Developing Transgenic Rice Plants Over-expressing Ascorbate-Glutathione Pathway to Overcome Oxidative Stress-disrupted Sexual Plant Reproductive Process

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Certificate

This is to certify that the research work embodied in this thesis entitled **"Developing Transgenic Rice Plants Over-expressing Ascorbate-Glutathione Pathway to Overcome Oxidative Stress-disrupted Sexual Plant Reproductive Process"** has been carried out in the Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India. This work is original and no part of this thesis has been submitted for the award of any other degree or diploma to any other university.

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Declaration

I hereby declare that the research work embodied in this thesis entitled **"Developing Transgenic Rice Plants Over-expressing Ascorbate-Glutathione Pathway to Overcome Oxidative Stress-disrupted Sexual Plant Reproductive Process"** has been carried out by me under the supervision of Dr. M. K. Reddy in Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi.

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To my parents

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Prachi

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
°C	Degree Celsius
E	Extinction coefficient
µ	Micron (micro meter)
µL	Miero liter
µM	Micro molar
β-ME	Beta-mercaptoethanol
2,4-D	2,4 –di-chlorophenoxy acetic acid
A	Absorbance
APX	Ascorbate peroxidase
AsA	Ascorbate
BAP	Benzylaminopurine
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
BLAST	Basic local alignment search tool
$CaCl_2$	Calcium chloride
cDNA	Complemetary DNA
Cm	Centimeter
CTAB	Cetyltrimethylammonium bromide
DEPC	Diethyl pyrocarbonate
DHAR	Dehydro Ascorbate Reductase
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleoside triphosphates
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol

DTNB	5,5'-dithiobis -2-nitrobenzoic acid
EDTA	Ethylene diamine tetra acetic acid
EST	Expressed sequence tag
EV-1	Entry vector 1
EV-2	Entry vector 2
FAD	Flavin adenine dinucleotide
FW	Fresh weight
G	Gram
GR	Glutathione reductase
GSH	Reduced Glutathione
GSP	Gene specific primer
GSSG	Oxidised Glutathione
GW	Genome walker
h	Hour
H_2O_2	Hydrogen peroxide
hpt	Hygromycin resistant phosphotransferase
IPTG	Isopropyl β-D-thio-galactopyranoside
kb	Kilo base pairs
kDa	Kilo Dalton
L	Litre
LB	Luria-Bertani
LF-PCR	Locus –Finding PCR
m	Meter
M	Molar
mA	Milliampare
MCS	Multiple cloning site
MDHAR	Monodehydro ascorbate reductase
mg	Miligram
min	Minutes

mM	Milli molar
mRNA	Messenger RNA
ms	Milli ssecond
MV	Methyl viologen
MW	Molecular weight
N	Normality
NAA	1-naphthaleneacetic
NADH	Nicotinamide adenine dinucleotide hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBT	Nitroblne tetrazolium
NLS	Nuclear localization signal
Ni-NTA	Nickel nitrilotriacetic acid
nM	Nano mole
nm	Nano meter
$^1O_2$	Singlet oxygen
$O_2^{\bullet -}$	Superoxide radical
$^\bullet OH$	Hydroxyl radical
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylenc glycol
Pg	*Pennisetum glaucum*
PMSF	Phenylmethane sulphonyl fluoride
PNDR	Pyridine Nucleotide-Disulphide Reductase
PVP	Polyvinyl-polypyrrolidone
RACE	Rapid amplification of cDNA ends

RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
ssDNA	Single stranded DNA
TE	Tris-EDTA
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TEMED	N,N,N,N-Tetramethylethylenediamine
UV	Ultraviolet
V	Volt
v/v	Volume by volume
wt	Wild type
w/v	Weight by volume

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INTRODUCTION

INTRODUCTION

Global warming and its associated climatic changes have emerged as a worldwide concern as it affects many aspects of crop productivity. The effects of the expected climate change mainly include an increase in average atmospheric temperature and an alteration of rainfall regimes that result in drought and/or floods. The climate change along with the abiotic stress factors like drought, salinity etc. is known to influence physiology, growth, development, yield, and quality of crops. Both short-and long-term stresses can drastically affect growth and yield processes especially during the reproductive stage of plant development. The examples of effect of the changing environmental conditions on crop yield include a 17% reduction in corn and soybean grain yields in the US, 15% reduction in rice grain yield in Philippines (Peng *et al.*, 2004), and a projected reduction in grapevine production by 81% in the US by the end of 21st century (White *et al.*, 2006).

Crops are generally known to be more sensitive to abiotic stresses like drought and/or heat stress during reproductive stages of development. These stresses can lead to inhibition of pollen development, failure of fertilization and shortening of grain filling duration. In some plants, drought stress inhibits the transition from vegetative to reproductive phase and plants remain vegetative until the stress is relieved (Craufurd *et al.*, 1993). The stage of male reproductive development has been found to be particularly vulnerable to the abiotic stress conditions. High-temperature and drought stress is known to cause male sterility in plant species, such as tomato, Arabidopsis, cowpea, wheat, barley soybean and rice (Saini *et al.*, 1984; Ahmed *et al.*, 1992; Peet *et al.*, 1998; Sakata *et al.*, 2000; Kim *et al.*, 2001; Matsui and Omasa, 2002; Abiko *et al.*, 2005; Koti *et al.*, 2005; Jagadish *et al.*, 2007). The pollen germination and germ tube elongation of maize (Hopf *et al.*, 1992) and the formation of pegs and pods in groundnut (Ketring, 1984; Wheelar *et al.*, 1997; Vara Prasad *et al.*, 2000) have also been found to be negatively affected by exposure to moderately high temperature.

The effect of environmental stress, at molecular level, on the reproductive phase is yet to be investigated in detail. In order to address this issue, it is necessary to identify the key components that plants use to deal with complex environmental stresses during the reproductive phase. Though a large number of gene products are known to play

putative roles in plant adaptation to environmental stresses, the ones that are involved in key oxidative stress detoxification are known to be the most crucial in preventing cellular-damage. Plants possess well-defined 'anti-oxidative' machinery, consisting of different enzymatic and non-enzymatic anti-oxidants for the detoxification of the excess reactive oxygen species (ROS) produced under various abiotic stress conditions. The major ROS-scavenging enzymes present in plants include superoxide dismutase (SOD) ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), catalase (CAT), glutathione reductase (GR), glutathione-S transferase (GST), and glutathione peroxidase (GPX). Among these enzymes APX, MDHAR, DHAR and GR together constitute the ascorbate- glutathione (AsA-GSH) cycle. The AsA-GSH cycle is known to play a critical role in combating oxidative stress by deactivation of ROS in multiple redox reactions.

The enhanced production of reactive oxygen species (ROS) during abiotic stresses can pose a threat to plants because plants cannot detoxify them effectively using the existing ROS scavenging machinery. Depending upon the efficiency of the ROS scavenging and/or avoidance mechanisms plants may differ in tolerance to different environmental stresses. Efficient ROS detoxification is thought to be one of the strategies used by stress tolerant plants to combat various abiotic stresses. *Pennisetum glaucum,* commonly known as 'pearl millet', is one such stress tolerant crop that grows well even under conditions of drought, high temperature and marginal soil fertility. Being a stress-tolerant crop, *P. glaucum* is considered to be equipped with better ROS-detoxification machinery. Therefore, we hypothesized that the SOD-AsA-GSH pathway encoding genes isolated from stress tolerant crop plant like *P. glaucum* could be over-expressed in stress-sensitive crop plants in order to enhance their stress tolerance.

Although substantial research has been conducted on the effect of abiotic stress during vegetative growth of plant, its impact on the reproductive development has not received its due attention. The effect of environmental fluctuations on the reproductive phase seems to be more pronounced in crop plants like rice that are at the limit of their climatic range of growth and, consequently, are highly sensitive to even short episodes of climatic fluctuations.

In an attempt to improve the stress tolerance, many plants have been genetically engineered to express genes that confer abiotic stress tolerance. The manipulation of multiple genes has emerged as a promising strategy to increase the stress tolerance of plants because many important traits and complex metabolic pathways depend on interactions among a number of genes. Various approaches have been used to introduce multiple genes into plant genomes and then to coordinate transgene expression (Halpin *et al.*, 2005). The application of stacking multiple genes into one T-DNA has an advantage over the other methods as it minimizes complex integration patterns, and reduces the transformation steps needed to engineer the desired genotype.

In the present study, the T7 RNA polymerase coupled SOD-AsA-GSH pathway was over-expressed in anthers of rice plants to protect the development of anther and quality of the pollen from the oxidative damage. The whole pathway was assembled in a single T-DNA hy *in vitro* gene pyramiding of *SOD*, *APX*, *DHAR*, *MDHAR* and *GR* genes isolated from *P. glaucum*. The regulated over-expression of all these genes can be an effective strategy to scavenge the ROS and minimize the oxidative damage caused to anthers under adverse environmental conditions which would in turn increase the reproductive fitness of plants.

The present study was undertaken with the following objectives:

1. Cloning and characterization of SOD-AsA-GSH pathway genes (SOD, APX, MDHAR, DHAR and GR) from a stress adapted plant, *P. glaucum*.
2. Construction of T7-promoter regulated SOD-AsA-GSH pathway encoding gene cassettes.
3. Isolation, cloning and *in silico* characterization of rice anther specific RA8promoter.
4. Isolation and cloning of T7 RNA polymerase gene from phage DNA.
5. Isolation and cloning of *bar* gene.
6. Construction of RA8 promoter-T7 RNA polymerase cassette.
7. *In vitro* gene pyramiding and construction of plant transformation vector with the *P. glaucum* gene cassettes, i.e. superoxide dismutase (*PgSOD*), ascorbate

peroxidase (*PgAPX*), monodehydroascorbate reductase (*PgMDHAR*), dehydroascorbate reductase (*PgDHAR*) and glutathione reductase (*PgGR*) cassettes along with the RA8 promoter-*T7 RNA polymerase* cassette.

8. Transformation of rice (*O. sativa* cv Swarna and *O. sativa* cv IR64) with the T7 RNA polymerase coupled SOD-AsA-GSH pathway construct through *Agrobacterium* mediated transformation.
9. Molecular analysis of transgenic rice plants to study the stable integration of all the transgenes.
10. Analysis of the morphological characteristics of the transgenic rice plants expressing the SOD-AsA-GSH pathway in the anthers.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Food production from crop plants faces serious threats due to the global climate change (Rosegrant and Cline, 2003; Stocking, 2003; Schmidhuber and Tubiello, 2007; Brown and Funk, 2008). The main cause of climate change is the increase in the concentration of green house gasses which has led to the rise in global temperature. The increase in the global temperature in turn leads to drought stress causing a huge damage to food production. In addition to its deleterious effects on crop growth, the climate change is also expected to threaten the conservation of cultivated land (Christensen *et al.*, 2007; Meehl *et al.*, 2007). Furthermore, the rising human population has resulted in increased demand for food. Thus, the global agriculture must produce more food while adapting to climate change.

