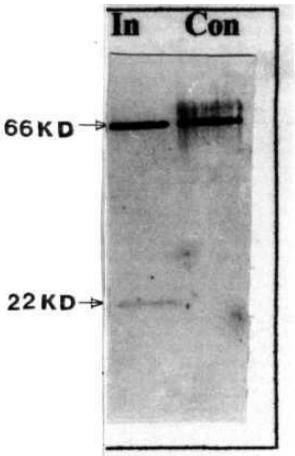


Fig 4.2.1 D. Immunodetection of induced *CT24* Protein as GST-like protein from *E.coli* cell extracts

Mw) Mol. Wt standards In) Induced *CT24* cells
Con) Uninduced *CT24* cells
Anti-*Pugb* antibodies are used.
Arrow represents induced GST-like protein

determination were denatured, run on an SDS-PAGE and immuno blotted. antibodies detected an immunoreactive 22-kDa *CT24* polypeptide, in the induced *E.coli* cell extracts (Fig.4.2.1D). No such immunoreactive protein was observed in the control un-induced cells. The absence of such a signal in the un-induced culture further confirms the observation that this induced 22kDa polypeptide is a GST-like protein. This probably is responsible for the enhanced GST activity. Interestingly, anti-*Pugh* antibodies from pumpkin also detect an intense 66kDa band in both the induced and un-induced cell extracts, probably representing the endogenous *E.coli* GSTs which might contribute to the GST activity observed in *E.coli*.

4.2.2 Partial purification of the GST-Like protein

The induced *E.coli* cells carrying *CT24* sequences were lysed, separated on preparative SDS gels and the GST-like protein was electro-eluted. The purity of the electro-eluted *CT24* protein was confirmed on SDS-PAGE (Fig. 4.2.2.A). This purified protein was used to generate polyclonal antibodies in rabbit. The identity of the polyclonal antibodies was confirmed by immonoblotting of the induced and un-induced culture extracts of *E.coli* cells. Anti-*CT24* antibodies detected a 22-24 kDa *CT24* protein in the induced cultures while no such protein was detected in the un-induced cultures (Fig.4.2.2B).

4.2.3 Detection of *CT24* specific genomic sequences in rice

In order to detect and map the *CT24* specific sequences in rice genome, the restriction digests of *Abhaya* and *Shyamala* with contrasting color phenotypes were probed with *CT24* cDNA. No polymorphism was detected at this locus among these lines with any of the eight enzymes used. However, the results of the genomic blot clearly reveal that there exists more than one *CT24* specific sequences in the rice genome (Fig. 4.2.3). These two parental lines were selected because we earlier used them for mapping the chalcone synthase gene of rice (Reddy *et al.*, 1996).

4.2.4 *CT24* like sequences in plants

The *CT24* sequence in rice is interesting mainly because of its structural similarities with several stress responsive sequences such as PR proteins, HSPs, and

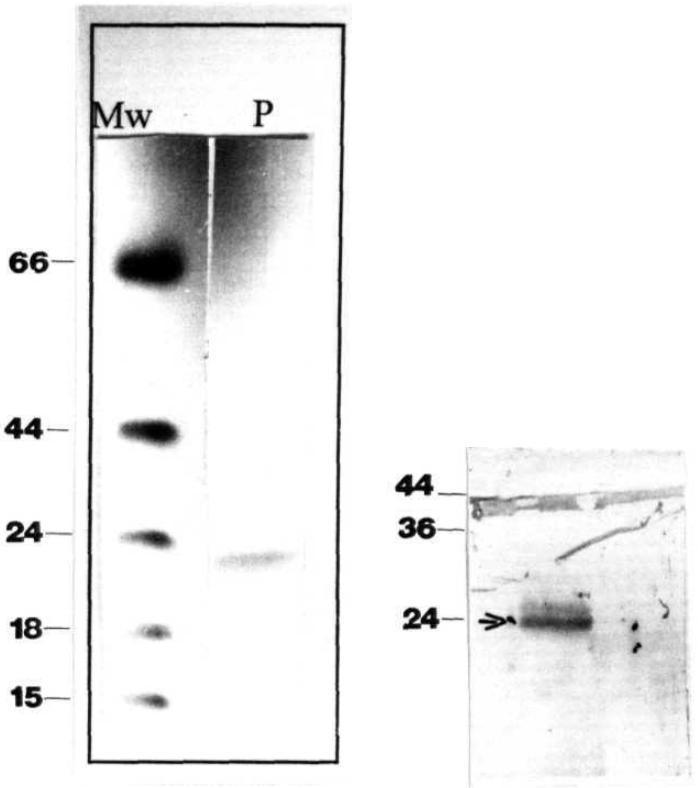


Fig. 4.2.2A. SDS-PAGE of purified 22kDa *CT24* protein from induced *E.coli* cell extracts.
Mw) Mol.Wt standards P) Purified protein

Fig. 4.2.2B. Western blot of induced 22kDa *CT24* protein using anti-CT24 antibodies
Mw) Mol.Wt standards P) Purified protein

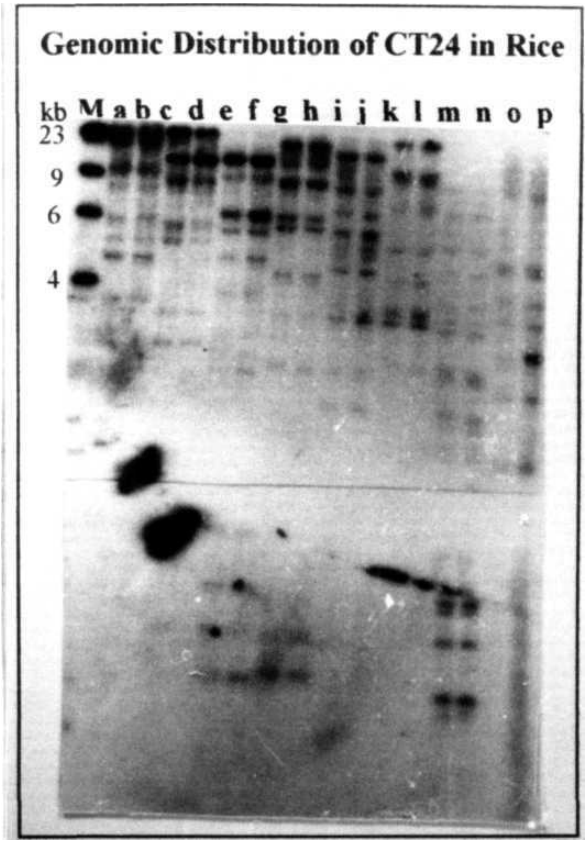


Fig. 4.2.3. Genomic Southern blot analysis of *CT24* locus in *Abhaya* and *Shyamala* digested with various enzymes and probed with 1kb *CT24* cDNA

M)Mol.wt in Kb a) Abhaya *Bam H1* b)Shyamala *Bam H1* c) Abhaya *EcoR1* d) Shyamala *EcoR1* e) Abhaya *Hind III* f) Shyamala *Hind III* g) Abhaya *Bgl II* h)Shyamala *Bgl II* I) Abhaya *EcoR V* J) Shyamala *EcoR V* k) Abhaya *Xbal* l) Shyamala *Xbal* m) Abhaya *Dra I* n) Shyamala *Dra I* o) Abhaya *Kpn1* p) Shyamala *Kpn1*

several auxin responsive sequences (Schmitz and Theres 1992). A recent BLAST search reveals that *CT24* shows extensive sequence identity with *Z.mays* mRNA (98%) for GST, *Oryza sativa* glutathione-S-transferase (82%), 2,4-D inducible glutathione-S-transferase (GSTa) of *Glycine max* (65%), *Aegilops squarrosa* GST (TS1-1) (68%), *N.tabaccum* auxin induced mRNA (*pCNT 107)* (58%), cold responsive gene *CoR* (56%), and several other cDNAs which are also probably GSTs. The similarity matrix of the *CT24* cDNA with other reported GSTs from the data base is shown in Fig. 4.2.4.

4.3 UV-B responsive CHS protein and transcript levels

The purpose of this experiment was to examine whether UV-B also enhances the levels of CHS transcript and protein in *PP* seedlings harvested at regular intervals after exposure. The total RNA prepared from these samples was separated on a 1.2% denaturing formaldehyde agarose gel and transferred to Hybond N+ and the blot was probed with 1.4kb maize *C2* cDNA fragment. The results of the UV-B induction reveals that the *C2* specific transcript was detected from 8h onwards upto 20h with a basal level of expression at Oh interval (Fig.4.3.1A). The Western blot analysis of the protein extracts of such seedlings when probed with anti-CHS antibodies from maize revealed a detectable 44kDa CHS protein from 4h onwards reaching a peak at 20h (Fig.4.3.2B). The increase in transcript levels coincide well with the CHS protein levels as shown in Western blots (Fig.4.3.1B). Thus mRNA accumulation profiles broadly agrees with the protein levels. Since the reaction catalysed by CHS is the committing step of the anthocyanin pathway its expression pattern reflects the level of anthocyanin biosynthesis.

4.4 Genetic engineering of the anthocyanin pathway in rice

Genetic engineering of rice for a regulated anthocyanin expression was attempted using a series of vectors. Plant expression vectors carrying the cDNA sequences cloned downstream of *Ubi* and *E35S* promoter were made. Both *Indica* and *Japonica* rice lines were used for transformation experiments. For the first time in rice, transient expression system for anthocyanin production was developed with *Tp309*.

Table 4.2.4. Alignment matrix of GSTs in plants

Pair Distances of Alignment, clustal

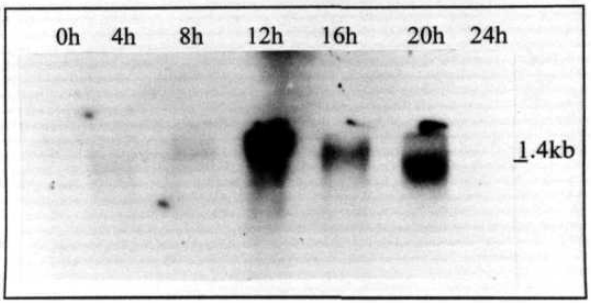
	Percent Similarity in upper triangle												
A *squarrosa* CT cDNA ORF	***	28.1	52 6	503	496	49.7	54.1	59 8	597	58	454	44.7	*A. squarrosa* CT cDNA ORF
B. juncea CT cDNA partial ORF?		***	276	290	292	29.4	28.8	30.5	30 5	29.5	27.0	25.2	*B. juncea* CT cDNA partial ORF?
E. globulus auxin-ind pep ORF			***	72.1	70.3	69.8	470	624	62.1	59 3	420	42 8	*E. globulus* auxin-ind pep ORF
G. *max* CT cDNA ORF				**.	797	79.6	482	608	60.8	56.8	388	39.3	G *max* CT cDNA ORF
N tabacum auxin-induced mRNA ORF						982	473	60.3	605	568	38.8	37 1	*N. tabacum* auxin-induced mRNA ORF
N. tabacum parC mRNA ORF						...	470	603	60 5	567	379	376	*N. tabacum* parC mRNA ORF
P. mariana CT cDNA? ORF								487	489	459	394	379	P. *mariana* CT cDNA? ORF
Z mays CT cDNA ORF								***	990	78.8	483	466	Z *mays* CT CdNA ORF
pCT24 ORF									***	78.7	482	47.1	*pCT24* ORF
O sativa CT cDNA ORF										...	456	45.8	*O sativa* CT cDNA ORF
0. *sativa* mRNA pep chilling tolerance ORF												41 8	*O. sativa* mRNA pep chilling Tolerance ORF
Z. mays Bz2 ORF												***	*Z mays Bz2* ORF

Fig. 4.3 Expression pattern of CHS in UV-B induced *PP* seedlings by Northern and Western blot analysis.

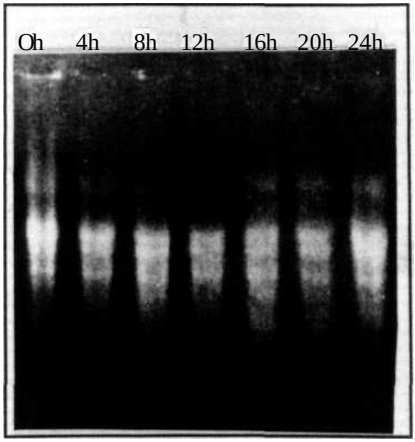
A) Northern Blot analysis using 1.4kb C2 cDNA as probe
B) RNA gel showing equal amount of RNA loaded
C) Western blot analysis using *Anti-CHS* antibodies form maize

Fig. 4.3 Expression pattern of CHS in UV-B induced *PP* seedlings by Northern and Western blot analysis.

