Abiotic Stress Response and Flavonoid Biosynthetic Pathway in Rice: Role of the Transcription Factor, C1-MYB, in Gene Expression

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that **Nagabhushana Ithal** has carried out the research work embodied in the present thesis entitled *"Abiotic Stress Response and Flavonoid Biosynthetic Pathway in Rice: Role of the Transcription Factor, Cl-MYB, in Gene Expression"* for the degree of **Doctor of Philosophy** under my supervision in the Department of Plant Sciences, School of Life Sciences, 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

1 hereby declare that the work presented in this thesis entitled ***"Abiotic Stress Response and Flavonoid Biosynthetic Pathway in Rice: Role of the Transcription Factor, Cl-MYB, in Gene Expression"*** **has** been carried out by me under the supervision of **Prof. Arjula Ramachandra Reddy** in the Department 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. All the assistance and help received during the course of the investigation have been duly acknowledged.

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CONTENTS

Abbreviations

ABA	Abscisic Acid
Abi	ABA Insensitive mutant
ABRC	ABA Responsive Complex
ABRE	ABA Responsive Element
ANS	Anthocyanidin Synthase
Apx	Ascorbate Peroxidase
bZIP	Basic Zipper protein
CBF	C-repeat Binding Factor
CE	Coupling Element
CHS	Chalcone Synthase
Cor	Cold Responsive
CRT	C-repeat
DFR	Dihydroflavonol Reductase
DRE	Dehydration Responsive Element
DREB	Dehydration Responsive Element Binding protein
EDTA	Ethylenediamine Tetra Acetic acid
ERD	Early Responsive to Dehydration
GA	Gibberellic Acid
GR	Glutathione Reductase
GST	Glutathione-S-transferase
HSP	Heat Shock Protein
Lea	Late Embryogenesis Abundant
LTRE	Low Temperature Responsive Element
MOPS	4-Morpholinepropanesulfonic acid
npt	Neomycin phospho transferase
PAGE	Poly Acrylamide Gel Electrophoresis
PAL	Phenylalanine Ammonia Lyase
PEG	Polyethylene Glycol
RD	Responsive to Desiccation
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TEMED	N,N,N,N (Tetramethyl) Aminomethyl ethylene Diamine
UFGT	UDP Flavonoid Glucosyl Transferase

1. Introduction

Plants use different adaptive mechanisms to acclimate to abiotic stresses through a cascade or network of events that begins with stress perception and ends with expression of a wide spectrum of stress responsive genes and gene products, and eventually the stress tolerant phenotype. In plants, there is an increasing evidence for the presence of several diverse metabolic pathways controlled by a large number of genes and gene families associated with stress response. However, these pathways appear to share common elements that are associated with adaptation of plants to a range of stresses. While, some of these well known pathways of stress response have been rigorously analyzed in selected plant species, others remained largely unknown. The association between the expression of genes belonging to such little known pathways and the drought tolerance phenotype has not been established in any major plant. Molecular dissection of the regulatory elements governing the expression of a host of genes, temporally and spatially, in rice will help in elucidation of the genetic basis of stress response.

Earlier studies in higher plants revealed that there exists a multitude of pathways that are associated with stress responses though the connection between some of them are not obvious from the data. Two such target pathways are abscisic acid (ABA) mediated abiotic stress response pathway and the flavonoid biosynthetic pathway. The ABA mediated response pathway has been thoroughly investigated in the model plant Arabidopsis (Abe et al., 1997), but very little is known about it in any agronomically important crop species. One common element between the ABA mediated pathway and the flavonoid pathway is the action of MYB and MYC class of transcription activators for target gene expression (Lloyd et al., 1992; Grotewold et al., 1994; Abe et al., 1997). While, *Atmyb2* and *rd22BP1* regulate the *rd22* gene expression in ABA-dependent abiotic stress response pathway, the *C1-myb* regulates expression of the flavonoid genes in cooperation with a *myc* family gene, the *R*. However, not much is known about the drought and salinity stress response of the genes belonging to flavonoid pathway, though, this pathway has been one of the most thoroughly investigated pathway and also been reported to be highly stress responsive (Chalker-Scott, 1999). The flavonoid biosynthetic pathway genes have been characterized and assigned with functions in crop plants like maize and rice (Kinoshita and Mackawa,

1986; Reddy et. al., 1987; Coe et al., 1988; Reddy 1996; Neuffer et al., 1997). The flavonoid pathway is known to respond to biotic and abiotic stresses. Flavonoid accumulation in response to UV-B (Reddy et al., 1994), cold (Christie et al., 1994), and osmotic stress induced by glucose (Tholakalabavi et al., 1997). sucrose (Do and Cormier 1991), mannitol (Tholakalabavi et al., 1994), and drought (Balakumar et al., 1993) were reported earlier.

In the present study, we have investigated into the abiotic stress response of flavonoid biosynthetic pathway genes and the role of transcription factor, the C1-MYB, in regulating the stress responsive gene expression in rice. We have chosen rice as a model for valid reasons. Drought and salinity tolerance in rice is an important trait and understanding the mechanisms underlying such responses will be of paramount importance in genetic improvement of this crop for such adverse conditions. With the availability of the complete genome sequence, saturated genetic maps and good EST coverage, rice serves as a model plant to study stress response mechanisms at the molecular level. Most importantly, the flavonoid biosynthetic pathway has several advantages in rice: availability of cDNA clones for all major genes of the pathway, a wealth of literature on gene expression and regulation, an array of specific mutants, clearly visible phenotypes and well established extraction and purification protocols.

A single transcription factor can orchestrate the expression of many genes in response to a given stress. However, acclimation to complex stresses such as drought and salinity must involve the simultaneous operation of many signaling pathways and networks. It is therefore, essential to characterize the regulatory genes that encode their role in regulation of genes and gene families. However, the available data from Arabidopsis indicate about 8000 genes encode for transcription factors, which is about 25% of the total coding sequence (Pastori and Foyer. 2002). Rice may not be an exception to this. Therefore, the task of characterizing transcription factor is exceedingly difficult. The *cis*-responsive elements in the promoters of target genes may hold the key with which to unravel the underlying mechanisms conferring tolerance to drought and salinity. The current study is aimed at addressing the role of a transcription factor, C1-MYB in stress responsive gene expression as well as its role in mediating cross talk between stress responsive pathways in rice using flavonoid biosynthetic pathway as a model. The major goal of this study is to understand the role of transcription factors and *cis*-acting elements in adaptive stress response mechanisms in

rice and to dissect the possible common features that these mechanisms share with other stress responsive pathways.

The specific objectives of the present study are

- Expression analysis of the major genes of the flavonoid **biosynthetic** pathway in rice under defined stress conditions.
- Comparative analysis of the promoter elements of major flavonoid biosynthetic pathway genes.
- Analysis of the role of the transcription factor Cl-MYB in regulating expression of genes of the two stress responsive pathways.
- Dissection of the common regulatory elements of the stress response pathways in the rice genome.

2. Literature Review

The genetic improvement of crops for abiotic stress tolerance is of paramount importance for the future of agriculture worldwide. Particularly, developing nations. including India, are increasingly suffering major losses in crop yield due to chronic and acute drought conditions in crop growing areas. While conventional breeding has contributed enormously to improvement of crops, the progress has been rather slow and release of cultivars for stress prone areas were insufficient mainly due to the complex nature of the stress response and tolerance traits and lack of appropriate tools to achieve this task. Application of advanced tools of modern biology such as marker assisted selection, genomics and transgenic technology has the potential to improve abiotic stress tolerance of cereal crops. The limiting factor for successful application of such powerful tools for improvement of stress tolerance in crops is the identification and isolation of candidate genes and their precise role in controlling the molecular process involved in plants* response to abiotic stress and tolerance. It has been well established across plant species that exposure to stress conditions triggers various protection mechanisms in plants leading to adaptation to a broad range of stress conditions. This means that the plants are able to perceive stress signals and that after perception signal transduction events take place leading to specific gene expression (Zhu et al., 1996). Consequently, various cellular mechanisms are set in place enabling plants to cope up with the stress imposed.

The present literature survey deals with the current information about complex molecular mechanisms underlying abiotic stress response and adaptation in higher plants. These include stress responsive gene expression and transcriptional regulation of stress related genes and the mechanism of cross-tolerance across plant genera. Also, recent advances in the analysis of stress responsive pathways and the cross talk between such pathways are described with an emphasis on the ABA dependent stress response pathway and the flavonoid biosynthetic pathways and their regulation.

2.1 Plants and environmental stresses

Plants can survive even in the most extreme environmental conditions. However, in areas where growing conditions are relatively good, like in many agricultural systems, environmental stresses can significantly affect photosynthesis and hence plant productivity is rarely optimal. Environmental conditions force plants to

adapt to surrounding conditions on a daily, or even on an hourly basis (Etherington, 1988). Precise definition of stress in plants is practically impossible, since no **fixed points** can be set. **In** a broad sense, plants' stress can be defined as "any unfavorable condition or substance that affect or blocks plant's metabolism, growth, or development" (Lichtentheler, 1996). In this case however, it is important to differentiate between low stress response, that can be partially be overcome by acclimation and repair, from strong or chronic stress effects, which may cause irreversible damage and cell death (Lichtentheler, 1996). Factors that can cause stress to plants and affect plant productivity can be classified into seven major classes (Listener and Oswald, 1994) namely, light (high and low), radiation (UV-B, UV-A etc.), temperature (high, low, chilling and freezing), hydration (drought and flooding), chemical factors (salt, heavy metals, pH etc.), mechanical factors, and biological influence. Plants adopt different strategies to adapt to these stress conditions. However, there exist common mechanisms through which plants can acclimatize to a broad range of stress conditions..

Of the different classes of stress factors mentioned above, freezing, drought, and salinity affect plant growth and productivity by disturbing the water balance of the cell. These abiotic stress factors and their impact on plant growth and productivity are described in detail in the following sections.

2.1.1 Drought stress

Drought stress is the major cause of yield instability in agriculture production across diverse crop ecosystems. Drought stress induces a plethora of biochemical and physiological responses in plants. The cellular concentration of phytohormone abscisic acid (ABA) increases as a result of water stress which inturn leads to the expression of several stress responsive genes. Expression pattern of these dehydration inducible genes is very complex. Some genes respond to drought very rapidly while others are induced slowly after accumulation of ABA. Most genes that respond to drought are also responsive to salt and cold stress as well as exogenous application of ABA (Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997). However, there are several genes that are induced by water stress which are not responsive to exogenous ABA treatment, which suggests existence of both ABA dependent and ABA independent signal transduction cascades between stress signal perception and gene expression (Shinozaki and Yamaguchi-Shinozaki, 1997).

The drought stress response in plants involves an array of different pathways associated with stress perception, signal transduction, gene expression, and synthesis of a number of proteins and other compounds. The products of water stress induced genes can be classified into two groups (Shinozaki and Yamaguchi-Shinozaki, 1997). The first group includes the proteins that are directly involved in the function of stress tolerance. Water channel proteins involved in the regulation of water movement across membranes, the enzymes required for the biosynthesis of various osmoprotectant molecules (sugars, Proline. Glycine-betaine etc.), proteins that protect macromolecules and membranes (LEA proteins, osmotin. antifreeze protein, chaperon, mRNA binding proteins etc.), and the detoxifying enzymes (GSTs. catalases, super oxide dismutase. Ascorbate peroxidase etc.) fall in this category. The second group contains protein factors involved in signal transduction and regulation of stress responsive gene expression. Examples include protein kinases, transcription factors and phospholipases. The transcripts for genes encoding several of these proteins are shown to accumulate under drought conditions. The roles of products of these genes are extensively reviewed (Shinozaki and Yamaguchi-Shinozaki, 2000).

2.1.2 Salinity stress

High salt or soil salinity is another major abiotic stress in plant agriculture worldwide. The problem of soil salinity has been aggravated by extensive adoption of modern agricultural practices such as irrigation and use of chemical fertilizers. Nearly 20% of the world's cultivated land and almost half of all irrigated lands are affected by salinity (Rhoades and Loveday, 1990). The primary consequence of salinity stress is the ion imbalance and hyperosmotic stress in plants, which often lead to secondary stresses such as oxidative damage. Like in the case of drought stress, high salt stress also disrupts homeostasis in water potential. The disruption of ion and osmotic homeostasis occurs at both the cellular and whole plant level, which leads to molecular damage. growth arrest and even death. To achieve improved salt tolerance in crop plants it is important to prevent cellular damage and to re-establish the homeostatic conditions in the new, stressful environment (Zhu. 2001). Plants adopt complex mechanisms to achieve tolerance by activating and/or accumulating several detoxification and protective molecules and compatible osmolytes (Zhu et al., 1996). Most reports of the transgenic approach for the improvement of salt tolerance in plants utilizes detoxification strategy through overexpression of enzymes involved in oxidative

protection, such as glutathione peroxidase, super oxide dismutase, ascorbate peroxidases and glutathione reductases (Roxas et al., 1997: Allen et al., 1997).

2.1.3 UV-B

Stratospheric ozone depletion has lead to a remarkable increase in the amount of UV-B (280-320 nm) irradiation on earth causing serious damage to plants (Teramura and Sullivan, 1994; Jordan. 1996). UV-B irradiation affects the leaf anatomical features differently in plants. Such damages include, reduction in plant height and leaf expansion (Sullivan et al.. 1996), tiller number (Barnes et al., 1990). Leaf thickness (Cen and Bornman, 1993), inhibition of hypocotyls elongation (Ballare et al., 1995), necrosis and formation of brown streaks (Lois. 1994: Sato et al., 1994). Such morphological changes are accompanied by alterations in the internal cellular and orgenellar structures such as stomatal disruptions, leaf surface deformation, hardening of epidermal layer, lamellar disorganization of the photosynthetic apparatus (He et al., 1993; Day 1993). In *Arahidopsis thaliana* leaf yellowing occurs as a response to UV-B irradiation (Lois, 1994). Conifers seem to be more tolerant to the irradiation and in broadleaf species tissue thickening in the palisade parenchyma layer is the most prominent anatomical response to UV-B irradiation (Nagel et al.. 1998). UV-B can induce DNA damage by the formation of covalent pyrimidine dimers (Rousseaux et al., 1999). The primary defence mechanism in plants against UV-B irradiation is the production of pigments, flavonoids. which absorb harmful UV wavelengths (Logemann et al., 2000), and therefore protect the plant DNA from damage (Kootstra, 1994). Reactive oxygen species (ROS) production in plant cells also takes place in UV-B treated plants (Green and Fluhr, 1995).

2.1.4 High and low temperature

Plants growing in temperate and boreal climates commonly experience freezing stress. Sub optimal temperature can cause serious damage to a number of plants. However, different plant species vary widely in their ability to tolerate low temperature stress (Levitt 1980; Sakai and Larcher, 1987). Chilling sensitive tropical species can irreparably damaged when temperatures drop below 10^{o} C while chilling tolerant but freezing sensitive species are readily damaged by subzero temperatures. Injuries are caused by impairment in cellular processes including alterations in membrane properties, interaction between macromolecules and enzymatic reactions. Freezing

stress is a composite stress caused by low temperature as such and mechanical stress due to formation of ice in the tissues. On cellular level freezing stress is mainly due to freeze induced cellular dehydration resulting in injuries in membranes (Steponkus et al., 1993) and linking freezing with other stresses (drought and high salinity) leading to cellular water deficit (Palva. 1994). Also at low temperatures, protein denaturation may occur (Guy and Li, 1998). Moreover, there is evidence that freezing stress can trigger the production of reactive oxygen species that cause membrane damage (McKersie and Bowley, 1997).

High temperature stress or heat shock causes cellular damage through protein denaturation, aggregation. increased fluidity of lipids in membranes leading to membrane degradation, inactivation of enzymes and block in protein synthesis (Levitt. 1980). Thus the crucial point in heat shock tolerance is the protection of proteins and enzymes from heat inactivation and denaturation. The heat shock leads to induction of a class of proteins called molecular chaperons that include chaperonin family proteins and heat-shock proteins. Molecular chaperones play an important role in protein folding, in which they form oligomeric structures that act as protein folding machinery (Gething and Sambrook. 1992; Gatenby and Viitancn. 1994). One of the essential functions of molecular chaperones seems to be to prevent the formation of, or to disassemble, incorrect structures generated by polypeptide chains during stresses such as heat shock (Ellis, 1987). The effect of high and low temperature on plants and the tolerance mechanism is extensively reviewed (Murata and Nishiyama, 1997; Schoffl et al., 1999; Thomashow and Browse, 1999; Palva et al., 2002).

2.2 Abiotic stress response

Plants respond to stresses by exclusion, tolerance, compensation and/or repair (Heath and Taylor, 1997). Plants' response to stresses is influenced by a variety of factors: the severity of stress and duration of exposure to the stress factor, environmental conditions, developmental stage of the plant and metabolic state of the plants (Guzy and Heath. 1993). Plants accumulate a large number of metabolites namely phenylpropanoids and flavonoid compounds, osmoprotectants, sugars, polyamines and antioxidant enzymes in response to environmental stress. However, we review only two compounds, phenylpropanoids and flavonoids in detail, as they are the subjects of this work. Emphasis is on the protective roles of these compounds in plants under stress and the pathways leading to their synthesis.

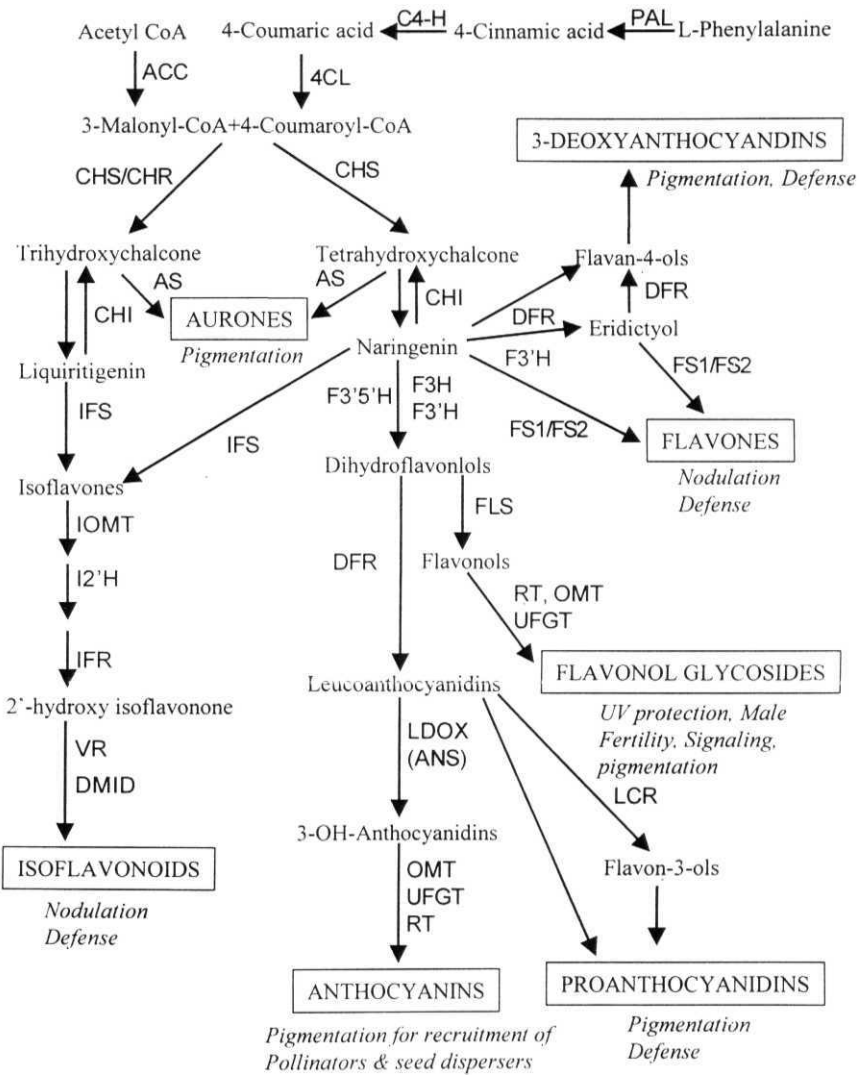
2.2.1 Phenylpropanoid and flavonoid synthesis

The general phenylpropanoid and flavonoid pathways are of great functional importance in protecting the plant cells from a variety of environmental stresses (Dixon and Harrison, 1990). These multi-step, multi-branch biosynthetic pathways lead to the synthesis of a vast array of structurally diverse and biologically active, low molecular weight secondary metabolites (Hahlbrock and Scheel, 1989), such as salicylic acid, phytoalexins (Smith, 1996), stilbenes, UV absorbing flavonoids and isoflavonoids, anthocyanins, and other structural molecules such as lignin (Figure 2.1), All these compounds have been reported to exhibit a wide range of protective functions in plants under stress. 7he key enzymes, phenylalanine ammonia lyase and 4-coumaryl: Co-A ligase of the phenyl propanoid pathway and chalcone synthase of the flavonoid pathway are of great importance since they determine the specific end product to be synthesized towards alleviating the deleterious effects of the stress.

Phenylpropanoid pathway leads to the synthesis of the basic precursor of flavoniods, 4-coumaryl-CoA. The biosynthesis starts from the deamination of phenylalanine by phenylalanine ammonia-lyase (PAL) to trans-cinnamic acid. PAL controls the flux of carbon into the pathway and therefore, efficiency of the whole pathway (Hahlbrock and Scheel, 1989). Cinnamic acid is then hydoxylated by cinnamate-4-hydroxylase to form 4-coumaric acid, which is activated by addition of co-enzyme A by 4-comarate:CoA ligase. The malonyl-CoA is synthesized from acetyl-Co A and CO_2 catalyzed by acetyl-CoA-carboxylase. From 4-coumaryoyl-CoA the pathway branches to the flavonoid and anthocyanin biosynthetic pathway. Regulatory circuits governing the channeling of malonyl-CoA into flavonoid pathway is yet to be established unequivocally.

Principal steps involved in the biosynthesis of flavonoids have been determined from a detailed analysis of a number of plant species (Harborne 1967). Flavonoid pathway begins with the stepwise condensation of three units of malonyl-CoA and one unit of 4-coumaryl-CoA catalyzed by the enzyme CHS, leading to the formation of the basic C-15 flavonoid skeleton, the chalconenaringenin. This is followed by isomerization of chalcone either non-enzymatically or by chalcone isomerase, to form naringenin (flavanone), the central intermediate of the flavonoid pathway. From this step onwards, the pathway diverges into distinct branches, each yielding a different

Figure 2.1 Phenylpropanoid and flavonoid biosynthetic pathway. The names of the some of the major classes of the flavonoid end products are boxed. Some of the known functions of the compounds in each class are indicated in italics. Figure adapted with modifications from Shirley, 2002. 4CL, 4-Coumarate:CoA ligase; ACC, Acetyl-CoA-corboxylase; ANS, anthocyanidin synthase; AS, aureusidin synthase; C4H, cinnamate-4-hydroxylase; CHR, chalcone reductase; DFR, dihidroflavonol 4-reductase; DMID, 7,2`-dihydroxy, 4`-methoxysoflavanol dehydratase; F3H, flavanone 3-hydroxylase; F3`H, flavonoid 3' hydroxylase; F3`5`H, flavonoid 3`5` hydroxylase; FS1/FS2, flavone synthase; I2`H, isoflavone 2`-hydroxylase; IFR, isoflavone reductase; IFS, isoflavone synthase; IOMT, isoflavone O-methyltransferase; LCR, leucoanthocyanidin reductase; **LDOX,** leucoanthocyanidin dioxygenase; OMT, O-methyltransferase; PAL, phenylalanine ammonia-lyase; RT, rhamnosyl transferase; UFGT, UDP flavonoid glucosyl transferase; VR, vestitone reductase.


Acetyl CoA
4-Coumaric acid
C4-H
4-Cinnamic acid
PAL
L-Phenylalanine
ACC
4CL
3-Malonyl-CoA+4-Coumaroyl-CoA
3-DEOXYANTHOCYANDINS
Pigmentation, Defense
CHS/CHR
CHS
Trihydroxychalcone
Tetrahydroxychalcone
Flavan-4-ols
AS
AS
CHI
CHI
DFR
DFR
AURONES
Pigmentation
Eridictyol
Naringenin
Liquiritigenin
F3'H
FS1/FS2
IFS
F3'5'H
F3H
F3'H
FS1/FS2
FLAVONES
Nodulation
Defense
IFS
Dihydroflavonlols
Isoflavones
FLS
IOMT
DFR
Flavonols
I2'H
RT, OMT
UFGT
IFR
FLAVONOL GLYCOSIDES
Leucoanthocyanidins
2'-hydroxy isoflavonone
UV protection, Male
Fertility, Signaling,
pigmentation
LDOX
(ANS)
VR
LCR
DMID
3-OH-Anthocyanidins
Flavon-3-ols
ISOFLAVONOIDS
Nodulation
Defense
OMT
UFGT
RT
ANTHOCYANINS
PROANTHOCYANIDINS
Pigmentation for recruitment of
Pollinators & seed dispersers
Pigmentation
Defense

class of flavonoids, the end products of the main pathway being anthocyanins. Therefore, this pathway is also referred generally as the anthocyanin pathway.

Structurally, flavonoid molecule consists of a typical A-ring benzoyl and B-ring hydroxycinnamoyl system composed of three planar rings A, C, and B. While, the fi-ring of the flavonoid skeleton originates from phenyl propanoid pathway, the A ring is derived from acetyl-malonyl pathway. The degree of oxidation of the C-ring determines the subclass of flavonoids formed such as isoflavonoids, dihydroflavonols, flavonols, flavones, catechins, proanthocyanidins and anthocyanins. Further, the overwhelming diversity of flavonoids observed in nature is attributed to different substitutions such as hydroxylation, methylation, glycosylation or acylation (Heller and Forkmann, 1988; Harborne. 1988). More than four thousand different kinds of flavonoids, including anthocyanins, have been reported in higher plants so far.