2.1 Challenges in Modern Agriculture: Climatic Changes and Abiotic Stress

Different abiotic stresses like drought, salinity, temperature extremes, nutrient deficiencies and mineral toxicities lead to reduction in plant growth and therefore have a major impact on crop yield (Langridge *et al.*, 2006; Munns and Tester, 2008; Witcombe *et al.*, 2008; Salekdeh *et al.*, 2009; White and Brown, 2010; Morison *et al.*, 2008). The increasing damage imposed by these stresses due to climate change and land degradation is of great concern (Tester and Langridge, 2010; Witcombe *et al.*, 2008). As is clearly evident from the Fourth Assessment Report (AR4) by the Inter-governmental Panel on Climate Change (IPCC), global climate change would cast an adverse effect on all the biological processes over the next decade (Christensen *et al.*, 2007).

Abiotic stress being an integral part of the complex phenomenon of climate change has a series of unpredictable impacts on the environment. Abiotic factors play a major role in limiting crop production worldwide (Fig. 2.1). It has been estimated that 51–82% of the potential yield of annual crops is lost due to abiotic stress (Bray *et al.*, 2000). The prolonged exposure of the plants to the abiotic stresses leads to altered metabolism and

damage to biomolecules thereby casting a negative effect on plant growth and development.

2.2 Abiotic Stress and Reproductive Development of Plants

Plants differ in their sensitivity to various abiotic stresses during different developmental stage of their life cycle. Sexual reproduction has been long recognized as being highly stress-sensitive, with reproductive stress tolerance often a limiting trait in crop productivity (Barnabas *et al.*, 2008; Hedhly *et al.*, 2008; Thakur *et al.*, 2010). Most crops are found to be highly sensitive to the abiotic stresses during flowering (Table 2.1). Even mild abiotic stress during the reproductive stage of plant can irreversibly affect grain yield, without considerably affecting the vegetative growth. In self-fertilizing cereals, such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and sorghum (*Sorghum bicolor*), successful pollen development is important for grain production, and abiotic stresses interfering with the early stages of pollen formation lead to dramatic losses in the number of grains formed (Satake and Hayase, 1970; Saini *et al.*, 1984; Briggs *et al.*, 1999; Matsui and Omasa, 2002; Abiko *et al.*, 2005; Jagadish *et al.*, 2007; Jain *et al.*, 2007; Endo *et al.*, 2009). Stress-induced pollen sterility is reported to occur in both the monocots and dicots (Aloni *et al.*, 2001; Karni and Aloni, 2002; Kim *et al.*, 2001; Pressman *et al.*, 2002; Ghanem *et al.*, 2009). The process of grain filling i.e. the accumulation of reserves in the developing and maturing grain has also been found to be sensitive to environmental conditions strongly affecting final yield quantitatively as well as qualitatively (Yang and Zhang, 2006).

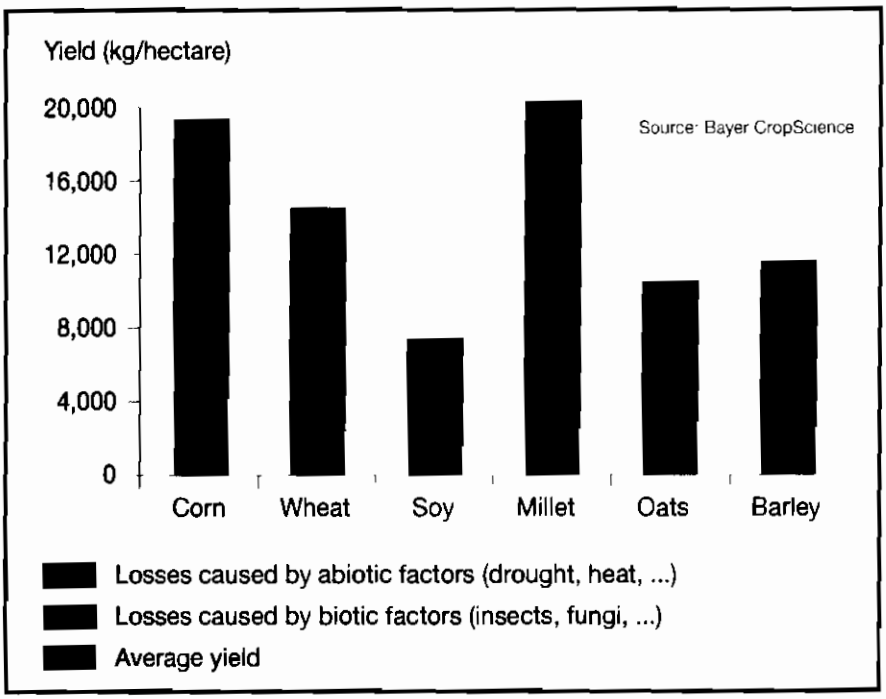


Figure 2.1 Graph representing loss in agricultural productivity due to abiotic stress (Adapted from Bayer Crop Sciences).

Table 2.1 Effect of abiotic stress on reproductive development of plants (Modified after Sakata and Higashitani, 2008).

Species	Abiotic Stress	Abnormal tissue	Reference
Arabidopsis	High temperature	Male organ	Kim *et al.*, 2001
Maize	Water deficit	Female organ	Westgate and Boyer, 1985
Maize	High temperature	Male organ	Mitchell and Petolino 1988
Maize	Water deficit	Male and female organ	Duvick, 2005
Rice	High temperature	Female organ	Takeoka *et al.*, 1991
Rice	High temperature	Anther	Matsui and Omasa 2002
Rice	High temperature	Male organ	Endo *et al.*, 2009
Rice	High temperature	Spikelet	Jagadish *et al.*, 2007
Rice	High temperature	Male and female organ	Satake and Yoshida 1978
Rice	Water deficit	Male organ	O'Toole *et al.*, 1981, Sheoran and Saini 1996
Rice	Low temperature	Male organ	Nishiyama 1970; Satake and Hayase, 1970; 1974; Nishiyama, 1976; Ito *et al.*, 1970, Mamun *et al.*, 2006,
Rice	Low temperature	Male and female organ	Hayase *et al.*, 1969
Rice	Low temperature	Pollen	Oliver *et al.*, 2005
Rice	Flooding	Male and female organ	Reddy and Mittra 1985
Wheat	Heat, water deficit, Abcissic acid	Male organ	Saini *et al.*, 1984
Wheat	Water deficit	Male organ	Lalonde *et al.*, 1997; Ji *et al.*, 2010
Wheat	Water deficit	Male organ	Bingham,1966
Wheat	Water deficit	Flower	Briggs *et al.*, 1999
Wheat	Salinity	Flower	Maas and Poss, 1989a
Tomato	Salinity	Flower	Ghanem *et al.*, 2009
Tomato	High temperature	Flower	Sawhney *et al.*, 1982
Tomato	High temperature	Male organ	Peet *et al.*, 1998; Pressman *et al.*, 2002
Rape	Low temperature	Male and female organ	Lardon and Triboi-Blondel, 1994
Barley	High temperature	Male organ	Sakata *et al.*, 2000; Abiko *et al.*, 2005; Oshino *et al.*, 2007
Snap bean	High temperature	Flower	Konsens *et al.*, 1991
Bell pepper	High temperature	Pollen	Aloni *et al.*, 2001; Karni et al., 2002
Rapeseed	High temperature	Male and female organ	Young *et al.*, 2004
Ground nut	High temperature	Flower	Vara Prassad *et al.*, 2000

2.2.1 Effect of Drought Stress

Drought stress is an important environmental factor limiting global crop productivity. In many crops, particularly cereals, reproductive development is the most stress-sensitive period after seed germination (Salter and Goode, 1967). Drought stress interferes with reproductive success of plants by arresting the development of the male gametophyte and sometimes the female gametophyte, preventing fertilization and/or inducing premature abortion of the fertilized ovule (Moss and Downey, 1971; O'Toole and Moya, 1981; Saini and Aspinall, 1981; O'Toole and Namuco, 1983; Westgate and Boyer, 1986; Sheoran and Saini, 1996). Drought during the period from stamen initiation to anthesis causes serious yield reduction in many cereal and dicot crops (Lewis *et al.*, 1974; O'Toole and Moya, 1981; Craufurd *et al.*, 1993; Turner, 1993; Westgate and Peterson, 1993). In the female tissue, the period most sensitive to drought corresponds to meiosis in the megaspore mother cell and the subsequent degeneration of three redundant megaspores in the tetrad (Bennett *et al.*, 1973). In maize, the anthesis-silking interval, (differences in the relative timing of male and female flowering) which is negatively correlated with yield, is found to be typically increased by water deficit (Duvick, 2005).

2.2.2 Effect of Salt Stress

It has long been recognized that a crop's sensitivity to salinity varies from one developmental growth stage to the other (Läuchli and Grattan, 2007; Maas and Grattan, 1999). In experiments with wheat (Maas and Poss, 1989a), sorghum (Maas *et al.*, 1986) and cowpea (Maas and Poss, 1989b), it was found that these crops were most sensitive during vegetative and early reproductive stages, less sensitive during flowering and least sensitive during the seed filling stage. Salinity often reduces shoot growth more than root growth (Lauchli and Epstein, 1990) and can reduce the number of florets per ear, increase sterility and affect the time of flowering and maturity in both wheat (Maas and Poss, 1989a) and rice (Khatun *et al.* 1995). Salinity stress is known to cause spikelet sterility in rice (Asch and Wopereis, 2001) and seed abortion in field-grown cotton (Davidonis *et al.*, 2000). The development of microspores is found to be very sensitive to salt stress (Namuco and O`Toole, 1986). As a result of salt stress, the microsporocytes become vacuolated and die instead of maturing into viable pollen grains (Sun *et al.*, 2004).

2.2.3 Effect of Temperature Stress

Temperature stresses have diverse effects on reproductive tissues leading to poor seed set and yield of plants (Fig. 2.2). High or low temperature stress can lead to-

(i) Early or delayed flowering (Balasubramanian *et al.*, 2006; Tonsor *et al.*, 2008; Craufurd and Wheeler, 2009),

(ii) Asynchrony between male and female reproductive development (Herrero, 2003; Hedhly *et al.*, 2008),

(iii) Defects in structure and function of reproductive tissues (stamens and carpels) (Takeoka *et al.*, 1991; Morrison and Stewart, 2002; Croser *et al.*, 2003; Whittle *et al.*, 2009) and

(iv) Defects in development of male and female gametes (Aloni *et al.*, 2001; Young *et al.*, 2004).

Short episodes of high-temperature stress, affecting many reproductive processes, including pollen viability, female gametogenesis, pollen-pistil interaction, fertilization, and grain formation, can lead to severe reduction in yield even when the seasonal average temperature is within a favorable range. Even a small increase in temperature above the optimum can very negatively affect pollen viability (Hedhly *et al.*, 2008). For example, a short exposure to high temperature during anthesis is known to greatly reduce the production of grains in cereals (Vara Prassad *et al.* 2000; Witcombe *et al.*, 2008). In addition to heat stress, cold stress is also known to negatively affect the reproductive development of plants. It is estimated that, worldwide, 7 million hectares of rice are prone to chilling damage (Oliver *et al.*, 2005). Cold-induced pollen sterility is the major factor decreasing the crop yields by an average of about 5 to 10% (Oliver *et al.*, 2005; Mamun *et al.*, 2006). Chilling leads to poor seedling establishment and arrest of microspore development at booting stage, resulting in reduced fertility (Mamun *et al.*, 2006).