A



B



C

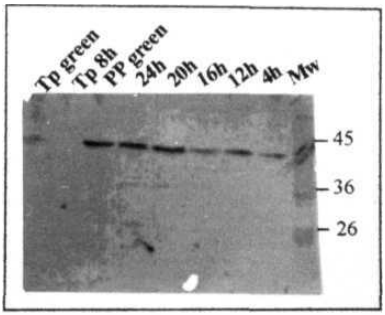
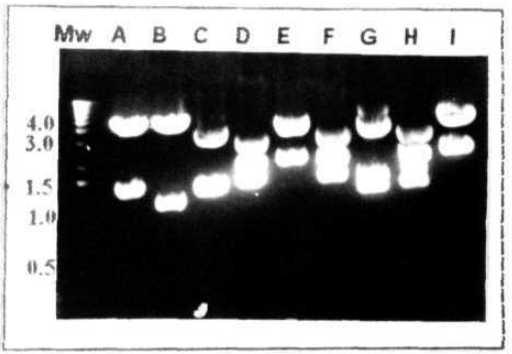


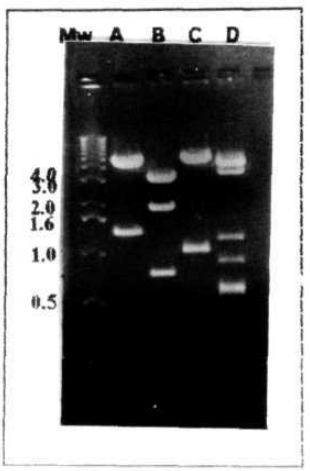
Table 4.4.1. List of plasmid constructs used in this study

Plasmid	ILTAB Number	Gene of Interest	Size (Kb)	Promoter	Source
pMAC1	374	2.5kb *cl* sense of maize	7.46	*UBI*	pUoH *Myb Cl*
pMAC2	354	1.4kb *C2* sense	6.3	*UBI*	pUoH C2
pMA*C1* (as)	359	2.5 *Kb c1 as* from maize	7.46	*UBI*	pUoH *Myb* C1 (as
pMA*Chs* (as)	360	800 bp *Chs* as rice	6.16	*UBI*	pUOH *Chs* (as)
pMAR (as)	375	800 bp *R* as *Pst1* frag.	6.025	*UBI*	pUOH *Myb* (as)
pMAR	375	2.5 kb *R* sense	5.9	*E35S*	pUOH *R*
pMA*A2*	**379**	12 *kb .42* of maize	5.1	*E35S*	pUOH *A2*
pMA*Bz2*	**376**	1 kb *Bz2* of maize	5.0	*E35S*	pUOH *Bz2*

Fig. 4.4.2. Restriction pattern of chalcone synthase gene constructs.



A) Sense constructs pMAC2
Mw) 1kb ladder A) *Bam H1* B)BamH1 + *Sma I* C) *Sal 1* D) *Pst 1*
E) *EcoR1 F)Bam H1+Pst1* G) *Sal 1+Sma 1* H) *EcoR1+Sma 1*



B) Anti-sense constructs - pMA*Chs* (as)
Mw) 1kb ladder A) *Nco1* B) *Pst 1 C) Hind III +Bgl II* D) *Hind III +Acc 1*

4.5 Transformation of *Indica* rice varieties

Rice lines *N22B* (genetically defined as pro-anthocyanidin accumulating mutant) and the *PP* (fully purple) were used in our attempts to establish transformation. The media used for culturing and their regeneration profiles are outlined in materials and methods. The culturing of both *N22* and *PP* was initially encouraging with *N22* being more responsive. However, the transformed calli failed to regenerate even though they survived in various selection media. Attempts to regenerate *PP* also proved to be futile. It is interesting that *PP* calli did not produce anthocyanins in any of the media tested. Due to the failure in our attempts to regenerate the *Indica* lines, we have concentrated on the transformation of a responsive *Japonica* rice line, *Tp309*.

4.6 Transformation of *Japonica* rice line *Tp309*

Genetic transformation of *Tp309* with anthocyanin genes was attempted to assess the feasibility of using these genes or a combination of genes as reporters in transformation. More importantly, our goal is to develop stable transgenic lines carrying anthocyanin genes accumulating the flavonoid products of interest which might have a role in conferring stress resistance to both biotic and abiotic. Results in the following paragraphs highlight our attempts at the genetic engineering of anthocyanin pathway in rice using maize genes.

4.6.1 Transient production of purple color in rice calli

Plant expression vectors carrying individually the maize anthocyanin regulatory genes, the *Cl* (pMAC1) and *R* (pMAR) under the control of *Ubi* and *E-35S* respectively, were co-bombarded into *Tp309* embryogenic calli. The calli were also co-bombarded with the *hph* (p35H) plasmid construct. No color production was observed initially in any of the calli co-bombarded with individual constructs or their combination. Since visual accumulation of anthocyanins did not occur it is most likely that *Tp309* requires additional genes to produce anthocyanins or *Tp309* might have a block in one or more steps in the anthocyanin biosynthetic pathway.

In the second set of experiments the calli (yellow and compact) were co-bombarded with the *Cl, R* and *C2* (pMAC2- maize chalcone synthase under the control

of the *Ubiqutin* promoter) with *p35H* as a selection marker. A few calli showed the production of bright pink color when placed on RNH50 medium (containing 50mg/l of hygromycin and supplemented with 2mg/ml of IAA) under a photo-period of 16h at 110-130 uM/m2 PAR (Fig.4.6.1). However, the frequency of the purple calli was very low. The pigmentation was localized and was first observed with in 48h after bombardment. In one particular case, it was also observed in calli grown in dark for 4 weeks and later placed in RNH50 medium supplemented with IAA for three days under illumination. Such pigmentation pattern was not observed in the control un-transformed calli. The pink color was stable for a week after which it faded off or was masked by rapidly growing green areas.

4.6.2 Transformation of *Tp309* with pMAC2

The genetic transformation of *Tp309* was carried out by co-bombardment of pMAC2 and *hph*. The map of pMAC2 was shown in Fig. 4.6.2. A total of 50 regenerants were obtained from three independent bombardment experiments. Of these, 10 RO seedlings (5 intense, and 7 pale pink) exhibited purple pigmentation to a varying degree (visually). The purple color was observed in the regenerated plants with in one week after placing on the rooting medium. The color persisted for a month until the plants were transferred to the growth chamber where the purple regions were overgrown by green areas. The primary transformants designated as *C2-5a*, C2-7a & C2-7b (siblings), showed a bright pigmentation in leaf blade and leaf sheath while C2-2, C2-3, C2-6, C2-8a, C2-9, C2-10, C2-11C and C2-25 seedlings showed a lighter pigmentation in localized areas on the leaf blade. The un-transformed *Tp309* plants did not show any purple color (Fig.4.6.2A). The pigmentation was prominent in leaf sheath and pale in leaf blade. In some plants, the pigmentation was barely detectable. The color seemed to be patchy in the leaf sheath of seedlings (Fig.4.6.2B). Some of the transgenic plants showed intense purple color in the leaf sheath (Fig.4.6.2C). The transformants 7a and 7b (siblings) that showed an early intense coloration died upon transfer to green house. The suspected connection between constitutively over-expressed *C2* cDNA and cell death remains to be tested. The growth and fertility of the other putative transgenic plants was found to be normal.

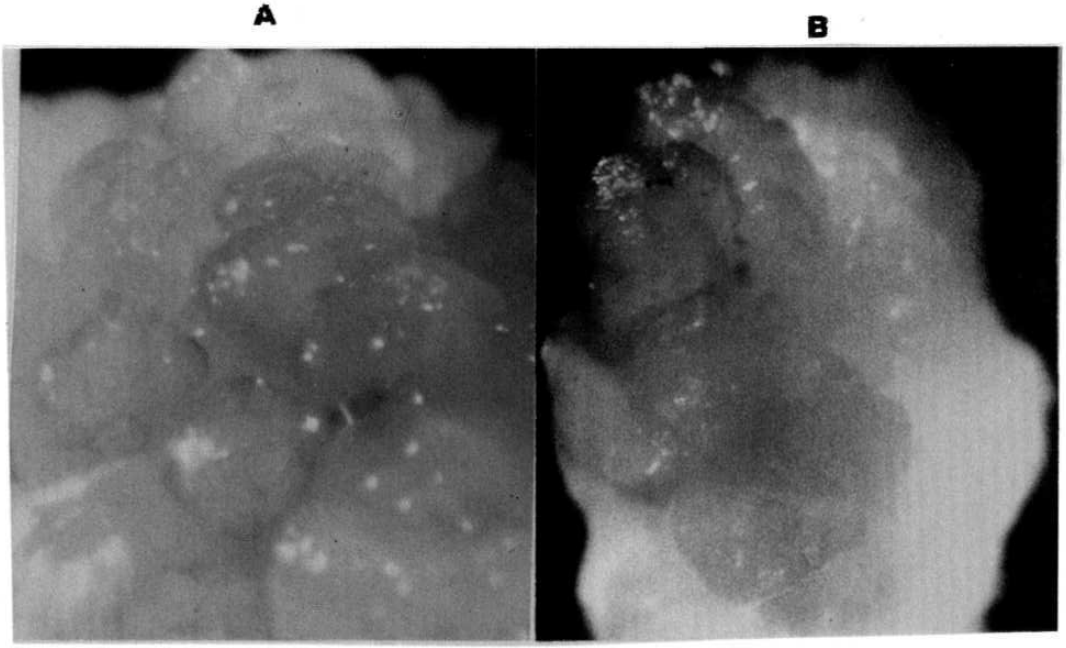


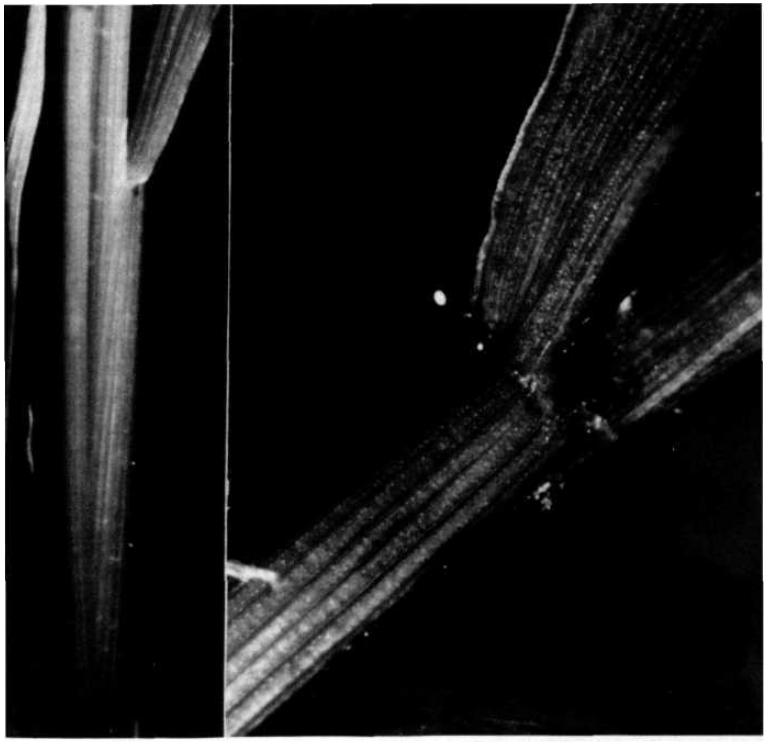
Fig. 4.6.1. Transient expression of anthocyanins in *Tp309* calli co- bombarded with pMAC*1*, pMAC*2*, and pMA*R*.

A) Untransformed control B) Transgenic calli

Development of purple calli in cells was observed 48h after the bombardment.

Fig.4.6.2.A. Chalcone **synthase transgenic *Tp309* exhibiting purple phenotype**

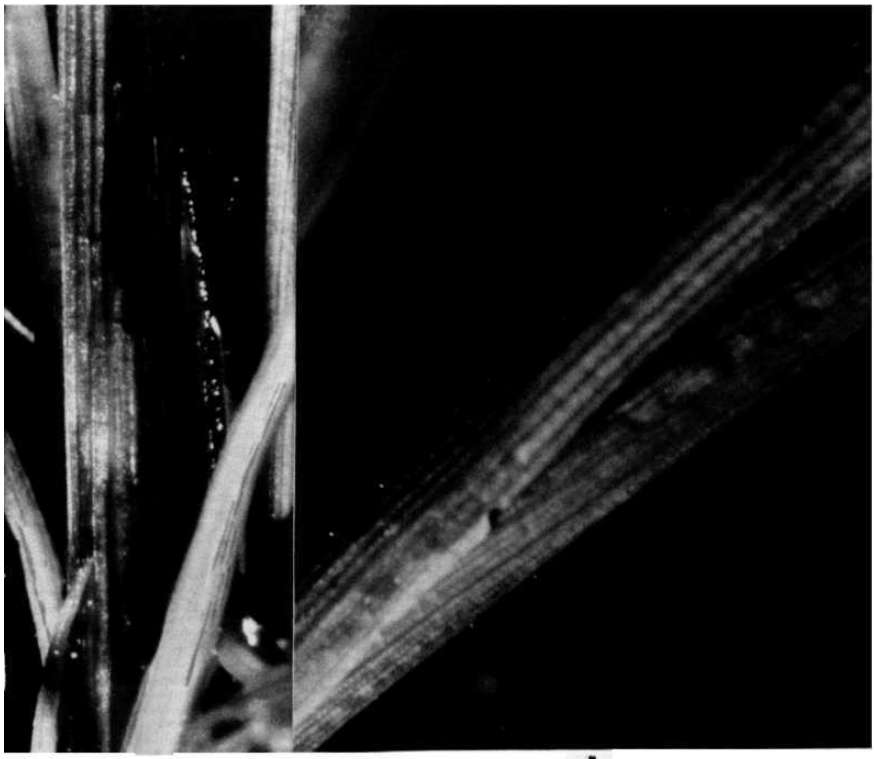
a) Untransformed *Tp309* b) Transgenic *Tp309*
Note: The pigmentation is seen as a purple region on the leaf



a b

Fig. 4.6.2.B. Chalcone synthase transgenic *Tp309* exhibiting purple phenotype

c) Transgenic *Tp309* showing a patchy pigmentation
d) Transgenic *Tp309* showing pigmentation in the leaf sheath



c d

Fig. 4.6.2.C. Chalcone synthase transgenic *Tp309* exhibiting purple phenotype

e) Transgenic *Tp309* showing a patchy pigmentation in one of the leaf sheath, while the other is green.
f) Transgenic *Tp309* showing pigmentation in the leaf sheath and some areas of the leaf

e f

4.6.3 Molecular analysis of the RO plants

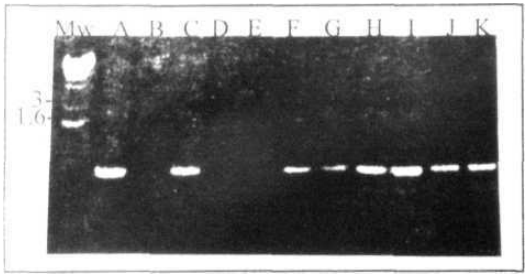
Genomic DNA extracted from the leaves of RO plants was subjected to PCR analysis. Two sets of primers were used, namely, *Ubi-Nos* for *C2* amplification and *H1* and *Hi* for *hph* amplification. The PCR experiments with *Ubi-NOS* were not successful probably because of a high GC proportion of the *C2* cDNA. However, the experiments with *H1* and *H3* primers for *hph* sequences gave positive results. All the thirty regenerants obtained in the first bombardment experiment showed the presence of the expected 850bp *hph* fragment (Fig.4.6.3A, B and C). The PCR data thus provide evidence for the transformation of *Tp309* with *hph* and also possibly the gene of interest. The RO plants were grown to maturity and selfed. The plants raised from the progeny seed were subjected to a detailed Southern blot analysis to check for the integration of *C2* sequence in the genome.