The anthocyanin pathway begins with the conversion of naringenin (flavanone), by hydroxylation at the C3 position by flavonone-3-hydroxylase (F3H) to dihydroflavonol, a pale yellow pigment. The next step is the reduction of dihydroflavonol to a colorless Icucoanthocyanidin by dihydroflavonol reductase (DFR) (Reddy et al., 1987). The leucoanthocyanidin is converted into a colored anthocyanidin in a two-step fashion, catalyzed by anthocyanidin synthasae (ANS), an NADPH dependent oxidoreductase (Menssen et aL 1990). UDP-glucose-flavonoid-3-oxy-glycosyl transferase (UFGT) catalyzes one of the last steps of the pathway, the glyocosylation of the anthocyanidin molecule, leading to the formation of anthocyanin. Further, acylation of the anthocyanin is catalyzed by the glutathione S-transferase (GST) that is associated with transport of anthocyanins into vacuoles (Marrs et aL 1995). Enzymes associated with distribution of flavonoids to various tissues are yet to be uncovered.

2.2.1.1 Genetics and molecular biology of flavonoid biosynthetic pathway

Flavonoid biosynthetic pathway for long has been a focal point of intensive research on gene organization, expression and regulation. Historically, this pathway is associated with the landmark discovery of plant biology, the identification and characterization of transposable elements (McClintock, 1948). This pathway, which results into vivid phenotypic expressions in flowers and foliage, played an important role in early mendalian genetic analysis. Further, flavonoid biosynthetic pathway

represents one of the oldest examples of coordinated gene and enzyme regulation in response to the environmental and developmental factors (Dooner, 1983).

The genes encoding enzymes of the general phenyl propanoid and flavonoid metabolism have been cloned from a number of plant species, particularly, maize. Petunia, Antirrhinum, and later Arabidopsis. The biochemistry, genetics, molecular biology and aspects of gene expression and regulation has been extensively worked out in maize. Petunia, Antirrhinum and Arabidopsis (De Vlaming et al., 1984; Coe et al., 1988; Dooner et al., 1991: Martin et al., 1991: Shirley and Goodman 1993, Shirley et al., 1995).

The genetic and molecular analysis of the maize flavonoid biosynthetic pathway revealed three functionally distinct classes of genes, acting sequentially in a temporal and spatial manner (Coe et al., 1988; Neuffer et al., 1997). Class I genes are the structural genes, encoding enzymes that control a single step in the biosynthesis and subsequent modifications. The class II genes are the regulatory genes encoding transcription activators that interact among themselves and with the promoter elements of the structural genes leading to activation of whole pathway or parts of the pathway, and determine spatial and temporal distribution of the pigments. The class III genes are largely known as modifying genes that influence the flavonoid concentration, intensity and distribution of the pigments. The gene action sequence in the biosynthesis of anthocyanin in maize has deciphered from inter-tissue complementation studies (Reddy and Coe, 1962)

The well-characterized structural genes of phenyl propanoid and flavonoid biosynthetic pathway from many plant species include, the *Pal* (encoding phenylalanine ammonia lyase), the *C2* (chalcone synthase), the *CHI* (chalcone isomerase), the *A1* (dihydroflavonol reductase), the *A2* (anthocyanidin synthase), the *Bz1* (flavonoid 3-0-glycosyltransferase), and the *Bz2* (glutathione-s-transferase). Activity of all the enzymes is obligatory for full expression of color phenotype and absence of any one of the enzyme leads to non-color or a modified phenotype. For instance, maize tissue devoid of F3GT, and GST is brown, where as tissue devoid of DFR or ANS is non-colored while the wild type being purple. The dominance-recessive relationships between alleles at individual loci, complementation pattern between non-allelic gene loci and the regulatory interaction, leading to predictable phenotype have

been elegantly demonstrated in maize (Reviewed by Coe et al., 1981; Neuffer et al., 1997).

In maize, the regulatory loci encoding transcription factors such as the *CJ* (colored-1), the *P* (pericarp), the *PI* (plant color), all encoding an R2R3 myb proteins, the *R* (red), the *B* (booster), the *Lc*, all encoding a basic helix-loop-helix (bHLH) protein of myc family, and the *Vp1* (viviparous 1), govern the spatial and temporal expression of the flavonoid pathway genes (Forkmann, 1993; Neuffer et al; 1997). The structure, expression and regulation of the genes of the pathway, as well as the role of the pathway in plant stress responses form the subject of a number of reviews (Hahlbrock and Scheel, 1989; Dixon and Harrison. 1990; Chalker-Scott, 1999; Madhuri and Reddy, 1999; Shirley, 2002).

One important avenue for elucidating the role of flavonoids in the stress response is to understand how the expression of the biosynthetic pathway is regulated temporally and spatially. A great deal has been learned from studies on transcriptional regulation in a variety of plant species, although evidence for other types of control also exists. The major regulatory genes were cloned and characterized from different species including the C7 and *R* family from maize (Paz-Ares et al., 1987; Ludwig and Wessler, 1990) and rice (Hu et al., 1996; Reddy et al., 1998), the *Delila*, *Eluta* and *Rosea* genes of Antirrhinum (Martin et al., 1991), *An1*, *An2*, *An3*, and *An4* in Petunia (Gerats et al., 1982, Beld et al., 1989), and *tt8* and *tt2* of Arabidopsis (Nesi et al., 2000; Nesi et al., 2001). An apparent complex of novel regulatory proteins that may interact with CHS as well as with other flavonoid genes, was recently identified in Parsley using South-western and yeast two-hybrid screening (Rugner et al., 2001). Analysis of strawberry R2R3 myb homologs by overexpressing in transgenic tobacco plants has uncovered what appears to be a negative regulator of flavonoid gene expression (Aharoni, 2001). The data emerging from studies on transcriptional regulation of flavonoid pathway from different species suggests that different plants use somewhat different mechanisms to control the expression of flavonoid genes, consistent with the diverse requirement of flavonoids among species. However, some of the transcription factors regulating flavonoid biosynthesis appear to be functionally conserved across species. The tobacco and Arabidopsis plants carrying maize transcription factors *R* and C7 driven by *CaMV* promoter showed an increased anthocyanin accumulation in tissues, which normally accumulate it, and also in three novel tissue locations (Lloyd et al.,

1992). Also heterologous expression of the maize *Lc* and *CJ* lead to increased flavonol in tomatoes (Bovy et al., 2002).

2.2.1.2 Flavonoid biosynthetic pathway in rice

In rice, the information on flavonoid biosynthetic pathway has been restricted largely to the earlier phenotypic descriptions, the inheritance of specific loci governing the pigmentation pattern and their classical map positions (Ramaiah and Rao 1953; Kinoshita and Maekawa 1986; Kinoshita and Takahashi 1991). Later studies included biochemical characterization of pigments and molecular isolation and characterization of major genes (Reddy et al., 1994; 1995; 1996a; 1996b; 1998; Reddy 1996). The anthocyanin gene pigment system in rice is not clearly elucidated. Based on the limited information, the rice anthocyanin pathway was 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 *PI* (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. Detailed phenotypic effects of the inhibitor alleles in rice have been described (Reddy et al., 1995) though the mechanism of action is yet to be clarified.

Recent studies on biochemical and molecular aspects of the pathway in rice are beginning to add much needed information on this pathway. The rice flavonoid pathway has been demonstrated to be highly responsive to UV-B and this property has been exploited for the molecular analysis of the pathway (Reddy et al., 1994). Several structural and regulatory genes of the pathway have been cloned and sequenced (Hu et al., 1996; Reddy 1996; Reddy et al., 1996a; 1996b; 1998). The antifungal and antibacterial activity of the pathway intermediates was demonstrated against major pathogens of rice (Padmavathi et al., 1997). Further, rice transgenics overexpressing the maize *C2* gene are shown to have improved resistance, though marginal, against the rice blast (Madhuri et al., 2001).

2.2.1.3 Phenylpropanoid and flavonoid pathway and stress response

Phenylpropanoid derivatives have been widely implicated in disease resistance (Halbrock and Scheel, 1989) and UV-light resistance (Caldwell et al., 1983) in many

plants. They accumulate primarily in the epidermal layers of plant organs and have high extinction coefficients in the biologically damaging UV-B range. Phenylpropanoids are also required for the biosynthesis of polymeric lignin. which is an important structural component in disease resistance mechanisms (Moerschebacher et al., 1990).

Apart from imparting color to various plant parts and facilitating pollination and seed dispersal in plants, flavonoids and anthocyanins are known to play several other physiological functions (Chalker-Scott, 1999; Shirley, 2002). They include, protecting the plants from harmful radiation, particularly UV-B radiation, plant defense responses against phytopathogens, auxin transport regulation, plant-microbe symbiosis, male gametogenesis and stress response mechanisms (Figure 2.1). Flavonoids are known to be synthesized as a response to various environmental stimuli. Most importantly they protect plant tissues from harmful UV-B irradiation by absorbing light in the UV region, and therefore prevent the UV-B induced DNA damage (Teramura et al., 1983; Kootstra, 1994). Cell walls are strengthened at the pathogen penetration sites by incorporation and oxidative cross-linking of proteins and various phenolic subunits (Grant and Mansfield, 1999). Fortifying the plant cell wall gives many advantages to the plant. It prevents leakage of cytoplasmic contents and creates an excellent barrier. Lignin precursor molecules and free radicals formed in the cross-linking reactions may as such disrupt pathogen membranes or inactivate bacterial enzymes and toxins (Hammond-Kosack and Jones. 1996). A localized oxidative burst is often detected during cell wall fortification, but the accumulation of ROS is much below the level seen during the hypersensitive response (Grant and Mansfield, 1999). Rapid oxidative cross-linking of basic hydroxyproline-rich glycoproteins (HRGPs) with pathogenesis related-proteins (PR-proteins) may be one of the earliest defence responses linked to the oxidative burst (Bradley et al.. 1992).

Flavonoids accumulation in response to UV-B in rice (Reddy et al., 1994), cold in maize (Christie et al., 1994) and sorghum (Shichijo et al., 1993) has been reported. Accumulation of anthocyanin pigments and expression levels of phenyl propanoid and anthocyanin biosynthetic genes were monitored in maize seedlings during the short-term low temperature treatment (Christie et al., 1994). These maize lines are genotypically well defined in terms of anthocyanin production. A significant increase in the transcript levels of the structural genes of the pathway, *PAL, 4CL, C2, CHI, Al, A2. Bz1*, and *Bz2* as well as regulatory genes, *C1* and *R* with a corresponding increase in the

levels of anthocyanins, was observed in 7-day-old maize (B37N) seedlings upon exposure to cold stress at 15° C (Christie et al., 1994). This has led to the conclusion that the anthocyanin biosynthetic pathway genes can be considered as *Cor* (cold-regulation) genes.

It is also known that osmotic stress induced by glucose (Tholakalabavi et al., 1997), sucrose (Do and Cormier 1991), mannitol (Tholakalabavi et al., 1994), and drought (Balakumar et al., 1993) lead to accumulation of anthocyanins in plants. Moreover, maize seedlings that accumulate anthocyanins are reported to show increased tolerance to field drought (Ronchi et al., 1997). It is suggested that such tolerance is probably due to decrease in the osmotic potential of leaves and perhaps contributing to decreased stomatal conductance (Choinski and Johnson, 1993).

The co-ordinate regulation of phenyl propanoid and anthocyanin pathway genes in response to developmental cues and environmental signals emerges out as an excellent model system to study the underlying molecular mechanisms of stress response and tolerance. Anthocyanin phenotypes serve as good markers in developmental biology and genetics.

2.2.2 Antioxidant enzymes

It is widely reported that much of the injury to the plants caused by stress exposure is associated with oxidative damage at the cellular level. In plants, many of the degenerative reactions associated with several biotic, abiotic and xenobiotic stresses are mediated by the toxic Reactive Oxygen Species (ROS) formed from the super oxide and hydroxyl radicals (Scandalios, 1993). Antioxidant enzymes either catalyze reactions where an antioxidant molecule(s) is able to quench ROS without being transformed into a destructive radical itself or to process ROS directly. Each of the antioxidant enzymes comprise of several isoforms (Noctor and Foyer, 1998). Although they are often induced in similar stress situations, their responses may be differential (Adam et al., 1995). Plants with high level of antioxidants, either constitutive or induced, are reported to have a relatively greater resistance to oxidative stress damage (Harper and Harvey, 1978; Madamanchi and Alcher, 1991). There are several reports on antioxidant enzymes in relation to the stress tolerance in crop plants (Bowler et al., 1992; Allen, 1995). Recently many transgenic plants carrying antioxidant genes have been reported to exhibit improved stress tolerance (McKersie et al., 2000). Since the

antioxidant enzymes are reported to modulate gene expression through the **generation** of appropriate signal molecules and the destruction of unnecessary signal molecules (Noctor and Foyer, 1998), the ROS are also considered as important signaling molecules in crop plants.

Plants use both enzymatic and non-enzymatic mechanisms for scavenging free super oxide radicals, hydrogen peroxide and singlet oxygen generated in cell under stress. Antioxidant systems of plants include enzymes such as super oxide dismutase (SOD), catalases and ascorbate peroxidase (APX) and non-enzymatic components such as ascorbic acid and glutathione. The dehydro ascorbate reductase (DHAR), along with glutathione reductase (GR) removes H_2O_2 through a mechanism known as Halliwell-Asada pathway (Foyer and Halliwell. 1976; Nakano and Asada, 1980). Super oxide molecules are produced by the reaction of molecular oxygen at photo system I via Mehler reaction, which is rapidly dismutated to H_2O_2 by SOD associated with thylakoid membranes, thus converting a harmful oxidant to a relatively less harmful species. The H_2O_2 produced is effectively scavenged by a thylakoid-bound ascorbate peroxidase (APX). Monodehydroascorbate radicals produced by APX can be quickly reduced to ascorbic acid via ferridoxin or stromal monodehydroascorbate reductase. Alternatively, they can spontaneously disproportionate into ascorbic acid and dehydroascorbic acid, which inturn converted to ascorbic acid by DHAR using reduced glutathione as an electron donor. Subsequent regeneration of reduced glutathione requires GR and NADPH (Bowler et a!., 1992). !n addition, plant cells contain relatively high levels of ascorbate, glutathione. and α-tocopherol. which are efficient oxyradical scavengers (Larson, 1988: Cadenas, 1989).

2.2.3 Osmolytes in stress tolerance

Accumulation of low molecular weight metabolites, acting as osmolytes, in response to osmotic stress such as drought and high salt is probably a universal phenomenon (Bieleski, 1982; Yancey et al., 1982; Ford. 1984). These compatible solutes accumulate in the cells under water stress without inhibiting the normal metabolic processes and their accumulation is widely reported to confer osmotic stress tolerance (McCue and Hanson. 1990; Delauney and Verma, 1993). The major osmolytes include sugar alcohols such as sorbitol and mannitol, iminoacid proline and the quaternary ammonium compound glycine betaine. Mannitol and sorbitol are reported to play a role in osmotic adjustment and osmoprotection. They act as

osmolytes facilitating water retention in cytoplasm and allowing sodium sequestration into the vacuole or apoplast. Mannitol reported to accumulate in the vacuoles of tobacco cells adapted to salt stress (Binzel et al., 1988). The overexpression of mannitol in transgenic tobacco chloroplansts resulted in an improved tolerance to oxidative stress (Shen et al., 1997). Similarly *Plantago* shoots and roots under salt stress show an eight fold and hundred-fold increase, respectively, in sorbitol concentrations (Ahmad et al., 1979). The iminoacid proline has been reported to form long lived adducts with the **free** hydroxyl radicals and thus prevent the damage inflicted by these reactive species (Floyd and Nagy, 1984). Proline thus performs multidimensional functions in organisms as a rescue molecule that is aptly accumulated under unfavorable conditions. Glycine betaine is reported to protect thylakoid membranes and plasma membranes against freezing damage or heat stabilization (Coughlan and Heber. 1982).

2.2.4 Signaling in stress responses

The understanding of the mechanism by which plants perceive environmental stress signals and transmit them to cellular machinery to activate adaptive response is vital for developing strategies to improve stress tolerance in crops. A generic signal transduction pathway starts with the signal perception, followed by the generation of second messengers such as inositol phosphates and reactive oxygen species. Second messengers can modulate intracellular Ca^{+2} levels, often initiating a protein phosphorylation cascade that finally targets proteins directly involved in cellular protection or transcription factors controlling specific sets of stress-regulated genes. The products of these genes may be involved in the synthesis of regulatory molecules like plant hormones abscisic acid (ABA), ethylene, and salicylic acid (SA). These regulatory molecules can, in turn, initiate a second round of signaling that may follow **the same generic** pathway, although different components are often involved.

It is expected that plants cells may have many different sensors of stress signals, though none have been confirmed. The drought, cold and salinity stresses have been reported to induce a transient Ca^{+2} influx into the cytoplasm (Sanders et al., 1999; Knight, 2000). Ligand sensitive Ca^{+2} channels control this internal Ca^{+2} release. Such ligands include, for example, inositol polyphosphates, cyclic ADP ribose, and nicotinic acid adenine dinucleotide phosphate. These molecules have been found to be able to induce Ca^{+2} release in plant cells and, in particular, guard cells (Schroeder et al., 2001). The important feature of Ca^{+2} signaling is the presence of repetitive transient bursts.

The first round of transient Ca^{+2} generation lead to generation of secondary signaling molecules like ABA and ROS which stimulate a second round of Ca^{+2} increase. These multiple rounds of transient bursts from various sources will have different signaling consequences and therefore, physiological meaning.

During exposure to stress, phospholipids, the backbone of cell membranes serve as a precursor for the generation of secondary messenger molecules (Munnik et al., 1998). Drought and salt stress up regulates the mRNA levels for certain phosphoinositide-specific phospholipase C (PI-PLC) isoforms, a major cleaving enzyme of phospholipids (Hirayama et al., 1995; Kopka et al., 1998). This increase in PI-PLC expression could contribute to an increased cleavage of phosphotidylinositol 4, 5-bispho-sphate (PIP2) to produce diacylglycerol and inositol 1,4,5-triphosphate (IP3) that serve as key second messengers that can activate protein kinase C and trigger Ca^{+2} release, respectively. The phospholipase D (PLD) is also involved in the transduction of stress signals. PLD hydrolyzes phospholipids to generate phosphotidic acid (PA), which mediates the ABA-induced stomatal closure in guard cell protoplasts (Jacob et al., 1999). Drought and salinity activate PLD and lead to transient increase in PA levels in plants (Frank et al., 2000; Munnick et al.. 2000; Katagiri et al., 2001). PLD appears to be activated through a G-protein (Frank et al.. 2000) independently of ABA (Frank et al., 2000; Katagiri et al.. 2001). However, excess PLD lead to negative impact through membrane destabilization at high concentration of PA (Wang, 1999).

The reactive oxygen species (ROS) such as super oxide, hydrogen peroxide, and hydroxyl radicals form for another class of signaling molecules in cells under stress. ROS serves as intermediate signals for ABA in mediating the Catalase I gene *(CATI)* expression (Guan et al., 2000), thermotolerance (Gong et al.. 1998), activation of Ca^{+2} channels in guard cells (Pei et al., 2000), stomatal closure (Zhang et al., 2001), and even ABA biosynthesis (Zhao et al.. 2001).

Evidence suggests the role of Ca^{+2} -dependent protein kinases (CDPKs) and SOS3 family of Ca^{+2} sensors in coupling the universal inorganic signals to specific phosphorylation cascades under stressed conditions. A number of studies have shown that the CDPKs are induced or activated under abiotic stresses (Urao et al., 1994; Pei et al., 1996; Hwang et al., 2000). Overexpression of OsCDPK7 resulted in increased cold and osmotic stress tolerance in rice (Saijo et al., 2000). In addition to CDPKs plants also use other kind of phosphoproteins for abiotic stress signal transduction such as

mitogen activated protein kinases (MAPKs) pathway. Several MAPK modules (i.e., MAPKKK-MAPKK-MAPK) that may be involved in abiotic stress signaling are identified in alfalfa (Kiegerl et al., 2000), and tobacco (Yang et al., 2001; Zhang and Klessig, 2001).

2.2.4.1 Role of ABA in signaling

Abscisic acid is a naturally occurring plant hormone that regulates several agronomically important plant processes such as synthesis of seed storage proteins and lipids, the promotion of seed desiccation tolerance and dormancy, and the inhibition of the phase transitions from embryonic germinative growth and from vegetative to reproductive growth (Leung and Giraudat. 1998; Rock, 2000; Rohde et al., 2000). In addition, ABA mediates several aspects *of* physiological responses to environmental stresses such as drought or osmotic induced stomatal closure, the induction of tolerance to water, salt, hypoxic and cold stress. and wound or pathogen response (Leung and Giraudat. 1998; Rock, 2000; Shinozaki and Yamaguchi-Shinozaki, 2000). Regulatory factors and signaling pathways that control ABA response have been deciphered using genetic. biochemical, and cell biological approaches (Rock, 2000; Finkelstein and Rock, 2002). The availability of the Arabidopsis and rice genome sequences (Arabidopsis Genome Initiative, 2000; Sasaki and Burr, 2000; Barry, 2001, Goff et al.. 2002; Yu et al., 2002), the existence of many expressed sequence tag (EST) projects in a variety of species (Yamamoto and Sasaki, 1997; Michalek et al., 2002; Reddy et al.. 2002), and the genome wide transcriptional profiling projects provide a fourth approach to dissect the ABA regulated cell signaling pathways in plants. Biochemical studies have identified a variety of gene promoter elements, kinases, kinase inhibitors, phosphatases, phospholipases, and transcription factors involved in ABA responsive mechanisms (Finkelstein et al., 2002). It is natural to expect that the first step in ABA response is involvement of some kind of recognition event. However, no ABA receptors have been identified till date, though the downstream elements of ABA regulatory pathway have been extensively studied.

A major route towards analysis of ABA signaling pathway opened up with the identification of ABA-regulated genes (Busk and Pages, 1998; Rock, 2000) in a few plants. In most vegetative tissues, these genes are activated in response to abiotic stresses that result in cellular dehydration (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 2000; Xiong et al., 2002). In maturing seed, two well-known

classes of ABA regulated genes include those required for the synthesis of storage reserves and the acquisition of desiccation tolerance (Thomas 1993; Rock, 2000). Overall, ABA regulated genes range from those with relatively high abundance transcripts, which are required for adaptation to stress or reserve synthesis, to those with low abundance transcripts, encoding signaling components.

ABA plays a major role in plants during vegetative growth to optimize growth under adverse conditions by maintaining osmotic homeostasis. ABA is a major mediator of stress responses including regulation of stomatal aperture and elongation of root and inhibition of shoot growth under mild stress condition. It also plays a role in inhibiting root growth under severe stress. All this is achieved mainly through ABA mediated induction of an array of stress responsive genes. The mechanisms of gene expression through ABA-dependent stress responsive pathways are well studied in Arabidopsis (Shinozaki and Yamaguchi-Shinozaki, 1999). The transcription factors of the class MYB. MYC. and bZIP proteins and their cognate *cis*-acting recognition elements on promoters of stress responsive genes form the major component of these pathways.

Studies on ABA- mediated cell signaling using biochemical and cellular biological approaches have shown that G-proteins. phospholipases, protein kinases, and protein phosphatases participate in early events in ABA signaling (Rock 2000; Assmann, 2002; Yang, 2002). The *Rop 9* and *Rop 10*. encoding Rho/Rac-related GTPases are shown to negatively regulate the ABA effects on seed germination and seedling growth (Yang, 2002). Phasphatidic acid (PA), which is produced by the action of phospholipase D (PLD), also mediates the ABA regulation of stomatal aperture (Jacob et al., 1999) and gene expression (Gampala et al., 2001). ABA is shown to sensitize the Ca^{+2}- permeable plasma membrane channels by enhancing the production of reactive oxygen species (ROS) that can serve as secondary messengers leading to channel activation (Pei et al., 2000; Zhang et al., 2001). ROS production is a common Rop-dependent response to several stresses leading to stomatal closure, including drought and pathogen attack (Lee et al.. 1999). and the ROS-dependant pathway of response may be shared by multiple stresses (Yang, 2002). Apart from above signaling molecules, many kinases also have been implicated in ABA signaling affecting gene expression and/or stomatal regulation. Some of them show ABA-inducible expression (Hwang and Goodman, 1995; Hong et al.. 1997; Mikami et al., 1998; Gomez-Cadenas

et al., 1999; Piao et al., 1999), whereas others are expressed constitutively but are activated by ABA (Li and Assmann, 1996; Burnett et al., 2000).

2.3 Stress responsive gene expression

Distinct sets of genes are induced upon various stresses or other stimuli in plants. The most studied groups of genes related to stress responses include genes encoding PR-proteins (Warner et al., 1992), phenylpropanoid pathway enzymes (Kangasjarvi et al., 1994), ethylene biosynthetic enzymes, antioxidant enzymes, lipid metabolism enzymes (Kangasjarvi et al., 1994), transcription factors (Abe et al., 1997; Busk and Pages, 1998) and genes involved in the regulation of mitochondrial responses, such as *Aox* (Murphy et al., 1999). Some of the stress responsive genes and their regulation are explained in the following sections.

2.3.1 *Pal*

The expression of the key gene of the phenyl propanoid pathway, *Pal*, and the enzyme activity of its product under stress situations has been intensively studied. PAL activity required for the production of various phenylpropanoid compounds involved in plant protection, but its main function in defence is to produce precursors for salicylic acid, a signal molecule derived from a branch of the phenylpropanoid pathway (Mauch-Mani and Slusarenko, 1996). Role of SA in PAL induction is considered to be the potentiation of the response induced by some other environmental cues (Mur et al., 1996; Shirasu et al., 1997). It was shown that upon UV-B radiation or elicitor treatment, transcription rates of the *pal* increases several folds in cultured parsley cells and protoplasts, with simultaneous concomitant of flavonoids and coumarins in epidermal cells of plants (Hahlbrock, 1981; Chappel and Hahlbrock, 1984; Dangl et al., 1987; Dangl, 1991). In Arabidopsis, the *Pal-1* gene is induced by wounding and pathogen attack (Davis and Ausubel, 1989; Davis et al., 1991; Dong et al., 1991) in a tissue specific manner (Ohl et al., 1990). Three different *Pal* genes, *gPal-1*, *gPal-2* and *gPal-3* have been cloned and characterized from bean (Cramer et al., 1989). It was shown that their transcript distribution in hypocotyls is altered by various stress factors. While wounding activates all the three genes, *gPal-1* and *gPal-2* are expressed by high intensity illumination of etiolated hypocotyls and fungal infection stimulates the *gPal-1* and *gPal-3* gene transcription (Liang et al., 1989a; 1989b). In parsley, atleast three of the four *Pal* genes are activated either by UV light or fungal elicitor in cultured cells

and in wounded roots. Interestingly, only the parsley *gPal-3* gene is active in wounded leaves (Lois et al., 1989). The role of ROS in mediating *Pal* expression has been studied in various plant systems. The results show vast differences between plant species. A significant increase in PAL enzyme activity in response to UV-B treatment of etiolated rice seedlings has been demonstrated (Reddy et al., 1994). H_2O_2 seems not to be involved in *Pal* induction in soybean (Levine et al.. 1994) or tobacco suspension cell lines (Dorey et al.. 1999; Sasabe et al., 2000), although it is clearly demonstrated that H_2O_2 alone is able to increasce *Pal* as well as *Gst* mRNA level in Arabidopsis suspension cell cultures (Desikan et al., 1998).