2.3 Abiotic Stress Disrupts Male Reproductive Development

Male gametophyte development is the stage of reproduction that appears to be most susceptible to disruption by drought, heat, and cold stresses (Saini, 1997; Mamun *et al.*,

2006). Abiotic stresses, particularly, high or low temperature results in a lower seed set due to male sterility in most crops, including tomatoes (Peet *et al.*, 1998; Sato *et al.*, 2002), cowpeas (Ahmed *et al.*, 1992), wheat (Saini *et al.*, 1984), barley (Sakata *et al.*, 2000; Koike *et al.*, 2003; Oshino *et al.*, 2007) and rice (Satake and Yoshida, 1978; Nishiyama, 1984; Prasad *et al.*, 2006). Cold treated rice plants show greater abnormalities in the anthers than in their pistils or any other floral organs (Satake and Hayase, 1974; Gothandam *et al.*, 2007).

2.3.1 Stages of Male Reproductive Development Sensitive to Abiotic Stress

The male reproductive development in higher plants is a complex biological process that involves the correlated differentiation of anther tissues leading to generation of haploid microspores or pollen (Fig. 2.3) (Liu and Qu, 2008). The developed anther consists of the meiotic cells (also called microsporocytes) at the center, surrounded by the anther wall with four somatic layers namely the epidermis, the endothecium, the middle layer, and the tapetum (Goldberg *et al.*, 1993). Tapetum, which forms the innermost layer of the anther wall, plays an important role in regulating programmed anther development, pollen formation, and pollen wall formation (Li *et al.*, 2006; Parish and Li, 2010). Tapetal cell development and differentiation are critical for the early events in male reproduction, including meiosis. During late pollen development, tapetal degeneration, triggered by an apoptosis-like process, is also vital for viable pollen formation (Papini *et al.*, 1999; Varnier *et al.*, 2005; Li *et al.*, 2006; Aya *et al.*, 2009).

Two peaks of sensitivity to abiotic stress, particularly drought stress, are encountered during male reproductive development. The first peak is centered on the period from meiosis to tetrad break-up in anthers (Fig. 2.4). This window of sensitivity has been extensively studied in plants like wheat, rice, barley, oat and maize (Moss and Downey, 1971; Saini and Aspinall, 1981; Namuco and O'Toole, 1986; Dembinska *et al.*, 1992; Sheoran and Saini, 1996). The stage of meiosis is perhaps the most stress-sensitive period of reproduction in most plants. In rice and wheat, cold and drought stress are also known to induce irreversible abortion of pollen development at the young microspore stage (Satake and Hayase, 1970; Saini *et al.*, 1984; Sheoran and Saini, 1996; Oliver *et al.*, 2005; Ji *et al.*, 2010; Zinn *et al.*, 2010). Morphological and histological examinations

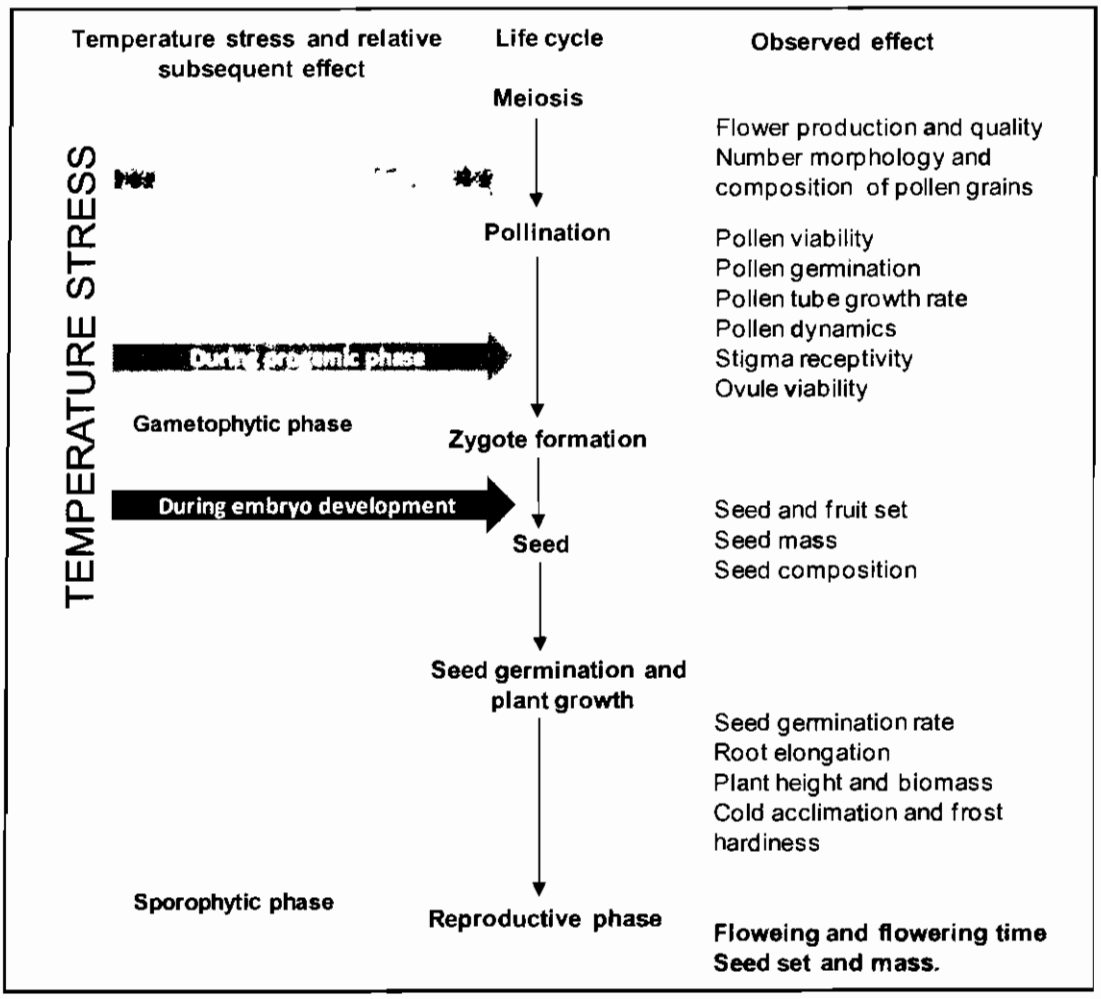


Figure 2.2 Temperature stress experienced during gamete development (green arrow), progamic or post-pollination–pre-zygotic stage (blue arrow) and embryo development stage (purple arrow). Gametophytic and sporophytic stages are shown with yellow and green background colours, respectively (Modified after Hedhly *et al.*, 2008).

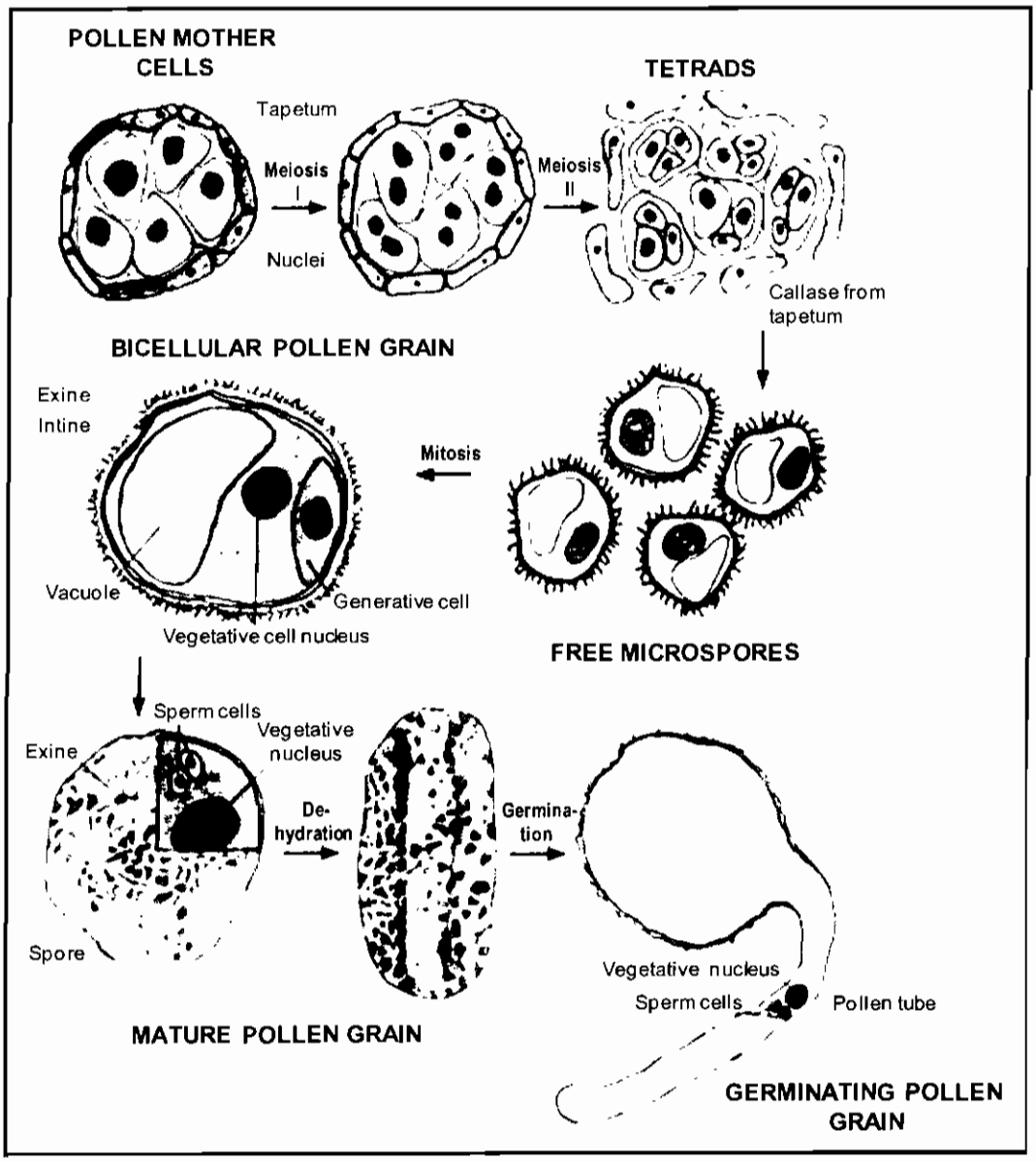


Figure 2.3 Scheme of microsporogenesis in ricc showing the various stages of development of pollen grains (Adapted from McCormick *et al.*, 2004).