4.6.4 Genomic DNA blot analysis of primary **transformants**

The integration of the cassette *Ubi-C2* in the primary transgenics was also tested by digesting the putative transgenics with *Hind III-Sma I* (intact cassette) and an additional digestion with *Hind HI* (number of integrated copies) analysed on 0.8% agarose gels and blotted on to the membrane. The blots were probed with the 3.2 kb *Ubi-C2* sequence Fig.4.6.4U. Figure 4.6.4 (L) is a genomic blot of the primary transformants showing extensive rearrangements. The data revealed that *Hind III* digests show a multi-copy integration (Lanes B, E, H and K) in the transgenic plants. The plants C2-17, C2-25, C2-24, C2-14b show a complex pattern of integration when tested with *Hind III + Sma I* (Lanes C, F, I and L). However, the transgenic plant C2-25 shows the presence of an intact cassette when digested with *Hind III + Sma I* (Lane F). The control *Tp309* samples in lanes M,N, O show the presence of a high molecular weight DNA which represents the endogenous *Chs*.

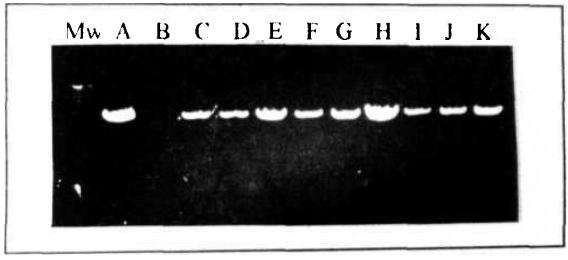
The Southern blot data of the primary transgenic plants having intact cassette is shown in the upper panel of Fig. 4.6.4.1. The transgenic plants C2-1, C2-6, C2-9, were digested with either *Hind III* or with *Bam H1* and loaded onto different wells. This was done in order to know the copy number of the insert and the presence of the gene of interest *(Bam HI* would excise the *C2* sequence from the cassette). The digested DNA was transferred onto a nylon membrane and probed with the pMAC2 **plasmid**. The

Fig.4.6.3. PCR analysis of transformants co-bombarded with *p35H* and pMAC2.



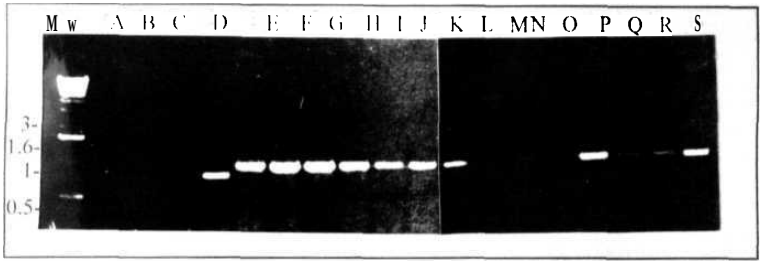
Mw) 1kb ladder A)+ plasmid B) -ve C) Tp + Con D) C2-15D E)C2-21 F) C2-12 G) C2-24 H) C2-5 I) C2-14 J) C2-7 K) C2-14B

H1 and *H3* primers for hygromycin phosphotransferase were used



B

A) +ve B) -ve C) C2-2 D) C2-3 E) C2-10 F) C2-8a G) C2-25 H) C2-12 I) C2-13 J)C2-27 K) C2-8b



Mw) 1kb ladder A) -ve B) Tp con C) Empty D) C2-14B E) C2-11 F) C2-4 G) C2-1 H) C2-17 I) C2-17b J)C2-21a K) C2-26 L) C2-24 M) C2-13a N) C2-15b O) C2-15 P) C2-6B Q) C2-15 R) C2-6 S) C2-7B

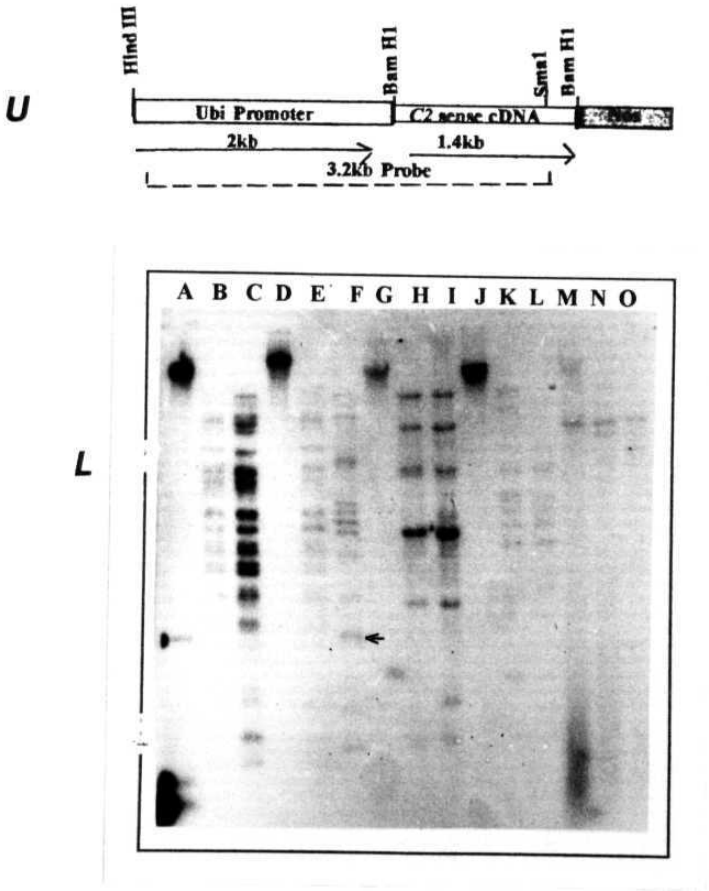
Fig. 4.6.4. Detection of multicopy *Ubi-C2* sequences inserted in the genome of the primary transformants

U) Physical map of the construct used for transformation showing the functional regions and restriction sites used for DNA analysis. The hybridization probes used is shown below the map as dotted lines, indicating the 3.2 kb *Ubi-C2* fragment.

L) Indicates the restriction pattern of the transgenic plants
A) *C2*-17 Un B) C2-17H C)C2-17HS D)C2-25Un E) C2-25H F) *C2*- 25HS G) *C2*-24Un H) *C2*-24H I)C2-24 HS J)C2-14Un K) C2-14H L)C2-14HS M) *Tp309* Un N) *Tp309* H O) *Tp309* HS

Un-Undigested ; H-*HindIII* ; HS- *Hind III +Sma 1*

Fig. 4.6.4. Detection of multi-copy *Ubi-C2* sequences inserted in the genome of the primary transformants



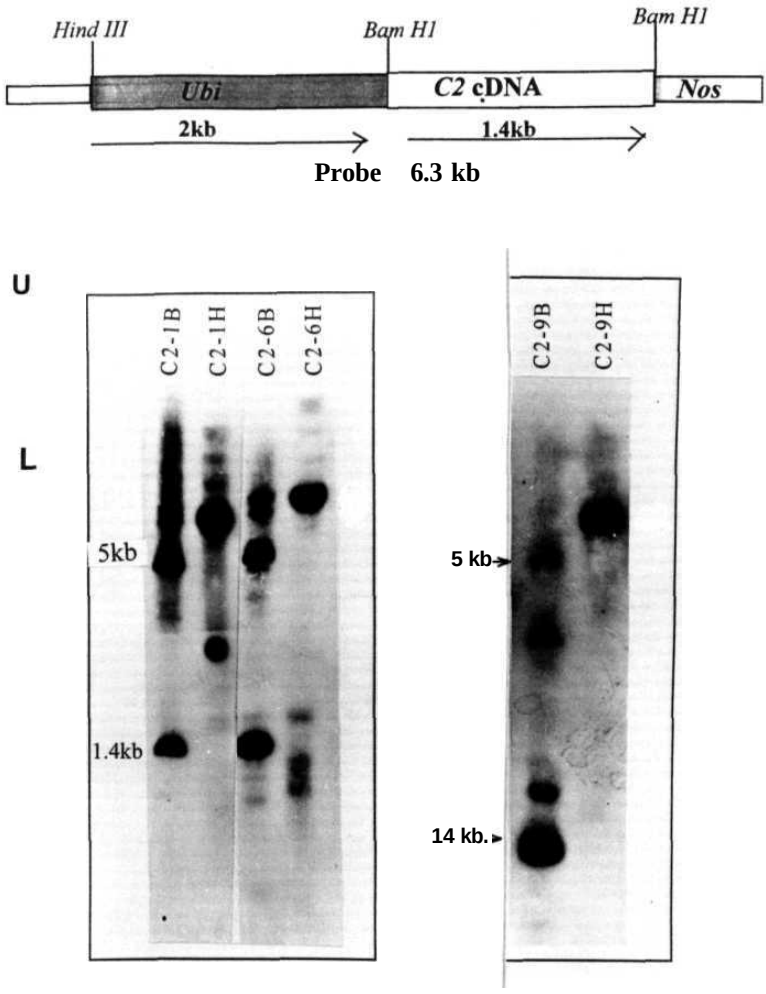


Fig. 4.6.4.1 Detection of intact *Ubi-C2* sequences in the genome of the primary transformants.

U) Physical map of the construct used for transformation showing the functional regions and restriction sites used for DNA analysis. The dotted line represents the hybridization probe.

L) 10 ugms of genomic DNA was digested either with *Hind III* or *Bam H1* and probed with pMAC2 plasmid. Arrows indicate the presence of 4.9kb *Ubi* promoter, and the 1 4kb *C2* fragment in the *Bam H1* digests of the transgenic plants.

Transgenic plant number and the restriction ensyme used is indicated on top (B=*Bam H1*; H=*Hind III*).

southern blots revealed that the transgenic plants C2-1, C2-6, and C2-9 showed the presence of the 1.4 kb *C2* fragment and 5 kb *Ubi* in the *Bam HI* digest. The *Hind III* digests of these plants show the presence of 3 copies (Fig.4.6.4.1). In summary the genomic blot analysis of the primary transgenics suggested that the plants C2-1, C2-6, C2-9 and C2-25 revealed the intact casette. Hence, these were followed in the subsequent generation. In summary, the genomic blot analysis of the primary transgenics suggested that four plants C2-1, C2-6, C2-9 and C2-25 revealed intact casette. Hence, these plants were followed to the subsequent generation. Interestingly, C2-6, C2-9 and C2-25 showed purple pigmentation.