2.3.2 *Chs*

The induction of the *Chs* gene expression in response to internal and external stimuli has been studied intensively. *Chs* expression is remarkably sensitive to UV- and blue light (Strid et al., 1994; Kalbin et al.. 1997; Logemann et al., 2000; Loyall et al., 2000). Increase in the fluence rate of white light increases the *Chs* mRNA level in Arabidopsis. as does the blue light. In some cases, red light may also affect the *Chs* transcript level (Jackson and Jenkins, 1995; Frohnmeyer et al.. 1998). By *in situ* hybridization analysis in parsley, it was shown that the expression of the *Chs* and accumulation of flavonoids were restricted to epidermal cells of UV-light treated etiolated seedlings (Jahnen and Hahlbrock. 1988). This general scheme for light regulation of the *Chs* expression holds true for Antirrhinum (Lipphardt et al., 1988), Petunia (Koes et al., 1989), and Arabidopsis (Feinbaum and Ausubel, 1988). In addition to UV-B, *Chs* up -regulation occurs as a response to ozone (Rosemann et al., 1991), fungal elicitor (Loake et al.. 1991; Kato et al.. 1995), pathogens (Cui et al., 1996) and wounding (Creelman et al., 1992). Of the known signaling molecules, *Chs* is induced by methyl jasmonate (MJ) (Creelman et al., 1992), and by the reduced form of glutathione (Wingate et al., 1988; Loyall et al., 2000). Glutathione and the oxidative status of the cells are possibly involved in the transcriptional regulation of UV-B induced *Chs* expression (Loyall et al.. 2000).

2.3.3 Arabidopsis desiccation responsive genes

Several genes responsive to desiccation were cloned from the cDNA libraries prepared from the 10h or 1h dehydrated Arabidopsis plants that were named as *rd* (Responsive to Desiccation) and *erd* (Early Responsive to Dehydration), respectively

(Yamaguchi-Shinozaki et al., 1992; Kiyosue et al., 1994). All these genes are found to be induced by dehydration and the sequence analysis suggested that these genes encode proteins that perform a protective function in cells under dehydration. For instance, these include putative proteinases that may degrade denatured or unnecessary proteins: chaperons that probably renature proteins. LEA proteins that may protect cells from dehydration, water channel proteins that can function in controlling osmotic potential of stressed cells, detoxicating enzymes etc. (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray. 1997). The expression analysis of these genes shows a broad variation in timing of induction of these genes in response to a given stress. Some of these genes responded to ABA treatment while several others did not respond suggesting existence of both ABA dependent and ABA-independent pathways in stress responsive gene expression (Shinozaki and Yamaguchi-Shinozaki, 1997). The expression of *rd22* requires protein biosynthesis for its induction by ABA. The inhibitor of protein synthesis, cyclohexiamide, inhibits the ABA induced gene expression of *rd22* but not that of *rd29A*, indicating that there are at least two independent signal transduction pathways between the production of ABA and gene expression in drought conditions (Yamaguchi-Shinozaki and Shinozaki, 1993). The mRNA levels for *rd29* gene are found to change differently in response to dehydration, low temperature, salt stress, or exogenous application of ABA (Shinozaki and Yamaguchi-Shinozaki, 1997).

2.3.4 Transcription factors

The major classes of stress responsive transcription factors that regulate the expression of stress responsive genes include, the basic leucine zipper proteins (bZIP) that bind to the G-box elements designated as ABA response elements (ABREs) and the functionally equivalent CE3 (Coupling Element)-like sequences, the MYB and MYC class of transcription factors and the DREB/CBF proteins that bind to DRE/CRT elements on promoters of stress responsive genes (Busk and Pages, 1998; Rock, 2000). There are 81 predicted bZIP factor genes in Arabidopsis (Riechmann et al., 2000), and only few are involved in ABA response. Only one bZIP family has been linked genetically to the ABA response: that composed of AB15 and its homologs, the ABRE binding factors, (ABFs and AREBs) (Choi et al., 2000: Uno et al., 2000), and AtDPBFs {*Arabidopsis thaliana Dc3* promoter binding factors). Homologs of these genes have been characterized in sunflower and rice (Kim et al., 1997: Kim and Thomas, 1998:

Hobo et al., 1999), in which they are correlated with ABA, seed specific, or stress induced gene expression.

The Arabidopsis genome encodes 43 members of the B3 domain family and only 14 of them are within ABA and stress responsive ABI3/VP1-related subfamily. The VP1 is also responsible for ABA dependent expression of the *C1* gene, an anthocyanin pathway regulatory gene belonging to MYB family of transcription activator, in maize seeds (Hattori et al., 1992).

Members of MYB and MYC family transcription factors are expressed in response to abiotic stresses (Urao et al., 1993, Abe et al., 1997). Both drought and ABA induce the expression of *AtMyc1* and three specific *MYB* family members (Abe et al., 1997). The MYB/MYC response system is somewhat slower than the bZIP-ABRE system, reflecting the need for de novo synthesis of MYB and MYC proteins (Shinozaki and Yamaguchi-Shinozaki, 2000).

In Arabidopsis, two groups of transcription factors, DREB1 (also called CBF), and DREB2, are involved in regulating expression of several transcription factors including the *rd29A* (Liu et al., 1998, Shinozaki and Yamaguchi-Shinozaki, 2000). The *DREB1* and *DREB2* genes encode structurally different proteins and are induced specifically by low temperature and by high salt or drought, respectively. DREB2A and DREB2B are produced in root only in response to salinity, but are produced in the stem and the root after drought treatment (Nakashima et al., 2000), offering a further level of specificity of response.

2.4 Transcriptional regulation of stress related genes: promoter context

Transcription is usually regulated by combinatorial control, meaning that several different proteins must bind to specific elements in DNA in a coordinated manner to achieve appropriate expression of the target gene. Transcriptional regulation therefore consists of transcription factor(s) (*trans*- acting factor) and the corresponding binding site(s) in the DNA (*cis*-element) (Wolberger, 1999). Not much is known about the transcriptional regulation of stress related plant genes. Some regulatory elements and *trans*- acting factors are, however, known and may be of importance in analyzing the regulation of the plant gene expression.

A low temperature responsive element (LTRE) identified in the Arabidopsis *LTI78* (also called *COR 78/RD29A*) gene promoter (Nordin et al., 1993) was shown to

be 9-bp element TACCGACAT, confers responsiveness to low temperature, drought. and high salinity, but not to ABA (Yamaguchi-Shinozaki and Shinozaki, 1994). This low temperature and dehydration-responsive element (LTRE/DRE) occurs also in several other promoters and has also been referred to as the C-repeat (CRT) (Baker et al., 1994). A small family of proteins called CBF1. CBF2, and CBF3 (CRT Binding Factor) also referred to as DREB1B, DREB1C, and DREB1A (DRE-Binding protein). respectively, activate the expression of genes carrying the DRE/CRT/LTRE element (Stockinger et al., 1997; Liu et al.. 1998; Gilmour et al., 1998; Shinwari et al., 1998). Another class of DRE was identified in maize with the consensus CCGGGCCACCGACGCACGG that was found to be ABA inducible (Busk et al.. 1997).

Several low temperature responsive genes are also induced by ABA and their promoter carry sequences closely resembling ABA response elements (ABREs). The ABREs with the consensus (C/T)ACGTGGC have been shown to confer ABA-regulated expression of many genes when present in more than one copy (Guiltinan et al., 1990; Leung and Giraudat 1998). Two bZIP proteins (basic domain leucine zipper) that bind specifically to the ABRE elements required in the dehydration and ABA induction of Arabidopsis *RD29B* (*LT165*) gene were identified (Uno et al.. 2000). These genes, encoding AREB1 and AREB2 (ABA responsive element binding protein) are drought, salt, and ABA responsive. Another small family of ABRE binding factors (ABFs) was characterized, also from Arabidopsis (Choi et al., 2000). *ABFs* respond differently to various environmental stresses including cold, suggesting they act in different stress responsive pathways.

The ABRE is not the only element with an ACGT core sequence. ACGT elements are found in many promoters and mediate induction by light (Guiliano et al.. 1988), anaerobiosis (McKendree and Ferl, 1992), UV-light (Weisshaar et al., 1991), and coumaric acid (Loake et al.. 1992). Furthermore, there are ACGT-containing elements that do not function as ABREs even in ABA inducible promoters (Straub et al., 1994; Kao et al., 1996; Busk et al., 1997). There are *cis*-elements called coupling elements (CE), which are active in combination with an ABRE but not alone (Shen and Ho., 1995; Shen et al., 1996). Dissection of the promoters of barley genes *HVA22* and *HVA1* defined the minimal ABA-responsive complexes (ABRC) consisting of a

coupling element and an ABRE capable of conferring ABA inducible activation of minimal promoter (Shen et al., 1996).

Analysis of Arabidopsis *rd22* gene established that a 67-bp DNA fragment in the promoter is sufficient for response to dehydration and ABA induced gene expression and that this DNA fragment contains two closely located putative recognition sites for the basic helix-loop-helix protein MYC, and an additional recognition site for MYB. The base substitution analysis of these MYB and MYC binding sequences, TGGTTAG and CACATG. respectively, revealed that the MYB and the first MYC binding elements are essential for dehydration responsive expression of *rdd22* promoter-*gus* fusion gene in transgenic tobacco (Abe et al., 1997).

The expression of another MYB class of genes from maize, the C7, was found to be activated independently by three factors. *VPl* expression. ABA and light (Hattori et al., 1992; Kao et al., 1996). The *sph* element on the *Cl* promoter with the consensus, CGTGTCGTCCATGCATGCAC has been shown to be sufficient and necessary for VP1 mediated activation of the *Cl* gene (Hattori et al., 1992; Kao et al., 1996). Such *sph* elements are present in promoters of several ABA and dehydration-stress responsive genes including *Em* of wheat (Guiltinan et al., 1990; Vasil et al., 1995), *RAB17* of maize (Vilardell et al., 1990), *RAB16A* of rice (Mundy et al., 1990), *DLEC2* of *Phaseolus* (Helmut et al., 1992). The *Cl* promoter element that confers a response to ABA is tightly linked to. but apparently distinct from. VPl responsive *sph* element (Kao et al., 1996) and the light inducibility of the C1 promoter is mediated by the promoter region, present downstream of the *sph* element, that comprises a light-responsive G-box elements (CACGTG), similar to ACGT elements (Kao et al., 1996).

The *cis* -elements responsive to ROS, such as the antioxidant responsive element (ARE), has been described (Dalton et al., 1999; Delaunay et al., 2000). However, regulation of gene expression by these regulators does not only occur through ROS, for they respond to, or are fine tuned by. other cellular signals as well (Dalton et al., 1999). ARE motif was found from the promoters of three maize *Cat* genes, and it seems to be involved in the expression *of Cat 1* during senescence related oxidative stress (Polidoros and Scandalios, 1999). More than 100 plant transcripts are regulated by light of different qualities. Numerous light responsive *cis*- elements are found in the promoters of many genes. Still, the assembly of elements varies greatly: Neither can any single element be found in all light regulated promoters nor do any

single element confer to light induced expression all by itself (Terzaghi and Cashmore, 1995). Several light and UV-B responsive elements have been described in the *Chs* promoters (Batschauer et al., 1996; Schafer et al., 1997). There are also numerous other stresses, or signaling molecule responsive, regions in the promoters of different stress related genes (Goldsbrough et al., 1993; Rouster et al., 1997; Dron et al., 1988; Chen and Singh, 1999). No clear, single stress related factors affecting plant transcription are known, although some of the primary defence responses are shared between stresses. It appears that the stress induced gene expression in plants is regulated through a complex network of transcription factors and their combinations binding to diverse mixture of stress related *cis* -elements in the promoter sequences. Nevertheless, some regulatory elements are recognized as responsible for certain responses, and the picture is getting clearer with the results of various EST and genome projects as well as with the transgenic approaches using promoter deletions.

2.5 Cross **talk between pathways and common elements**

In nature, plants are exposed to a combination of stress factors concurrently or separated temporally thus necessitating an integrated response to them. Plants make use of common pathways and components in the stress response relationship. This phenomenon, which is known as cross tolerance, allows plants to adapt or acclimate to a range of different stresses after exposure to one specific stress (Pastori and Foyer, 2002). Different stress responsive pathways also often share some common components that enable pathways to converge, thus achieving the same end or, pathways interacting and affecting each other's outcome. This phenomenon often referred to as cross-talk (Knight and Knight, 2001). The mechanism of cross-tolerance and cross-talk enables plants to adapt to diverse range of stress conditions and hence understanding this phenomenon is of importance in developing crop plants tolerant to multiple stress factors. The cross-talk between pathways is likely to depend upon specific and multiple protein-protein interactions, mostly at the level of transcriptional regulation. The genetic interactions among various hormone-signaling pathways in Arabidopsis have been described (McCourt, 2001). For example, *abi3* encodes a seed-specific transcription factor having a number of different domains that is involved in regulation of seed dormancy. Analysis of a set of mutant alleles of *abi3* having a mutation in different domains shows that ABI3 is a complex protein that is involved in responses to a number of different signals including resistance to exogenous ABA

application and the sugar signaling. Another example of protein-protein and/or protein-DNA interaction dictating the rate of transcription of specific genes under particular environmental conditions is the interaction between the bZIP and Dof transcription factors in the expression of Arabidopsis glutathione-S-transferase-6 (*GST6*). The *GST6* promoter contains Dof-binding sites closely linked to a 20-bp octopine synthase *(ocs)* element. The *ocs* element is not only the binding site for bZIP proteins but it is also responsive to H_2O_2 and pathogens (Chen and Singh. 1999).

Gene expression profiling using cDNA microarrays has identified many more genes that are regulated by multiple stresses like cold, drought, and salinity (Bohnert et al., 2001; Kawasaki et al., 2001; Seki et al., 2001). One group of such genes is represented by *RD29A* (also called *COR78/LT178*). The promoters of this class of genes contain both ABRE and the DRE/CRT (Yamaguchi-Shinozaki and Shinozaki. 1994; Stockinger et al.. 1997). The DRE related motifs are found in several cold and drought responsive genes and DRE-binding transcription factors like DREB1B/CBF1, DREBC/CBF2, and DREB1A/CBF3 are shown to be induced early and transiently by cold stress as well as osmotic stress (Liu et al.. 1998). Several basic leucine zipper (bZIP) transcription factors (also called ABF/AREB) that can bind to ABRE are cloned and characterized (Choi et al., 2000; Uno et al.. 2000). The ABRE family found in promoters of many ABA responsive genes is similar to the G-Box sequence group that is present in many promoters responsive to environmental stimuli such as UV, wounding, and anaerobiosis (Menkens et al.. 1995). This is presumably a major component of cross-talk.

In Arabidopsis several two-component response regulators have Myb-like DNA binding motifs (Urao et al., 2000). The promoter of *RD22* gene of Arabidopsis carry binding sites for MYB and MYC class of transcription factors which form critical domain for ABA, drought, and salinity responsive expression of *RD22* (Abe et al., 1997).

A single transcription factor can orchestrate the expression of many genes to improve stress tolerance in plants. However, acclimation to complex stresses such as drought and cold must involve the simultaneous operation of many signaling and biosynthetic pathways and networks. It is therefore, essential to identify stress regulated transcription factors and to characterize the proteins, and the signaling mechanisms that control their function. The understanding of complex interactions between transcription

factors and the c/.v-responsive elements in the promoters of the stress responsive genes is vital to unravel the underlying mechanisms conferring tolerance to multiple environmental stress factors on plant life.

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals and reagents

Agarose, EDTA. calf-thymus DNA, ethidium bromide, glucose, (3-mercaptoethanol, SDS, sodium acetate. NaCl, PEG, Tris, PVP, formamide, formaldehyde, guanidine isothiocyanate, glycerol, ABA were obtained from Sigma chemicals, USA. The streptavidin linked paramagnetic beads were purchased from Novagen, USA. Chemicals for bacterial media preparation and solvents phenol, ethanol, glacial acetic acid, acetone, chloroform, methanol. and DMSO of analytical grade, purchased from local sources. Oligotex suspension was purchased from Qiagen GmbH, Germany. The DNA labeling kits were obtained from Life Technologies, Rockville, Md. or BARC. India. Hybond N+ for nucleic acid transfer and DYEnamic ET terminator kit for automated DNA sequencing were from Amersham Pharmacia Biotech. Sweden. X-ray films and intensifying screens were purchased from Kodak, USA. DNA restriction and modifying enzymes, DNA and protein molecular weight markers and RNAse were purchased from New England Biolabs, Beverly, Mass. USA, Bangalore Genei Pvt. Ltd., Bangalore and MBI Fermentas, Lithuvania. Nickel- CL agarose was obtained from Bangalore Genei Pvt. Ltd.. India.

3.1.2 Rice and Arabidopsis stocks

Rice cultivars, showing clear differences in levels of anthocyanin pigmentation in different plant parts, were obtained from various sources as indicated in Table 3.1. The plants were grown in clay soil maintaining upland conditions in greenhouse at a day/night temperature of 35 ±1°C / 25±1°C. and Relative Humidity varied from 60-80%. Under the conditions at Hyderabad (longitude 78° 4` E; latitude 17° 3` N; altitude 600m above mean sea level) the sunlight intensity was nearly 2800 µmol/sec.

Arabidopsis ecotype Colombia was obtained form Nottingham Arabidopsis Stock Center (NASC), UK And WT2A from Purdue University.

Table 3.1 Distribution of purple/red pigmentation in indica rice lines

Rice line[1]	Phenotype[2]
Purpleputtu	All plant parts are purple except the node
G962	All plant parts are purple except collar, auricle and node
R27	All plant parts except node are purple
N22	All plant parts are green except red apiculus and brown pericarp
Prasanna	All plant parts are green
Hamsa	All plant parts are green

[1]The Purpleputtu rice cultivar was obtained from the Tamil Nadu Agricultural University, Coimbatore and others from the Directorate of Rice Research, Hyderabad. India

[2]The presence of purple/red pigments was checked in the leaf blade, leaf sheath, node. internodc, corolla, auricle, ligule and apiculus and pericarp.

3.1.3 Plasmids, cDNAs clones and antibodies.

The description of the cDNA clones and the hybridization probes used in this study, their accession number and the source are given in table 3.2. All cDNA clones for the rice flavonoid genes, *chs, dfr, ans* and *C1-myb* arc from our lab and previously described in detail (Reddy 1996; Reddy et al., 1996a; 1996b; 1998). The *pal* and *act in* cDNAs were as explained (Minami et al., 1989 and McElroy et al., 1990 respectively). All cDNA clones of Arabidopsis flavonoid genes were gifted by Dr. Brenda Winkel-Shirley, Virginia Tech. USA, and the *At-rd22* clone was from Dr. Kazuo Shinozaki, RIKEN, Japan. The *At-lea* was a PCR amplicon from Arabidopsis genomic DNA obtained by using specific primers designed for cDNA sequence from the database. Monoclonal antibody against the maize C1-MYB protein was obtained from Dr. Erich Grotewold, Ohio State University, USA. The super promoter cassette, pE1945, was from Dr. Stanton Gelvin, Purdue University, USA. Cloning vector pBluescript SK+ was from Stratagene, La Jolla, USA and- the T7 RNA polymerase promoter vector pRSET-B used for protein expression in *E. coli* was from Invitrogen Life Technologies. USA.

Table 3.2 Description of the cDNA clones and hybridization probes used in the present study

Gene	Description	**Source** Reference	Accession #
Os-pal	cDNA 2.5 Kb *Eco* RI fragment	Minami et al., 1989	X16099
Os-chs	cDNA 2.2 Kb *Eco* RI fragment	Reddy et al., 1996a	X89859
Os-dfr	cDNA 1.4 Kb *Eco* RI-*Xho* I fragment	Reddy et al., 1996b	Y07956
Os-ans	cDNA 1.6 Kb *Eco* RI-*Xho* I fragment	-	Y07955
OsC1-Myb	cDNA 1.2 Kb *Eco* RI-*Xho* I fragment	Reddy et al., 1998	Y15219
actin	cDNA 1.2 Kb *Eco* RI fragment	McElroy et al., 1990	X16280
At-chs	cDNA 1.1Kb *Bam* HI-*Xho* I fragment	Pelletier and Shirley, 1996	-
At-fls	cDNA 1.2 Kb *Sal* I-*Not* I fragment	Pelletier et al., 1997	-
At-rd22	cDNA 1.1 Kb	Yamaguchi-Shinozaki and Shinozaki, 1993	D10703
At-lea	PCR amplicon 800bp	-	X92115

3.2 Methods

3.2.1 Rice culture and stress treatment

We have previously classified the rice cultivars on the basis of visible levels of anthocyanin pigmentation (Reddy et al., 1994). The lines belonging to all three classes, i.e., cyanic (Purpleputtu and R27), moderately cyanic (G962), and acyanic (Nagina 22, Prasanna and Hamsa) were screened for accumulation of anthocyanins under given stress treatments. All rice lines used in this study were repeatedly selfed to obtain genetic homozygosity. Rice seeds were imbibed in water, surface sterilized with 5% sodium hypochlorite (v/v) for five minutes, thoroughly washed with sterile water and germinated in water upon filter papers in dark. Two-day-old germinated seeds were transferred to growth chambers and were supplemented with Hogland's medium. The average temperature during seedling culture was 28 ± 1° C and a photoperiod of 16 hours light and 8 hours dark was maintained. Seedling trays were examined twice daily to maintain constant moisture content. Ten-day-old seedlings were treated with either 20% poly ethylene glycol (PEG 8000, simulating water stress) or 150 mM sodium chloride or 100 μM ABA solutions prepared in Hogland's solution. Control plants received only Hogland's solution. The treatments were given three hours after

beginning of light period. Samples were collected at different intervals up to 48 hours after treatment,

3.2.2 UV-B irradiation experiments

For the experiments on UV-B irradiation, 10-day-old seedlings of Nagina 22 grown on 0.4% agar were exposed to midday sunlight for 30 min (which mostly contains UV-B) and returned to dark. Samples were collected after specific time periods upon dark incubation. The method of treatment was essentially same as explained earlier (Reddy et al., 1994).

3.2.3 Anthocyanin estimation

To determine the extent of anthocyanin accumulation upon stress treatment, a pair of seedlings of uniform height was harvested and anthocyanins were extracted in 2 ml of acidified 1% (v/v) HCl/ methanol for 24 hour at 4°C with occasional shaking. The absorbance of the extract at 530 nm per milligram fresh weight of tissue served as an arbitrary measure of anthocyanin content (Harborne, 1967). All the data points indicated in figures represent the mean of values obtained from at least three independent measurements each with three replicates and the standard errors were calculated thereof.

3.2.4 Extraction of total cellular DNA

Genomic DNA was isolated from leaf tissue by the procedure described by Murray and Thompson, 1980. Freshly harvested leaf tissue (8 g) was quick frozen in Hquid nitrogen and 20 ml of pre-heated (to 90°C) 2 X CTAB buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 40 mM 2-mercaptoethanol), was added. This mixture was incubated in a water bath at 65°C for 90 min with mild shaking and 20 ml of chloroform: isoamylalcohol (24:1) was added and further incubated for 20 min at room temperature (25°C-28°C) with mild shaking. The sample was centrifuged at 6000 rpm for 10 min at room temperature. The upper phase was separated, and mixed with 1/100 volume of RNase A stock solution (10 mg/ml), and incubated at 37°C for 30 min. The DNA was precipitated with 0.7 volumes of isopropanol at room temperature for 15 min. The DNA pellet was spooled out with a glass hook, washed with 5 ml of 76% aqueous ethanol, 0.2 M sodium acetate followed by 5 ml 70% ethanol. The DNA pellet was air dried for 15 min and dissolved in an appropriate volume of sterile TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0)

and stored at 4°C. The concentration of DNA was determined spectrophotometrically and the quality of DNA was checked through agarose gel electrophoresis.

3.2.5 Extraction of total cellular RNA

Total RNA was isolated from shoot and leaf samples collected at different time intervals after stress treatment using guanidine isothiocyanate method (Chomczynski and Sacchi, 1987) with minor modifications. The harvested tissues were quick frozen in liquid nitrogen and stored at -70°C. Five grams of freshly harvested or frozen tissues were ground in liquid nitrogen to a fine powder, transferred to tubes with extraction buffer (4M guanidine isothiocyanate, 20 mM EDTA. 20 mM MES. 50mM 2-mercaptoethanol). The extracts were incubated for 10 min at room temperature and centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was extracted twice with phenol-chloroform and once with chloroform. Finally, the aqueous upper phase was precipitated with 1/10 volume of 3 M sodium acetate and 2 volume of ice-cold ethanol. kept at -80°C for 2 hours and centrifuged at 8500 rpm for 30 min at 4°C. The resultant pellet was resuspended in the RNA resuspension buffer (2M LiCl, 10 mM Sodium acetate. pH 5.2), incubated at 4°C for 1 hour and centrifuged at 8500 rpm for 10 min at 4°C. The resultant pellet was resuspended in RNase free water. The quality of the total RNA was determined through 1.2% denatured agarose formaldehyde gel and concentration estimated spectrophotometrically.