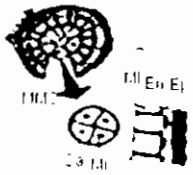
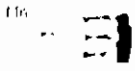
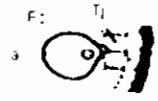
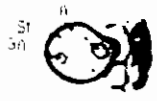
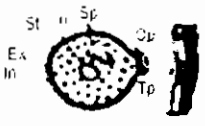
Stages during normal development	In plants stressed during meiosis and then re-watered
 Meiosis and tetrad	Callose present; normal meiosis Anther development normal; tetrads formed
 Young microspores	Occasional microspore disorientation or abnormal vacuolation of tapetum
 Vacuolate microspores	Tapetal vacuolation progresses, microspores loosing contact with tapetum which degenerates or persists
 First pollen grain mitosis	Pollen grains fail to accumulate starch, have poorly formed or no intine. All abnormal pollen grains are detatched from tapetum. Anthers smaller and paler than normal
 Secondpollen grain mitosis	Shrivelled pollen grains devoid of starch, have thin or no intine. Pollen grains non- viable and fail to germinate

Figure 2.4 Sequence of events during failure of pollen development in response to transitory episode of water deficit during meiosis (First stage of sensitivity). Abbreviations denote AW, anther wall; Ca, callose; En, endothecium; Ep, epidermis; Ex, exine; Gn, generative nucleus; In, intine; Inf, inflorescence, Mi, microspores; Ml, middle layer, MMC, Microspore mother cell; Mn, microspore nucleus; Op, operelum; Po, pore; Sp, Sperm; St, starch; Tp, tapetum; Va, vacuole; Vn, vegetative nucleus (Modified after Koonjul *et al*., 2005).

have shown that the transition of the tetrad to a uni-nucleate stage of male reproductive development is particularly sensitive to chilling (Ito *et al.*, 1970; Satake and Hayase, 1974; Wada *et al.*, 1990).

The second peak of sensitivity occurs during anthesis and initial stages of grain development, and is conspicuous in rice, maize, wheat, barley and oats (Saini *et al.*, 1997; Westgate and Boyer 1986; Ekanayake *et al.*, 1989; Ekanayakc *et al.*, 1990; Turner, 1993; Westgate and Peterson, 1993). In fact, the main cause of floret sterility induced by high temperatures at flowering is anther indehiscence (Satake and Yoshida, 1978; Mackill *et al.*, 1982; Matsui *et al.*, 1997a; b; 2001). High temperature during flowering inhibits swelling of the pollen grains (Matsui *et al.*, 2000) which is the driving force behind anther dehiscence in rice (Matsui *et al.*, 1999a; b). Anthers of high temperature- tolerant cultivars dehisce more easily than those of susceptible cultivars and contribute to pollination under high temperature conditions (Satake and Yoshida, 1978; Mackill *et al.*, 1982; Matsui *et al.*, 2000; 2001).

2.3.2 Factors Responsible for Male Sterility in Plants

The loss of reproductive fertility due to drought, heat, and cold stresses imposed during flowering and early stages of seed growth has been attributed to a range of metabolic causes (e.g. inadequate supply of photosynthates, excessive accumulation of abcissic acid (ABA), changes in the cell cycle) acting during gametogenesis and/or during the early stages of grain development (Zinselmeier *et al.*, 1999; 2002; Saini and Westgate, 2000; McLaughlin and Boyer, 2004a; 2004b; 2007; Fresneau *et al.*, 2007; Collins *et al.*, 2008). The alterations in the availability and metabolism of carbohydrates appear to be involved in the effects of stress during meiosis and anthesis.

2.3.2.1 Disturbance of Sugar Metabolism in Anthers

The disturbance in carbohydrate availability and metabolism plays a key role in abiotic stress; particularly drought stress induced male sterility (Dorion *et al.*, 1996). The sterile pollen grains are always devoid of starch, a feature commonly observed in pollen affected by various types of oxidative stress (Saini *et al.*, 1984). Sheoran and Saini revealed that drought inhibits the starch accumulation in anthers (Sheoran and Saini, 1996). Recently,

Fu et al. reported that oxidative stress and soluble sugar content is responsible for drought stress induced pollen abortion in rice (Fu *et al.*, 2010).

2.3.2.2 Premature Degeneration of Tapetum

Tapetum, the innermost cell layer of the anther wall plays a crucial role in microspore maturation by providing nutrients to them and regulating their release (Balk and Leaver, 2001) thereby controlling pollen development and, ultimately, plant fertility. Abiotic stress during tapetal development leads to aborted microgametogenesis and male sterility (Kapoor *et al.*, 2002; Higginson *et al.*, 2003; Jung *et al.*, 2005; Oliver *et al.*, 2005). It has been observed that under different low temperature conditions, the tapetum swelled severely and the cellular divisions between tapetal cells were greatly altered (Gothandam *et al.*, 2007). Cold treatment at this stage was found to result in reduced activity of cell wall-bound invertase, leading to accumulation of sucrose that causes tapetal swelling in rice anthers (Nishiyama, 1995; Kawaguchi *et al.*, 1996; Sheoran and Saini, 1996; Oliver *et al.*, 2005). Similarly, drought stresses was also shown to trigger a premature cell death response in the tapetum (Oliver *et al.*, 2005; Nguyen *et al.*, 2009; Ji *et al.*, 2010).

2.3.2.3 Oxidative Stress

It is a well established fact that abiotic stress triggers the formation of superoxide radicals and hydrogen peroxide which can directly attack membrane lipids and inactivate sulfhydrl containing enzymes (Navarri-Izzo *et al.*, 1994). Higher activities of anti-oxidative enzymes like superoxide dismutase, ascorbate peroxidase, glutathione reductase as well as increased content of anti-oxidants like ascorbic acid and glutathione were observed in panicles of drought tolerant genotype of rice as compared to drought susceptible ones (Sairam and Saxena, 2000; Devarshi and Chopra, 2004; Fu *et al.*, 2010). Nguyen *et al.* reported that there occurs increased concentration of hydrogen peroxide and down-regulation of anti-oxidant transcripts in anthers due to drought stress (Nguyen *et al.*, 2009). These results clearly indicate that along with the other factors, oxidative stress in anthers might be the cause of pollen sterility due to drought stress. However, the relationship between pollen sterility and oxidative stress has not been studied in detail.

2.4 Oxidative Stress and Male Sterility

Almost all sorts of abiotic stress conditions are known to negatively affect the male reproductive development of crop plants. As all the abiotic stress conditions lead to the over-production of reactive oxygen species (ROS) in plants (Jaspers and Kangasjärvi, 2010), it can hence be assumed that the disruption of male reproductive development occurs due to oxidative stress. Moreover, evidences indicating production of ROS and altered anti-oxidant enzymes activity in anthers during drought stress (Nguyen *et al.*, 2009) also support the fact that oxidative stress may be responsible for male sterility. The section ahead provides a brief description about the source of oxidative stress and the anti-oxidant mechanism present in plants.

2.4.1 Source of Oxidative Stress: ROS

ROS are partially reduced forms of atmospheric oxygen (O_2). ROS typically result from either the excitation of O_2 to form singlet oxygen (O_2^1) or from the transfer of one, two or three electrons to O_2 to form, respectively, a superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) or a hydroxyl radical ($HO^{\bullet}$) (Asada and Takahashi, 1987; 1999; Mitler, 2002; Apel and Hirt, 2004) (Fig. 2.5). The reactions involved in normal metabolism, such as photosynthesis and respiration are one of the potential sources of ROS in plants, thus making the ROS inevitable byproducts of aerobic metabolism (Asada and Takahashi, 1987). Other sources of ROS include pathways triggered during abiotic stresses, such as glycolate oxidase in peroxisomes during photorespiration (Dat *et al.*, 2000), NADPH oxidases, amine oxidases and cell-wall-bound peroxidases (Hammond-Kosack and Jones, 1996; Grant and Loake, 2000). ROS have also been acknowledged as central players in complex signaling pathways (Mittler *et al.*, 2011) and research has proved that they act as signals for the activation of stress-response and defense pathways (Knight and Knight, 2001).

Though, the production of ROS in cells is low under physiological steady state conditions (240 $\mu M\ s^{-1}$ O_2^- and a steady-state level of 0.5 μM H_2O_2 in chloroplasts), abiotic stresses like drought, salt stress, chilling, heat shock, heavy metals, ultraviolet radiation, air pollutants, mechanical stress, nutrient deprivation, pathogen attack and high light stress

that disrupt the cellular homeostasis enhance the production of ROS (240–720 μM s^{-1} O_2^- and 5–15 μM H_2O_2) (Polle, 2001; Mittler, 2002). These ROS are capable of causing uncontrolled oxidation of various cellular components and can lead to the oxidative destruction of the cell (Asada *et al.*, 1999; Dat *et al.*, 2000) (Fig.2.6). Thus, enhanced production of ROS during stress can be hazardous to cells. Therefore, in order to protect themselves against the toxic ROS, plant cells and its organelles like chloroplast, mitochondria and peroxisomes utilize anti-oxidant defense systems which form the major defensive strategy of plants against oxidative stress.

2.4.2 Anti-oxidant Mechanism

A great deal of research has established that the induction of the cellular anti-oxidant machinery is important for protection against various stresses (Apel and Hirt, 2004; Gill and Tuteja, 2009). The term anti-oxidant can be considered to describe any compound capable of quenching ROS without itself undergoing conversion to a destructive radical (Nishikimi and Yagi, 1996; Rose and Bode, 1993). The anti-oxidant defense system which is responsible for maintaining a balanced state of ROS in plants basically comprises of the non-enzymatic and enzymatic components (Noctor and Foyer, 1998) (Fig. 2.7).

2.4.2.1 Non-enzymatic Components

The non-enzymatic components of the anti-oxidative defense system include the major cellular redox buffers: ascorbate (AsA) and glutathione (γ-glutamyl-cysteinyl-glycine, GSH) as well as tocopherol, flavonoids, alkaloids and carotenoids. AsA and GSH are low molecular weight anti-oxidant abundantly found in plants (Barnes *et al.*, 2002). AsA provides membrane protection, preserve enzyme activities (Noctor and Foyer, 1998) and has a key role in removal of H_2O_2 via Ascorbate- Glutathione (AsA-GSH) cycle (Pinto *et al.*, 2003). Being present virtually in all the compartments of cell (Foyer and Noctor, 2003), GSH functions as an anti-oxidant in many ways. It functions as a free radical scavenger and participates in regeneration of another potential anti-oxidant AsA, via the AsA-GSH cycle (Asada, 1994).

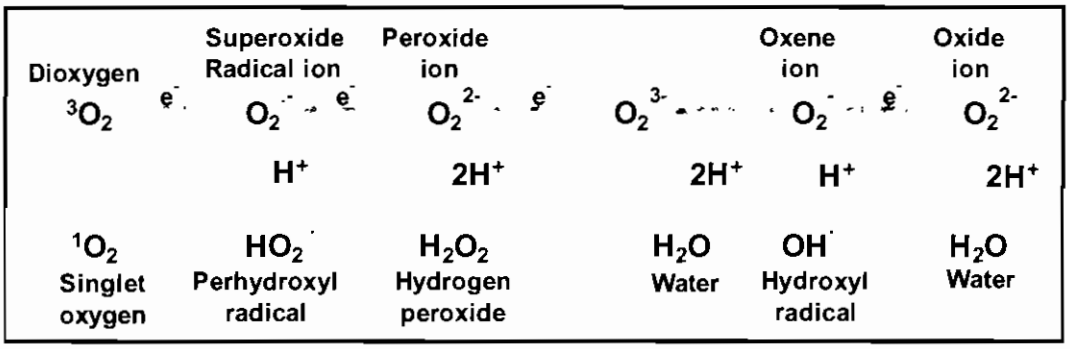


Figure 2.5 Generation of different ROS by sequential univalent reduction of ground state triplet oxygen (Modified after Apel and Hirt, 2004).