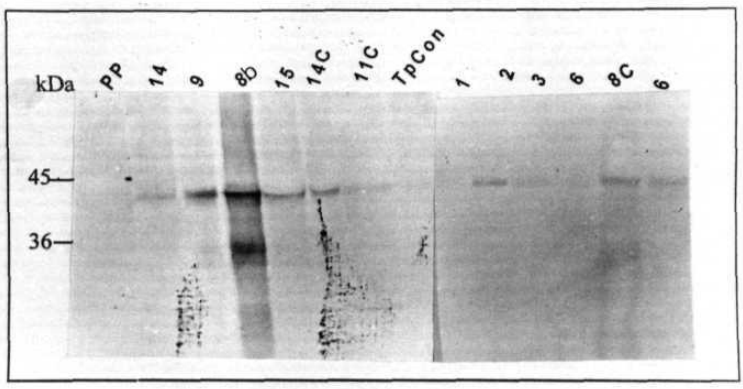
4.6.5 Western blot analysis of primary transgenics

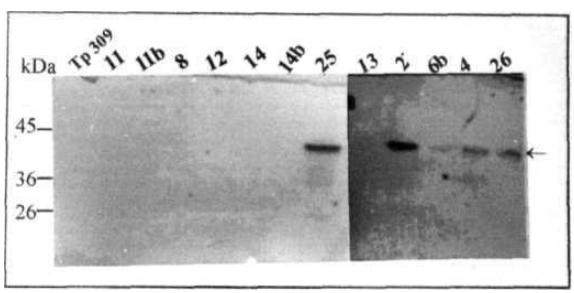
One month old primary transgenic seedlings were tested for the presence of CHS protein using the anti-sera raised against maize chalcone synthase protein (Courtesy, Loverine Taylor, Univ. of Georgia). The data clearly revealed that CHS of the primary transgenic plants reacts with maize chalcone synthase antibodies (Fig.4.6.5 A and B). The un transformed *Tp309* did not show any detectable levels of CHS. The transgenic plants *C.2A. C2-2, C2-3, C2*-4, *C2-6,* C2-6b, C2-8b, C2-8C. C2-9, *C2*-11C, *C2*- 14a, C2-14C, *C2*-15, *C2-25* and C2-26 showed a immuno-detectable signal. Interestingly, the immunoreactive 44kDa polypeptide observed in these plants exhibited the same electrophoretic mobility confirmed earlier as CHS by us in other rice lines (Reddy *et al.*, 1996). Incidentally, the plants *C2-2, C2-3, C2-6, C2*-8b, C2-9 and C2-25 showed purple color in leaf sheath. These results provide evidence that the accumulation of CHS protein in these lines is due to the over expression of *C2* cDN A under the control of *Ubiquitin* promoter.

4.6.6 Molecular analysis of R1 plants

In order to study the inheritance of the gene of interest, the RO plants were selfed seeds were harvested and planted. The Rl plants were further analyzed for the presence and expression of CHS. Surprisingly, none of the Rl transgenic plants in general showed detectable levels of purple color. However, the young seedlings of *C2*-1-1 exhibited pale pink color in young leaf sheath which faded off later. Plants were similar to the wild type with respect to the morphology, vegetative growth and flowering

Fig. 4.6.5. Western blot analysis of primary transgenic plants showing the immuno-detectable CHS protein.





The transgenic plant number is indicated on the top.
PP- Purpleputtu (+ve control), *Tp309- Tp309* (-ve control)
Equal amounts (30 *u* gms) of total soluble protein was loaded into each slot. *Anti*-CHS anti-body from maize were used.

behavior.

4.6.7 DNA slot blot Analysis of R1 plants

Total genomic DNA of the twenty one independent transgenic Rl plants was slot blotted and hybridized with 2kb *Ubi* promoter sequence as a probe. Un transformed Tp 309 served as a negative control and 100ng of *Ubi* was the positive control included. The data revealed the presence of positive *Ubi* signal in sixteen out of the twenty one Rl plants (Fig 4.6.7 A and B). The differences in the intensity of the *Ubi* positive signal probably represent the differences in the copy number. Those plants which are found to be positive for *Ubi* in slot blots were used for further Southern analysis. It may be pointed out that C2-1 showed intact casette in the R0 generation, with a immuno-detectable CHS protein.

4.6.8 Genomic DNA blot analysis of Rl plants

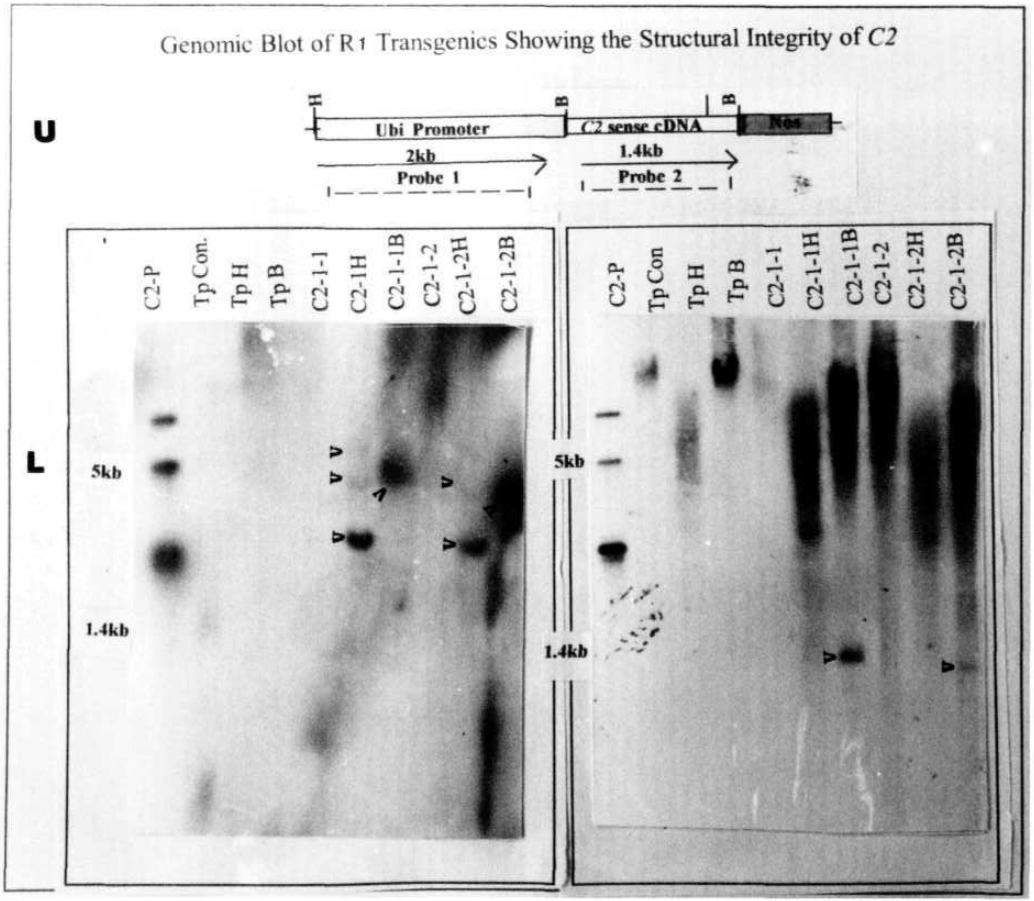
The *Ubi* positive Rl transgenic plants were subjected to a detailed Southern analysis as follows: Genomic DNA were digested with either *Hind III* or with *Bam HI* and loaded onto different wells in order to know the copy number of the insert and the presence of the gene of interest *(Bam Hl* would excise the *C2* sequence from the cassette). The hybridization pattern of the pooled samples (*C2*-1-1 and *C2*-1-2) revealed three copies of the transgene with *Hind III* when probed with the *Ubi*-promoter (lower left panel Fig.4.6.8). The same blot was stripped off and re probed with the 1.4kb *C2* cDNA fragment. The transgenic C2-1-1 and *C2*-1-2 plants revealed a clear *Bam Hl* fragment of the expected size (1.4kb) (Fig.4.6.8, lower right panel). It is clear from the Southern blot that *C2*-1-1,and *C2*-1-2 plants represent different progeny of the Rl population and do not exhibit chimerism. They were therefore pooled and used in subsequent analysis.

The DNA digests *of Hind III* and *Bam Hl* of several other Rl plants that were positive for *Ubi* by slot blot when probed with pMAC2 revealed a complex hybridization pattern. The transgenic plant *C2*-1-6 shows the presence of the 4.9kb *Ubi* fragment and 1.4 kb *C2* when digested with *Bam Hl*. In contrast, the transgenic plants C2-1-3, C2-l-23b, C2-1-24, C2-1-17 do not show the fragments of the expected

Fig. 4.6.8. Genomic blot analysis of R1 plants: Detection of *Ubi-C2* sequences inserted in the genome of the transformants

U) Upper panel indicates the physical map of the construct used for transformation showing the functional regions and restriction sites used for DNA analysis, H- *Hind III*, B- *Bam H1.* The hybridisation probes used are shown below the map as dotted lines. Probe I is 2kb *Ubi* fragment and Probe II is 1.4kb C2 cDNA fragment.

L) First half of the lower panel represents the blot probed with *Ubi* while the next half of the panel represents the blot probed with *C2.* 10 ugms of genomic DNA was digested either with *Hind III* or *Bam H1* and probed with 2kb Ubi stripped and re-probed with 1.4 kb *C2.* Note the arrows with *Hind III* digests showing three copies of the transgene with *Ubi* as a probe. The 4.9kb intact *Ubi* and 1 4kb *C2* fragment was seen in the lanes digested with *BamH1 .*
Note: C2-1-1 and C2-1-2 are siblings.
Transgenic piant number and the restriction enzyme used is indicated on top


Genomic Blot of R1 Transgenics Showing the Structural Integrity of C2
U
H
B
B
Ubi Promoter
C2 sense cDNA
Nos
2kb
Probe 1
1.4kb
Probe 2
L
C2-P
Tp Con.
Tp H
Tp B
C2-1-1
C2-1-1H
C2-1-1B
C2-1-2
C2-1-2H
C2-1-2B
5kb
1.4kb
C2-P
Tp Con
Tp H
Tp B
C2-1-1
C2-1-1H
C2-1-1B
C2-1-2
C2-1-2H
C2-1-2B
5kb
1.4kb

molecular weight of either 4.9kb *Ubi* or 1.4kb *C2* in their *Bam H1* digests (Fig.4.6.8.1). The transgenic plant *C2*-1-24 (digested with *Bam H1*) shows the fragments of higher molecular weight while the plants C2-1-3, C2-1-23 and C2-1-17 show the fragments of lower size. In summary, the R1 southern blots indicated the intact casette in C2-1, C2-6 (data in Fig. 4.6.8 and 4.6.8.1) C2-25, and C2-9b (data not shown).

4.6.9 Western blot analysis of Rl plants

The total protein extracts of one month old transgenic plants, *C2*-1-1, C2-1-6 and *C2*-1-9, C2-25 showed the correct integration and also plants showing rearrangements (C2-1-14, *C2*-2-16, *C2*-1-24) were subjected to Western analysis using the maize anti-CHS antibodies. Equal amounts of the total proteins were separated by SDS-PAGE and used for Western Blot. *C2*-1-1, C2-1-6 and *C2*-1-25 revealed the presence of detectable levels of CHS protein of the expected size (Fig. 4.6.9). However, the other transgenic plants did not show immuno-detectable CHS protein.

4.7 Defense response of Rl transgenics to fungal blast

The Southern positive Rl plants (representative of intact and rearranged cassette) were screened for their performance to infection by blast fungus *P. oryzae* pv *oryzae (IC9).* The screening was done in the nursery beds at the Directorate of Rice Research, Rajendranager, Hyderabad. Forty day old Rl and R2 plants of uniform vigor and growth and also positive for the intact cassette were subjected to *Pyricularia oryzae* infection. *IR64* and *HR12* were used as the resistant and susceptible controls respectively. Non-transgenic *Tp309* was used as the negative control. Disease scoring was done on the SES (Standard Evaluation Scale, IRRI) based on the infection, type and number of disease lesions including the overall performance of the plant under infection conditions.

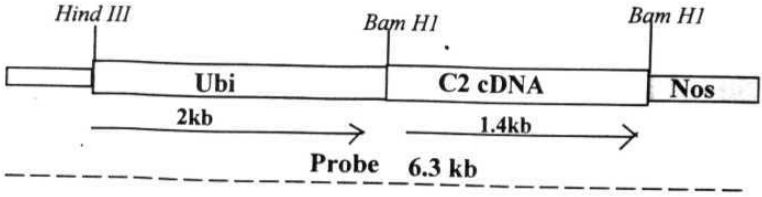
Pots a, b, and c show the uninfected *Tp309,* infected *Tp309* and infected *IR64* plants respectively (Fig.4.7A). It is clear that *IR64* is resistant where as *Tp309* is moderately resistant. The response of the infected transgenic plants C2-1-1, C2-1-23, and C2-1-25 is displayed in pots d, e and f in Fig.4.7 B. The spread of the infection in a single leaf is shown in Fig. 4.7 C as HR12(g), Tp control (h), C2-l-l(i), C2-1-25 (j) and

Fig. 4.6.8.1 Genomic blot analysis of R1 plants: Transformants showing extensive rearrangements

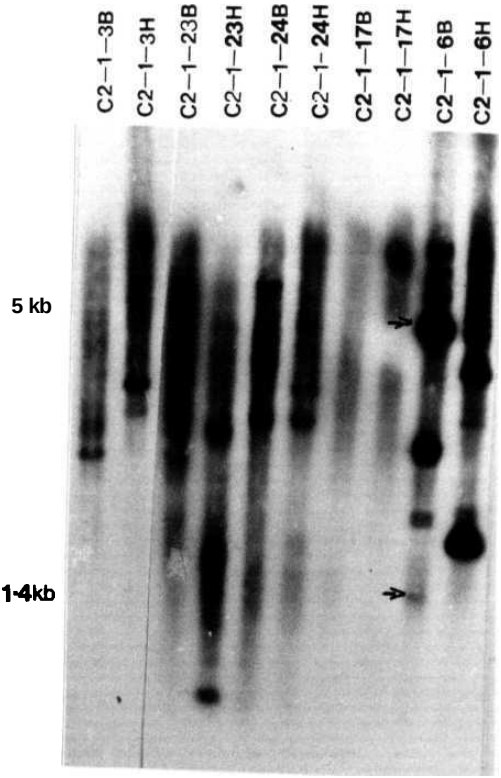
U) Physical map of the construct used for transformation showing the functional regions and restriction sites used for DNA analysis. The dotted line represents the hybridization probe

L) 10 *u*gms of genomic DNA was digested either with *Hind III* or *Bam H1* and probed with pMAC2 plasmid. Arrows indicate the presence of 4.9kb *Ubi* promoter, and the 1.4kb *C2* fragment in the *Bam H1* digests of the transgenic plants C2-1-6.
Transgenic plant number and the restriction enzymes used is indicated on top (B=*Bam H1*; H=*Hind III).*

U


Hind III
Bam H1
Bam H1
Ubi
C2 cDNA
Nos
2kb
1.4kb
Probe
6.3 kb

L


C2-1-3B
C2-1-3H
C2-1-23B
C2-1-23H
C2-1-24B
C2-1-24H
C2-1-17B
C2-1-17H
C2-1-6B
C2-1-6H
5 kb
1·4kb

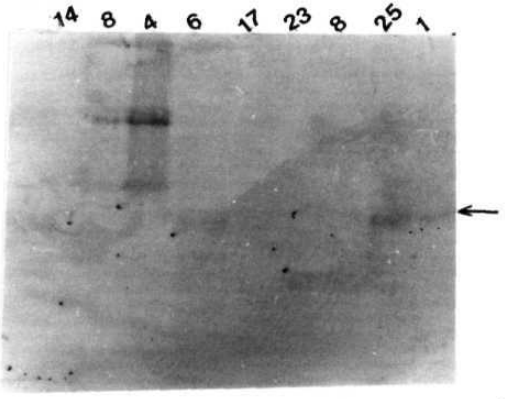


Fig. 4.6.9. Immuno-blot analysis of R1 plants
The transgenic plant number is indicated on the top.
Anti-CHS antibodies are used.