3.2.6 Isolation of poly $(A)^+$ mRNA

Poly $(A)^+$ mRNA was purified from the total RNA using Oligotex suspension. Nearly 250 µg of total RNA dissolved in 250 µl of RNase-free water was mixed with 250 µl of buffer OBB (20 mM Tris.Cl, pH 7.5, 1M NaCl, 2 mM EDTA and 0.2% SDS) and 15 µl Oligotex suspension. The mixture was incubated at 70°C for 3 min and then at room temperature for 10 min. The mixture was spun at 12000 rpm for 2 min and the pellet was washed twice with 1 ml of buffer OW2 (10 mM Tris.CI. pH 7.5, 150 mM NaCl, 1mM EDTA) by mixing and centrifugation. After the final wash, poly $(A)^+$ mRNA bound to Oligotex particles were eluted by resuspending the pellet in 100 µl of hot (70°C) buffer OEB (5 mM Tris.CI, pH 7.5) followed by centrifugation at 12000 rpm for 2 min and the step was repeated once for maximum recovery. The supernatants. containing the eluted poly $(A)^+$ mRNA, were pooled, precipitated with ethanol, washed in 70% ethanol, dried and dissolved in an appropriate volume of RNase-free water.

3.2.7 Northern Analysis

Equal concentrations of poly $(A)^+$ mRNA were **separated through** 1.2% **agarose** formaldehyde gel [each 100 ml gel consisting of 1.2 g of agarose, 10 ml of 10 X MOPS buffer (200 mM MOPS, 100 mM sodium acetate, 10 mM EDTA, pH 7.0), 73 ml of water, boiled, cooled to 40°C and added 17 ml of 37% formaldehyde], transferred to **Hybond** N+ nylon membrane and baked at 80°C for 2 hours. Pre-hybridization and hybridization to radioactively labeled DNA sequences were carried out at 42°C in **a** buffer containing 50% formamide, 5X Denhardt's reagent, 5X SSPE, 1.5% SDS and 50 µg/ml denatured salmon sperm DNA. After hybridization for 15 hours, membranes were washed twice with 2X SSC, 0.5% SDS at room temperature followed by two washes of 0.5X SSC, 0.2% SDS at 62°C. The filters were exposed using an X-ray film with intensifying screens at -70°C. After autoradiography, northern blots were stripped of the probe by incubation in boiling 0.1X SSC and 1% SDS for 5 minutes. The blots were probed sequentially with the rice cDNAs for *pal*, *chs*, *dfr*, *am* and *C1*. The rice *actin* cDNA probe was used as an internal control to ensure equivalent loading of poly $(A)^+$ mRNA.

3.2.8 Random-primer labeling

The cDNA fragments were used as templates for preparation of probes by random primer labeling reaction. Nearly 100 ng of template DNA in 21 µl of water was denatured by boiling for 5 minutes and snap cooled To this, 20 µl of 2.5 X random primer solution, 10 µM each of dATP, dGTP, and dTTP, 50 µCi of [α-^{32}P]dCTP (3000 Ci/mM) and 4 units of Klenow were added and incubated at 37°C for 10 min. Sephadex G-25 spin column was run to remove unincorporated [α-^{32}P]dCTP. The specific activity of the resultant probe was calculated as follows: cpm/µg = cpm X 2.5 X total volume of the probe X 1000/40; where "2.5" is a Czrenkoff factor. The probe was denatured by boiling for 5 min, snap cooled and added to the hybridization solution.

3.2.9 Cloning promoter regions

We used directional genome walking technique using PCR (Reddy et. al., 2002b) to amplify and clone the 5' upstream region of rice *am* gene. Ten micro grams of rice (Variety Nagina 22) genomic DNA was separately digested to completion with 60 units *of Bam* HI, *Bgl* II, *Sau* 3AI and *Bcl* I at 37°C (except for *Bcl* I incubated at 55°C). The restriction-digested fragments were partially filled in by using 10 units of

Klenow fragment in the presence of dGTP and dATP. Specific partially double stranded genome walker adapters were made by annealing walker adapter primer 1 (WAP-1) (5`-CTA ATA CCA CTC ACA TAG GGC GGC CGC CCG GGC-3`) with its complementary walker adapter primer 2 (WAP-2) (5`-TCG CCC GGG CG-3`) in 3:1 ratio by mass. The WAP-2 primer was phosphorylated at the 5' end and its 3` OH group was blocked by a primary amine group. The partially end-filled restriction fragments were ligated separately with 1 μg of genome walker adapter. Approximately 50 ng of the adapter-ligated fragments were PCR amplified separately using 5' biotinylated gene specific primer and the walker primer 1 (5`-CTA ATA CGA CTC ACT ATA GGG-3`). PCR conditions were 94°C, 1 min; 55°C, 1 min; and 72°C, 4 min for 30 cycles. A total of 50 μl of streptavidin linked paramagnetic beads were washed thrice in 1X PCR buffer, the amplified biotinylated PCR products were immobilized on streptavidin linked paramagnetic beads, and the nonbiotinylated DNA was washed off. The immobilized double-stranded PCR products were then denatured with 0.15 M NaOH for 10 min at room temperature. After the magnetic separation of the biotinylated strands, the unbound complimentary strand was collected and alcohol precipitated and finally dissolved in 20 μl of TE buffer. Nested PCR was carried out using 1 μl of single stranded primary PCR products, purified from the previous step as a template, along with appropriate target gene specific inner primer and the walker primer 2 (5`-GGG CGG CCG CCC GGG CGA TC-3`). The ends amplified nested PCR products were blunted by treating with 1 μl (5 U) of 14 DNA polymerase in the presence of 0.1 mM each of dNTPs, 4 μl of 5X reaction buffer [335 mM Tris-HCl pH 8.8, 33 mM $MgCl_2$ 5 mM DTT, 84 mM $(NH_4)_2SO_4$] in a total reaction volume of 20 μl, and digested with *Not* 1. The restricted fragment was ligated into *Eco* RV and *Not* 1 digested pBluescript SK+. The cloned ampilcons were sequenced on MegaBace 500 automated DNA sequencing system using the DYEnamic ET Terminator chemistry.

3.2.10 Restriction digestion and ligation of fragments

Restriction digestion of the plasmid DNA and the ampiicons was carried out in a total volume of 20 μl containing 0.5 μg of plasmid DNA, 2 μl of appropriate 10 X restriction enzyme buffer. 1 μl BSA (1 μg/μl, if necessary), 15 (or 16) μl of double distilled water and 1 Unit of restriction enzyme. The reaction was incubated at 37°C (or

at an appropriate temperature according to the restriction enzyme used) for 1 hour and the digestion pattern was analyzed on 1% agarose gel.

The following principle was used to calculate the concentration of fragment and plasmid DNA needed (3:1 ratio of fragment to vector) for ligation reaction (Sambrook and Russel 2001). Fragment size/vector size X 100 X 3 where "100" denotes the amount (ng) vector DNA and "3" denotes the number of times of fragment DNA (ng) required for ligation. The ligation reaction was carried out in a total reaction volume of 20 µl containing 100 ng of restriction digested vector DNA, appropriate amount of fragment DNA, 2 µl 10 X ligase buffer, 1 µl of T4 DNA ligase (5 U/µl) and sterile double distilled water to make up the volume. The reaction was incubated at 16°C for 2 h or overnight where necessary. After completion of the reaction, an aliquot of 10 µl was used for transformation.

3.2.11 Bacterial transformation

E. coli strain DH5 a was grown at 37°C either on solid (1.5% agar) or in liquid LB medium (1% tryptone. 1% NaCl. 0.5% yeast extract). Liquid cultures were grown initially in 2 ml of LB medium in a test tube, and later in 1-liter flasks for plasmid isolation.

Competent cells of *E. coli* were prepared as follows. One ml of DH 5 a cells from an overnight grown culture was inoculated in 100 ml of LB medium without antibiotic. The cells were grown till they reached an A_{600} of 0.4-0.6. Cells were then harvested into precooled 50 ml falcon tubes by centrifugation 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 resuspended in 15 ml 0.1 M $CaCl_2$ and incubated on ice for 10 min. This suspension was centrifuged at 3000 rpm for 10 min. The resultant pellet was resuspended in 4 ml of 0.1 M CaCb (in 10% glycerol) for every 100 ml of original culture, dispensed into 200 µl aliquots, frozen and stored at -70°C for future use.

Transformation of the competent cells was done as follows: Frozen *E. coli* cells were thawed on ice to which 1 ng of plasmid DNA or 100 ng of ligation mix were added. The suspension was carefully mixed with pipette tip and incubated on ice for 30 min. A heat shock of 42°C for 45 sec was applied followed by incubation on ice for another 2 min. 800 µl of LB was added and the bacterial suspension was incubated at

37°C with shaking for 1 h. Aliquots of the suspension were spread evenly on LB supplemented with an appropriate antibiotic. The plates were incubated at 37°C overnight. Following day. single colonies were picked up and inoculated for plasmid mini preparation.

3.2.12 Miniprep of plasmid DNA

A single colony of the *E. coli* strain DH 5 a, carrying the plasmid of interest, was inoculated into 5 ml of LB medium containing the appropriate antibiotic and incubated overnight with shaking at 37°C. An aliquot of 1.5 ml of the culture was transferred to a 1.7 ml tube and spun in a microcentrifuge for 1 min at 14,000 rpm at 4°C. The supernatant was removed by aspiration. The pellet was suspended in 100 µl of GTE solution (50 mM glucose, 25 mM Tris.CI pH 8.0. 10 mM EDTA pH 8.0) by vortexing. Then 200 µl of freshly prepared lysis solution (0.2 N NaOH. 1% SDS) was added and the contents were mixed and stored at room temperature for 5 min. Then the solution was neutralized by 150 µl of 3 M potassium acetate pH 4.8. mixed by inversion and stored on ice for 10 min. The cellular debris was removed by centrifugation at 14,000 rpm for 10 min at 4°C. The supernatant was transferred to a fresh tube and DNase free RNase was added at a final concentration of 20 µg/ml and incubated at 37°C for 20 min. After the RNase treatment, the suspension was extracted twice with phenol: chloroform (1:1) and once with chloroform. Then the plasmid DNA in the aqueous phase was precipitated with 0.6 volume of isopropanol. The DNA pellet was washed with 70% ethanol, dried. dissolved in TE and stored at -20°C. High quality plasmid DNA for sequencing purpose was isolated using Qiaprep spin mini prep kit according to manufacturer's instructions.

3.2.13 DNA sequencing

DNA sequencing was carried out on a MegaBace 500 automated DNA sequencing system by the dideoxy termination method (Sanger et al., 1977) using DYEnamic ET dye terminator cycle sequencing kit for MegaBace. A total reaction volume of 20 µl containing 400 ng of purified template plasmid DNA, and 8 µl of DYEnamic ET terminator reagent premix and 1 µl of 5 mM sequencing primer was used. The reaction tubes were placed in a thermal cycler with the following cycling parameters: 95°C for 20 seconds. 50°C for 15 seconds and 60°C for 1 minute for 25 cycles and stopped the thermal cycler with rapid thermal ramp to 4°C and hold.

After completing the thermal cycling, the reaction products were precipitated with 0.75 M ammonium acetate and 2.5 volume of ethanol, centrifuged, pellet was washed with 70% ethanol, air dried and dissolved in 10 µl formamide loading solution (70% formamide, 1 mM EDTA). The samples were injected at 3 KV for 40 sec and run at 9 KV for 110 minutes.

3.2.14 *In-silico* analysis

Nucleotide sequences were analyzed using standard bioinformatics tools. BLASTN and BLASTX were used to search NCBI non-redundant database for rice sequences. CLUSTALW (Thompson et al.. 1994) for multiple sequence alignment and the NCBI Conserved Domain Database for identifying conserved domains on protein sequences. The sequences were analyzed for the presence of putative motifs using the plant *cis* acting elements database at PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/) and PlantCARE (http://sphinx.rug.ac.be:8080/PlantCARE/cgi/index.html).

3.2.15 Construction of the bacterial expression vector

The 819 nucleotide open reading frame from the *OsCl-myb* cDNA was PCR amplified using the forward primer carrying *Bam* HI restriction site (5`-CGC GGA TCC.AGG GAG AAT GGG GAG GAG AG-3`) and the reverse primer with *Eco* RI restriction site (5`-CGG GAA TTC CGA ACT AAT GTC ACG CAC ACA-3`). PCR amplification was carried out in a MJ research thermal cycler. 20 ng of plasmid DNA carrying the *OsCl-myb* cDNA was added to the 50 µl PCR mix containing 5 µl of 10X PCR buffer, 2 µl each of 10 µM forward and reverse primers, 2 µl of dNTP mix (5 mM each) and 2 units of *pfu*DNA polymerase. DNA was denatured at 94°C for 5 min. followed by 25 amplification cycles of 55°C for 1 min annealing, extension at 72°C for 2 minutes, and denaturation at 94°C for 1 min. The terminal delay was set at 72°C for 5 min. The PCR amplicon was digested with *Bam* HI and *Eco* RI and cloned into *Bam* HI and *Eco* RI sites of T7 RNA polymerase expression vector pRSET-B. The restriction map of the recombinant plasmid and their identities were confirmed by restriction analysis using suitable restriction endonucleases. The recombinant plasmid designated as pOsC l -Myb-ORF was mobilized into *E. coli* strain BL21 (DE3) through transformation.

3.2.16 Growth of *E. coli* and induction of OsC1-MYB

E. coli strain BL21 (DE3) containing the expression vector carrying the recombinant pOsC1-Myb-ORF was grown in LB medium at 37°C in an orbital shaker until the A_{600} of the culture reached 0.6-0.9. At this stage the *E. coli* cells were induced with 1 mM IPTG and the cultures were allowed to grow for an additional three hours. Cells were harvested by centrifugation at 5000 rpm, 15 min at 4°C, suspended in 4 ml of binding buffer (20 mM sodium phosphate, 500 mM NaCl. pH 7.8). The cell suspension was incubated with 1 mg/ml of lysozyme for 30 min on ice followed by 10 min on a rocking platform at 4°C. The mixture was further incubated with 1% Triton X-100, 5 µg/ml DNase, 5 fig/ml RNase on a rocking platform at 4°C. The insoluble cellular debris was removed by centrifugation at 5000 rpm for 30 min at 4°C. The supernatant was subjected to Nickel- CL agarose affinity chromatography for purification of the hexahistidine tagged recombinant OsC1-MYB protein.

3.2.17 Purification of recombinant OsCl-MYB protein

The crude total protein extract from *E. coli* was applied onto a nickel CL agarose column that was pre equilibrated with binding buffer (pH 7.8). The column was washed with 6 volumes of binding buffer followed by 4 volumes of wash buffer (20 mM sodium phosphate, 500 mM NaCl, pH 6.0). The column was further washed with the wash buffer until the A_{280} of flowthrough was less than 0.01. The bound protein was eluted using the wash buffer with a continuous gradient of increasing imidazole concentration from 10 mM to 100mM. The fractions of interest were pooled and assayed for recombinant OsC1-MYB by 10% SDS-PAGE. The identity of the purified protein was confirmed by western blotting using monoclonal antibody raised against the maize C1-MYB protein.

3.2.18 SDS-PAGE

SDS-PAGE was performed according to Laemmli et al., (1970). About 20 µg of crude protein or 5 µg of partially purified protein was loaded on mini gels. The separation and stacking gel composition is as follows: Separating gel solution (30 ml) contains 16 ml of 28% acrylamide solution, 4.5 ml of 3M Tris.CI pH 8.9, 3 ml of 1% SDS, 6.45 ml of distilled water, 100 µl of 10% ammonium persulphate (APS), and 30 µl of TEMED. Stacking gel solution (5 ml) contains 0.9% acrylamide (28%), 2 ml of 0.5M Tris.CI pH 6.7, 0.5 ml of 1% SDS, 0.6 ml of water. 40 µl of 10% APS, and 10 µl

of TEMED. Electrophoresis was carried out at 150V after which the gels were stained with Coomassie blue. Gels were destained with a solution containing 7.5% methanol and 7% glacial acetic acid. From these gels the induced OsC1-MYB protein was identified by comparison with that of the control sample.

3.2.19 Western blot analysis

The SDS-PAGE gels were electrophoretically blotted onto nitrocellulose filters with an electroblotting buffer (25 mM Tris.Cl. pH 8.3, 100 mM glycine and 20% methanol) using Bio-Rad transfer apparatus. After the transfer, the proteins were visualized by Ponceau-S (Sigma) solution. The membrane was then blocked with 5% non fat milk solution dissolved in PBS buffer containing 0.01% Tween -20 for one hour and transferred to a blocking solution containing anti maize C1 monoclonal antibody at 1:150 dilution for one hour at 4°C. The blot was then washed thrice with PBS buffer containing 0.01% Tween 20 for 10 minutes, each wash. After the third wash the blot was incubated for 10 minutes at RT in a solution containing 150 mM NaCl and 50 mM Tris.Cl pH 7.5 and then transferred to the phosphate free, azide free blocking solution (5% non fat dried milk, 150 mM NaCl and 50 mM Tris.Cl pH 7.5) containing alkaline phosphate conjugated anti-mouse secondary antibody at a dilution of 1:20000 and incubated for one hour at RT. The blot was washed three times. 10 min each, with a buffer containing 150 mM NaCl and 50 mM Tris.Cl (pH 7.5). The bands were visualized using BCIP and NBT in AP buffer (100 mM Tris.Cl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$. NBT solution was prepared by dissolving 50 mg of nitro blue tetrazolium in 700 µl of dimethylformamide and 300 µl of AP buffer. BCIP solution was prepared by dissolving 50 mg of 5-bromo-4-chloro-3-indolyl phosphate in 1 ml of dimethylformamide).

3.2.20 Amplification of putative MYB binding domains on the *dfr* and *ans* promoters

Primers were designed to amplify 80-120 nucleotide regions flanking different putative MYB binding domains identified on the rice *dfr* and *am* promoters. The" sequences of the primers were given in Table 3.3. The PCR conditions were as follows: initial denaturation at 94°C for 5 min followed by 94°C, 1 min; 61°C, 1 min; and 72°C, 1 min for 30 cycles. The amplicons were phenolyzed, ethanol precipitated, washed in 70% ethanol, dried and dissolved in appropriate quantity of DNase free water.

Table 3.3 **Primers** to amplify putative MYB binding domains on **the** *dfr* **and** *ans* promoters

Promoter Domain	Primer	Sequence (5'-3')
ZMPMYB on *Os-dfr*	Forward	GTA CTA AAT GCA CCG GCC CT
	Reverse	CAA GCT AGA ACA GAA TGA CCA GC
GAMYB on *Os-dfr*	Forward	ATT TTG AGA CGG AGG GAG TG
	Reverse	TTT GTT TTA GTA TAC AAT TTT TCC *CC*
AtMYB2 on *Os-dfr*	Forward	TGG CGG GTG AGA CGT AAA
	Reverse	GAT AGC TTG CCT GTT GCT CCT
MYBC1-Plant on *Os-dfr*	Forward	GCG GAT GCA GCG ATC TAA A
	Reverse	TTT TTG GAC GGA CGG AGT AG
MYBRD22 on *Os-ans*	Forward	GAG CTA TGA CGG AAG CGA CT
	Reverse	GGA TGC GAA AAC AGG AGA AC
MYBC1-Plant on *Os-ans*	Forward	AGC ACG AGG ACT AAA ATG AGA A
	Reverse	ATA GGC CCA CTC CTA TCC AA

3.2.21 Gel mobility shift assay

PCR amplicons of 80 to 120 nucleotides flanking different putative MYB binding domains from cloned promoters of the rice *dfr* and *arts* genes were used for DNA binding studies. Five pmol of the PCR amplicons were end labeled in a reaction volume of 25 µl using 5 units of T4 polynucleotide kinase, 5 µl of [γ-^{32}P]dATP (10 µCi/µl, 3000 Ci/mmol) and 5 µl of 5 X exchange reaction buffer (250 mM imidazole-HCl pH 6.4, 60 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 350 µM ADP). The reaction was incubated at 37°C for 10 min and heat inactivated in the presence of 5 mM EDTA at 65°C for 10 min. The binding reaction and electrophoretic mobility shift assays were essentially same as explained in Sambrook and Russel (2001) with a few modifications. Binding reaction containing 1 ng of labeled DNA, 1 µl of Poly (dI-dC) (1mg/ml), 5 µl of 20% ficoll 400, 1 µg of purified recombinant OsC1-MYB protein, 4 µl of 5X binding buffer (20% glycerol, 100 mM Tris-HCl pH 8.0, 300 mM KCl, 25 mM $MgCl_2$ 500µg/ml BSA) in a reaction volume of 20 µl was incubated on ice for 30 minutes. After 30 minutes, samples were mixed with 3 µl of 6X gel loading buffer (0.25% bromo phenol blue, 0.25% xylene cyanol FF, 40% sucrose). EMSA to separate free and bound DNA was performed in a 5% polyacrylamide gel cast and run in 0.5X TBE (12.5

mM Tris-Cl, 125 mM glycine, 0.05% SDS). For competition studies, the **binding reaction** was **incubated** for 10 minutes on ice before adding 10 or 100 fold excess of unlabeled competitor DNA and the reaction mixture was further incubated for 30 minutes on ice before loading onto a 5% native polyacrylamide gel.

3.2.22 Construction of plant expression vector

The 1.2 kb *OsCl-myb* cDNA was excised from the cloning vector pBluescript KS+ by digesting with *Eco* RI and *Xho* I. The cDNA fragment was separated on an agarose gel **and** eluted and purified using Concert gel extraction kit (Life technologies). The ends of cDNA fragments were filled in using Klenow fragment. The plant expression vector pE1945 was digested with *Sma* I and the 5* ends were dephosphorylated using calf intestine alkaline phosphatase. The filled-in *OsCl-myb* cDNA was cloned into *Sma* I site of pE1945. The sense orientation of *OsCl-myb* cDNA in the recombinant plasmid was confirmed by restriction analysis.

The filling-in reaction was carried out in a total volume of 20 µl reaction containing 1 µg of cDNA fragment, 2 µl of 10 X Klenow buffer (500 mM Tris-HCl pH 8.0, 50 mM $MgCl_2$, 10 mM DTT), 0.05 mM each of 4 dNTPs, 5 units of Klenow fragment and double distilled water to make up the volume. The reaction was incubated at 37°C for 10 minutes and the enzyme was heat inactivated at 70°C for 10 min. The reaction mixture was phenolyzed, alcohol precipitated, washed in 70% ethanol, dried and dissolved in an appropriate volume of TL buffer.

The dephosphorylation reaction was performed in a total volume of 50 µl containing nearly 20 Pico moles of DNA, 5 µl of 10X reaction buffer (0.1 M Tris-HCl pH 7.5, 0.1 M $MgCl_2$), one unit of calf intestine alkaline phosphatase and sterile double distilled water. The reaction was incubated at 37°C for 30 minutes, enzyme was heat inactivated at 85°C for 15 min. The mixture was phenolyzed, ethanol precipitated, washed in 70% ethanol, dried and dissolved in appropriate quantity of TE. The recombinant plasmid was mobilized into Agrobacterium strain AGL-1 by electroporation and used for Arabidopsis transformation

3.2.23 Electro-transformation *of Agrobacterium tumefaciens*

Electro-competent cells of Agrobacterium were prepared as follows. One ml of Agrobacterium strain AGL-1 cells from a saturated culture was inoculated in 100 ml of LB medium without antibiotic. The cells were grown till they reached an A_{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 washed sequentially in 100 ml of 0.1 M HEPES, 50 ml of 0.1 M HEPES and 50 ml of 10% glycerol by resuspension and centrifugation. The resultant pellet was resuspended in 300 µl of 10% glycerol for every 100 ml of original culture, dispensed into 40 µl aliquots, frozen and stored at -70° C for the future use.

Electro-transformation of the competent cells was done as follows: Frozen *E. coli* cells were thawed on ice to which 1 ng of plasmid DNA or 100 ng of ligation mix were added. The suspension was carefully mixed with pipette tip and transferred to a prechilled 2 mm cuvette. Electroporation was carried out at 2.5 KV voltage, 25 µF capacitance and 201 Ω resistance for nearly 5 msec. Immediately after the electroporation, 1 ml of LB was added and the bacterial suspension was incubated at 28°C with shaking for 1 h. Aliquots of the suspension were spread evenly on LB medium supplemented with an appropriate antibiotic. The plates were incubated at 28°C for 48 hours. Single colonies were picked up from the plate and inoculated for plasmid mini preparation.

3.2.24 Arabidopsis culture

Arabidopsis seeds were sown in pots filled with a compost mixture of 1:1:1 perlite: vermiculite: soilrite. Pots were covered with nylon window screen after sowing. The germination was synchronized by cold treatment for 48 h at 4°C, and the pots were placed in a controlled environment chamber (16 hours day photoperiod, 20±1°C constant day and night temperature), sub-irrigated with the standard nutrient solution [5 mM KNO_3, 2.5 mM KH_2PO_4, 2 mM $MgSO_4$, 2 mM Ca $(NO_3)_2$, 1 X Fe-EDTA and 1 X MS micronutrient mix (Murashige and Skoog 1962)]. Four to five week old plants with adequate number of bolts were used for transformation using vacuum infiltration.

3.2.25 Transformation by vacuum infiltration

Agrobacterium tumefaciens strain AGL-1 carrying the *OsC1-myb* construct was grown in LB medium supplemented with 50 µg/ml kanamycin. Cells were harvested from 500 ml log phase culture (A_{600} of >2.0) by centrifugation at 5000 rpm for 10min at 4°C.and resuspend in 3 volumes infiltration medium (½ strength MS medium. 2% sucrose, 200 µl/l silwet and 0.05 µM benzylamino purine). Agrobacterium in the infiltration medium was added to a beaker and plants (pot, soil, and all) were inverted

into liquid solution making sure that bolts and the entire rosettes are submerged. Beaker was placed into vacuum desiccator and a vacuum was applied until bubbles form on leaf and stem surface and solution starts to bubble a bit (550-650 mmHg for 10 min), and then released the vacuum very rapidly. Plants were removed from the beaker, laid them on their side into a plastic tray and covered with saran wrap to maintain humidity. The next day, plants were uncovered and set upright. Plants were grown approximately four weeks, keeping bolts from each pot together and separated from neighboring pots. When siliques on plants were very dry, seeds were harvested and plated on a selection medium to select transformants.