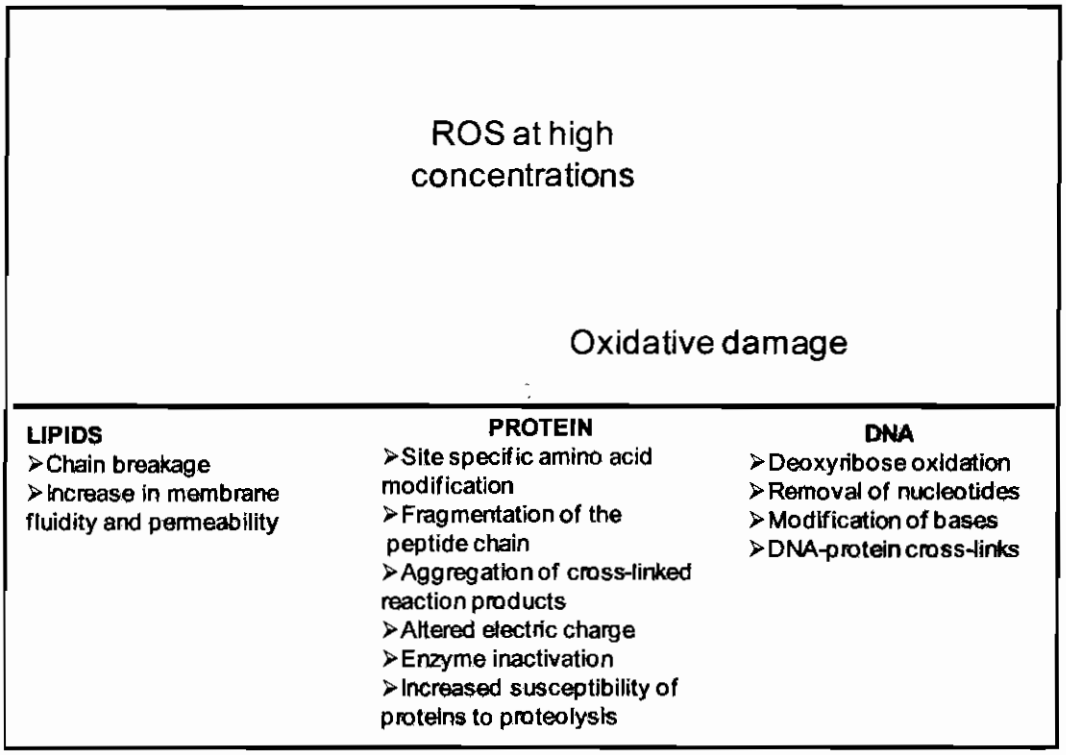


Figure 2.6 ROS induced oxidative damage to lipids, proteins and DNA.

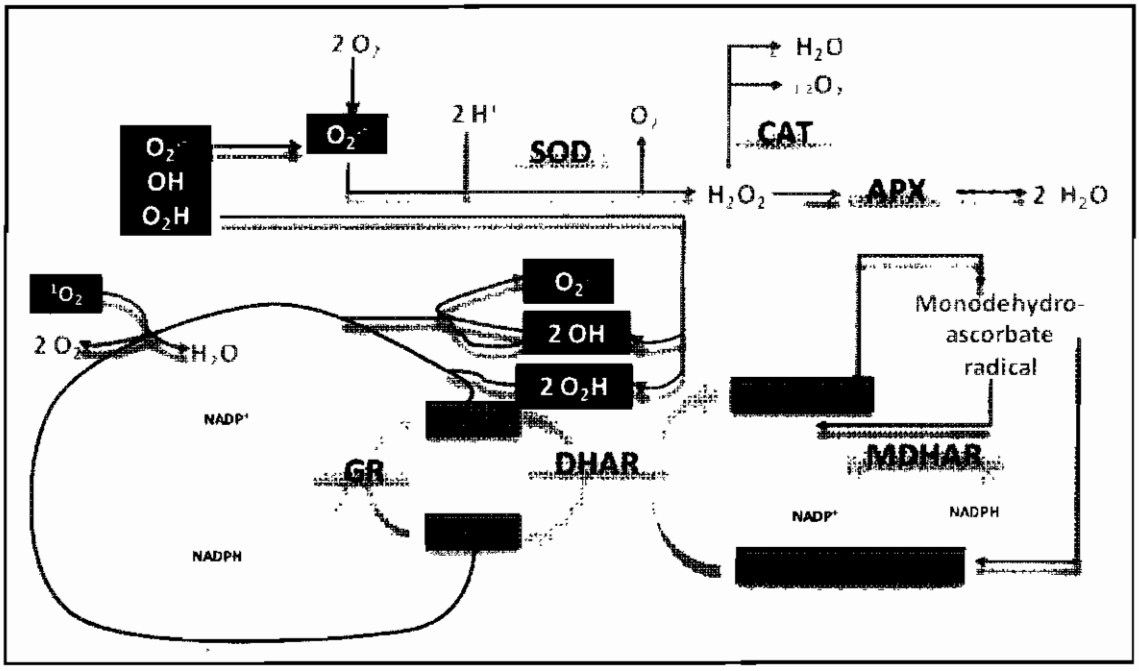


Figure 2.7 Sehematic representation of enzymatic and non enzymatic components of plants anti-oxidant mechanism.

Tocopherols (α, β, γ, and δ) and carotenoids represent a group of lipophilic anti-oxidants involved in scavenging of oxygen free radicals, lipid peroxy radicals, and $^1O_2$ Tocopherols are known to protect lipids and other membrane components against oxidative stress (Diplock, 1989; Young, 1991). Carotenoids scavenge $^1O_2$ to inhibit oxidative damage and quench triplet sensitizer (3Chl*) and excited chlorophyll (Chl*) molecule to prevent the formation of $^1O_2$ for protecting the photosynthetic apparatus (Gomathi and Rakkiyapan, 2011). Phenolics are diverse secondary metabolites (flavonoids, tannins, hydroxyl-cinnamate esters, and lignin) which possess anti-oxidant properties (Grace and Logan, 2000). Polyphenols can chelate transition metal ions, directly scavenge molecular species of active oxygen, inhibit lipid peroxidation and modify lipid packing order to decrease fluidity of the membranes (Arora *et al.*, 2000).

2.4.2.2 Enzymatic Components

The enzymatic components of the anti-oxidative defense system consist of a number of anti-oxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), and the enzymes of ascorbate-glutathione (AsA-GSH) cycle namely ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Noctor and Foyer, 1998) (Table 2.2). These enzymes operate in different sub-cellular compartments and respond in concert when cells are exposed to oxidative stress.

Among all the anti-oxidant enzymes, SOD together with the enzymes of AsA-GSH pathway plays a crucial role in disposal of ROS. Before describing in detail about the role of SOD-AsA-GSH cycle in protecting cell against oxidative stress, the role of other important anti-oxidant enzymes is briefly discussed below.

Table 2.2 List of different anti-oxidant enzymes, their localization and the primary ROS detoxified by them.

Anti-oxidative mechanism	Localization	Primary ROS	Reference
Superoxide dismutase	Chloroplast, Cytoplasm, Mitochondria, Peroxisomes, Apoplast	O_2^-	Bowler *et al.*, 1992
Ascorbate peroxidase	Chloroplast, Cytoplasm, Mitochondria, Peroxisomes, Apoplast	H_2O_2	Asada and Takahashi, 1987; Asada,1999
Catalase	Peroxisomes	H_2O_2	Willekens *et al.*, 1995
Glutathione peroxidase	Cytoplasm	H_2O_2, ROOH	Dixon *et al.*, 1998
Peroxidases	Cell wall, Cytoplasm, Vacuole	H_2O_2	Asada and Takahashi, 1987
Thioredoxin peroxidase	Cell wall, Cytoplasm, Mitochondria	H_2O_2	Baier and Dietz,1996

2.4.2.2.1 Catalase

Catalase (CAT, EC 1.11.1.6) is a tetrameric heme-containing enzyme ubiquitously present in all organisms and catalyzes the dismutation of two molecules of H_2O_2 into water and oxygen. It has high specificity for H_2O_2, but weak activity against organic peroxides. CAT scavenges H_2O_2 generated in the peroxisomes which are major sites of H_2O_2 production during photorespiratory oxidation and β-oxidation of fatty acids (Del Rio *et al.*, 2006; Scandalios *et al.*, 1997; Corpas *et al.*, 2008). To date, all angiosperm species studied contain three CAT genes: Class I CATs are expressed in photosynthetic tissues; Class II CATs are expressed at high levels in vascular tissues, and Class III CATs are highly abundant in seeds and young seedlings (Willekens *et al.*, 1995). Environmental stresses cause enhancement of CAT activity, depending on the intensity, duration, and types of the stress (Sharma and Dubey, 2005; Han *et al.*, 2009; Moussa *et al.*, 2008).

2.4.2.2.2 Guaiacol Peroxidase

Guaiacol peroxidase (GPX, EC 1.11.1.7), a heme containing protein, performs the oxidation of aromatic electron donors such as guaiacol and pyragallol at the expense of H_2O_2. GPX can function as effective scavenger of reactive oxygen species and peroxy-radicals under stressed conditions (Vangronsveld and Clijsters, 1994). Various

isoenzymes of GPX have been found in vacuoles, cell wall and cytosol (Asada, 1992). GPX also performs a crucial role in many important biosynthetic processes like lignification of cell wall, degradation of Indole Acetic Acid (IAA), biosynthesis of ethylene, wound healing, and defense against abiotic and biotic stresses (Kobayashi *et al.*, 1996). Various stressful conditions of the environment have been shown to induce the activity of GPX (Shah *et al*; 2001; Sharma and Dubey, 2005; Han *et al.*, 2009, Verma and. Dubey, 2003; Moussa and Abdel-Aziz, 2008; Radotic *et al.*, 2000).

2.4.2.3 Superoxide Dismutase-Ascorbate-Glutathione Pathway

Superoxide dismutase and the enzymes of AsA- GSH pathway are among the most important components of the ROS detoxification machinery of plants. Since the discovery of the AsA-GSH cycle in the mid-1970s, the enzyme-catalyzed reactions of this pathway have attracted considerable interest and still are a matter of intensive research (Noctor and Foyer, 1998; Noctor *et al.*, 1998; Asada, 1999; Polle, 2001). Besides chloroplasts, the constituents of the AsA-GSH cycle have also been localized in other sub-cellular compartments (Jiminez *et al.*, 1997). Studies with mutants or transgenic plants over- or under-expressing enzymes or metabolites of the AsA-GSH pathway have proved the co-relation between the pathway and stress tolerance (Scandalios, 1993; Allen, 1995). The AsA-GSH cycle does not only combat oxidative stress, but also plays important roles in regulating photosynthesis in response to light conditions (Foyer and Harbinson, 1994; Asada, 1999).