Fig.4.7. Performance of transgenic plants under artificial infection against *Pyricularia oryzae*.

A)Performance of the non-transgenic plants
a) *Tp 309* control b) *Tp 309* infected c) *IR64* infected
B} Performance of the transgenic plants
d) C2-1-23 e) C2-1-25 f)C2-1-1
C) Type of lesion during infection
g) *HR12* h) *Tp 309* i) C2-1-1 j)C2 1-25 k)C2-1-6

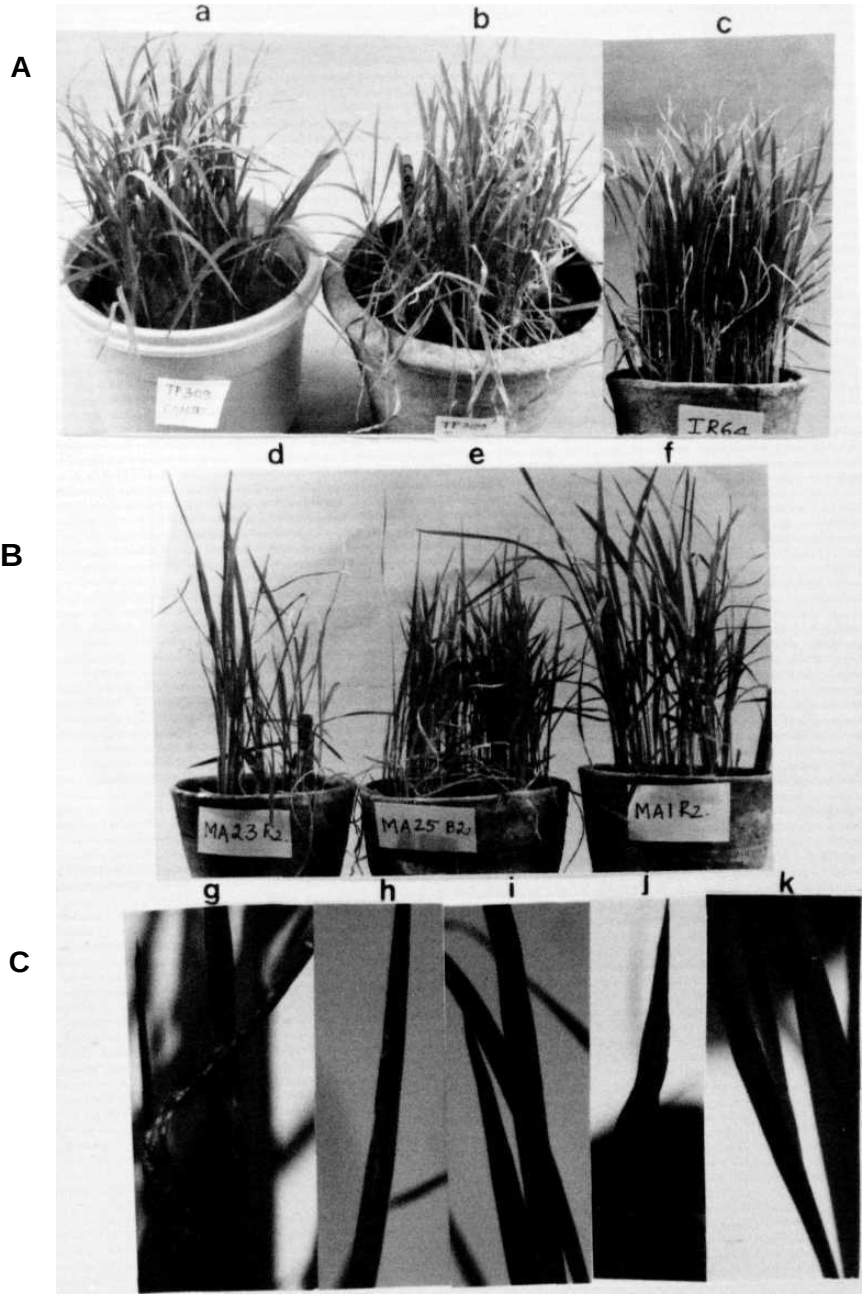

A
a
b
c
IR64
B
d
e
f
MA23R2
MA25 B2
MA1R2
C
g
h
i
j
k

Table: 4.7.1. Screening of transgenics against *Pyricularia oryzae*

Plant No	**No. Inf/tot.**	No. **lef./tot**	**Transngenic Plant Number/Score**											
			1	2	3	4	5	6	7	8	9	10	O.S	(
C2-1-1a	7/10	8/28	4	3	3	2	1	3	5	1	1	4	4-5	II
C2-1-lb	6/10	8/30	3	4	1	3	4	4	1	5	1	1	4	
C2-l-25Ba	7/10	11 25	!	5	1	1	5	2	5	4	1	3	4-5	I
C2-l-25Bb	9/10	9/41	2	3	5	?	3	2	5	5	4	2	4	
C2-l-17Ba	8/10	8/41	3	5	4	3	6	5	5	5	1	1	5	III
C2-l-17Bb	3/5	3/12	4	4	1	1	2						5	
C2-l-24Ba	8/10	12/41	4	4	6	5	5	4	3	3	1	1	6	III
C2-l-24Bb	9/10	9/43	2	3	5	3	3	2	5	5	4	2	5	
C2-l-6a	7 10	8/35	1	6	5	2	3	2	3	4	3	3	5	II
C2-J-6b	1010	16/41	5	3	2	5	5	4	4	4	3	5	4	
C2-1-9ba	9/10	10/36	3	3	4	1	3	5	5	3	3	5	5	1
Tp309	9/10	14/38	4	1	4		4	6	6	3	4	5	5-6	
Tp309	10/10	13/32	4	2	5	4	5	5	5	3	2	4	5-6	
IR64	0/10	None	1	1	1	1	1	1	1	1	1	1	1	
IR64	0/10	None	1	1	1	1	1	1	1	1	1	1	1	
HR12	8/10	18/25	9	8	8	8	9	9	9	8	9	9	9-10	
HR12	9/10	25/38	9	8	8	9	9	9	9	8	7	8	9-10	

No.Inf/tot total no. of plants infected out of the 10 randomly chosen plants.

No. lef./tot number of leaves infected out of the total number of infected plants

Note: Score of 1-3 is Resistant: Score of 8-10 is Susceptible; 5-7 Moderately sensitive; 3-5 moderately resistant

O.S = Overall score; Gp. classified based on western and phenotypic data in R0 plants.

a & b represents duplicate pots of each of the experiments done.

Data represents the mean values of three independent experiments done in two seasons. Field experiments were done at the Directorate of Rice Research, Hyderabad.

C2-1-6 (k). The leaves of the susceptible *HR12* show severe necrotic lesions (g), while that of *Tp309* (h) and the transgenic plants C2-1-25; C2-1-1; C2-1-9 (i to k) show smaller and less severe lesions. The scores of the 10 individual plants of each of the representative transgenic line are given in Table 4.7.1. On the basis of SES scoring , on a 0 to 10 scale non-transgenic *Tp309* showed a score value of 5-6 for, the resistant *IR64* 1, *HR12* 10, and the transgenic plants showed a score of 4-5. The table also includes some transgenic plants (C2-1-17b and C2-l-24b) showing rearrangements. It is interesting here that these plants show the score values reaching those of the control plants. Interestingly, the plants C2-1-1 and C2-1-25 has the functional casette and shows immuno-detectable protein. The scores suggest that the transgenics show marginally improved response against the pathovar. It is to be noted that the screening conditions were more severe for the seedlings compared to the mature plants. The resistance pattern would be more clear if screened at later stages of seedling growth and the improvement in the transgenics performance might be more realistic.

4.8 Genomic blot analysis of R2 **transformants**

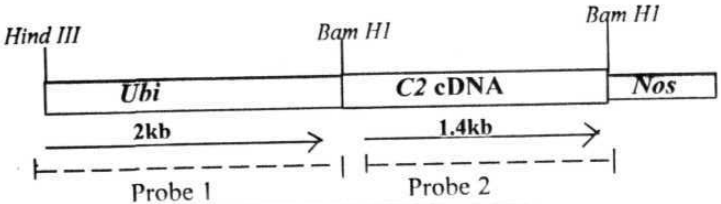
The transgenic plant having an intact cassette in the R1 generation (C2-1-1, C2-1-9 and C2-1-1-25) and plants showing rearrangements (C2-1-1-17 and C2-1-1-24) were selfed and used for genomic blot hybridizations in the R2 generation. The R2 plants were digested with *Hind III* to analyse the inheritance of the insert, *Bam H1* to know the inheritance of the gene of interest (*Bam H1* would excise the *C2* sequence from the cassette). The schematic representation of the plasmid used and the restriction sites are shown in the upper panel. (Fig. 4.8.1 U). The hybridization pattern of the R2 plant DNA probed with *Ubi* is shown on the lower left side of the panel. The plants C2-1-1-24, C2-1-1-9 and C2-1-1-25 show the presence of 4.9kb *Ubi* fragment in their respective *Bam HI* digests . The plant C2-1-1-25 show the presence of two copies while C2-1-1-9 and C2-1-1-24 show the presence of a single copy transgene as seen in *Hind III* digests. Right side of the panel shows the same blot stripped and re probed with 1.4kb *C2* cDNA fragment. Interestingly, the *Bam HI* digest reveals a 2.8kb *Ubi* hybridizable fragment while the *Hind III* reveals the fragments of 4.9kb (Fig.4.8.1 right panel). It is to be noted here that plants C2-1-1-9 and C2-1-1-25 does show the presence of the intact casette in the Rl Southerns. Assuming a rearrangement of the transgene, and in order to

Fig 4.8.1 **Detection of single copy *Ubi-C2* sequence inserted in the genome of the R2 plants**

U) Indicates the physical map of the construct used for transformation showing the functional regions and restriction sites used for DNA analysis. The hybridization probes used is shown below as dotted lines.

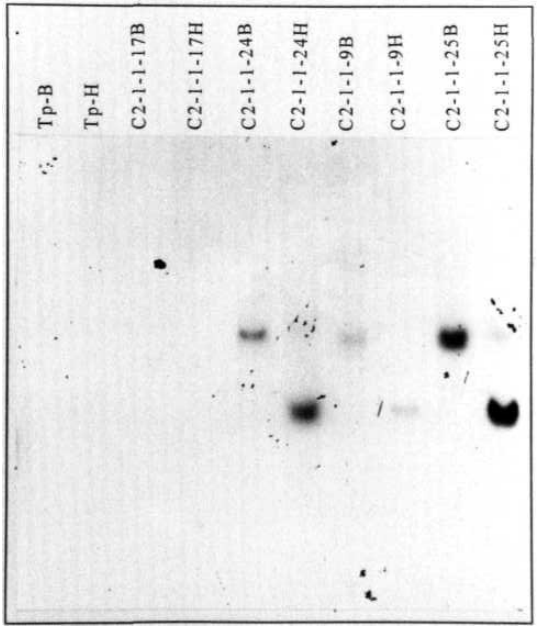
L) 10 *u*gms of genomic DNA was digested either with *Hind III* or *Bam H1* and used for analysis. Lower left panel represents the blot probed with 2kb *Ubi* . The arrows with *Hind III* digests showing the copy number of the transgene. Lower right panel is the duplicate gel containing the same samples and was probed with 1.4kb *C2.* Note the detection of 2.8kb band in the *Bam H1* digests.