3.2.26 Transgenic selection and analysis

Seeds were surface sterilized by treating them once with absolute ethanol for 5 min and 0.05% $HgCl_2$ for 3 minutes followed by 3 washes in sterile double distilled water. Surface sterilized seeds were suspended in sterile, room temperature 0.1% agarose and spread onto selection plates (standard nutrient medium with 0.7% agarose and 50 μg/ml of kanamycin). Plates were dried in a laminar flow hood until seeds no longer flow when plate is tipped. Plates were stored at 4°C for 48 hours and moved to a controlled environment chamber (16 hours day photoperiod, 20± 1 ^{0}C day and night temperature). After about 7 days, transformants that were clearly identifiable as dark green plants with healthy green secondary leaves and roots that extend over and into the selective medium were transplanted onto soil. The putative transgenic plants were analyzed for the integration of transgene by PCR. The sequences of primers used for amplification of selectable marker gene *npt II* were as follows:

Forward primer: 5`-GAG GCT AAT CGG CTA TGA CTG-3`

Reverse primer: 5`-ATC GGG AGC GGC GAT ACC GTA-3`

The primers for amplification of *OsC1-Myb* gene are:

Forward primer: 5`-ACA CCG CAC AGA GAC AGA GA-3`

Reverse primer: 5*-CTC AAG AAA CGA CGC CAA G-3`

The expression of the transgene in transgenics carrying *OsCI-Myb* was confirmed by northern analysis.

4. Results

4.1 Comparative analysis of the rice *C1-Myb* sequence

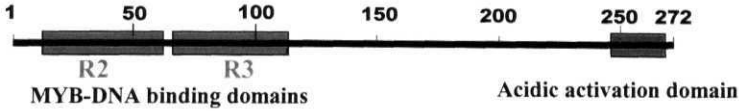
4.1.1 Structure of the *OsC1-Myb* gene product

The rice homologue of the maize *C1* gene, designated as *OsCl-Myb*, encodes a transcriptional activator belonging to the MYB family of proteins. The DNA sequence of this gene, cloned earlier from our lab (GenBank accession number Y15219; Reddy et al., 1998), was further analyzed to study its homology with that of the other members of stress responsive MYB family proteins. The amino acid sequence of the OsC1-MYB protein was deduced from the nucleotide sequence of *OsC1-Myb* gene. The protein is 272 amino acids long, with a predicted molecular weight of 37 kDa. The comparison of the deduced amino acid sequence of the *OsCl-Myb* gene with sequences in the NCB1 conserved domain database revealed the presence of two repeats of DNA binding domains similar to the R2 and R3 repeats of mammalian MYB class of proteins (Fig. 4.1). We found an acidic region between the amino acids 248 and 270 (Sequence DDWMDDVRALASFLDTDDAWNL) which could be a candidate for the transactivation domain, as is reported in the human c-MYB protein (from 286 to 308 Sequence- DEDPEKEKRIKELELLLMSTENE) (Kanai-Ishii et al., 1990)

4.1.2 Comparison of sequence of the OsCl-MYB with other MYB family proteins

The amino acid sequence of different MYB related proteins from rice, maize, Arabidopsis, cotton, oats and human were aligned with that of the OsC1-MYB in the figure 4.2. A comparison of the amino acid identity between the OsC1-MYB and other MYB family proteins in the entire protein region as well as the DNA binding domain region is summarized in table 4.1. Analysis of these data shows the degree of conservation of amino acids in DNA binding domain is significantly higher than that of entire protein. As expected, the DNA binding domain of the OsCl-MYB protein shows a significantly higher identity (~89%) to its orthologs from maize, Zm-P1 and Zm-C1. The DNA binding domain of the OsCl-MYB also shows a good sequence homology with the gibberellic acid responsive GAMYB protein (~59%) from rice and oats, the drought responsive Arabidopsis AtMYB2 (-56%) and a human MYB (40%) protein.

A



B

R2 domain

RGAWTSKEDDVLASYIKSHGEGKWREVPQRAGLRRCGKSCRLRWLNYL

R3 domain

RGNIDDDEEELIVRLHTLLGNRWSLIAGRLPGRTDNEIKNYWNSTL

Figure 4.1 Structure of the OsCl-MYB protein.

The amino acid sequence deduced from the nucleotide sequence of the *OsC1-Myb* gene (GenBank accession # Y15219; Reddy et al., 1998) was analyzed using the NCBI Conserved Domain Database.

A. Physical map of the OsC1-MYB protein showing the highly conserved R2R3 MYB-DNA binding domains and the acidic activation domain. R2R3 domains are shown in red and the acidic activation domain is shown in green.

B. Amino acid sequence of the R2 and R3 domains on the OsCl-MYB protein.

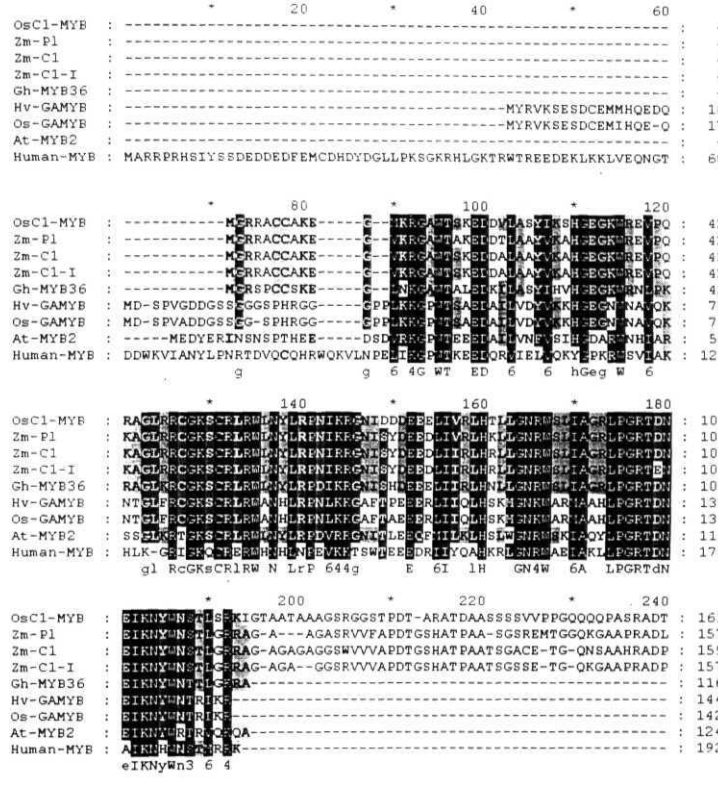


Fig 4.2 Alignment of the protein sequence of the OsC1-MYB with other MYB family proteins.

The sequence of the OsC1-MYB (Acc. # Y15219) is aligned with that of Zm-Pl (Acc. # T03972). Zm-C1 (Acc. # P10290), Zm-Cl-I (Acc. # P23592), Gh-MYB36 (Acc. # AAK19617), Hv-GAMYB (Acc. # AY008692), Os-GAMYB (Acc. # X98355), At-MYB2 (Acc. # BAA03534) and Human MYB (Acc. # AF104863).

Table 4.1 Comparison of homology of OsC1-MYB with other MYB domain carrying proteins

MYB Proteins	Entire Sequence (%)	MYB Domain (%)
Zm-Pl	56.39	89.89
Zm-C1	55.88	88.88
Zm-C1-I	55.15	87.87
Gh-MYB36	40.59	82.82
Hv-GAMYB	29.04	59.79
Os-GAMYB	28.30	59.79
At-MYB2	25.00	56.12
Human MYB	22.05	40.40

The high degree of similarity between the OsCl-MYB and the AtMYB2 proteins indicates the possibility that the transcript and protein encoded by the *OsC1-Myb* also accumulates in response to water stress. If water stress activates the *OsC1-Myb*, then it is logical to expect activation of the entire flavonoid pathway leading to an increased accumulation of anthocyanins in competent genotypes under water stress conditions. To test this hypothesis we screened several rice lines for changes in anthocyanin content under various stress regimes.

4.2 Anthocyanin accumulation in response to various stresses **in rice seedlings**

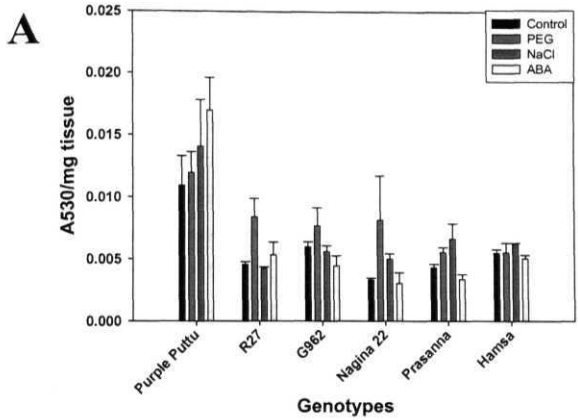
Effect of PEG, NaCl and ABA on anthocyanin accumulation in different rice genotypes was investigated. Rice genotypes were selected on the basis of their ability to accumulate anthocyanin pigmentation in different plant parts. Ten days old seedlings were treated separately with 20% PEG. 150 mM NaCI and 100 uM ABA. Shoot samples were harvested 48 hours after treatment and anthocyanins were extracted in acidified methanol. The estimation of anthocyanin content as A_{530} value revealed that the rice lines genetically competent to produce anthocyanins responded to the stress treatments by enhanced accumulation of the pigment in shoot tissues (Figure 4.3A). while the colorless lines carrying mutant alleles did not. Significant variation in pigment accumulation under stress was observed among different genotypes. The fully colored rice genotype, Purple Puttu. showed a significant increase in accumulation of anthocyanins under PEG. NaCI and ABA treatments. While, the other two tested

colored lines, R27 and G962, showed only a moderate increase in pigment accumulation under stress conditions. Treatment with 20% PEG appears to be more effective in triggering the anthocyanin accumulation among the genotypes that are genetically competent to produce anthocyanin. The rice line Nagina 22, which also showed a moderate increase in pigment accumulation under stress treatments, was proved suitable for detailed studies because it accumulates brown pigments in seed pericarp indicating that the line is genetically competent to produce anthocyanins under stress. Moreover, this line is found to be drought tolerant and does not accumulate any visible pigmentation in vegetative tissues under natural conditions of growth thus showing a low basal level of pigments against which changes during various stress treatments could be scored. The non-purple genotype, Hamsa, that does not show any pigment deposition in any plant part under normal conditions, did not show anthocyanin accumulation before or after the treatment where as another non-pigmented rice line, Prasanna, showed only marginal increase in pigment accumulation in leaves (Fig. 4.3A).

We further analyzed the effect of stress treatments on rate of accumulation of pigments over time in rice genotype Nagina 22. Samples were collected at different time intervals after the stress treatment and the anthocyanin content was estimated. Seedlings exposed to PEG, NaCl and ABA accumulated anthocyanins at a relatively constant rate to reach a maximum level by 24 to 48 hours after the treatment (Figure 4.3B). The increase was significant with 20% PEG treatment. Only marginal increase in anthocyanins levels was observed with 150 mM NaCl and 100 µM ABA.

4.3 Analysis of expression of flavonoid pathway genes in response to abiotic stresses

We investigated the effect of various stress treatments on accumulation of transcripts of the major genes of phenyl propanoid and flavonoid biosynthetic pathways in the rice line Nagina 22. The results of time course of gene expression by northern analysis using the Poly $(A)^+$ mRNA isolated from treated seedlings at different time interval after the treatment are presented below.



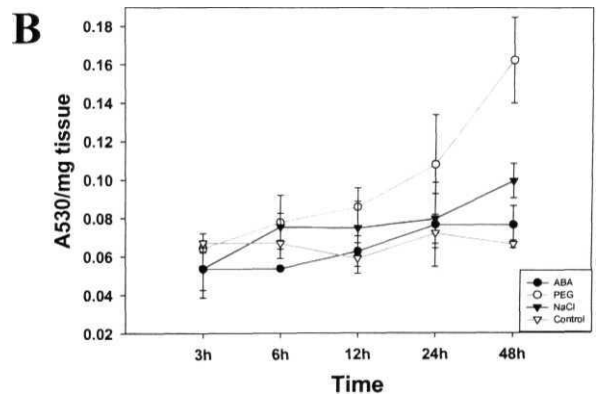


Figure 4.3 Stress responsive anthocyanin accumulation in rice.

A) Effect of 20% PEG, 150 mM NaCl and 100 µM ABA on anthocyanin accumulation in different in rice lines. Anthocyanin content is presented as A530 value of acidified methanol extract of leaf samples of 10 day old rice seedlings subjected to different treatments.

B) Time course analysis of anthocyanin accumulation under 20% PEG, 150 mM NaCl and 100 µM ABA treatments in rice line Nagina 22. Samples were collected at indicated time intervals after the treatment.

4.3.1 Transcript accumulation of genes encoding **phenylalanine ammonialyase and chalcone** synthase

We have analyzed transcript accumulation of key genes of the phenyl propanoid and flavonoid pathways using northern analysis. The blots were probed with cDNAs for the rice *pal*, encoding phenylalanine ammonialyase that catalyses the first step of phenyl propanoid pathway and the rice *chs* (orthologue of the maize *C2* gene) encoding chalcone synthase that catalyses the first committed step for flavonoid and anthocyanin biosynthesis. Results show that the transcript levels of these two genes were abundant in control seedlings and there were no significant changes in transcript levels upon stress treatment (Figure 4.4).

4.3.2 Message levels of the **late'** genes of the anthocyanin biosynthetic pathway, ***Os-dfr* and *Os-ans***

We further studied the status of the transcripts of two genes of the anthocyanin pathway under stress condition. The cDNA probes for the rice *dfr* (an orthologue of the maize *A1* that encodes dihydroflavonol reductase involved in conversion of dihydroxyquercitin to leucocyanidin) and the rice *am* (an orthologue of the maize *A2*, encoding anthocyanidin synthase that converts colorless leucocyanidin to the colored cyanidin) were used (Figure 4.5). The message levels of the *Os-dfr*, which was less abundant in control plants, increased dramatically with in three hours of treatment reaching a peak by about six hours. The transcript level declined thereafter reaching the background level by 48 hours. Treatment with 20% PEG appears to be more effective in activating the *Os-dfr* gene. The *Os-ans* transcript, that was not detected by northern analysis in leaves of Nagina 22 under normal condition, transiently accumulated at around three to six hours post treatment and then disappeared by 12 hours after treatment. The levels of transcript accumulation for both the *Os-dfr* and *Os-ans* were much higher in case of PEG treatment (Fig. 4.5A) than that of NaCl and ABA treatments (Fig. 4.5B and C). The changes were drastic leading to a many fold increase in the transcript level by about 3 hours after the treatment in case of PEG, while it was slow leading to a gradual increase in the transcript level in case of ABA treatment. The level of both the *Os-dfr* and *Os-ans* transcripts reached to the maximum by around 6 hours post treatment in case of PEG and by about 3 hours post treatment in case of NaCl, then declined gradually to reach the basal level by 48 hours. However, in case of ABA treatment, the *Os-ans* transcript level reached to its maximum by 12 hours, while

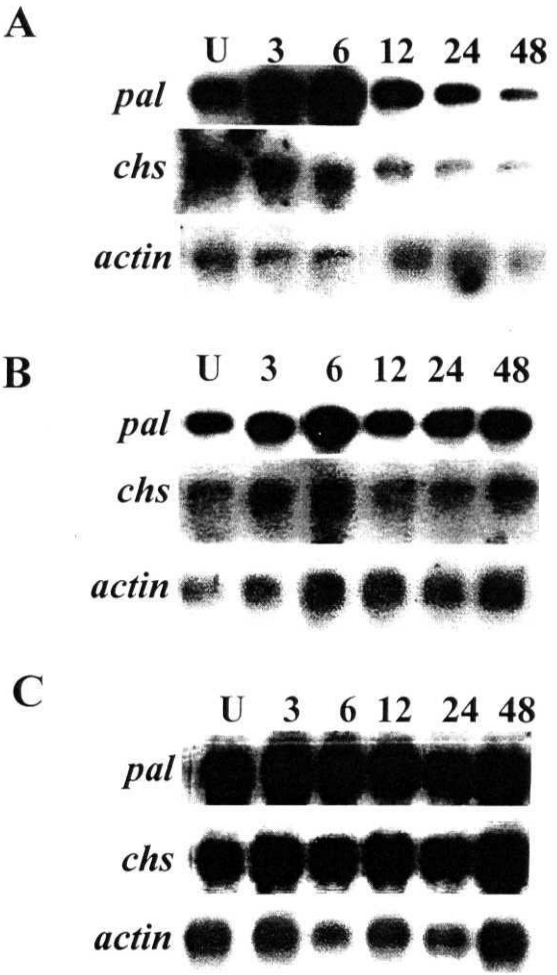


Figure 4.4 Stress responsive expression of the rice *pal* and *chs* genes. Northern analysis of effect of A) 20% PEG, B) 150 mM NaCl, and C) 100 μM ABA on expression of the rice *pal* and *chs* genes. 10 day old Nagina 22 seedlings were treated with PEG, NaCl or ABA and the shoot samples were collected at different time interval. 1 μg of poly A^+ mRNA was loaded in each lane. The rice *actin* was used as an internal control to confirm the equivalent loading. U- Untreated control; 3, 6, 12, 24, and 48- Time in hours, post treatment.

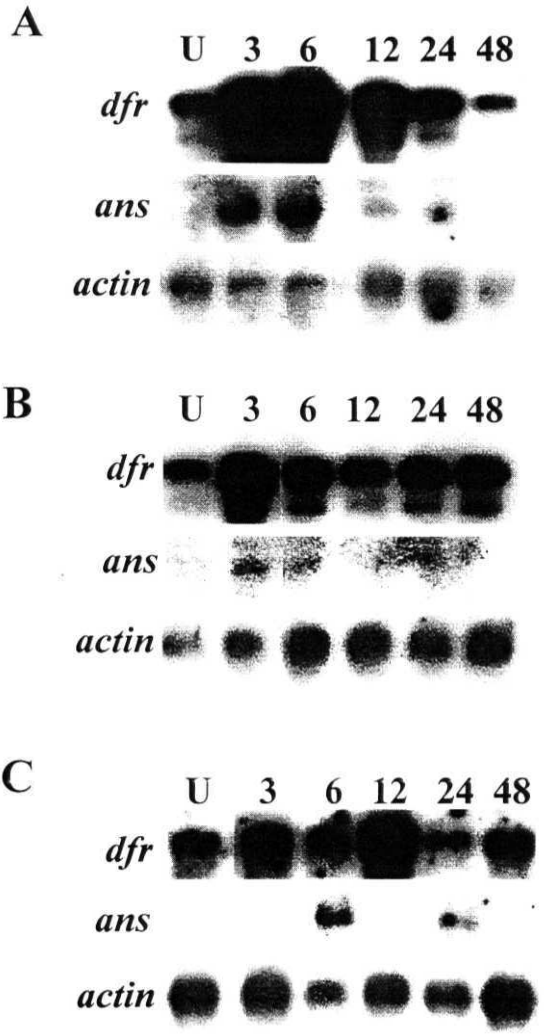


Figure 4.5 Stress responsive expression of the rice *dfr* and *ans* genes.

Northern analysis of effect of A) 20% PEG. B) 150 mM NaCl, and C) 100 μM ABA on expression of the rice *dfr* and *am* genes. 10 day old Nagina 22 seedlings were treated with PEG. NaCl or ABA and the shoot samples were collected at different time interval. 1 fig of poly A^+ mRNA was loaded in each lane. The rice *actin* was used as an internal control to confirm the equivalent loading. U-Untreated control; 3, 6. 12, 24, and 48- Time in hours, post treatment.

that of the *Os-ans* by 6 hours after treatment, then declining back to background level by 48 hours.

4.3.3 Changes in transcript level of the *OsC1-Myb*, a regulatory gene of the flavonoid biosynthetic pathway

It is known that the R and C genes, each encoding MYC and MYB class of regulatory elements, respectively, control the expression of the flavonoid structural genes (Goff et al., 1990; Roth et al., 1991; Grotewold et al., 1994; Tuerck and Fromm. 1994). These classes of proteins are known to be involved in the regulation of expression of several stress responsive genes in higher plants (Abe et al., 1997). We examined the effect of water stress, salinity and ABA treatment on expression of the *OsC1-Myb* homologues in rice seedlings. The transcripts of the *OsC1-Myb* homologue, which was not detectable in control plants accumulated at around three hours after the treatment, increased up to six hours and dropped down to background level by 48 hours (Figure 4.6). The pattern of expression of the transcripts of each gene tested was similar in all three-treatment regimes.

4.3.4 Flavonoid biosynthetic pathway genes in rice- response to UV-B treatment

To compare the water stress and high salt responsive expression of the flavonoid biosynthetic pathway genes with that of the well-known phenomenon of UV-B stress . we analyzed the expression pattern of rice flavonoid genes in dark grown rice seedlings upon exposure to short term UV-B light. Results show that the UV-B treatment triggers expression of all the flavonoid biosynthetic pathway genes tested (Fig 4.7). However, the key gene of the phenyl propanoid pathway, *pal*, did not show any change in the transcript level with UV-B treatment. The *Os-chs* and *Os-dfr* transcripts showed a significant increase in their levels immediately after the treatment and remained constant up to 12 hours post treatment. The transcript of the *Os-ans*. which was not detected in dark grown control seedlings, appeared only at about 12 hours after treatment. While, the *OsC1-Myb* transcript, which was also not detected in dark grown seedlings, appeared immediately after UV-B treatment and remained constant up to 12 hours post treatment. The results confirm the multiple stress responsive nature of the flavonoid biosynthetic pathway genes.

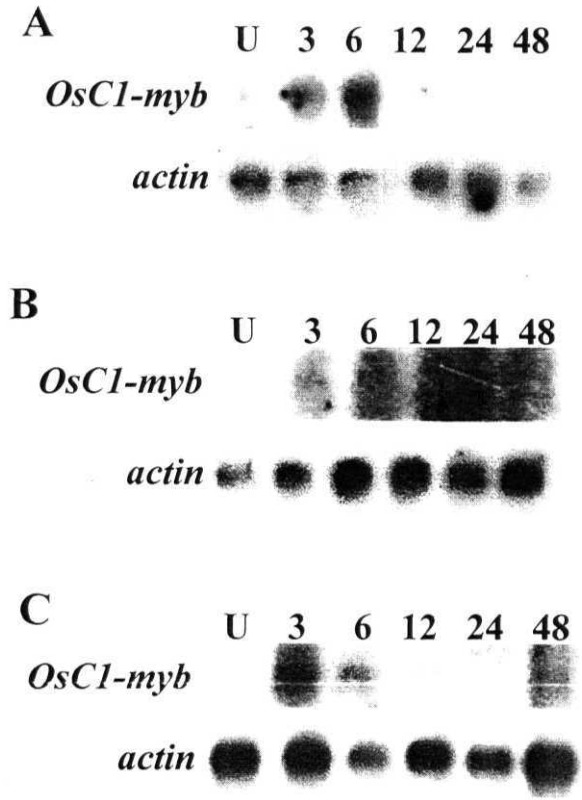


Figure 4.6 Stress responsive expression of the rice *OsCl-myb.*

Northern analysis of effect of A) 20% PEG. B) 150 mM NaCl. and C) 100 μM ABA on expression of the *OsC1-Myb*gene. 10 day old Nagina 22 seedlings were treated with PEG. NaCl or ABA and the shoot samples were collected at different time interval. 1 μg of poly A* mRNA was loaded in each lane. The rice *actin* was used as an internal control to confirm the equivalent loading. U-Untreated control; 3. 6. 12, 24. and 48- Time in hours, post treatment.

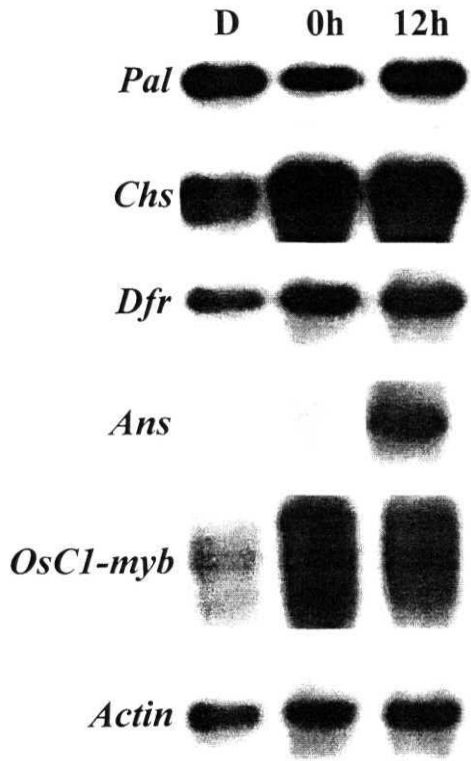


Figure 4.7 UV-B responsive expression of the rice phenylpropanoid and flavonoid pathway genes.

Northern analysis of effect of UV-B treatment on expression of the rice *pal chs, dfr, cms* and *OsC1-Myb*genes. 10 day old dark grown Nagina 22 seedlings were exposed to mid day sunlight for 30 minutes and returned to dark. Shoot samples were collected at different time intervals after returning to dark. 1 μg of poly A^+ mRNA was loaded in each lane. The rice *actin* was used as an internal control to confirm the equivalent loading. C-Dark control; 0. 12, Time in hours, post treatment.

4.4 Analysis of the promoter regions of the abiotic stress-responsive rice flavonoid pathway genes, *dfr* and *ans*

To understand the stress responsive expression of the *Os-dfr* and *Os-ans* genes in terms of regulation of gene expression, the 5" upstream region of these two genes were analyzed in detail. The Plant *cis* acting elements databases, PLACE and PlantCARE were searched using the *Os-dfr* and the *Os-ans* promoter sequences as a query to identify putative stress responsive motifs present in these two promoters. Visual inspection of the sequences was used to further characterize the domain organization in comparison with well-characterized and confirmed stress responsive regulatory domains described in the literature.