2.4.2.3.1 Superoxide Dismutase

Superoxide dismutase (SOD; EC 1.15.1.1) is a ubiquitous metallo-enzyme that catalyzes the first step in scavenging of ROS i.e. the dismutation of superoxide anion radical to hydrogen peroxide and molecular oxygen (Fridovich, 1978). Superoxide dismutases function as dimers with a catalytic metal ion in each monomer (Abreu *et al.*, 2010). On the basis of the metal co-factor present at the catalytic center, SOD isoforms are classified into four types: copper/zinc (CuZnSOD), nickel (NiSOD), manganese (MnSOD) and iron (FeSOD). Ni-dependent enzymes have been reported in *Streptomyces* species and are not found in eukaryotes (Youn *et al.*, 1996). The different isoforms of SOD are distributed in

different sub-cellular locations. CuZnSODs are widely found in the cytosol, plastids and periplasm of prokaryotes (Steinman *et al.*, 1990) as well as in eukaryotes (Bordo *et al.*, 1994; Kliebenstein *et al.*, 1998; Bueno *et al.*, 1995). MnSOD is confined predominantly to the mitochondrial matrix and the peroxisome (Wolfe-Simon *et al.*, 2005; Perry *et al.*, 2010) while FeSOD is found mainly in prokaryotes and chloroplasts of plants (Bridges and Salin, 1981; Van Camp *et al.*, 1990; Bowler *et al.*, 1994; Kliebenstein *et al.*, 1998; Myouga *et al.*, 2008).

Multiple forms of SODs have been characterized in plants which include seven *Arabidopsis* SOD genes comprising of three CuZnSODs, one MnSOD and three FeSODs (Kliebenstein *et al.*, 1998); five wheat SOD genes including two CuZnSODs (Zhang *et al.*, 2008) and three MnSODs (Wu *et al.*, 1999); seven rice SODs consisting of four CuZnSOD, two MnSODs (Kanematsu and Asada, 1989) and one FeSOD and nine maize SODs including four CuZn cytosolic isoenzymes, four mitochondrial associated MnSODs and one chloroplastic CuZnSOD (Guan and Scandalios, 1998).

The function of SOD is frequently associated with tolerance to various abiotic stress conditions (Bowler *et al.*, 1994). This view is supported by the observation that microorganisms such as yeast and cyanobacteria become more sensitive to ROS when they lack SOD (Wallace *et al.*, 2004; Thomas *et al.*, 1998). SODs have been reported to be regulated on the transcriptional as well as translational level in multiple ways (Perl-Treves *et al.*, 1991; Tsang *et al.*, 1991; Kampfenkel *et al.*, 1995; Kurepa *et al.*, 1997; Kaminaka *et al.*, 1999; Sreenivasulu *et al.*, 2000; Wang *et al.*, 2004; Sunkar *et al.*, 2006; Abercrombie *et al.*, 2008).SODs have also been found to be activated by various environmental stress conditions (Table 2.3).

2.4.2.3.2 Ascorbate Peroxidase

Ascorbate peroxidase (APX; EC 1.11.1.11), a class I peroxidase, catalyzes the conversion of H_2O_2 to H_2O and O_2 using ascorbate as specific electron donor (Asada, 1999). APX, an important component of AsA-GSH cycle, prevents the accumulation of toxic levels of H_2O_2 in photosynthetic organisms. APX has been identified in a number of higher plants and comprises of different isoenzymes with different characteristics. So far, five APX

isoforms have been identified in plants: cytosolic isoforms, mitochondria isoforms, peroxisomal/glyoxysomal isoforms and chloroplastic isoforms (Dąbrowska *et al.*, 2007). All the forms of APX are thought to function as scavengers of H_2O_2 which is generated continuously in cells (Miyake and Asada, 1996). In *Arabidopsis thaliana*, the presence of eight isoenzymes has been confirmed: soluble cytosolic (APX1, APX2 and APX6), bound to the microsome membrane (APX3, APX4 and APX5) and chloroplastic (sAPX and tAPX) (Jespersen *et al.*, 1997; Panchuk *et al.*, 2002). Similarly, the identification of APX gene family in tomato revealed the presence of seven APX genes: three cytosolic, two peroxisomal and two chloroplastic (Najami *et al.*, 2008). In rice, eight members of the *APX* gene family have been reported; encoding two cytosolic, two peroxisomal, three chloroplastic and one mitochondrial isoforms (Texeira *et al.*, 2004; 2006).

Like SODs the expression of APX genes can also be activated by specific factors such as pathogen attack, mechanical pressure, injury, UV-B radiation, salt stress, too low or too high temperature, atmospheric pollution excess metal ions and herbicides (Table 2.3).

2.4.2.3.3 Monodehydroascorbate Reductase

Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) recycles monodehydroascorbate molecules into ascorbate. Under environmental stress conditions like high light exposure, the pool of AsA in the chloroplast is oxidised to monodehydroascorbate within a few minutes (Polle, 2001). It is therefore necessary for the survival of plants that monodehydroascorbate is reduced, thereby regenerating AsA. In the chloroplast monodehydroascorbate is reduced to AsA by photoreduced ferredoxin at a high rate and this is likely to constitute the main pathway in the vicinity of the thylakoid membrane (Miyake and Asada, 1994). Away from the thylakoid membrane, reduction of monodehydroascorbate can occur via two enzymes in the AsA-GSH pathway; dehydroascorbate reductase (DHAR) and MDHAR (Asada, 1999). MDHAR reduces monodehydroascorbate directly by using NAD(P)H as an electron donor. Alternatively, two molecules of monodehydroascorbate can react non-enzymatically and spontaneously generate ascorbate and dehydroascorbate. The majority of monodehydroascorbate is however found to be reduced by MDHAR (Polle, 2001). The MDHAR enzyme activity is found across the entire plant and animal kingdom (Arrigoni

et al., 1981). Plant MDHAR exhibits the highest level of sequence similarity with prokaryotic flavoenzymes such as iron-sulphur protein reductases. Expression studies in a number of plant species have shown the modulation of MDHAR gene expression during abiotic stresses like salt, drought, oxidative stress and cold (Table 2.3).

2.4.2.3.4 Dehydroascorbate Reductase

Ascorbic acid, which is a major anti-oxidant in plants, is oxidized to dehydroascorbate (DHA) via successive reversible electron transfers with monodehydroascorbate as a free radical intermediate (Foyer and Mullineaux, 1998). DHA, so produced, is reduced to AsA with glutathione as electron donor by dehydroascorbate reductase (DHAR; EC 1.8.5.1). DHAR proteins have been isolated and characterized from higher plants like *Arabidopsis*, tobacco and agricultural crops such as spinach and rice (Urano *et al.*, 2000; Shimaoka *et al.*, 2000; Eltayeb *et al.*, 2006; Ushimaru *et al.*, 2006).

DHAR is a key enzyme to regenerate AsA. Additionally, DHAR is known to be important for plant growth (Chen and Gallie, 2006). It is reported that lack of DHAR caused the quick loss of AsA from plants and as a consequence affected plant growth and development (Ye *et al.*, 2000). The suppression of DHAR gene expression resulted in a slower rate of leaf expansion. DHAR is a physiologically important enzyme in the ascorbate-glutathione recycling reaction for most of the higher plants.

DHAR also plays important roles in plant adaptation to environmental stresses and their expression is found to be activated by a number of abiotic stress factors (Table 2.3). Moreover, enhanced tolerance to various abiotic stresses was observed in plants over-expressing the DHAR protein (Eltayeb *et al.*, 2006; Kwon *et al.*, 2003; Ushimaru *et al.*, 2006).

2.4.2.3.5 Glutathione Reductase

Glutathione reductase (GR, NADPH: oxidized glutathione oxidoreductase; EC 1.6.4.2), is an anti-oxidant enzyme which maintains the cellular redox state by regenerating the reduced form of glutathione thereby maintaining the balance between reduced glutathione and ascorbate pools (Mullineaux and Creissen, 1997; Noctor and Foyer, 1998; Reddy and Raghvendra, 2006). GR is a flavo-protein oxidoreductase ubiquitously present in both

prokaryotes and eukaryotes (Mullineaux and Creissen, 1997; Romeo-Puertas *et al.*, 2006). GR has been purified and characterized from a variety of organisms (Rao and Reddy, 2008). Although localized mainly in the chloroplasts (Connell and Mullet, 1986), GRs are also found in cytosol (Drumm-Herrel *et al.*, 1989; Edwards *et al.*, 1990), mitochondria and peroxisomes (Jimenez *et al.*, 1997; Romeo-Puertas *et al.*, 2006). In general, all GRs have high specificity for their substrates although some glutathione conjugates and mixed glutathione disulphides can also be reduced by them (Gaullier *et al.*, 1994).

Multiple isoforms of GR have been reported in tobacco (Creissen and Mullineaux, 1995), spinach (Guy and Carter, 1984), red spruce (Hausalden and Alscher, 1994) eastern white pine (Anderson *et al.*, 1990), scot pine (Wingsle and Karpinspi, 1996), mustard (Drumm-Harrel *et al.*, 1989), pea (Edward *et al.*, 1994), cow pea (Contour-Ansel *et al.*, 2006) and wheat (Lascano *et al.*, 2001).

GR and GSH play a crucial role in determining the tolerance of plants during the different abiotic stresses and the increase in GR activity under varying environmental stresses has been shown by several studies (Table 2.3).

Table 2.3 Activation of anti-oxidant enzymes in response to oxidative stress induced by various environmental stresses (Modified after Sharma *et al.*, 2012).