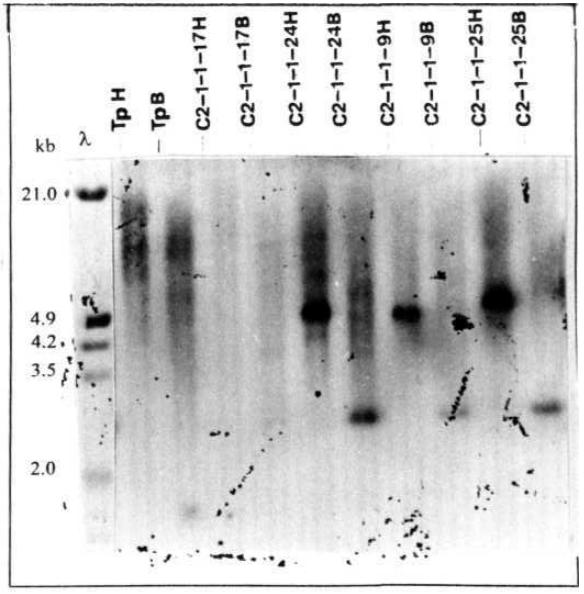
Plant number of the transgenic rice and the restriction enzyme used is indicated on top of the lane. Arrows indicate the location of the fragments detected.


Hind III
Bam H1
Bam H1
Ubi
C2 cDNA
Nos
2kb
1.4kb
Probe 1
Probe 2

U

L


Tp-B
Tp-H
C2-1-1-17B
C2-1-1-17H
C2-1-1-24B
C2-1-1-24H
C2-1-1-9B
C2-1-1-9H
C2-1-1-25B
C2-1-1-25H


kb
λ
Tp H
Tp B
C2-1-1-17H
C2-1-1-17B
C2-1-1-24H
C2-1-1-24B
C2-1-1-9H
C2-1-1-9B
C2-1-1-25H
C2-1-1-25B
21.0
4.9
4.2
3.5
2.0

know the orientation of the transgene we have tested C2-1-1-9, C2-1-1-25 with *EcoRl* (*EcoRl* digest is expected to give a 2.2 and a 4kb fragment when probed with 2kb *Ubi* fragment). The results of the *EcoRl* digests of C2-1-1-9 and C2-1-1-25 are shown in Fig. 4.8.1.1 (Right panel). Interestingly, these plants show the presence of 850bp and 4.2kb fragment indicating a rearrangement.

The genomic blot analysis of C2-1-1-1 digested with *Hind III, Bam Hl* and *EcoRl* were probed with 2 kb *Ubi* and the pattern is shown in the left panel of the Figure 4.8.1.1. The Bam Hl fragment reveals the expected 4.9kb fragment and the *EcoRl* digest also reveals the expected 4.2 kb and 2.2kb fragments. In summary, based on our R2 analysis we conclude C2-1-1 having an intact cassette with the right orientation. In contrast the plants C2-1-1-9 and C2-1-1-25 show an unexplainable pattern of hybridization.

4.8.2 Western blot analysis of R2 plants

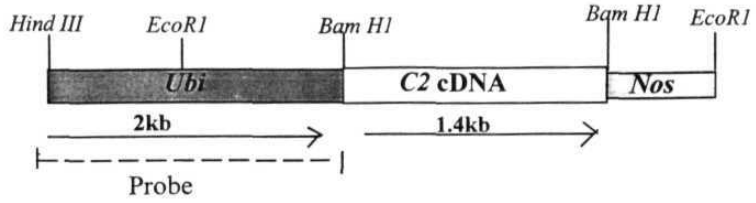
In order to confirm the presence/absence of the CHS protein in R2 plants, the total soluble protein extracts of the three week old R2 plants were run on 12% SDS-PAGE (Fig. 4.8.2A) transferred to the membrane and probed with anti-CHS antibody using the *PP* protein extracts as the positive control. None of the R2 transgenics show the expression of CHS protein, while detectable protein levels were found in the *PP* control (Fig.4.8.2B).

Fig.4.8.1.1 Genomic blot analysis of the R2 plants showing the orientation of the integrated transgene.

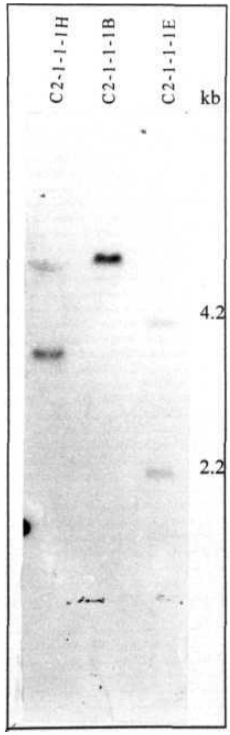
U) Indicates the physical map of the construct used for transformation showing the functional regions and restriction sites used for DNA analysis. 2 kb *Ubi* fragment was used as a probe is shown below as dotted lines.

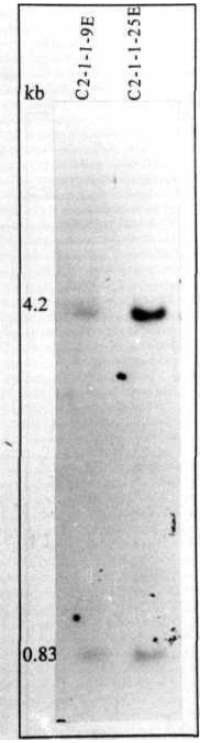
L) Lower left panel indicates the hybridization pattern of the C2-1-1-1. Note the detection of the expected 4.2kb and 2.2kb fragments in the *EcoR1* digest. Right panel shows the digestion pattern of C2-1-1-9 and C2-1-1-25 showing the unexpected size (4.2kb and 830bp).
Transgenic plant number and the restriction enzyme used is indicated on top.
(B= *Bam H1,* H= *Hind **III;** E=EcoR1)*

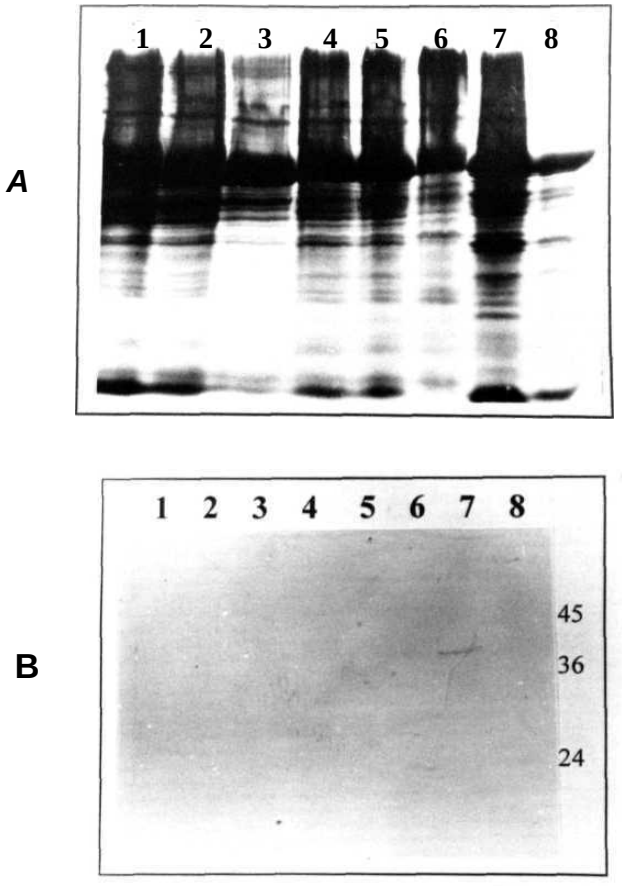
U


Hind III
EcoR1
Bam H1
Bam H1
EcoR1
Ubi
C2 cDNA
Nos
2kb
1.4kb
Probe

L


C2-1-1-1H
C2-1-1-1B
C2-1-1-1E
kb
4.2
2.2


kb
C2-1-1-9E
C2-1-1-25E
4.2
0.83



Fiq 4 8 2. Protein analysis of R2 plants

A) 12% SDS-PAGE analysis of total proteins

1) Tp309 2) C2-1-1-1 3) C2-1-1-4 4) C2-1-1-6 5) C2-1-1-8 6) C2-1-1-25 7) *PP* 8) Mw

B) Immuno-blot analysis of R2 transgenic plants
Note the detection of CHS protein in *PP* seedlings.

5. DISCUSSION

5. Discussion

5.1 Glutathione-S-Transferase activity and its correlation with anthocyanin biosynthesis in rice seedlings

The results highlight the identification of GST activity in seedlings of both *Indica* and *Japonica* rice lines (Table.4.1.1). The interesting aspect of this work is that the UV-B induces GST activity in rice seedlings (Figs. 4.1.1 and 4.1.2). The basal levels of this enzyme differ among rice lines and also between roots and shoots. Further, the constitutive levels of GST are significantly higher in shoots compared to roots (Fig.4.1.1). While the increased GST activity in shoots can be associated with anthocyanin sequestration, we cannot apply this criteria for root GST. It is to be noted that roots of rice normally do not accumulate anthocyanins, yet purple lines accumulate significantly higher GST levels in roots. Thus, in roots, the GST activity does not seem to be associated with purple pigments although we cannot rule out such an association with the colorless flavonoids. However, higher GST activities were reported in maize roots owing to their proximity to the herbicide compounds in the rhizosphere (Holt *et al,* 1995). This is in support of the hypothesis that GSTs are associated with a multitude of functions in addition to purple pigment accumulation.

Earlier studies by Marrs *et al,* (1995) conclusively proved that glutathione conjugation and anthocyanin sequestration are mediated by GST. Our data on time scan experiments with UV-B induced rice seedlings show a discernible relationship between enhanced GST specific activities and anthocyanin levels, particularly between 12-20h (Fig.4.1.2). Such a relationship implies the sequestering role of GST. Again, there is a considerable genotypic variation in rice in terms of UV-B response, anthocyanin accumulation, and GST activities. Given the vast differences in the genetic background of these tested rice lines, it is not surprising to find such variations. The enhanced levels of GST activity in *N22* indicates its possible association with flavonoids since *N22* is known to accumulate flavonoids, but not anthocyanins. The spatial distribution pattern of GST activity in seedlings showing higher GST levels in base and tip compared to the middle is similar to the purple

pigmentation pattern in induced seedlings (Fig.4.1.4). Thus, the data substantiate the relationship of GST activity with anthocyanin levels in this rice line.

Western analysis confirms that the *in vivo* GST-like protein levels correlate with the enzyme levels. This holds true for both constitutive and induced levels (Fig.4.1.3). Changes in the enzyme and transcript levels of the genes of the anthocyanin pathway upon UV-B treatment was observed in maize and *Arabidopsis* (Taylor and Briggs 1990; Kubasek *et al*, 1992). We have also shown previously that the young rice seedlings accumulate anthocyanins, in a time dependent manner, in response to UV-B mediated stress which is correlated with the increased PAL activity in *PP* seedlings (Reddy *et al*, 1994). Recent evidences indicate that several enzymes of the anthocyanin pathway including flavanone-3'hydroxylase show such a pattern of increase both in transcription, translation and enzyme activities in seedlings of *Petunia* and rice (Markham *et al.*, 1998). However, in none of those experiments, GST was assayed. Enhanced accumulation of anthocyanins and flavonoids requires to be followed by mobilization into vacuoles in order to prevent possible anthocyanin toxicity, particularly under stress, and GST activity becomes relevant in this context. We propose that the enhanced GST levels that accompany enhanced levels of pigmentation in UV-B induced seedlings suggest a role for GST in stable anthocyanin accumulation in seedlings. It is therefore reasonable to assume that GSTs have a role to play in protecting plants from UV-B damage through stabilization of pigments.

5.2 GST activity in rice is responsive to PEG mediated water stress cold and ABA

GST-like protein and the enzyme activity are both increased in rice seedlings under PEG mediated water stress. Interestingly, the pattern of enzyme activity during PEG treatment is same in all the tested lines (Fig. 4.1.5A and 4.1.5.B). Besides, enhanced levels of GST-like protein was observed under ABA stress and cold in addition to PEG stress (Fig. 4.1.5B). Transcriptional and translational changes of several genes encoding GST in response to abiotic stress have been demonstrated in many plant species including rice. Our group has

demonstrated such changes in the enzymatic activity profiles and the protein levels of SOD (superoxide dismutase), GR (glutathione reductase), and AR (aldose reductase) (Karunasree 1998). The present work showing the increase in GST enzymatic activity and protein levels during various abiotic stress conditions indicates that in rice GST is stress responsive. The stress responsive feature of GST has been reported in many plants. Roxas *et al,* (1997) showed constitutive over-expression of *Nt107* cDNA encoding glutathione-S-transferase with additional peroxidase activity in tobacco results in improved performance under cold and salt stress. Further, two dehydration inducible genes *(ERD 11* and *ERD 13)* have sequences encoding putative glutathione-S-transferase in *Arabidopsis thaliana* (Kiyosue *et al,* 1993). To date, in plants, several cDNAs/genes involved in diverse functions such as auxin response, pathogen response, response to diverse environmental conditions also encode GST and are generally designated as MSR (multiple stimulus responsive) genes.

In summary, GSTs in plants are associated with different response pathways to both biotic and abiotic stresses. Exact role of GSTs in specific pathways, however, remains to be established. With the demonstration of GST in rice, one can now use rice system for evaluation of GST role in stress resistance in their important crop species.