4.4.1 Source of the *Os-dfr* promoter sequence

The NCBI non-redundant sequence database was searched for rice genomic sequences using the *Os-dfr* cDNA sequence (GenBank accession # Y07956) as a query. The *Os-dfr* showed a complete homology to a region on a BAC sequence of 29877 bp in the database (GenBank accession # AF101045; Chen and Bennetzen, 1996). This BAC sequence was previously annotated to carry four putative protein coding regions; an ADP-glucose pyrophosphorylase subunit, *SH2*, two transcription factors, *X1* and *X2* and a NADPH- dependent reductase, *A1 (Os-dfr)* in that order (Chen et al., 1998). The region encoding NADPH- dependent reductase, *AL* spanning between nucleotides 27146 and 28764 on the BAC clone showed 100% homology with the *Os-dfr* cDNA sequence spanning all the three-exon regions. The intergenic region between gene *X2* and *AL* which is 11858 bp long was analyzed for putative promoter region of the *Os-dfr* gene. Nucleotide sequence of 1.8 kb 5* upstream region of the *A1* gene on the BAC clone was closely analyzed for the presence of putative promoter elements and transcription activators binding sites.

4.4.2 Cloning of the *Os-ans* promoter

Since no genomic clone with sequence homology for the *Os-ans* cDNA sequence (GenBank accession # Y07955) was found in the database at the time of this work, we used a method of directional genome walking through polymerase chain reaction to amplify and clone *5"* upstream region of the *Os-ans* gene. The method uses a restriction digestion of genomic DNA and the partial fill in of the fragment to avoid the self-ligation between the fragments. Use of specific genome walker adapters and

biotinylated primers aided in the enrichment of the specific template prior to nested PCR. An amine group was added to 3' end of the adapter strands to prevent the extension of the DNA sequence. Using this strategy we were able to clone a 3078 bp region corresponding to 5* upstream of the *Os-ans* gene using primers specific to the *Os-ans* cDNA sequence and the adapter specific primers. The cloned PCR amplicon was sequenced and the sequences were assembled. The assembled sequence of 5` upstream region of the *Os-ans* gene is presented in figure 4.8.

4.4.3 Comparative analysis of the promoter sequences of the *Os-dfr* and *Os-ans* genes

The *Os-dfr* promoter sequence obtained from the database and the *Os-ans* promoter sequence cloned by directional genome walking in this study were subjected to further analysis to identify and characterize the putative domains with relevance to abiotic stress response. Comparison of these promoter sequences with plant *cis* acting elements database at PLACE and PlantCARE as well as the visual inspection of the sequences by comparing with promoters of known stress responsive genes revealed several interesting features. Putative sites of significance with reference to stress response that were identified on the promoter region of the *Os-dfr* and the *Os-ans* genes and their relative positions from transcription start site are listed in table 4.2. The C1-MYB binding sequences that resemble the consensus site A(C/A)C(T/A)A(C/A)C present in most of the anthocyanin gene promoters studied (Sainz et al., 1997), were identified on both the *Os-dfr* and the *Os-ans* promoters (Fig 4.9). A MYB binding domain (TAACAAA) with a proximal pyrimidine rich domain resembling the Gibberellic Acid Responsive Complex (GARC) of rice and the barley a-amylase gene promoter (Gubler et aL 1995; Morita et aL 1998) was found in 5` upstream region of the *Os-dfr* gene (Fig. 4.10). Also present on the *Os-dfr* and *Os-ans* promoters is a domain sharing homology with the Arabidopsis *Atmyb2* binding region (CAGTTA on the *Os-dfr* and TGGTTAG on the *Os-ans)* that is present in several drought stress responsive genes (Urao et aL 1993) including drought, ABA and high salt responsive *rd22* gene of Arabidopsis (Abe et aL 1997; Busk and Pages; 1998). Several putative ABRE/G-Box like elements with a central ACGT core that is implicated in gene expression in response to a variety of environmental and physiological cues (Marcotte et aL 1989; Mundy et aL 1990; Shen et aL 1996) were also present in the promoter region of the *Os-ans* gene (Table 4.2). A Low Temperature Responsive Element

Figure 4.8 Nucleotide sequence of 5' upstream region of *Os-ans* gene. The putative transcription start site and the translation start sites are shaded.

TGTGTAGCATGAATTGGTATGTACTTATGCATTAATTCTGAAGTTCACTTGCAAGTTT
TGGCAAAAGTGCTTTTAGTAACATGTATGTACAAGACTGAAAGATGAAATATAAATGT
GGCTTGACTCTGAAGTTGCATGAATTTCAGTTTGCTTCACCCAGAGAGACCACTTAAC
GTTTTTTTACGAAATTTGAGTGACTTGATTTAGGGTAATCTATTTATCACAAATATTA
TGCCACATGGATTCCACGTGCATACTTACTAGCTGTGCCTGGCAACCTTTATTGAAAC
GCTTAGCCTGCACGTATTTATACAACAATATCTATTTAGCTTACCAATTTCGTGAAAA
CATATTGTTCTTTAGCAGTAAATTGCCATAGTTCATTCGTGGTCCATGTGCATGCAGT
CATGGTGTTGCCTTGGTTCTAAGTTTCTAACAGAGGGCCATGGAATGATCATGACCTT
TTGAAGGTACGATTAAATTTGTAAGTGTACCCTTACTTTTATGCAATAGCCCCAACAC
TTCAAAAAATGCTCTATGGAGGTTTACTTGTGACAGTTCTAGAGATGAATGTTCTTCG
GATGCTATTACGAAGGATTCTTTTGTAGCTCACACTCTGTTTAATTTATTTTTATAGC
AGCCACCTTTTGTGATGGTAGCAGGATGAAGAGCGGATTAGTACAGGTGAGAGATGAG
GATGACACAGTGGTGTTGGACAAGTCTATATCTCCGTATCTTGCTTGCGGTGGTCGAT
TGCATCAAGGGGAAACTTCATTGGAGGGTGTCAGCTGTCATGGATGCAAACGTGGTGA
CCCTTCTAATCCAACGGTTTGAGTGGCCACAATACTGGTTAATATTGATTTTGGTTCC
TAACCATCTGACGGCTGACGAGCCGGCAAGTACGAGCAATGTTTTTAGGTGTGCATCT
TTGTTACATGTTGCTATAATATTTCTGTGTGTTTAAGCACCATTGATCAGCGGCAAGA
AGGTGTGTATCTCGTTGCTCCTTGTCTAGATGTGCATATCTAATCTCTGGTAGTTTCA
ACAGTCTGTGTTTTGGGCAATGGGGAGTCCATCCTTTTTTGGGAGGACAACTGGTTGG
AGGGTTCCTCCATTCGCTACATATCTCCGGCGGTTTGGGCGTCTGTCCCAACACGGCT
TCGCTGTCGCAGAACGGTCGCCAAGGCCCTTCAAGACCGGAGATGGATTAGAGACTGC
ACCGGAGCGCTGGGTTTGCAAGCTATTCTTCAATATCTTCAACTCTGGAGTCTCCTGA
GGTCGTCGGTGCGGCTCTCTGACCACCCCGACTCTTTCATTTGGAAGTGGGAAGCATC
GGGAGTCTACTCTTAACGTTCGGCATACCCGTGCACTCTTTCTAGGTAGGGCTCCTTT
CCACTCCGAACCCATCTAGAAGACTCCCCCCCCCTCGAGATGCCGGTTCTTTGCCTGGC
TGGTCGCAATGAGGCGCTGTTGGACGGTGGACCGCCTGTGTTCTAGGGGTTTGCCTCA
CCCGGATGGATGTGTGCTCTGCGACCAACATGAAGAGACTATTGATCACATCTTGGTT
GCCTGTCCGGAGTCTCATCAGCTTTGGTGGGTCCTCCTTTCCAGCACTGGTTTGCCAT
AGTTTCTCCCCTTGAATGAAGATTCCTTTTATCTCTGGGTCTACAATTCCTGCCTTAA
AGTGGGGAGGGCTAGCAGGCGGGGATTTGATACAATAGCAACCCTTACTGCGTGGACA
ATCTGGAAGGAGAGGAACAATAGGGTCTTCAACTCTCAGCAAAGGCCCTGGTCAGAGA

TAGCCCGAGCTATGACGGAAGCGACTCTCTGGC:GGTTGGCACACGAGGTGCTGCCGGT
GCTAACCATTTAGGTCTTGTTCTAGGTCGTTTCCTTCTGATGTCGCGAGAATAGGCTT
AAGCTTTTTATTGTTCTCCTGTTTTCGCATCCCCCTCCTAATTTTCTTGTTTGGCTTC
GCTTGTACATACTCTTTATTTCTCTTAATACAAA7ATGCGTGCCTTGCGTATTCCCAA
ACAAAAAAAAAACTCTGGTAGTAATATATTTTTTTGTCATATCCCTTGTACATTCTAT
AAGTTTGTTGAATTGTATTTTAGGATGGCAATCTGTGGATGATAAATCAAACCCGTGA
TATGAAGCTTTTAGTTTTTCCAGACTTTTTAGTACTACTATTTGCTAGATGATTCTGA
GTAGATACAATTGCTGATGGTTTTGATTAATTTTTAGACAATGTGATAGTTTTGTACT
TTTTCTTAAATATATTTCATATATGACATGGATCAAATGGACAGAATTTGGGAAATTT
AATTTGATTAACATGAATTTAATAATTTATTAACACAAATTTTATAGTGCCGTAGCGT
TAGCACGGACAGATTACTAGTAGTGTAAAATTTTAAATCTTCTTGTAAAATTTAGAAG
CACGAGGACTAAAATGAGAAGTGCCCAAAAGGGTAGGTTTTTTTTAGAGGTGTTGCCT
CAGCGCCTACCTGTTATTTGAGGCCATTGGATAGGAGTGGGCCTATATTGTTCATGCC
CTCGTGCGCGTACACAATCATAGTTTCACATAGTAGAAATGGTGCTTAAGACTTTTAA
ACTTTCGTTCTTCACTGTCCAAATATATGCTAATTAAGGTTGGAAACTAGAAAACAAT
AGAAAAAATTCAACTACAAAATAAGTTCTACAATTTAAATTTCTAACTTGTAAAAGTC
AAATTCTTGTTATGCCTTATATAGGCTAACGAGCATGCAATGAGTTTATACATACGGA
TCGTATCATCTGAATTAAGAAACAACAGGGGCATTATTGACAGGGACCAATCAACCAA
TGTTACGAACGCGCACGTGATTGATGGATGGAGCTAACCGCGCGTGCCCGACACCGCT
GGTTGTTGTTAGCTACAAACGTAACACATGCATGCACCGATCCATGGATGGAGCAAAG
CGGCGGCAGCGCCGGAGTATAAATCTACCCGCGCTCTGCCTGCCTCGCCATCACCGGC
CGCCGATCGAGTACGTGCGCACGCAGGTCATTACTAGCCTACTTCGGGAGGGCGACA
TGGAC

Gene	Position	Sequence
Os-dfr	-314	ACCTACCTATC
Maize *A1*	-65	ACCTACCAACC
Os-ans(Rev)	-578	ACCTACCCTTT
Maize *A2*	-105	ACC_ACCAGTC
Maize *Bz2*	-100	ACCAACCCGCA
Consensus		A(C/A)C(T/A)A(C/A)C

Figure 4.9 The C1-MYB binding domains on the *Os-dfr* and *Os-ans* promoters

Comparison of putative Cl-MYB binding sites identified on the *Os-dfr* and *Os-ans* genes promoter with that of maize *A1* (Sainz et al.. 1997), *A2* (Lesnick and Chandler, 1998), and *Bz2* (Bodeau and Walbot, 1996). The sequence on the *Os-ans* is depicted in reverse orientation. The consensus sequence for the C1-MYB binding site is boxed.

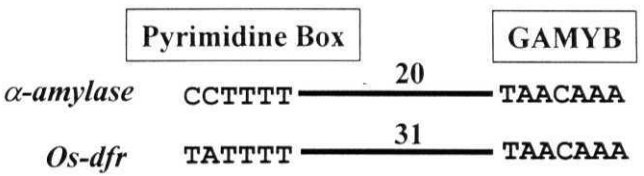


Figure 4.10 The GARC on the *Os-dfr* promoter

Comparison of putative Gibberellic Acid Responsive Complex (GARC) present on the *Os-dfr* promoter with that of the rice and barley *α-Amylase* gene promoter (Gubler et al., 1995, and Morita et. al., 1998). GARC consists of a pyrimidine rich box and a GAMYB binding domain. The number indicates distance in nucleotides between the pyrimidine box and GAMYB binding domain.

(LTRE) with a consensus sequence CCGACC flanked by ATGC like regions in the *Os-ans* promoter was similar to that of the multiple stress responsive Arabidopsis *cor 15a* and the cold responsive Brassica *BN115* genes promoter (Fig. 4.11) (Baker et al., 1994; Jiang et al., 1996). Another interesting feature found in the *Os-ans* promoter is the presence of an *sph* element (CACATGCATGCAC) (Fig. 4.12) that is implicated in ABA responsive gene expression (Hattori et. al.. 1992).

Table 4.2 Putative promoter domains and stress responsive motifs on 5' upstream **region** of **the** *Os-dfr* **and** *Os-ans* genes

Motif	Consensus	**Seq** in *os-ans*	Location	Seq in *os-dfr*	Location
ELRECORE	TTGACC			TTGACC	-290. -558
ATMYB2	TAACTG			TAACTG	-989
MYBCORE	CNGTTR	CTGTTG	-145, -214	CG/AGTTG/A	-989. -1229
MYBGAHV	TAACAAA	TAACAAA	-2094	TAACAAA	-1647
MYBPLANT	MACCWAMC	AACCTACC	-554	C/AACCTACC	-315.-1789
ZMPMYB	CCWACC	CCTACC	-522. -554	CCA/TACC	-313, -475
MYCATRD22	CACATG	CACATG	-97	CACATG	-572
ABRE	ACGTSSSC	ACGTGCGC	-169		
LTRECORE	CCGAC	CCGAC	-132		
MYBATRD22	CTAACCA	CTA A CCA	-1164		
TATABOX	TATATA	TATAAAT	-46	TATATA	-29

4.5 Analysis of interaction of the OsCl-MYB protein **with putative MYB binding domains on the** *Os-dfr* **and** *Os-ans* promoters

The objective of these experiments was to examine whether the OsCl-MYB protein binds to the different putative *myb* responsive domains identified in the *Os-dfr* and *Os-ans* promoters. A recombinant 6His-OsC1-MYB-fusion protein was used in these studies. The protein was over expressed in *E, coli* and the partially purified recombinant protein was tested for its ability to bind to the PCR amplicons of specific *myb* responsive elements using Electrophoretic gel Mobility Shift Assay (EMSA).

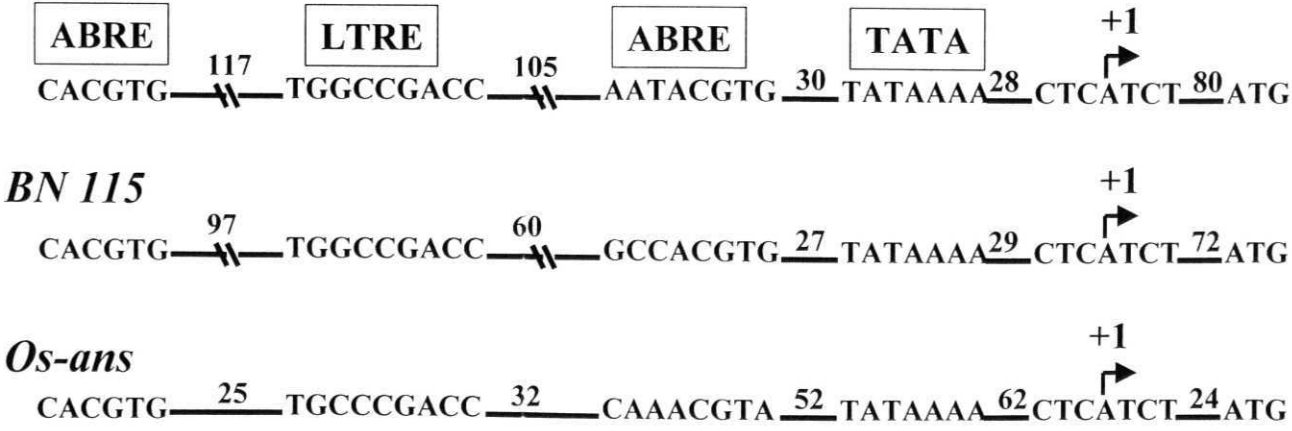


Figure 4.11 Comparison of the conserved regions in the promoter of the *Os-ans* with that of the multiple stress responsive genes, Arabidopsis *cor15a* (Baker et al., 1994) and Brassica *BN 115* (Jiang et al., 1996). The highly conserved ABRE like elements, an LTRE element, TATA box and the transcription (+1) and translation start (ATG) sites are shown. Numbers indicate the distance in nucleotides between the domains.

Gene	Position	Sequence
Os-Ans	-121	CACATGCATGCAC
Cl (Maize)	-145	TCCATGCATGCAC
	-258	TGCATGCATGCAC
RAB17 (Maize)	-111	TCCACTCATGCAT
	-105	CTCATGCATGCCC
RAB16A (Rice)	-79	TCCACCCATGCCG
Em (Wheat)	-341	TGCATGCATGCAA
DLEC2 (Phaseolus)	-114	ACCATGCATGCTG
Consensus		TCCATGCATGCAC

Figure 4.12 The *sph* element on the *Os-ans* promoter.

Comparison of putative *sph*-like element identified on the *Os-ans* genes promoter with that of maize *Cl* (Hattori et al., 1992; Kao et al., 1996), *RAB17* (Vilardell et al., 1990), rice *RAB J6A* (Mundy et al., 1990), Wheat *Em* (Vasil et al., 1995; Guiltinan et al., 1990), and *Phaseolus DLEC2* (Helmut et al., 1992) promoters. The consensus sequence for the *sph* site is boxed.

4.5.1 Construction of the bacterial expression vector

Bacterial expression vector for the *OsC1-Myb* was constructed under the control of T7 RNA polymerase promoter. The expression vector pRSET-B was chosen for ease of purification as it gives a fusion protein with N terminal hexa histidine tag. A PCR amplicon of the open reading frame of the *OsCl-Myb* cDNA was directionally cloned into *Bam* HI/ *Eco* RI sites of the pRSET-B in the correct reading frame. The ligated products were used for transforming the DH5α cells using ampicillin as a selectable marker. The resultant construct was verified for the expected fragment sizes by restriction analysis. The scheme of construction of bacterial expression vector is outlined in Fig. 4.13 and described in materials and methods section. The PCR amplification of *OsCl-Myb* ORF is shown in Fig. 4.14B. The physical map and the restriction analysis of the bacterial expression construct are shown in Fig. 4.14A and 4.14C, respectively. The *Bam* HI-*Eco* RI double digest releases the entire 819 bp fragment of the *OsC1-Myb* form the expression construct pOsC 1-myb-ORF (Fig. 4.14B lane 1). The confirmed construct was mobilized into *E. coli* strain BL21 (DE3) and used for recombinant protein induction and purification.

4.5.2 Overexpression and purification of the recombinant OsCl-MYB protein

The *E. coli* strain BL21 (DE3) cells carrying the recombinant plasmid pOsC1-myb-ORF were induced with IPTG for recombinant protein over expression. The total proteins extracted from the control and the induced cells were analyzed by SDS-PAGE and the electrophorogram is shown in Fig. 4.15A. The expression of the recombinant OsC1-MYB protein was confirmed by western analysis using a monoclonal antibody against the maize C1 protein. The western blot analysis showed the presence of a protein with an apparent molecular weight of 38 kDa corresponding to the recombinant OsCl-MYB protein, in the induced cells extract (Fig 4.15B, lane 2), which was absent in the un-induced cells extract (Fig 4.15B, lane 1). Since the expression level was poor, no bands indicating over expression of the recombinant OsCl-MYB protein was observed in the Coomasie-stained gel of induced cells extract (Fig. 4.15A, lane 2). However, the protein extracts from induced cells, partially purified using Nickel CL agarose affinity chromatography, showed a clear band of about 38 kDa on coomasie-stained gel (Fig. 4.15A, lane 3). The identity of this band was confirmed as the OsCl-MYB protein by western blotting (Fig. 4.15B, lane 3).

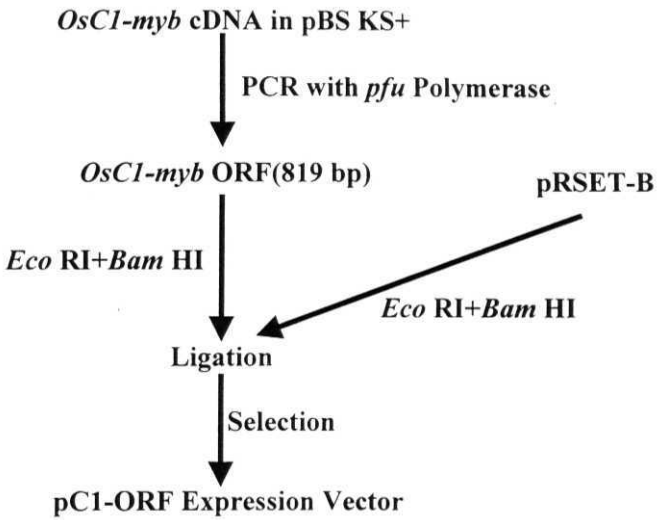


Figure 4.13 Construction of bacterial overexpression vector for isolation and characterization of the OsC1-MYB protein.

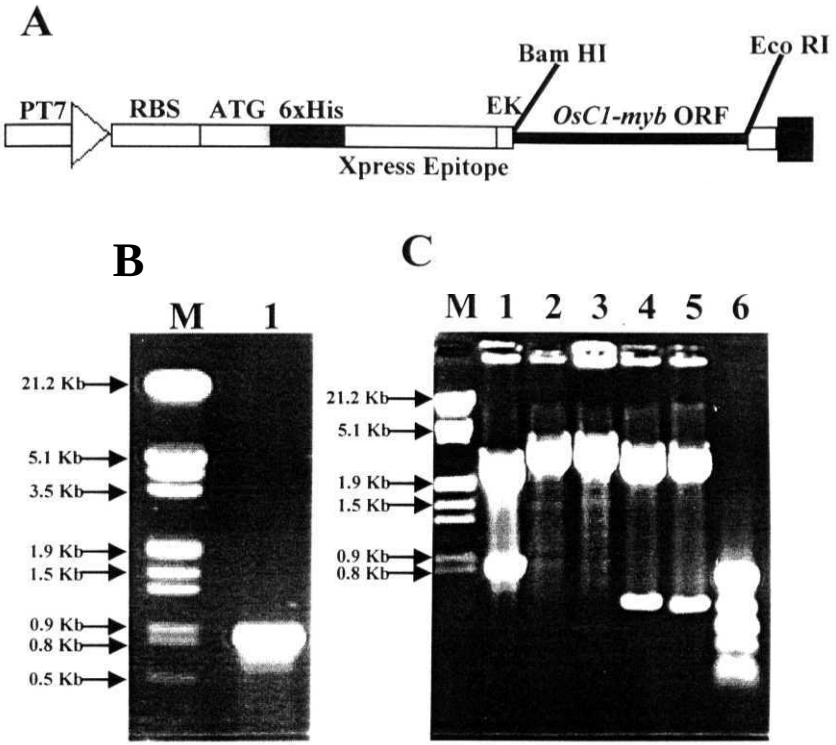


Figure 4.14 Physical map and restriction analysis of the pOSCl-Myb-ORF expression construct.

A) Physical map of the pOsC1-Myb-ORF, the bacterial expression construct of the *OsC1-Myb*. The 819 bp PCR amplicon of the *OsCI-Myb* cDNA was cloned into the *Bam* HI-*Eco* RI sites of the T7 promoter expression vector pRSET-B.

B) PCR amplification of the 819 bp ORF of the *OsC1-Myb* gene. *OsCI-Myb* cDNA was used as a template for PCR amplification using the *pfu* DNA polymerase. M- DNA molecular weight marker, λ *Eco* RI-*Hind* III double digest; 1- PCR amplicon of *OsC1-Myb* ORF.

C) Restriction analysis of the bacterial expression construct, pOsC1-Myb-ORF. M- DNA molecular weight marker. *X Eco* RI-*Hind* III double digest; \-*Bam* HI-*Eco* RI double digest; *2-Bam* HI; 3-*Eco* RI; 4-*Pst* I; 5- *Sac* I; 6- *Sau* 3A.

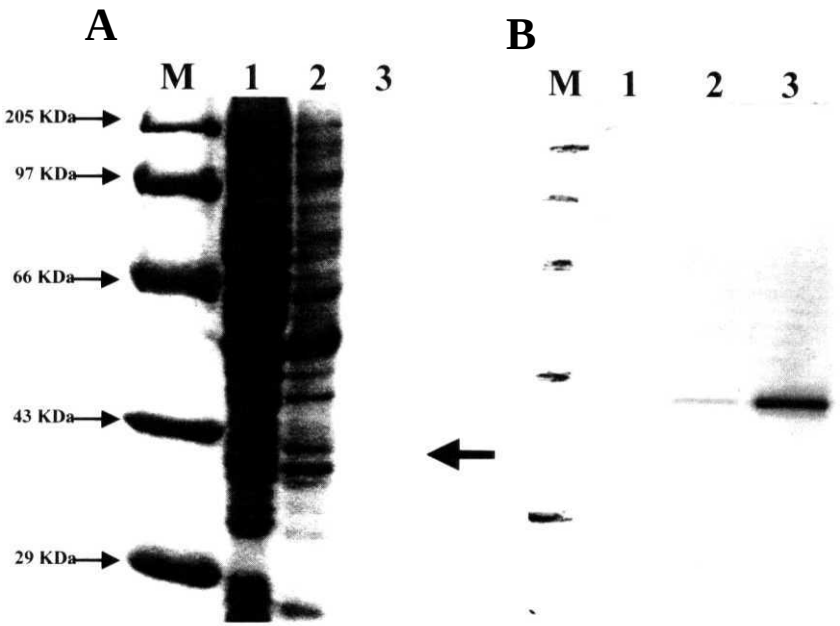


Figure 4.15 Expression and purification of the recombinant OsC1-MYB protein from *E. coli*.

A) SDS-PAGE showing protein extracted from *E. coli*. Arrow indicates the partially purified recombinant OsC1-MYB protein.

B) Western analysis of the recombinant OsC1-MYB protein. Monoclonal antibody raised against the maize C1 protein was used to detect the recombinant OsC1-MYB protein.

M-Protein molecular weight marker; 1- Crude extract from un-induced cells; 2-Crude extract from induced cells; 3-Partially purified protein.