Stresses	Plant Species	Anti-oxidant enzyme	References
Drought	*Oryza sativa*	SOD, GPX, APX, MDHAR, DHAR,GR	Sharma and Dubey, 2005
	Beta vulgaris	SOD, CAT, GPX	Sayfzadeh and Rashidi, 2011
	Triticum aestivum	SOD, APX, GR	Sairam *et al.*,1998
	Oryza sativa	SOD, CAT, GPX, APX, GR	Mishra *et al.*, 2013
Salinity	*Olea europaea*	CAT, SOD, GR	Valderrama *et al.*, 2006
	Oryza sativa	GPX	Mittal and Dubey, 1991
Chilling	*Zea mays*	APX, MDHAR, DHAR, GR SOD	Fryer *et al.*, 1998
	Fragaria X ananassa	APX, MDHAR, DHAR, GR SOD	Zhang *et al.*, 2008
Aluminum	*Oryza sativa*	SOD, GPX, APX	Sharma and Dubey, 2007
	Glycine max	SOD, GPX, APX	Cakmak and Horst, 1991
Nickel	*Oryza sativa*	SOD, GPX, APX	Maheshwari and Dubey, 2009
Arsenic	*Oryza sativa*	SOD, GPX, APX	Mishra *et al*, 2011
Manganese	*Oryza sativa*	SOD, GPX, APX, GR	Srivastava and Dubey, 2011
UV-B	*Picea asperata*	SOD, APX, CAT, GPX	Han *et al.*, 2009
	Arabidopsis thaliana	GPX, APX	Rao *et al.*, 1996
Pathogen			
Odium lini	*Linum usitatissimum*	GPX, CAT	Ashry and Mohamed, 2012
Bean yellow mosaic virus	*Vicia faba*	POD, CAT, APX, SOD	Radwan *et al.*, 2010

2.5 Biotechnological Approaches for Developing Oxidative Stress Tolerant Crops: Transgenics Over-expressing AsA-GSH Pathway Genes

The defensive action of the various enzymes of AsA-GSH pathway has been explored by transgenic approaches primarily by over-expressing them and relating their expression to the degree of stress tolerance conferred to the plant. SOD gene was used to transform plants for the improvement of abiotic stress tolerance in a number of cases. Van Camp *et al.* transferred MnSOD genc from tobacco into clover (Van Camp *et al.*, 1994). The transgenic plants showed a significant increase in growth, vigor and yield under drought conditions. Meanwhile, oxidative stress resistance under drought stress was reported to significantly increase in transgenic tobacco over-expressing a CuZnSOD gene from rice (Badawi *et al.*, 2004a). Over-expression of tobacco MnSOD gene in maize chloroplast was found to decrease the oxidative stress in leaves (Frank *et al.*, 1999). Du *et al.* reported that over-expression of MnSOD could alleviate oxidative stress in maize (Du *et*

al., 2006). Similarly, expression of cytosolic APX gene (cAPX) in tomato improved tolerance of the transgenic plants to direct sunlight exposure under field conditions (Wang *et al.*, 2006) and expression of *katE* gene in rice resulted in improved growth and yield under salt stress conditions (Nagamiya *et al.*, 2007). The numerous studies reporting the production of stress tolerant transgenic plants over-expressing different anti-oxidant enzymes have been summarized in Table 2.4.

The outcome of the over-expression of genes depends on number of factors including the the source of the gene and the type of promoter used. In many cases, simultaneous over-expression of more than one transgenes has been reported to give better results. All these factors are hereby discussed in brief.

2.5.1 Role of Source Organisms: Importance of Introduction of Genes from Stress Adapted Species

One important strategy for strengthening the ability of plants to resist the abiotic stresses is the introduction of genes from stress-adapted species such as desert and halo-tolerant plants. The over-expression of proteins isolated from stress tolerant plants in the crop plants may possibly give the later the necessary additive advantages and enable them to resist stress conditions better than the non-modified parental plant. The large number of plant genome sequencing projects, as well as the sequencing projects of other organisms from extreme environments, could generate a rich database of genes that can be used for the manipulation of crops for increasing their tolerance to abiotic stresses (Mitler and Blumwald, 2010).

2.5.1.1 *Pennisetum* – A Stress Adapted Food Crop

Pearl millet (*Pennisetum glaucum* L.) is the fourth most important cereal crop in India, after rice, wheat and sorghum. Although, pearl millet grows best on well-drained light sandy soils, it can withstand water limited conditions relatively well compared to other crops like sorghum and maize (Kholová, 2010; Burton, 1983). Therefore, it is considered as a drought tolerant crop. It can also tolerate high ambient temperature, low soil fertility, low soil pH and high concentration of aluminium (National Research Council, 1996). It is considered more efficient in utilization of soil moisture and has a higher level of heat

tolerance than sorghum and maize (FAO, 2010). Therefore, the stress-adapted pearl millet plant can be used for isolation of various stress responsive genes.

2.5.2 Role of Promoters

Most of the genes engineered into crops in order to improve abiotic stress tolerance are driven by constitutive promoters, the most common promoter used for manipulation of gene expression being Cauliflower mosaic virus 35S (CAMV35S; Odell *et al.*, 1985), Ubiquitin (UBI1; Holtorf *et al.*, 1995) or actin (McElroy *et al.*, 1990). Although these promoters have been effective in production of transgenic plants with increased stress tolerance, the constitutive expression of the transgenes is not often desirable because of negative pleiotropic effects on growth and development of plants under control conditions (Ori *et al.*, 1999; Hsieh *et al.*, 2002). Moreover, the use of strong constitutive promoters could accelerate the process of trangene silencing (Dietz-Pfeilstetter, 2010). A solution to this problem is the use of stress inducible promoters that results in expression of the transgenes only under the stress conditions (Peleg *et al.*, 2011; Rivero *et al.*, 2007, 2010; Moller *et al.*, 2009). In case of multi- gene manipulation the co-ordination of expression of the transgenes needs to be considered. The T7 based expression system has recently emerged as a probable strategy facilitating co-ordinated and high expression of the transgenes in various plants (Nguyen *et al.*, 2004).

2.5.3 Number of Transgenes: Advantages of Multigene Transformations

Most metabolic processes which serve as targets for genetic manipulation depend on the interaction between a number of genes, and flux through biochemical pathways is often co-ordinated with that of competing pathways; therefore, the most effective strategy for enhancing tolerance of plants to any stress can be by controlling multiple genes on the same, or interconnected, pathways. Multi-gene transformations have been successfully used in order to increase the oxidative stress tolerance of plants. Pyramiding of anti-oxidant enzymes has been done and the transgenic plants so obtained exhibit enhanced tolerance as compared to the over-expression of single enzymes (Table 2.4). Transgenic tobacco plants over-expressing CuZnSOD (Gupta *et al.*, 1993), after retransformation with the chloroplastic APX, showed enhanced tolerance to paraquat (Kwon *et al.*, 2002).

This double transgenic plant when transformed with chloroplastic DHAR gene showed further enhanced tolerance to oxidative stress (Lee *et al.*, 2007). Likewise, transgenic tobacco plants developed through *in vitro* pyramiding of cytosolic CuZnSOD and APX showed enhanced tolerance to drought stress (Faize *et al.*, 2011). The pyramiding of CuZnSOD and chloroplastic APX in potato (Tang *et al.*, 2006) and in sweet potato (Lim *et al.*, 2007) enhanced the tolerance of the transgenic plants to chilling, high temperature and paraquat. Transgenic tobacco over-expressing GST (from cotton) and GPX (from *Chlamydomonas*) showed enhanced resistance to paraquat as well as chilling (Yu et al., 2003; Yoshimura *et al.*, 2004). Furthermore, gene pyramiding with double transgenic plants over-expressing both GST and GPX enhanced the seedlings growth during chilling and salt stress (Roxas *et al.*, 1997).

Table 2.4 List of transgenic plants over-expressing different anti-oxidant enzymes (Modified after Gill and Tuteja, 2010).

Gene	Source	Target transgenic	Reference
Single gene transgenics			
CuZnSOD	*Oryza sativa*	*Nicotiana tabacum*	Badawi *et al.*, 2004
CuZnSOD	*Avicennia marina*	*Oryza sativa*	Prasanth *et al.*, 2008
MnSOD	*Nicotiana plumbaginifolia*	*Triticum aestivum protoplast*	Melchiorre *et al.*, 2009
MnSOD	*Tamarix androssowii*	*Populus davidiana x P. bollean*	Wang *et al.*, 2010
MnSOD	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Wang *et al.*, 2004
CAT	*Triticum aestivum*	*Oryza sativa*	Matsumura *et al.*, 2002
CAT3	*Brassica juncea*	*Nicotiana tabacum*	Guan *et al.*, 2009
katE	*Escherichia coli*	*Nicotiana tabacum* '	Al-Taweel *et al.*, 2007
cAPX	*Pisum sativum*	*Lycopersicon esculentum*	Wang *et al.*, 2005, 2006
APX3	*Arabidopsis thaliana*	*Nicotiana tabacum*	Yan *et al.*, 2003, Badawi *et al.*, 2004b
APX1	*Hordeum vulgare*	*Arabidopsis thaliana*	Xu *et al.*, 2008
GR	*Escherichia coli*	*Triticum aestivum protoplast*	Melchiorre *et al.*, 2009
GR	*Arabidopsis thaliana*	*Gossypium hirsutum*	Kornyeyev *et al.*, 2003, Mahan *et al.*, 2009, Logan *et al.*, 2003
MDAR1	*Arabidopsis thaliana*	*Nicotiana tabacum*	Eltayeb *et al.*, 2007
DHAR	*Arabidopsis thaliana*	*Nicotiana tabacum*	Eltayeb *et al.*, 2006
DHAR	*Arabidopsis thaliana*	*Nicotiana tabacum*	Ushimaru *et al.*, 2006
DHAR	*Oryza sativa*	*Arabidopsis thaliana*	Chen *et al.*, 2005
DHAR	*Homo sapiens*	*Nicotiana tabacum*	Kwon *et al.*, 2003
GST	*Suaeda salsa*	*Oryza sativa*	Zhao and Zhang, 2006
GPX	*Chlamydomonas*	*Nicotiana tabacum*	Yoshimura *et al.*, 2004
GPX-2	*Synechocystis*	*Arabidopsis thaliana*	Gaber *et al.*, 2006
Multiple genes- transgenic			
MnSOD + APX	*Nicotiana tabacum*	*Festuca arundinacea*	Lee *et al.*, 2007
MnSOD +CAT	*Escherichia coli*	*Brassica campestris*	Tseng *et al.*, 2007
GST + GPX	*Nicotiana tabacum*	*Nicotiana tabacum*	Roxas *et al.*, 2000
CuZnSOD+ APX and ***DHAR***	*Arabidopsis thaliana*	*Nicotiana tabacum*	Lee *et al.*, 2007
MnSOD+FeSOD	*Nicotiana tabacum*	*Medicago sativa*	Rubio *et al.*, 2002

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Materials and Chemicals

The plant materials, bacterial strains, vectors, enzymes, chemicals, etc. used for the gene isolation, cloning, protein expression and purification, plant transformation, transgenic screening and evaluation, and their sources are listed in Table 3.1.

3.2 DNA Vectors

The vectors used in the cloning and transformation studies are listed below.

3.2.1 pCR 4.0 TOPO

This vector was used for cloning of the PCR amplified genes. It has bacterial selectable marker genes for kanamycin and ampicillin.

3.2.2 pET-28a

This bacterial expression vector was used for the expression of Ascorbate-Glutathione (AsA-GSH) pathway genes. It has kanamycin gene for bacterial selection.

3.2.3 pET-14b

This vector was used for construction of the T7 gene cassettes.

3.2.4 Gateway cloning vectors

Gateway cloning vector system comprises of a destination vector and two types of entry vectors.

3.2.4.1 Entry Vectors

Entry vectors allow restriction based cloning of the gene of interest into a vector for entry into the Gateway® System (Invitrogen). Two types of entry vectors were used: Entry vector 1 (pL12R34H-Ap) having ampicillin and gentamycin selection marker and Entry vector 2 (pL34R12H-CmR-ccdB) having chloramphenicol and gentamycin as selection marker

3.2.4.2 Destination vector

The destination vector used in this study was pMDC99 having kanamycin as a bacterial selection marker and hygromycin as the plant selection marker.

Table 3.1 List of materials used and their sources.