5.3 The *CT24* product is a GST-like protein

The *CT24* overexpression experiments have demonstrated that it encodes a GST-like protein as revealed by its enzymatic activity (Fig. 4.2.1C) and immunological cross reactivity with *anti-Pugb* antibodies (Fig. 4.2.1D). This is not surprising in view of its extensive sequence similarities with GST. Infact, several cDNAs involved in diverse functions are found to be GSTs and were renamed as *GST*. Examples include potato *Prp1* protein renamed as GST1 (Hahn *et al,* 1994), the tobacco *Nt107/ParA* protein, now renamed as GST3-1 (Droog *et al,* 1995, Takahashi *et al,* 1995), and the maize *Bz2* protein (Marrs *et al,* 1995).

Several auxin-regulated tobacco genes are responsive to stress (Boot *et al,* 1993; Droog *et al,* 1993; Takahashi *et al,* 1991; 1992; 1990). The entire auxin regulated tobacco gene family and the other homologous genes could be *Gsts* and the protein product of the auxin responsive gene *NT 103* was renamed as GST-1. Further, GST (CDNB) activity was shown by genes involved in heat shock responses in soyabean such as *GH2/4* or *GmGst26-A* (Ulmasov , 1995).

In addition, several cDNAs originally identified to be involved in other functions such as ethylene responsive property and floral senescence in carnation were found to be due to *GSTs* (Meyer *et al.,* 1991; Itzahaki *et al.,* 1994). Such GST (CDNB) activity was shown by *Hyoscyamus muticus* auxin binding protein (Bilang *et al,* 1993), *Par b* auxin responsive gene from tobacco, (Takahashi and Nagata 1992), *NT103* another auxin responsive cDNA (Droog *et al,* 1993), *GH2/4* or *GmGST26-A* heat shock responsive genes from soybean, and the *An9* locus of *Petunia* involved in the last steps of anthocyanin biosynthesis (Alfenito *et al,* 1998).

It is interesting that anti-*Pugb* antibodies detect a 65kDa protein in *E.coli.* This intense band may belong to GST *of E.coli* which might be responsible for the observed GST activity *in vitro.* In literature, several cDNAs involved in different functions were identified to be encoding a 65 kDa protein with GST activity. For instance, mung bean 65kDa auxin binding protein shows extensive N-terminal amino acid homology to *HmGst-1* and other type I GSTs. In addition *Antirrihinnum.* maize, wheat, tomato, *Petunia* and Soybean also have homologous genes for the 65-kDa protein (Marrs 1996).

Sequence alignment at both DNA and protein level show that the *CT24* shows extensive sequence similarities with several sequences encoding *GST,* including auxin binding *Par C* - whose mRNA is strongly induced by 2,4,D, weakly by IAA, NAA, Cadmium and Salicyclic acid (Boot *et al,* 1993; Droog *et al,* 1993; 1995; Takashashi *et al,* 1990; 1991; 1995) *N.Tabacum* auxin responsive mRNA *NT107* (57%) (Boot *et al,* 1993; Droog *et al,* 1993; 1995; Takashashi et al 1992);

and maize glutathione S-transferase mRNA III, (Grove *et al,* 1988; Moore *et al,* 1986; Shah *et al,* 1986; Wiegand *et al,* 1986); III A and II1B (Moore *et al,* 1986; Wiegand *et al,* 1986: Shah *et al,* 1986) and also the potato pathogen related protein *Prp1* (Hahn *et al,* 1994; Taylor *et al,* 1990).

The homology shown here opens up new avenues for understanding the relationship between different GSTs in plants associated with detoxification as well as other normal cellular process. Further, this may reflect to a greater extent the functional significance of the conserved aminoacids domains in GST. The evolutionary origin of the *CT24* or similar sequences and their relatedness to the other genes like auxin responsive genes, metal binding genes, however, remains to be clarified.

5.4 *CT24* like sequences in rice

Southern analysis of rice revealed more than one copy of *CT24* hybridizable sequences in rice genome (Fig. 4.2.3). Further, there was no polymorphism in two parents differing in anthocyanin phenotype even with eight restriction endonucleases. That the *CT24* and *Gst* like sequences flourish in cereal genomes is well known, and in fact, as discussed in the earlier paragraphs such sequences are involved in diverse activities such as auxin binding, plant's secondary metabolism and numerous other stress responses arising from pathogen attack, oxidative stress and heavy metals stress. Although, it has been difficult to pin point the function of *CT24* like sequences in rice, their abundance does provide several interesting possibilities.

5.5 UV-B **responsive CHS in rice**

Our results reveal that the *Chs* transcript is induced in rice seedlings upon UV-B exposure. This increase is correlated with the induction pattern of anthocyanin pigment accumulation in seedlings (Reddy *et al.,* 1994). The UV-B inducibility of *Chs* gene has been amply demonstrated in maize and *Arabidopisis* (Taylor and Briggs 1990; Kubasek *et al.,* 1992). Most of these studies have concentrated at the transcript level. In the present study we show not only the

increase in the transcript levels (Fig. 4.3A) in addition we show the increase in the CHS protein levels in response to UV-B in a time dependent manner (Fig. 4.3B). Based on the response pattern of the committing step of the anthocyanin pathway, it is proposed that CHS may be a good marker to study the mechanism of UV-B mediated seedling response.

5.6 Transient expression of maize transgenes produce **purple color** in *Tp309* **calli**

Two important observations emerge from the transient expression experiments. First, the *C* and *R* constructs separately or in combination do not produce color in callus. Second, the co-bombardment of the same constructs with the *C2* construct results in color (Fig.4.6.1). The failure of *Tp309* transformed calli to produce color with C and *R* constructs is presumably due to the fact that *Tp309* is genetically deficient in chalcone synthase function or it may be a mutant at one or more loci controlling the individual steps of the pathway. Another reason is the expression level of C and *R* genes to activate the pathway in callus tissue under defined culture conditions may not be adequate. This is unlike the earlier observations on *C* and *R* activation of the anthocyanin pathway both in homologous and heterologous systems (Llyod *et al*, 1992; Ludwig *et al*, 1990; Bradley *et al*, 1998).

During the establishment of a transient expression system for the production of anthocyanins in *Tp309*, two parameters were found to be important. One is the exposure of the bombarded calli to controlled light and the other is the type of media used including the concentration of hormones, in particular IAA. However, addition of IAA to the medium has a disadvantage, that is, regeneration of the plants is not possible at that concentration. Such auxin supplemented media promote extensive greening of the callus and inhibit differentiation. The transformed purple calli of *Petunia* with the *Lc* allele of maize failed to regenerate (Quattrocchio *et al*, 1993). It was proposed that *Lc* encoded *Myc* protein at a certain restricted level of expression could be toxic or may regulate another pathway leading to the inhibition of growth (Bradley *et al*, 1998). Thus, in rice, the production of purple calli and

their successful regeneration are found to be mutually exclusive, and probably high level of expression of transgene(s) producing purple color, may lead to the disturbances in the shoot development. Most interestingly, we found that even a naturally purple line *PP* did not produce any anthocyanins in calli grown under various media. More detailed investigations on the production of purple calli using the gene(s) would provide a non-destructible marker system for *Tp309* transformation experiments. The present result demonstrate the activation of anthocyanin pathway in *Tp309* calli by maize transgenes providing the first step towards the development of the anthocyanin marker system in Tp309.

5.7 Chalcone synthase is sufficient to restore the pigmentation in *Tp309*

Stable transformation experiments using the *C2* constructs resulted into the production of RO plants exhibiting purple color although the calli were devoid of any color (Fig. 4.6.2A; 4.6.2B; 4.6.2C). The absence of color in the calli may be attributed to the fact that the regeneration medium was not supplemented with IAA - an important prerequisite for the production of purple colored calli in the transient studies. It appears that the *C2* overexpression, leading to the enhanced CHS activity could complement the *C2* deficiency in RO plants. The color in RO plants was stable for about a week after which it faded. Two of the intensely colored transformants designated as C2-7a and C2-7b died in the green house by the fourth week, while the other, C2-5a, showed severely stunted growth with no seed set. Transformants exhibiting seedling lethality or a low seed set is a common observation in transgenic research. Such lethality, indirectly associated with purple color, was also observed in *Arabidopsis*, tomato and sugarcane (Goldsbrough *et a!.,* 1996; Lloyd *et al.,* 1994; Bower *et al.,* 1996) suggesting the deleterious effect of the respective overexpressed transgene(s). It is also possible that the young transgenic seedlings have low threshold levels of pigmentation.

One important observation was the appearance of color in a patchy pattern in RO plants in contrast to fully colored purple seedlings of the competent genotypes. Such a patchy distribution of color can be speculated to result form the site of insertions, or the altered behavior of the promoter sequences. For instance,

insertion of the transgene, down stream of a developmentally regulated promoter, will have altered expression. The exact reason for the localized distribution of color in transgenic plants is not yet clear. However, a genetic explanation is sought on the basis of the occurrence of a series of known gene loci that inhibit color production in rice. The extent and the tissue specificity of their distribution has been well documented in rice (Ramaiah and Rao 1953; Dhulappanavar 1973; Kinoshita and Takahashi 1991; Takahashi 1982) these include several classes of inhibitor genes showing their characteristic inhibition over a range of tissues in rice plants. While some alleles such as *I-P-1* and *I-lb* (Reddy *et al*, 1995) are known to inhibit the color in leaf, others such as *I-Pl-4, I-Pl-5* inhibits color in pericarp. *I-Pl-6* inhibit color in leaf sheath, ligule, internode, collar and auricle (Chang and Jordan 1963; Takahashi 1982; Kinoshita and Takahashi 1991).

The genetic constitution of *Tp309* with respect to anthocyanin pathway is not known. From the available information on the pathway, in rice the anthocyanin gene complement includes three major genes, namely, C, *A* and *P*. Based on the nature of action of the genes (Kinoshita and Takahashi 1991; Reddy *et al.*, 1995) and the behavior of *Tp309*. we predict that *Tp309* is deficient in *P* function. In addition, we already suggested that the *Tp309* carries inhibitor alleles for the elimination of purple color in leaf blade. With the assumption that *Tp309* has a functionally active inhibitor allele, which might down regulate or completely inhibit the expression of other genes controlling down stream steps of the pathway, one would expect incomplete coloration or total elimination of color. The RO plants, expressing patchy pigmentation represent the former case. It is well known in maize, that the classical dominant inhibitor allele, the *C-I*, eliminates color even in heterozygous condition. However, aleurone cells that are genetically *C1-IC1C1* shows specks of color but *Cl-1 Cl-1 Cl-1* do not normally show any color (Coe 1962; Neuffer *et al.*, 1997). Our results show that chalcone synthase transgene expression in *Tp309* leads to the production of color, though patchy which is analogous to the behavior of C-ICC. The deciphering of *Tp309* genotype is required for clear interpretation of this observation. The Fl progeny of the cross *Tp309* X *PP* are colorless suggesting that *Tp309* carries a dominant inhibitor allele which eliminates color in the Fl generation.

5.8 RO plants show single copies to multiple-copy integration

The southern blot analysis of the RO plants C2-1, C2-6, and C2-9 revealed the presence of an intact cassette as was seen in their respective *Bam HI* digests. Moreover, these plants showed a few copies (three) as observed in their *Hind HI* digests (Fig.4.6.4.1). C2-25 shows the intact casette, however has multicopy integrations. (Fig. 4.6.4 lane F). It is interesting here that the plants C2-6, C2-9 and C2-25 show the presence of purple phenotype. In addition, the genomic distribution showed a complex pattern of hybridization commiserate with the lack of visible phenotype in C2-14. C2-17, and C2-24 (Fig.4.6.4). The interpretation of stability and integrity of the insert and its functionality is given along with the R1 data.

5.9 *Tp309* is **deficient in CHS function**

The genetic interpretation predicted that *Tp309* is deficient in CHS protein. This is indeed confirmed by the Western analysis of RO plants. The data revealed that those RO plants which are colored show the presence of CHS protein. Only those rice lines transformed with *C2* cDNA under the control of *Ubi* promoter displayed an immuno-reactive signal of the expected 44kDa protein. In addition, some lines which do not show any indication of purple color, however, show the cross reacting 44kDa signal (4.6.5 A and B). Further, some of the tested lines lacked immunoreactive signal. The protein appears to be of similar size to that of *PP* as reported earlier (Reddy *et al.*, 1996). Based on the phenotypic and Western data, RO transgenics are classified into four groups (Table. 5.1). Group I (C2-2, C2-3, C2-6, C2-8b, C2-9, C2-11C, C2-25,) plants are positive for both phenotype and Westerns, Group II (C2-1, C2-4, C2-6b, C2-8, C2-8C, C2-11, C2-14C, C2-15, C2-26) are colorless but have detectable levels of CHS protein, Group HI plants are neither positive for phenotype nor CHS like protein.