4.5.3 PCR amplification of the putative MYB binding domains from the promoter regions of the *Os-dfr* and *Os-ans* genes

The regions carrying different *myb* responsive domains on the *Os-dfr* and *Os-ans* promoters were PCR amplified and used as templates in EMSA. Specific sets of primers were designed for 80 to 120 bp flanking regions of each putative *myb* responsive domains on the promoter regions and the domains were PCR amplified using Nagina 22 genomic DNA as a template. The size and purity of the PCR amplicons were checked on a 10% native polyacrylamide gel (Fig 4.16). The amplicons were end labeled using [γ-^{32}P]dATP and used in protein-DNA binding reactions.

4.5.4 Binding of the recombinant OsC1-MYB to the putative MYB binding domains on promoters

Electophoretic gel Mobility Shift Assay was used to study the interaction between the purified C1-MYB protein and the PCR amplified oligonucleotide probes carrying different putative MYB binding domains. The recombinant OsCl-MYB protein was incubated with the end labeled oligonucleotides carrying different *myb* responsive elements separately and the binding reaction mixture was separated on 5% native polyacrylamide gel. The result shows that the C1-MYB fusion protein bound to different oligonucleotide probes carrying the putative MYB binding domains, resulting in the formation of a lower mobility complex (Fig 4.17. lane 4, 6, 8, 10 and 12). However, the recombinant OsC1-MYB protein failed to bind to the putative ZmPMYB binding domain identified on the *Os-dfr* promoter (Fig 4.17. lane 2).

4.5.5 Relative binding affinities of the MYB binding domains *in vitro*, for the recombinant OsCl-MYB protein

To assess the relative affinity of each domain to which the OsCl-MYB protein binds, each oligonucleotide probe was competed with an excess of cold oligonucleotide carrying a different MYB responsive domain. The figure 4.18 shows that among all the oligonucleotides carrying different MYB binding domains tested, the oligonucleotide probe carrying putative GAMYB-binding domain showed the highest affinity to the recombinant OsCl-MYB protein that could be effectively competed out only by addition of other cold competitors in excess by nearly 100 times over the labeled probe (Lane 1 to 8). The recombinant OsCl-MYB protein bound to the putative AtMYB2-binding and C1MYB-binding domains present on both the *Os-dfr* and *Os-ans*

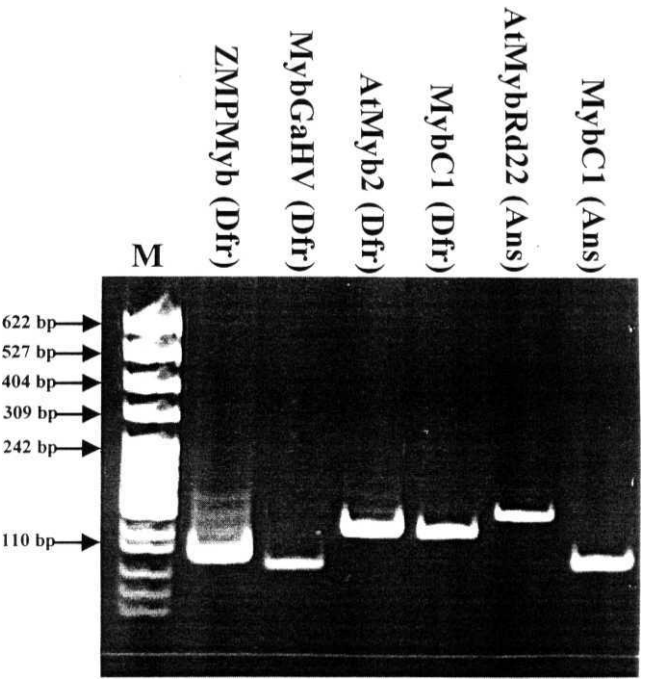


Figure 4.16 PCR amplification of putative MYB binding domains identified on the promoter regions of the *Os-dfr* and *Os-ans* genes.

Primers were designed to amplify 80-120 nucleotides flanking the putative MYB binding domains. M- DNA molecular weight marker, pBR322 DNA-*Msp*I digest.

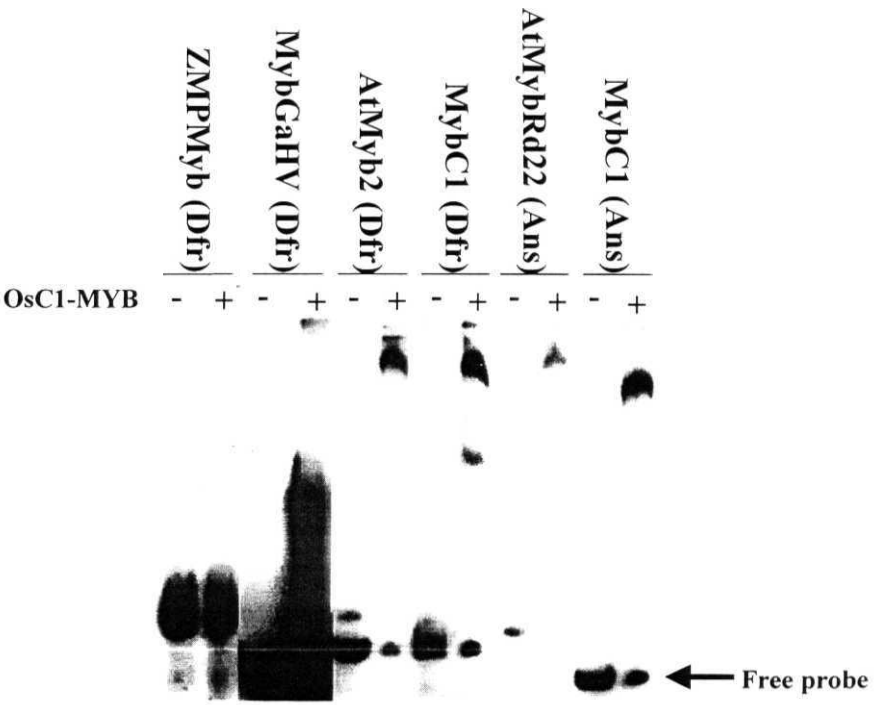


Figure 4.17 Interaction of the OsCl-MYB protein with putative MYB binding domains.

Electrophoresis Gel Mobility Shift Assay using the end labeled PCR amplicons carrying different putative MYB binding domains from the *Os-dfr* and *Os-ans* promoters and the purified recombinant OsCl-MYB protein.

+ and - indicate presence or absence of the recombinant OsC1-MYB protein in the binding reaction.

promoters with almost same affinity where just 10 times excess of cold probes could reduce the extent of binding significantly (Fig 4.18 lane 9 to 40).

4.6 Analysis of *in vivo* functions of the *OsC1-myb*

To understand the *in vivo* role of the *OsCI-Myb* gene product vis-a-vis regulation of stress responsive gene expression. we used a transgenic approach using Arabidopsis as a model system. The *OsCI-Myb* gene was over expressed in Arabidopsis and the effect of it on expression of both flavonoid genes and the known stress responsive genes in Arabidopsis were studied. The results are presented below.

4.6.1 Construction of the plant expression vector

Plant expression vector for the overexpression of the *OsCI-Myb* in Arabidopsis was constructed under the control of a super promoter. The scheme of vector construction is outlined in the Fig. 4.19. The source of the super promoter is the vector pE1945. The super promoter consists of a trimer of the *Agrobacterium* octopine synthase *(ocs)* activator affixed to the Agrobacterium mannopine synthase *(mas)* activator plus promoter. This promoter was chosen because this was shown to be approximately 70-fold stronger than that of the CaMV promoter. The promoter cassette carries *npt II,* encoding neomycin phospho transferase conferring resistance to antibiotic kanamycin, as both bacterial and plant selection marker.

The cDNA sequence of the *OsCI-Myb* excised from the cloning vector pBluescript KS+ was cloned down stream of the super promoter into *Sma* I site of pE1945. The ligated products were used for transforming the *E. coli* strain, DH5α using kanamycin as a selectable marker. The resultant construct was verified for the desired orientation and expected fragment sizes by restriction analysis. The physical map and the restriction analysis of the sense construct carrying the *OsCI-Myb* gene are shown in Fig 4.20A and 4.20B, respectively. The *Xba* 1 digestion (Fig 4.20B, lane 6) excises nearly 1 kb fragment confirming the sense orientation of the insert. The confirmed construct was mobilized into the Agrobacterium strain AGL-1 and used for Arabidopsis *in planta* transformation.

4.6.2 Development of Arabidopsis transgenics carrying the *OsCl-myb* gene

Arabidopsis ecotype Columbia was transformed using the *Agrobacterium lumefaciens* strain AGL-1 harboring the *OsC1-Myb* plant expression construct.

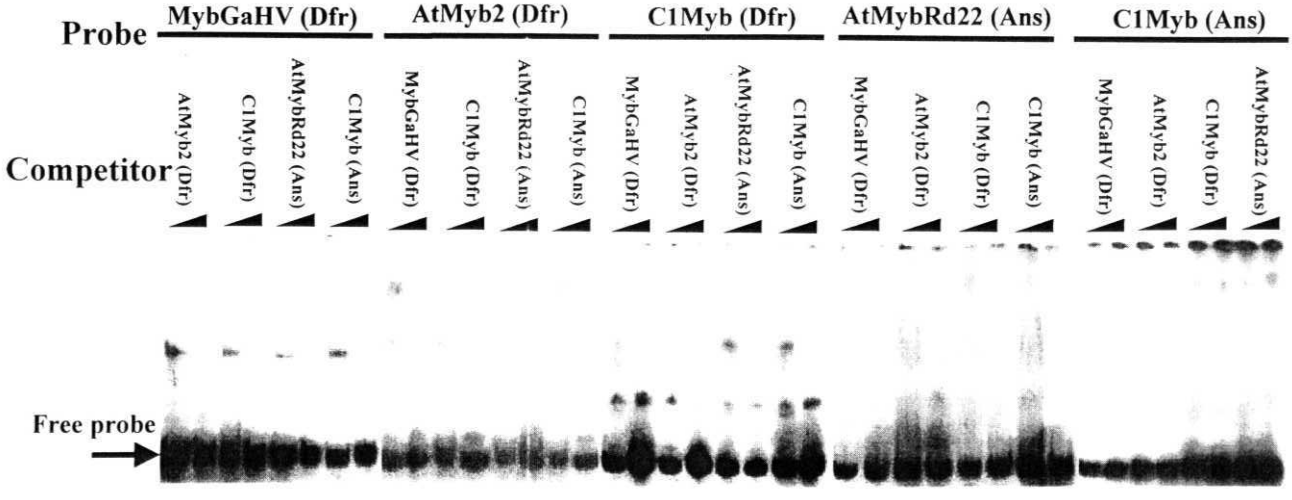


Figure 4.18 Analysis of relative affinities of the OsC1-MYB protein to different MYB binding domains identified on the *Os-dfr* and *Os-ans* promoters. Each end labeled domain was competed with 10 or 100 fold excess of competing unlabelled domain. Black triangle indicates the increasing concentration of the competing domain.

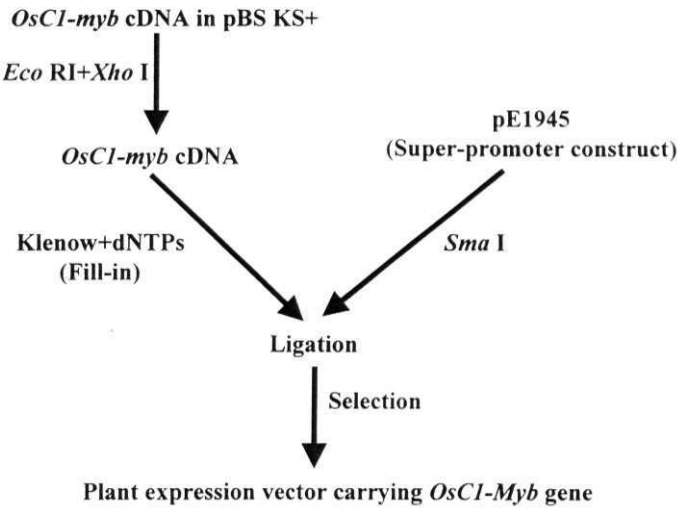
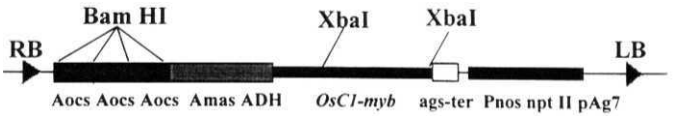


Figure 4.19 Construction of the plant expression vector carrying the *OsCl-Myb* cDNA for Arabidopsis transformation.

A



B

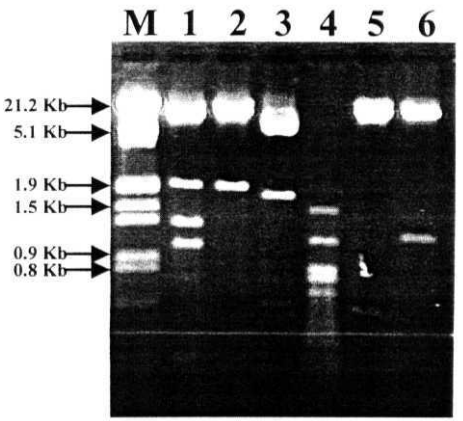


Figure 4.20 Physical map and the restriction analysis of the plant expression construct.

A) Physical map of the plant expression construct of the *OsC1-Myb*. The end filled *Eco* RI-*Xho* I fragment of the *OsCI-Myb* cDNA was cloned into the *Sma* 1 site of the plant super promoter expression vector pE1945.

B) Restriction analysis of the *OsC1-Myb* plant expression construct. M-DNA molecular weight marker, *X Eco* RI-*Hind* III double digest; 1-*Bam*HI; *2-Eco* RI; 3-*Pst* I; 4- *Sau* 3AI; 5-*Sal* I; 6-*Xba* I.

Agrobacterium mediated vacuum infiltration of floral buds was used for efficient transformation of Arabidopsis. The seeds from the transformed siliques were harvested and plated on a medium containing kanamycin for the selection of putative transgenics.

4.6.3 Effect of over expression of **the** *OsCl-Myb* on Arabidopsis phenorype

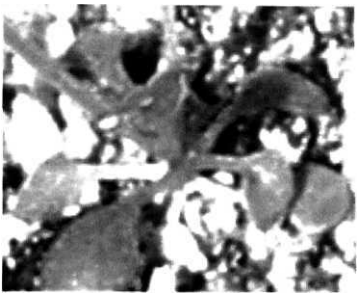
The effect of over expression of the *OsCl-Myb* on Arabidopsis was investigated. The kanamycin resistant plants (T1 generation) were transferred to the pots and grown under controlled conditions as explained in materials and methods. One of the putative transgenic plants accumulated an intensive purple pigment in the rosette (Fig. 4.21B). The colored plant was slender, short and weak with very small leaves compared to that of control non-transgenic plants. The pigmented plant did not survive beyond three weeks. However, some of the putative transgenics were normal in their phenotype, survived and produced seeds. These Tl plants were further analyzed for integration and expression of the transgene.

4.6.4 Effect of over expression of the ***OsC1-myb*** on expression of flavonoid genes and other stress responsive genes in Arabidopsis

A total of 9 kanamycin resistant Arabidopsis plants, which were normal and healthy, were obtained from transformation experiment. These Tl plants were designated as C1S-1 to C1S-9. These plants were subjected to PCR analysis for confirmation of transgene integration. Two sets of primers were used, one set specific for the *npt II* gene and another set specific for the *OsCl-Myb* sequence. Of the 9 plants tested for presence of the *npt II* sequence, only 5 turned out to be positive (C1S-2, C1S-4, C1S-5, C1S-7 and C1S-9) (Fig 4.22A). These five plants were tested for the integration of the *OsCl-Myb,* As shown in the Fig. 4.22A, only two plants, C1S-5 and C1S-9, showed the presence of the expected 819bp *OsCl-Myb* fragment. The total RNA made from these two plants were subjected to northern analysis to confirm the expression of the transgene. The results show that the *OsCl-Myb* gene is expressed only in one transgenic Arabidopsis plant, designated as the C1S-9 (Fig. 4.22B)

The transgenic plant, C1S-9 was further analyzed to understand the effect of over expression of the *OsC1-Myb* on expression of flavonoid biosynthetic genes and target stress responsive genes. Nearly 10 μg of total RNA was used for northern blotting. The blots were sequentially probed with cDNA probes of the two major flavonoid biosynthetic pathway genes from Arabidopsis, *At-chs* and *At-fls* as well as

A



B

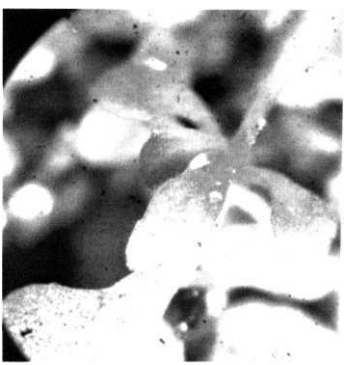


Figure 4.21 Effect of overexpression of the *OsCl-Myb* on Arabidopsis phenotype.
A) Control untransformed plant.
B) Transgenic Arabidopsis carrying the *OsCl-Myb* gene exhibiting dark purple color in the rosette.

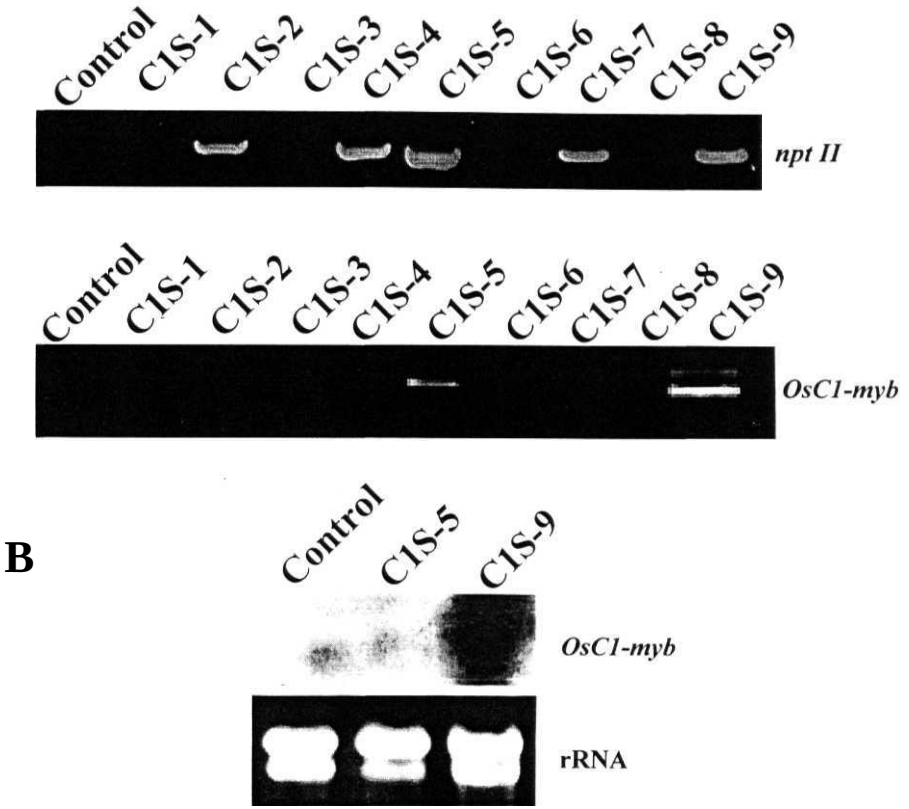


Figure 4.22 Analysis of putative Arabidopsis transgenic plants.

A) PCR analysis of putative Arabidopsis transgenic plants. Specific primers were used to amplify the *npt II,* the selection marker gene and the *OsC1-Myb*, the gene of interest.

B) Northern analysis of Arabidopsis transgenic plants carrying the *OsC1-Myb* sequence. 10 µg of total RNA was used and the *OsC1-Myb* cDNA was used as a probe. Ethidium bromide stained gel showing rRNA bands are shown to depict the equivalent loading of total RNA.

Control-Untransformed plant, C1S-5 to C1S-9-Independent putative Arabidopsis transgenic plants.

two stress responsive genes, *At-rd22* and *At-lea*. The results show an increased accumulation of transcripts for all the four genes in the transgenic plant expressing *OsC1-Myb*gene compared to the control plant (Fig. 4.23A and B).

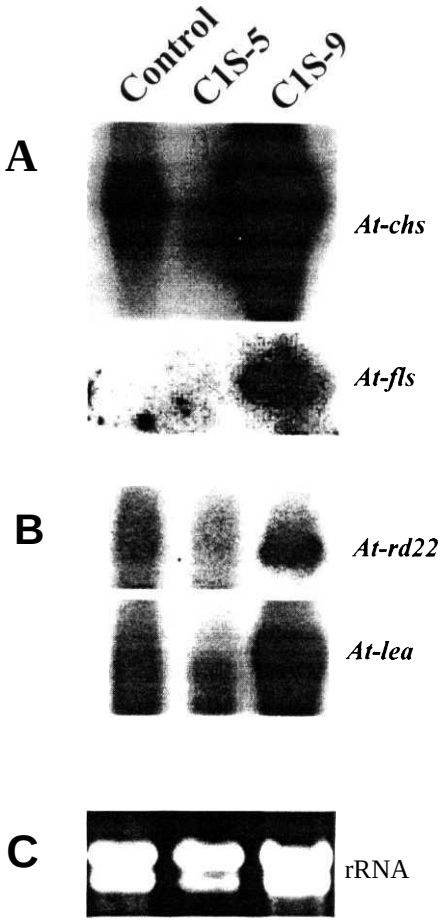


Figure 4.23 Effect of the overexpression of the *OsC1-Myb*gene on flavonoid pathway genes and stress responsive genes in Arabidopsis.

A) Arabidopsis flavonoid pathway genes, *At-chs* and *At-fls.*

B) Stress responsive genes of Arabidopsis, *At-rd22* and *At-lea.*

10 μg of total RNA was used and the cDNA clones of Arabidopsis flavonoid pathway genes, *At-chs* and *At-fls,* the cDNA clone of stress responsive gene, *At-rd22,* and the PCR amplicon of the stress responsive gene *At-lea* were used as probes.

C) Ethidium bromide stained gel showing rRNA bands are shown to depict the equivalent loading of total RNA.

5. Discussion

The present study demonstrated, for the first time in rice, that the key flavonoid biosynthetic pathway genes are responsive to drought stress and high salt. The promoter regions of these genes are shown to carry many classes of stress responsive domains. Studies on the role of transcription activator, the OsC1-MYB, in regulation of the abiotic stress responsive gene expression in rice led to several important observations. We have convincingly demonstrated the role of the *OsCl-Myb* product in mediating 'cross-talk' between the flavonoid biosynthetic pathway and the ABA dependent stress responsive pathway in rice. A detailed analysis of gene organization, expression, and regulation under stress, of both these pathways are discussed beloAv.

5.1 Analysis of the *OsCl-Myb* sequence

The amino acid sequence of the OsCl-MYB protein was deduced from the nucleotide sequence of the *OsCl-Myb* cDNA. Like other MYB related gene products reported in plants, the OsC1-MYB contains putative DNA binding domains consisting of two adjacent α-helix structures of 44 and 46 amino acids (Fig. 4.1) containing three and two conserved tryptophan residues, respectively. Comparison of this DNA binding domains with R2 and R3 repeats of human c-MYB protein revealed that the tryptophan residue at 147 of human c-MYB is replaced by isoleucine residue in the OsCl-MYB, another common feature observed in plant MYB class of proteins (Shinozaki et al., 1992). Also, we found an acidic region between amino acids 248 and 270 (Sequence DDWMDDVRALASFLDTDDAWNL) which might be a candidate for the transactivation domain, as was reported in the human c-MYB protein (from 286 to 308; Sequence- DEDPEKEKRIKELELLLMSTENE) (Kanai-Ishii et al., 1990). Interestingly, the OsC1-MYB shares significant homology in its DNA binding domains with many functionally diverse MYB like proteins of different plants including the stress responsive Arabidopsis AtMYB2 (Table 4.1; Fig. 4.2). The OsCl-MYB shares slightly better homology with AtMYB2 in R3 domain than in R2 domain. Earlier studies revealed a broader DNA binding specificity of the maize C1 protein as suggested by the diversity of sequences identified by binding site selection (Sainz et al., 1997). This, in conjunction with the observed significant similarity of the OsCl-MYB with other MYB class of proteins in this study, suggests that the OsCl-MYB protein, when present in higher titer in plant cell, presumably regulates many genes that carry a

MYB binding domain in their promoters. The OsC1-MYB is a well-known transcription activation factor for flavonoid and anthocyanin biosynthetic pathway genes. Anthocyanin accumulation could be used as an ideal phenotype for delineation of the role of the OsC1-MYB in the regulation of flavonoid pathway that would allow us to dissect the possible common mechanisms of stress responsive expression of genes belonging to different pathways.

5.2 Genotypic variation in anthocyanin accumulation and stress response

Flavonoids are ubiquitous plant secondary metabolites that are implicated in diverse metabolic functions, including their role in stress protection. A wide range of stress conditions, both biotic and abiotic, such as pathogen attack and UV radiation, wounding, chilling are known to activate expression of genes of the pathway leading to the accumulation of flavonoids in almost all tissues. (Dixon and Harrisson, 1990: Reddy et al., 1994; Christie et al., 1994). Our studies on the effect of water stress, salinity and exogenous ABA on anthocyanin accumulation clearly revealed a genotype dependant response. These genotypes were selected on the basis of their ability to accumulate anthocyanin pigmentation in different plant parts. The genetic variation in anthocyanin content among different rice lines has been described earlier (Reddy et al., 1995). Our studies show that the exposure of rice seedlings to water stress (20% PEG treatment), high salt (150 mM NaCl treatment), and 100 µM ABA lead to a rapid accumulation of anthocyanins in leaves (Fig. 4.3). However, the phenotypic manifestation of this accumulation depends on genetic constitution of the rice line since many genes and interactions between them are required for the visible expression of anthocyanins in target tissue. Genotypes homozygous recessive at any of these loci or heterozygous for the dominant color inhibitor will lead to either a modified colored or a colorless phenotype, respectively. Therefore, we have assayed both anthocyanin accumulation and transcript levels of the pathway genes since phenotypically indistinguishable tissue might show differences at transcription level under a given stress. Among treatments, water stress found to be significantly more effective in activating the flavonoid pathway, in comparison to NaCI and ABA treatments (Fig. 4.3). The data presented in the figure 4.3 clearly reveal the enhanced levels of anthocyanin pigments under stress in all the competent genotypes. The colorless lines, as expected, did not show any detectable accumulation under stress. The data allow us

to conclude that anthocyanin accumulation in competent rice genotypes is stress responsive.

Earlier genetic analysis showed that the rice genotype Nagina 22 is a homozygous dominant for leaf color inhibitor allele *I-lb* (Reddy et al., 1995) and hence does not accumulate any visible pigmentation in any vegetative plant part. Interestingly. Nagina 22 was found to accumulate anthocyanins upon stress treatments as indicated by the A530 values of acidified methanol extract (Fig 4.3 A and 4.3B). This is possibly due to stress-induced overexpression of the genes of the flavonoid pathway in comparison with that of the inhibitory genes. Evidence for the overexpression of the regulatory gene of the flavonoid biosynthetic pathway, *OsC1-Myb*, in response to the given stress treatments, is discussed in the following section.

5.3 Abiotic stress responsive expression of flavonoid biosynthetic pathway genes

The expression pattern of key genes of the phenylpropanoid and flavonoid pathway has been investigated in seedlings under stress and normal growth conditions. Northern analysis showed significant differences in expression pattern of the individual genes. There arc significant differences in the level of expression between the genes and the treatments (Fig 4.4, 4.5, 4.6). Interestingly, the transcripts of the *Os-pal* and the *Os-chs* did not show any detectable change in their accumulation under all the given stress regimes (Fig. 4.4). Infact, the transcripts of these two genes were found in abundance in untreated control plants. PAL is a basic cellular enzyme associated with a host of functions and biosynthesis of diverse phenylpropanoids and thus constitutively expressed in young seedlings (Hahlbrock and Scheel, 1989). It is also possible that with the technical limitation in the present assay, a subtle increase, if any, would have gone undetected. Similarly, the CHS is the first enzyme that commits the pathway for flavonoid biosynthesis and appears to be present in significant amounts in young seedlings under normal conditions. Basal constitutive accumulation of CHS is not surprising since flavonoids are known defense molecules in plants with high antioxidant activity. In contrast, the key genes of the anthocyanin pathway, *Os-dfr* and *Os-ans*, respond to various stress treatments by rapidly accumulating the transcripts in about 3 hours after treatment (Fig. 4.5). This is explained by the fact that the products of the *Os-dfr* and the *Os-ans*, dihydroflavonol reductase and anthocyanidin synthase, respectively catalyze steps towards the end of the flavonoid pathway and therefore unique to the production of anthocyanins. It is reasonable to argue that these enzymes

determine the kinetics of anthocyanin production in rice, although we do not yet have any evidence. Though the transcripts of these two genes accumulate in 3 hours after treatment and continue up to 12 hours, they revert to almost basal level by 24 hours after treatment, indicating an adaptive role in stress response process.

The pattern of transcript accumulation of the regulatory gene, *OsCl-myb* (Fig. 4.6), is by and large similar to that of the structural genes of the pathway, *Os-dfr* and *Os-ans* (Fig. 4.5). This agrees well with the earlier studies in maize, which conclusively showed that the expression of the *dfr* and *ans* orthologs is regulated by the *C1* locus (Sainz et al., 1997; Lesnick and Chandler 1998) and the *OsCl-myb* is homologous to the maize C1 locus (Reddy et al 1998).

Studies in Arabidopsis suggest the existence of two major classes of transcriptional control of ABA and drought responsive genes. One class is the bZIP-ABRE system, where the transcription factor bZIP regulates the expression of stress responsive genes carrying an ABRE element (Uno et al., 2000). Another system requires the action of MYB/MYC class of transcription factors in regulation of stress responsive gene expression (Abe et al., 1997). The MYB/MYC response system is somewhat slower, taking few hours reflecting the need for de novo synthesis of MYB and MYC proteins, compared to that of bZIP-ABRE system, which lead to gene expression within an hour after the stress treatment. It has been suggested that the MYB/MYC system regulates the slow adaptive responses to dehydration stress (Shinozaki and Yamaguchi-Shinozaki, 2000). In this background, our results, where stress responsive transcript accumulation of the flavonoid pathway genes, *Os-dfr* and *Os-ans* take place at about 3 hours post treatment and follow a pattern with the *OsC1-Myb*, perhaps shows that the transcriptional activation of structural genes of the flavonoid pathway genes under stress conditions is also mediated by the transcription factor, OsC1-MYB.

The observed differences in the pattern of message accumulation of the anthocyanin pathway structural genes, *Os-chs, Os-dfr* and *Os-ans* under stress are somewhat similar to the reported differences in gene expression patterns of the maize orthologs under light and cold treatments of seedlings (Taylor and Briggs, 1990 and Christie et al., 1994). The pattern of message accumulation for each gene studied was more or less qualitatively similar under three different stress regimes (PEG, NaCl and ABA treatments). However, there were differences in the levels of message

accumulation under stress. These results, taken together indicate the role of a common signaling and regulatory network of stress response pathways.

To compare the water stress and high salt-responsive expression of flavonoid genes with UV-B responsive expression pattern, we analyzed transcript levels of flavonoid genes in dark grown rice seedlings upon exposure to short term UV-B light. Results (Fig. 4.7) show that, except the *pal*, all other genes tested were activated by UV-B treatment. The transcript level for the *pal* remained same as in the control even after 12 hours of UV-B exposure. Earlier studies showed an increased activity of PAL enzyme, in a specific phase of response, upon UV-B treatment in rice (Reddy et al., 1994). Probably the PAL activity is regulated by posttranslational modifications or enzyme stabilization and not at the transcriptional level under UV-B treatment. It is also possible that transcripts for different alleles of the *pal* behave differently under various stress conditions.

The observed increase in transcript level of the flavonoid pathway genes upon UV-B treatment (Fig. 4.7) correlates with the earlier reported induction pattern of anthocyanin pigment accumulation in rice seedlings (Reddy et al., 1994). The UV-B inducibility of the *chs* gene has been amply demonstrated in maize and Arabidopsis (Taylor and Briggs 1990; Kubasek et al., 1992). The interaction of UV-B radiation with water stress in crop plants leading to changes in target enzyme activity levels has been reported earlier (Teramura et al., 1983; Balakumar et al., 1993). Such interactions may naturally involve common mechanisms, such as production of free radicals leading to the activation of antioxidative pathways. Earlier experiments showed an increased accumulation of anthocyanins under blast infection in rice that was qualitatively similar to the anthocyanins accumulated under UV-B treatment (Padmavati 1999). This confirms the multiple stress responsive nature of the flavonoid biosynthetic pathway genes in rice.

The observed accumulation of transcripts of the flavonoid pathway genes under stress goes well with the earlier reports that flavonoids and anthocyanins form the first line of defense molecules under UV-B stress and pathogen attack (Hahlbrock and Scheel, 1989; Kootstra, 1994; Chalker-Scott, 1999). Flavonoids and anthocyanins are implicated in protection function of the photosynthetic machinery under high light, protecting storage reserve from photo-oxidative damage during senescence and as osmotic adjusters in plants under osmotic stress (Chalker-Scott, 1999).

5.4 Analysis of the promoter regions of **abiotic stress responsive flavonoid genes of rice**

Molecular basis of stress-induced expression of target genes in rice under stress is investigated and the results highlight the role of promoter sequences. Analysis of specific promoter domains of the *Os-dfr* and the *Os-ans* genes revealed the presence of several putative motifs sharing homology with the known MYB binding domains in plants (Table 4.2). The comparable pattern of the timing of induction of all the three genes, *Os-dfr, Os-ans* and *OsC1-myb*, in conjunction with the presence of the consensus sequences in their promoter for the maize C1 binding site (ACCTACCTATC on the *Os-dfr* and ACCTACCCTTT on the *Os-ans)* (Fig. 4.9) and the binding domains for Arabidopsis AtMYB2 protein (TAACTG on the *Os-dfr* and CTAACCA on the *Os-ans)* (Table. 4.2) indicates role of the MYB class of proteins in triggering the flavonoid gene expression in response to different stresses. In addition, DNA-protein binding studies by electrophoretic mobility shift assays with the recombinant OsC1-MYB protein and the PCR amplicons carrying the putative MYB binding sequences of the *Os-dfr* and the *Os-ans* showed that the OsC1-MYB protein binds to these domains with almost equal affinity (Fig. 4.17 and Fig. 4.18). This interaction pattern is in agreement with the one reported earlier showing two of the Arabidopsis drought stress responsive genes, *rd22* and *rd29B*, that show a pattern of gene expression similar to that of a *myb* family gene, *Atmyb2* (Abe et al., 1997). All the three genes in Arabidopsis are also induced by dehydration and salt stress as well as by exogenous ABA treatment (Yamaguchi-Shinozaki and Shinozaki, 1993). The 5" flanking region of *rd22* and *rd29B* contains MYB recognition sequences (TGGTTAG) to which AtMYB2 protein binds (Abe et al., 1997).

The *Os-dfr*, *Os-ans* and *OsC1-Myb* genes are also induced by the application of exogenous ABA (Fig. 4.5 and 4.6). Water stress and salinity also lead to an increase in the intracellular levels of ABA, which inturn leads to the induction of various ABA-responsive genes. Therefore, many genes that respond to dehydration stress and salinity are also induced by exogenous ABA. A highly conserved sequence, namely PyACGTGG, has been found in the 5` upstream region of many ABA responsive genes, and it is thought to function in the ABA-responsive transcription of the wheat *Em*, rice *rab16* and barley *HVA1* genes (Marcotte et al., 1989; Mundy et al., 1990; Shen et al., 1996). Such sequences, also known as ABRE or G-Box elements, are implicated

in a variety of other signaling pathways in plants, including plants' response to red light (Donald and Cashmore, 1990), UV light (Weisshaar et al., 1991), anaerobiosis (McKendree and Ferl, 1992). Similar conserved sequences were also found in the 5' upstream region of *Os-ans* (ACGTGCGC) (Table 4.2). Such G-Box elements in conjunction with an LTRE (CCGAC) is also known to form critical domains in cold induction of Brassica *BIN 115* (Jiang et al., 1996) and cold-, drought- and ABA-regulated Arabidopsis *cor 15a* (Baker et al.. 1994) genes. Such LTRE element identified in the low temperature responsive *LT178* gene promoter (Nordin et al., 1993) was subsequently shown, by deletion analysis. conferring responsiveness to cold, drought. and high salinity, but not to ABA (Yamaguchi-Shinozaki and Shinozaki, 1994). An LTRE domain flanked by G-Box like elements was found in the 5' upstream region of *Os-ans* gene (Fig. 4.11). The conserved ACGT domains present in the *Os-dfr* and *Os-ans* genes may also play a critical role in mediating the multiple stress responsive flavonoid and anthocyanin accumulation in rice.

In maize, the *C1* gene is induced by ABA, and it is regulated by the *Viviparous-1 (Vp1)* allele, which encodes a transcription activator protein (McCarty et al., 1991). A *cis*-acting element involved in ABA-responsive, *Vpl* -regulated gene expression of *Cl* was identified as GGTCGTGTCGTCCATGCATGCAC (The underlined element called *SphI* element) by cotransfection assay with maize protoplasts (Hattori et. al.. 1992). We found one such sequence similar to the *SphI* element in the 5* upstream region of *Os-ans* gene (CACATGCATGCAC) (Fig. 4.12). Such *sph* elements are also present in promoters of several ABA and dehydration-stress responsive genes including *Em* of wheat (Guiltinan et al., 1990; Vasil et al., 1995), *RAB17* of maize (Vilardell et al., 1990), *RAB16A* of rice (Mundy et al.. 1990), and *DLEC2* of *Phaseolus* (Helmut et al., 1992). The *sph* element has been shown to be sufficient and necessary for the VP1 and ABA mediated activation of these genes.

Earlier studies have shown that the *dfr* gene in Petunia is induced by treatment with exogenous gibberellic acid (Weiss et al., 1995). The rice *Os-dfr* also has a Gibberellic Acid Responsive Complex (GARC) though much upstream, sharing a strong sequence homology to the GARC of rice and barley *α-amylase* gene promoters (Fig. 4.10). Earlier, the maize *Cl* expression was shown to repress GA activation of GUS expression by the high-pi a-amylase promoter of barley while the GAMyb. another member of the *myb* family of transcription activators, is shown to transactivate

the *α-amylase* gene (Gubler et al., 1995). Our gel mobility shift studies revealed a strong interaction between putative GARC on the *Os-dfr* promoter and the recombinant rice C1-MYB protein (Fig 4.17 and 4.18). Probably the C1-MYB competes with GAMyb for the binding at GARC. The mutually antagonistic effects of GA and ABA at the level of gene expression may involve competitive binding of the MYB class of transcription activators. The ability of both the hormones to activate the *dfr* gene may be attributed to the complex organization of the promoter with different classes of MYB binding domains.

EMSA clearly showed that the recombinant OsC1-MYB protein did not bind to one of the putative MYB binding domains on the *Os-dfr* promoter, ZmPMYB, the core of the consensus maize P (Pericarp color) binding site (Fig 4.17, lane 1 and 2). This confirms the specificity of the OsC1-MYB protein binding to responsive domains tested in this study. The maize *P* is a paralogue of the maize *C1*, which have most likely arisen by gene duplication. The maize *P* controls phlobaphene biosynthesis in pericarp tissues (Grotewold et al., 1991). Phlobaphenes are derived from the flavonoid pathway that also gives rise to anthocyanins, and the maize *P* is known to activate some, but not all of the target genes of the maize *C1*, although it is thought to bind to the sites in the promoters of the structural genes with differing affinity to the maize *C1* (Grotewold et al., 1994). The maize *P* is quite closely related structurally to the maize *C1*, suggesting that structurally related proteins perform related, although not identical, functions (Jin and Martin, 1999). Such an allelic divergence in regulatory genes with varied DNA binding specificity is of significance in spatial, temporal and stress responsive regulation of different pathways in plants.

5.5 *in vivo* functions of the *OsC1-Myb* gene product

To further dissect the role of the rice C1-MYB on stress responsive gene expression and to asses the spectrum of genes that are regulated by it *in vivo*, we took a transgenic approach using Arabidopsis as a model system. Earlier reports revealed that the maize regulatory loci *Lc* and *C1* activate the expression of flavonoid pathway genes and accumulation of anthocyanins in Arabidopsis (Lloyd et al., 1992). We transformed Arabidopsis with the Agrobacterium strain, AGL-1 harboring a plant expression vector carrying the *OsC1-Myb* cDNA driven by a super promoter as explained in materials and methods. Of the 10 kanamycin resistant putative transgenic plants, nine did not manifest the purple/red coloration of the seedling. The remaining one transgenic plant

exhibited intensely purple colored rosette (Fig. 4. 21 B) indicating the transactivation of the entire flavonoid pathway genes by the OsC 1 -MYB.

PCR analysis of the 9 kanamycin resistant putative transgenic plants, namely, C1S-1 to C1S-9, revealed presence *of np t II* (neomycin phospho transferase) in 5 plants (C1S-2, C1S-4, C1S-5, C1S-7 and C1S-9) (Fig. 4.22 A). The Arabidopsis plants, C1S-1, C1S-3, C1S-6, and C1S-8, that did not show presence of *npt II,* as revealed by the PCR analysis appears to be escapes from the kanamycin selection. Further analysis of the 5 plants, which are positive for *npt II* gene, by PCR using the specific primers for *OsC1-Myb* cDNA showed that only two plants, C1S-5 and C1S-9, carried the PCR amplifiable transgene (Fig. 4.22 B). The absence of the amplification in the remaining 3 plants, C1S-2, C1S-4, and C1S-7, may be attributed to rearrangements or fragmentation of input DNA. Presence of multi-copy and rearranged sequences is a common phenomenon among the transgenic plants. Such multicopy sequences are found to be a favorite target for methylation and gene silencing in several dicots (Matzke and Matzke., 1995; Park et al., 1996). Transcriptional silencing of the *bar* coding region driven by the *Ubi* promoter due to extensive methylation leading to herbicide sensitive plants has been reported earlier (Cornejo et al., 1993; Kumpatla et al., 1997). In addition they showed that plants having complex integration patterns displayed aberrant segregation indicating homology mediated gene silencing (Kumpatla et al., 1997; Kumpatla and Hall, 1998).

The transgenic plants, C1S-5 and C1S-9, which carried the *OsC1-Myb* sequence, as revealed by PCR analysis were further analyzed for the expression of transgene by Northern analysis. Of the tvvo transgenic plants, only one, C1S-9, showed the presence of transcript for the *OsCl-Myb* gene (Fig 4.22 B). The absence of the expression of transgene in the transgenic plant C1S-5 may again be attributed to gene silencing due to methylation and rearrangements as discussed above. The absence of color in the transgenic plant C1S-9 could be attributed to copy number or gene dosage. For instance, earlier studies showed the correlation between the copy number of the transgenes and the coloration of the Petunia RL101 transformed with *Al* gene, wherein single copy integration correlates with the red phenotype while multiple copy integration was observed mainly in the variegated and white plants (Linn et al., 1990). In addition, the lack of phenotypic expression may be due to the co-suppression, where

overexpression of the product of a gene up to a threshold level trigger for degradation, presumably with the target being the RNA (Elmayard and Vauchert, 1996).

Earlier studies clearly established the requirement of both *mvb* and *myc* family of genes for the coordinate induction of structural genes encoding enzymes involved in anthocyanin biosynthesis in maize (Dooner, 1983; Neuffer et al., 1997). In another study, tobacco and Arabidopsis transgenic plants carrying the maize *Cl* driven by *CaMV* promoter (Lloyd et al., 1992) were phenotypically wild type. However, transgenic tobacco and Arabidopsis plants carrying maize *Lc*, paralogue of maize *R* showed enhanced levels of anthocyanin accumulation in tissues that normally accumulate anthocyanins. The F1 plants of Arabidopsis developed by crossing the original transformants carrying *Cl* and *Lc* separately, showed an increased anthocyanin accumulation in tissues that normally accumulate it, and in addition, in three novel tissue locations (Lloyd et al., 1992). While these studies show an absolute requirement of the product of *R* family genes in anthocyanin pigmentation in tobacco and Arabidopsis plants, our studies demonstrate that overexpression of the *OsC1-Myb* alone is sufficient for color production in Arabidopsis. This may be attributed to a hyper-expression of the *OsC1-Myb*, as we have used a much stronger promoter than commonly used *CaMV* promoter to drive the transgene expression. It is also possible that the transcription activation mechanisms of the *OsCl-Myb* in rice is different from that of the maize *Cl*. Another possibility is the presence of products of the *R* family gene in sufficient tiler in Arabidopsis genotype used, which in interaction with the transgene product was able to activate the entire pathway. However, a clear understanding of these aspects needs generation of a large number of transgenic plants and extensive analysis.

The intensely pigmented Arabidopsis transgenic plant was slender, short and weak with very small leaves, compared to the control non-transgenic plants. The overall growth of this plant was impaired and eventually died at the end of the third week. 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, sugarcane and rice transgenics (Lloyd et al.. 1994; Goldsbrough et al., 1996; Bower et al.. 1996; Madhuri. 1998) suggesting deleterious effects of intracellular flavonoid or anthocyanin overload resulting from the overexpression of the regulatory transgenes. Such severe growth retardation was also

observed in the Arabidopsis transgenics upon constitutive overexpression of stress **responsive** transcription factor DREB1A (Kasuga et al., 1999). It is also possible that **the** Arabidopsis plants have low threshold levels of pigmentation.

Further, we analyzed the status of transcripts of the two flavonoid pathway genes and some other well-known stress responsive genes in transgenic Arabidopsis **plant.** C1S-9, overexpressing the *OsC1-Myb* gene. Northern analysis showed an increased accumulation of transcripts of the flavonoid genes, *chs* (chalcone synthase) and *fls* (flavonol synthase) (Fig. 4.23 A), as well as the two Arabidopsis stress-responsive genes, *rd22* (responsive to desiccation) and *lea* (Late Embryonic Abundant protein) (Fig. 4.23 B). This clearly shows the ability of the C1-MYB in mediating the cross talk between diverse stress responsive pathways in rice.

Plants are often exposed to different environmental stresses concurrently, and thus adopt common mechanisms for stress adaptation and tolerance against diverse stress conditions. This perhaps involves instances where two signaling pathways from different stresses converge and then lead to common response events. Such instances are often referred to as "cross-talk" (Knight and Knight, 2001). This phenomenon of cross talk allows plants to acclimate to diverse kind of stress factors after exposure to any one kind of stress. The mechanism of cross talk generally depends on specific and multiple protein-protein interactions (Pastori and Foyer. 2002). Our studies suggests a role for MYB classes of transcription factors that appears to serve as common regulatory switch between stress responsive pathways, regulating the expression of a wide array of stress responsive genes across the pathways. It also appears that such a mechanism is highly conserved across plant species.

Finally, our present study allows a comparative evaluation of mechanisms of stress responsive gene expression in higher plants. It allowed us to decipher the role of regulatory elements in promoter sequences that share a high sequence homology across stress responsive genes and gene families, Further, these studies demonstrate that the *OsC1-myb* is a common transcription factor regulating the expression of anthocyanin pathway genes and also the ABA mediated stress responsive pathway. The rice *C1-myb* regulation of different pathways in the dicot Arabidopsis plants confirms the conservation of genetic elements of stress response mechanisms across angiosperms. Further, these studies indicate that the anthocyanin biosynthetic pathway is an ideal pathway to study mechanisms of multiple stress response at genetic and molecular level

in diverse plants. Ideally, a large number of mutant forms of regulatory element **sequences** as well as transcription factors are needed to elucidate the molecular mechanisms and genetic basis of plants' responses to various environmental stresses.

In summary, the results of this study highlight the cross talk between different pathways under water stress and high salt. The rice *C1-myb* gene is shown to mediate such a cross talk between the stress responsive anthocyanin pathway and the ABA dependant transcription activation of other genes such as *rd22* and *lea*. The interaction between the OsCl-MYB protein and the target domains on promoter region of studied genes of flavonoid pathway has been conclusively demonstrated for the first time in rice. We presented evidence for the possible involvement of more than one transcription factor in regulation of gene expression associated with stress response and flavonoid biosynthesis in rice. We show here that the *myb* class of transcription factors is conserved across plants suggesting their central role in varied stress response processes in plants. Further, the presence of other putative stress responsive domains in promoter region of these genes associated with seemingly unrelated pathways in plants suggest a combinatorial regulation of stress responsive gene expression.

6. Summary

The following is the summary of results from the present investigation

- The *OsC1-Myb* gene from rice encodes a transcription factor belonging to the MYB family of proteins that shares homology with the *Atmyb2*, a multiple stress responsive transcription factor from Arabidopsis.
- Flavonoid biosynthetic pathway in rice is responsive to water stress, salinity, and exogenous ABA treatments.
- Stress responsive anthocyanin accumulation in rice is genotype dependent. However, stress responsive accumulation of transcripts for various genes of the flavonoid and anthocyanin pathway appears to be universal.
- Transcripts of the rice phenylpropanoid pathway gene, *pal* and the flavonoid pathway gene, *chs* do not respond significantly to water stress, salinity, and exogenous application of ABA.
- Transcripts of the structural genes of the rice anthocyanin biosynthetic pathway. *Os-dfr, Os-ans,* and the regulatory gene. *OsCJ-myb,* accumulate in response to water stress, salinity and exogenous application of ABA.
- Analysis of transcript accumulation of the phenylpropanoid and flavonoid pathway genes upon UV-B treatment reveals that the rice*pal* does not respond to the treatment, while the transcripts of other genes, *Os-chs*, *Os-dfr*, *Os-ans*, and *OsCJ-Myb* accumulate in response to UV-B.
- The pattern of the stress responsive expression of the anthocyanin pathway genes, *Os-dfr*, *Os-ans*, and *OsC1-Myb*, indicates involvement of a common mechanism in activation of gene expression under all the treatment regimes. Further, the activation of the transcripts of the structural genes of the pathway. *Os-dfr* and *Os-ans,* appears to be regulated by the regulatory gene, *OsCJ-Myb.*
- Analysis of the promoter region of the stress responsive anthocyanin pathway. *Os-dfr* and *Os-ans* revealed the presence of the C1-MYB binding domain that is conserved in promoters of many flavonoid pathway genes studied from different species. The Electrophoresis Mobility Shift Assay (EMSA) confirmed binding

of the recombinant OsC1-MYB protein to the C1-myb binding domains **identified** on the *Os-dfr* and *Os-ans* genes promoter.

- Apart from the C1-MYB binding domain, three other putative MYB binding domains similar to the binding sites of ZmPMYB, MybGaHV, and AtMYB2 were identified on the *Os-dfr* and *Os-ans* gene promoters. The EMS A confirmed the binding of the recombinant OsCl-MYB protein to MybGAHV and AtMYB2 binding sites, but not to that of ZmPMYB.

- The analysis of relative binding affinity of the recombinant OsCl-MYB to different MYB binding domains identified on the *Os-dfr* and *Os-ans* promoters indicated that the recombinant OsCl-MYB binds with a higher affinity to the MybGAHV binding domain on the *Os-dfr* gene promoter compared to that *of* other MYB binding domains identified. All other MYB binding domains identified showed almost comparable relative binding affinity for the OsCl-MYB protein.

- Promoter regions of the stress responsive anthocyanin pathway genes, *Os-dfr* and *Os-ans*, also carry several domains that are common among known stress responsive genes. They include, putative ABRE (ABA Responsive Element) like elements, LTRE (Low Temperature Responsive Element), and *sph* like element.

- Overexpression of the *OsC1-myb* in Arabidopsis lead to transactivation of the flavonoid pathway leading to anthocyanin accumulation resulting in colored phenotype. However, such hyper-accumulation of anthocyanins leads to seedling lethality in Arabidopsis.

- The overexpression of *OsC1-Myb* in Arabidopsis also resulted in transactivation of the Arabidopsis flavonoid biosynthetic pathway genes, *At-chs* and *At-fls* as well as stress responsive genes *At-rd22* and *At-lea*.

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