Group IV (C2-7a, C2-7b and C2-5a) plants are intensely colored and could not survive in the growth chamber. The basis for the group I and group III transgenics showing color as well as CHS protein is straight forward in that the color is due to the expression and activation of CHS protein. However, group II plants

Table.5.1. Summary of *Chalcone Synthase* Transgenic Analysis

Plant No.	**Phenotype R0/R1**	Western **R0/R1**	Slot Blot **R0/R1**	Southern **R0/R1**	South/ **Wes** R2	Group
C2-1	+	+ +	NA +	+ I + I	+ I	II
C2-2	+	+ NA	+ NA	- NA		I
C2-3	+	+ NA	NA +	+		I
C2-4	- NA	+ NA	NA			II
C2-5a	+ NA	Stunted No seed set				IV
C2-6 b	-	+	+ +	NA		II
C2-6	+	+ +	+	+ I + I	RA	I
C2-6C	-	- NA	+ +	NA	NA	III
C2-7a&b	+++	Very purple so plants d ed in green House				IV
C2-8	-	-	+ +	+		II
C2-8b	+	+	+			I
C2-8C	-	+				II
C2-9	+	+	NA +	+ I +I	+ R	I
C2-10	+ NA	NA	NA			UC
C2-11	-	+	NA			II
C2-11b	-	- NA	NA			III
C2-11C	+	+				I
C2-12	-	-	NA	-		III
C2-13	-	NA NA	+			UC
C2-14a	-	+	NA +	+		III
C2-14b	-	-				III
C2-14C	-	+				II
C2-15	-	+	NA			II
C2-16	-	- NA	+ +			III
C2-17	-		+ +	NA + R	+ R	III
C2-23	-	-	+			III
C2-24		-	NA +			III
C2-25	+	+ +	+ +	+ I + I	+ R	I
C2-26		+				II
C2-27		-	NA +			III
C2-29	-	-	+			III

Note: All Western are done using *Anti-CHS* antibodies from maize.
All Slot blots are done using 2kb *Ubi* probe
Southerns are done with both 2kb *Ubi* ;1 4kb *C2* and with the 6.2kb plasmid
NA and empty cells represent that Data is Not Available.
UC: Unclassified; RA: Result awaited
+ I - Intact cassette
+ R - Rearranged cassette

are atypical, because we have no explanation for the behavior of these plants showing no color but the presence of CHS protein. It may be speculated that this protein may represent an enzymologically defective but immonologically active protein resulting from the rearranged sequences. The observation that the seedlings belonging to group IV with intense purple color are severely affected in their growth is an interesting but complex phenomenon. There is no unambiguous evidence for toxicity nor the inhibitory effect of anthocyanins. On the other hand anthocyanins are associated with several functions some of which are involved with the plant's survival under adverse conditions. The generation of chalcone synthase rice transgenics thus offers us an opportunity to investigate the role of overexpressed flavonoids in conferring disease resistance.

5.10 Inheritance and expression of transgene in R1 plants

A detailed Southern analysis and characterization of Rl plants was done to assess the integrity and organization of the *C2* gene in order to eventually incorporate the plants in breeding programmes. The molecular analysis revealed that 75% of the plants show the presence of the *Ubiquitin* promoter (based on the slot blot analysis in Fig. 4.6.7 A and B). However, color expression was not seen in Rl plants except in C2-1-1, which showed a very pale pink color during early seedling growth. It is interesting here to note that the C2-1-1 plant belongs to class II plants which show the presence of detectable CHS protein but do not exhibit purple phenotype. The detailed Southern blot analysis revealed the presence of intact cassette in C2-1-1 and C2-1-2 and the absence of chimeras in these plants (Fig. 4.6.8). The Western blot analysis shows the presence of immuno-reactive signal in these plants (Fig.4.6.9). Further, the performance of these plants under artificial inoculation conditions revealed a marginally improved performance with a score of 4. In summary, based on the Rl data, the C2-1-1 is found to be a promising transgenic plant having the functional transgene. In addition, C2-1-25 and C2-1-6 were also found to have the functional casette. All the other Rl plants examined are negative for CHS protein. While the basis for the lack of CHS protein in Rl

generation in other plants is not known at present, gene silencing could be one of the potential reasons.

Among the tested class HI plants, C2-1-17, C2-1-23, C2-1-24 show extensive rearrangements consistent with the lack of phenotype or expression in RO and R1 generation. In the present study, we have come across many transgenic plants showing multiple, fragmented integration with extensive rearrangements lacking an intact functional transgene. This is probably due to the fragmentation of the input DNA. Such a fragmented DNA would undoubtedly result in the lack of functional product and the lack of functional copies in the transgenic plants results in the absence of the protein product and consequently the phenotype. While there are numerous reports on the generation of transgenic cereals with various agronomical traits, not much is known about the structural integrity, organization, inheritance and expression of the transgene introduced by direct DNA delivery method (Mc Elroy and Brettel 1994). Presence of multi-copy and rearranged sequences is a common phenomenon among the transgenic plants obtained by microprojectile bombardment. Such multicopy sequences are found to be the favorite targets not only for methylation but also for gene silencing in several dicots (Matzke and Matzke, 1995, Park *et al,* 1996,). Cornejo *et al,* (1993) established that *Ubi* promoter used to drive the *bar* coding region was extensively methylated and transcriptionally silent in herbicide sensitive plants. Kumpatla *et al,* (1997) also show that methylation of the *Ubi*-promoter and the consequent susceptibility to herbicide in *Ubi-bar* transgenics in rice. In addition they showed that plants having complex integration patterns displayed aberrant segregation indicating homology mediated gene silencing (Kumpatla *et al,* 1997; 1998).

Insertion of multiple copies of the maize *A]* gene in *Petunia* line *R101* resulted in the lack *of A]* specific pigmentation (brick Red pelargonidin pigmentation) as a consequence of methylation in the promoter region of the *Al* gene (Linn *et al,* 1990). Alternately, multiple copy sequences exert variable effects on the phenotype. For instance, Linn *et al,* (1990) showed correlation between the copy number of the transgenes and the coloration of the *Petunia* RL101 transformed

with *Al,* wherein single copy integration correlates with the red phenotype while multiple copy integration was observed mainly in variegated and white plants. Further, Prole and Meyer (1992), showed that methylation state around the chromosomal environment influences the methylation of the transgene while integration into unmethylated region would leave the transgenic DNA hypomethylated and transcriptionally active. Methylation of the transgene can also result in transcriptional silencing (Meyer *et al.* 1993; Vauchert *et al,* 1993; Park *et al,* 1996; Ye and Singer *et al,* 1996), or post-transcriptional silencing (Ingel brecht *et al,* 1994; Smith *et al,* 1994; English *et al,* 1996). The silencing state is transmitted to the subsequent generations with variable degrees of heritability (Napoli *et al,* 1990; Mittelsten Scheid *et al,* 1991; Meyer *et al,* 1993; Assaad *et al,* 1993; Vauchert *et al,* 1993; Jorgenssen *et al,* 1996). In some cases the post-transcriptional state of silencing is not meiotically transmissible (Decarvalho *et al,* 1992; Dehio and Scheel 1994; Kunz *et al,* 1996). Although there are numerous reports on the silencing phenomenon, the actual silencing mechanisms are not known.

In this study we show that *Tp309* is a functional mutant for CHS and probably the introduced transgene behaves as a dominant Mendelian gene. This interpretation is in agreement with the observation that the *Wx* transgene in maize transgenics behaved as a dominant allele over the recessive *wx* allele (the transgenics were originally *wx wx)* (Itoh *et al,* 1997).

In addition to silencing, the lack of expression could be attributed to co-suppression, where over-expression of the product of a gene up to a threshold level itself is a trigger for degradation, presumably with the target product being the RNA (Elmayard and Vauchert, 1996).

5.11 Performance of *C2* transgenics under artificial infection

Screening of *Chs* transgenics (Southern and Western positive) for their performance against attempted infection by the major blast pathogen, *Pyricularia oryzae* revealed a marginal improvement over the untransformed *Tp309* control

plants. Earlier, several *in vitro* studies have indicated that production of flavonoid naringenin is inhibitory to the growth of certain rice pathogens (Kodama 1991; 1992; Kramer 1984; Padmavathi *el al.*, 1997). It would mean that plants accumulating such chalcones are expected to show better performance under pathogen attack. Our primary objective is to generate rice plants that contain stably expressing *Chs* gene, accumulate flavonoids and consequently show enhanced resistance *in vivo*. The results of seedling screening demonstrated that the transgenic plants C2-1-1 and C2-1-25 displayed marginally improved performance against a major rice pathogen *Pyricularia oryzae*. Interestingly, the plants C2-1-1 and C2-1-25 showed the presence of functional cassette (intact casette and immuno-detectable signal). Interestingly, the plants belonging to the group I and II showed improved performance consistent with the expectation. However, the correlation between the accumulation of CHS product and enhanced resistance in transgenic plants is yet to be established. The screening results thus point at the need to evaluate mature plants by field testing or in plant breeding programs.

Further, elaborate field screening of transgenic plants is required for elucidating the mechanisms by which such improved performance is brought about. Infact, young seedlings may be more fragile and prone to severe damage by the pathogens. The present study, however, reiterates the role of flavonoid pathway in defense response in rice.

5.12 Stability of the transgene in R2 plants

Genomic Southerns of R2 plants. C2-1-1-9 and C2-1-1-24, confirmed the presence of a single copy where as C2-1-1-1 and C2-1-1-25, plants carry two copies of the transgene. However, in all plants, the 1.4kb *C2* hybridisable *Bam HI* fragment was not detected although 4.9 kb *Ubi* hybridizable fragments are present. Further, R1 parents of C2-1-1 and C2-1-25 were showing the 1.4kb *C2* specific *Bam HI* fragment. These results suggest rearrangement in the R2 genome. The *EcoRI* digests of C2-1-1 gave the expected 4.2kb and 2.2 kb *Ubi* hybridizable fragment suggesting the presence of intact casette. This does not however, explain the reason for the presence of 2.8kb C2 specific *Bam HI* fragment. On the other hand the

plants C2-1-9 and C2-1-25 plants showed fragments of unexpected size. Western analysis of all the above R2 plants did not reveal the accumulation of CHS in these plants.

In summary, the present work demonstrates that the Chalcone synthase of maize activates the anthocyanin pathway in rice. Some of the primary transgenics expressed the CHS protein and exhibited purple color in the transgenics in seedlings and showed the presence of an intact transgene. Also, several transgenic plants were identified which showed extensive rearrangements. Further, several R1 plants were found to harbor the intact cassette, show detectable levels of CHS protein and exhibited a marginally improved performance under artificial inoculation conditions.

6. CONCLUSIONS

6. Conclusions

1. Glutathione-S-transferase activity was demonstrated in various purple and green rice lines. The tested lines show considerable variation in GST levels. Purple lines showed higher levels compared to that of non-purple lines. In addition, all the tested lines show significant differences in GST activity between roots and shoots.
2. Based on the activity levels of GST under UV-B, the rice varieties were classified as highly responsive (*PP*), moderately responsive *{R27* and *G962)* and the least responsive lines (*N22*).
3. Six day old etiolated seedlings were found to accumulate significant amounts of GST activity upon exposure to sun light (UV-B). The UV-B mediated GST activity and GST protein levels were found to be the highest at 20h after induction .
4. GST activity and its protein levels were increased in seedlings during PEG mediated water stress. Further, GST protein levels were also increased during cold and ABA mediated stress, suggesting that GST is a stress responsive enzyme.
5. The maize *CT24* cDNA (a homologue of maize *Bz2* gene) was over-expressed in *E.coli* and the protein was isolated, partially purified and the *anti-CT24* antibodies raised.
6. The over-expressed *CT24* protein product was found to be immunologically related to the pumpkin-GST. In addition, the induced *CT24* cells show a two to four fold increase in their enzymatic activity.
7. Rice genome was found to have several *CT24* like sequences based on the genomic Southern blot analysis.
8. The CHS protein and transcript levels in seedlings were enhanced during the UV-B mediated anthocyanin synthesis in a time dependent manner.
9. As a first step towards the characterization of the role of anthocyanin pathway genes in stress response, several plant expression vectors of structural and regulatory genes of the anthocyanin pathway in sense and anti-sense direction were constructed with *Ubi* or *EJ5S* as the promoter elements.

10. The *Tp309* calli were co-bombarded with pMA*C1*, pMAR and pMA*C2* carrying the maize *C1*, *R* and *C2* genes respectively, and the appearance of color was monitored in a transient expression system. Further, these studies demonstrated that the chalcone synthase over expression is required to produce purple color in the calli of the *Tp309*.
11. Primary transformants carrying only *C2* construct exhibited a localized purple color in seedlings which suggested that *C2* can serve as a 'red reporter' in *Tp309* transformation experiments. More experiments are needed for precisely defining the system for future use in rice transformation experiments.
12. The stable integration of the chalcone synthase gene was demonstrated by Genomic Southern blot analysis of R0 transgenics. Several R0 transgenics showed the presence of multiple integration events.
13. The Western blot analysis of these transgenics revealed the presence of an immuno-detectable 44kDa CHS protein which was absent in the non-trangenic *Tp309* suggesting that *Tp309* might be a functional mutant at the *Chs* locus. It is concluded that the *C2* transgene is overexpressed in these transgenics.
14. F1 analysis of the cross *Tp309* X PP revealed the presence of dominant inhibitors of plant color in the *Tp309* genome.
15. The integration of the transgene was followed for three subsequent generations. The R1 transgenics Southerns showed the presence of an intact transgene in few plants C2-1-1 and C2-1-6. Several other Rl transgenics revealed multiple copy insertions as well as rearranged versions.
16. Western analysis of the above Rl plants showing the intact cassette revealed the CHS protein in C2-1-1, C2-1-6, C2-1-25. Several other Rl plants did not show the presence of the immuno-detectable CHS protein.
17. Screening of the Rl transgenic seedlings against a fungal pathogen *Pyricularia oryzae* in nursery beds indicated a marginal improvement over the non-transgenic control plants.

18. Genomic Southern Blot analysis of R2 Southerns confirmed the stable integration of the C2 gene . However, two of the transgenics C2-1-9 and C2-1-25 show rearrangements.
19. Surprisingly, the Western blot analysis of all the R2 plants did not detect the CHS protein, suggesting the gene inactivation in these plants.

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