	MATERIALS	SOURCES
1	**Plant Material**	
i	*Pennisetum glaucum*	ICRISAT, Hyderabad
ii	*Oryza sativa* L IR64	IARI, New Delhi
iii	*Oryza sativa* L Swarna (MTU-5072)	DRR, Hyderabad
2	**Bacterial Strains**	
i	*Escherichia coli* (DH5α)	Invitrogen Life Technologies, USA
ii	*Escherichia coli* (Top10)	New England Biolabs
iii	*Escherichia coli* (BL21{DE3})	Novagen
iv	*Rossetta* 2 (DE3) PLyses	Novagen
v	*Agrobacterium tumefaciens* (EHA105)	Stratagene
3	**Cloning Vectors**	
i	pCR-TOPO	Invitrogen Life Technologies, USA
ii	pET28a(+), pET14b(+)	Invitrogen Life Technologies, USA
iii	pMDC99 (Plant Transformation Vector)	Novagen, Germany, ABRC
iv	Entry vector 1 and 2	Xue-chen Wang lab, China
4	**Markers**	
i	1kb DNA Ladder,	Invitrogen Life Technologies, USA
ii	Protein markers	Fermantas, GmbH, Germany Biorad, USA
5	**Antibiotics**	
i	Kanamycin, Ampicillin, Streptomycin, Rifampicin, Hygromycin, Cefatoxime, Chloramphenicol, carbenicillin	Sigma Chemical Company, St. Louis, USA
6	**Restriction Endonuclease and other Enzymes**	
i	Restriction enzymes	New England Biolabs Inc. MA, USA
ii	LR Clonase	Invitrogen Life Technologies, USA
	Klenow, T4 DNA ligase	Roche applied science, Germany
iii	RNase A	Promega
iv	Pfu DNA Polymerase	Fermentas
7	**Membrane and Filter Papers**	
i	Nylon membrane, nitrocellulose,	Amersham Biosciences, UK
	3mm Whatman sheet	Whatman Co., USA
8	**Antibody**	
i	Secondary antibody (anti-rabbit IgG)	Sigma Chemical Company, St. Louis, USA
9	**Media and Hormones**	
i	MS medium, Hoagland's medium, YEM medium	Duchefa Biochemie,Netherland; Himedia, Bombay;
	2,4-D, 6-BAP, NAA, Zeatin, TDZ	Sigma, St. Louis, USA
10	**Others**	
i	Primer	IDT, Belgium
ii	X-ray films	Kodak, Amersham Biosciences, UK
iii	Radioactive material	Perkin-Elmer, USA
iv	Agarose and PAGE Gel electrophoresis	BIO-RAD
v	DNA Sequencing	Macrogen (South Korea)
11	**General Chemicals**	
i	Buffers and Solutions	Sigma Chemical Company, St. Louis, USA; USB (Amersham International plc.), Buckinghamshire, UK; Amersham Biosciences, UK; BIO-RAD, USA; Boehringer Manheim, GmbH, Germany; Promega Life Science, Madison, WI, USA

The maps of the vectors used for cloning are shown in Fig. 3.1 and 3.2.

3.3 Sterilization Procedure

All glass wares, bacterial culture media, tissue culture tools and tissue culture media were sterilized by autoclaving at 120 °C under 15 lb psi pressure for 15 min. The antibiotics and other heat labile components were filter sterilized with an autoclaved cellulose nitrate filter of 0.22 µM pore size (Millipore).

3.4 *In silico* Analysis of DNA and Protein Sequences

3.4.1 BLAST Search

Similarity search of the ESTs, DNA and protein sequences was performed against different DNA and protein related databases (NCBI, PDB, Swissport, TAIR, Rice Genome Annotation at MSU) using BLASTN and BLASTX programmes respectively (Altschul *et al.*, 1990). The similarity and identity percentage among DNA and protein sequences were obtained with the help of Mac Vector software (Accelerys, GmbH, Germany).

3.4.2 Multiple Alignments

Though most of the DNA and protein alignments were performed using MAC Vector and CLC Free Workbench software package, some DNA and protein sequences were aligned using ClustalW version 2.0 program (Larkin *et al.*, 2007) at EMBL. The phylogenetic trees were constructed by using the UPGMA algorithm available in Mac Vector.

3.4.3 Restriction Analysis of DNA

Restriction map of a given DNA fragment was prepared by using Mac Vector to identify the restriction sites in the gene for further cloning into different vectors or for the creation of restriction sites in primers.

3.4.4 Domain Search

The conserved domain searches among different protein sequences were performed at many protein related databases like Pfam and CD- search (NCBI). The fingerprints in the protein sequences were identified by scanning PRINTS database (Attwood *et al.*, 2003) with the protein sequence.

3.4.5 Primer designing

Most of the primers were designed by using Mac Vector (Acceleris, GmbH). For quantitative real-time PCR analyses, the primers were designed with the help of web-based primer design software, Primer 3 at http://frodo.wi.mit.edu/primer3/ using customized parameters. Most of the primers were synthesized by IDT and Sigma-Aldrich, India.

3.4.6 *In silico* Promoter Analysis

In order to identify putative conserved plant *cis*-acting regulatory elements the promoter sequences were analyzed using PLACE (Higo *et al.*, 1999), plantCARE (Lescot *et al.*, 2002) databases and motifs reported in the literature.

3.5 Plant Growth Conditions

P. glaucum seedlings were grown on wet germination paper under a 14/10 h light/dark cycle at room temperature (30±2 °C) for two weeks. After two weeks the leaves were quickly frozen in liquid nitrogen before protein isolation.

Rice seeds (*Oryza sativa*. L, cv Swarna) were sterilized and germinated on soil in green house at 28 °C under 14 h light/10 h dark cycle until flowering .Developing panicles were harvested just before emergence and were dissected away from the tightly rolled leaf sheath and were quickly frozen in liquid nitrogen before total RNA isolation. Spikelets were separated into anthers, carpels, and paleas/lemmas under a dissecting microscope. Leaves and roots were collected from two-week-old seedlings. All the parts were individually frozen in liquid nitrogen before RNA isolation.

3.6 Bacterial Growth Conditions

Different strains of *E. coli* were incubated and cultured either in LB broth with continuous shaking at 220 rpm or on the LB agar at 37 °C (Table 3.2). *Agrobacterium* cells were incubated in YEM medium (Table 3.3) with continuous shaking at 220 rpm or in the YEM agar at 28 °C. These bacterial strains were incubated and selected with appropriate antibiotics (Table 3.4).

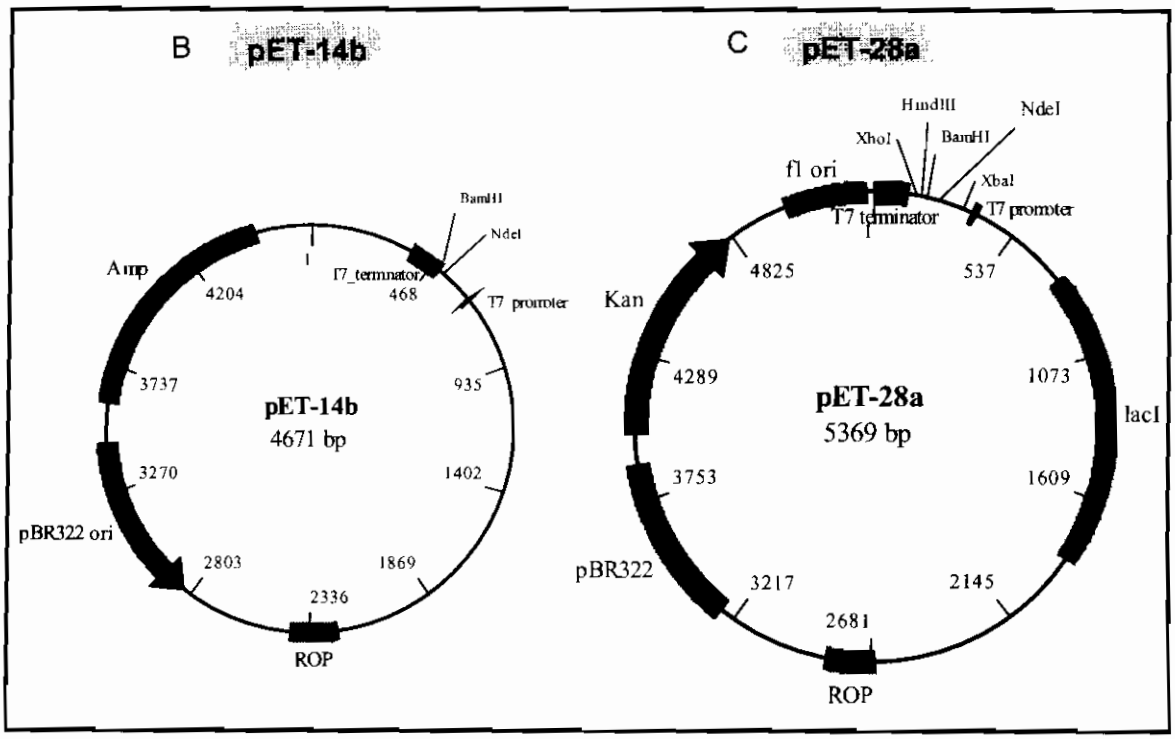
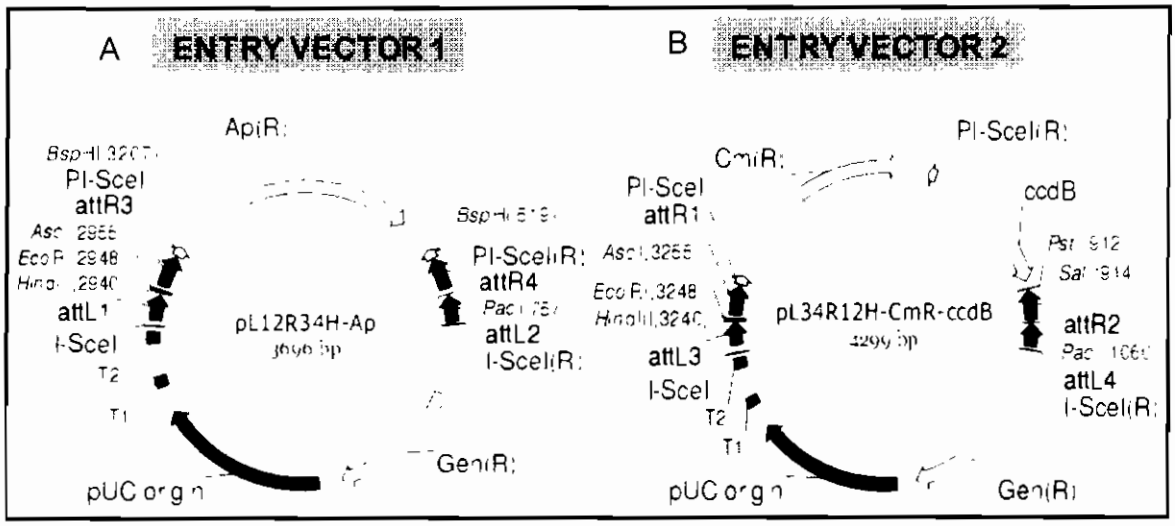


Figure 3.1 Different vectors used for cloning. **(A)** Vector diagram of TA cloning vector pCR4-TOPO. **(B)** Vector diagram of expression vector pET-14b. **(C)** Vector diagram of expression vector pET-28a.



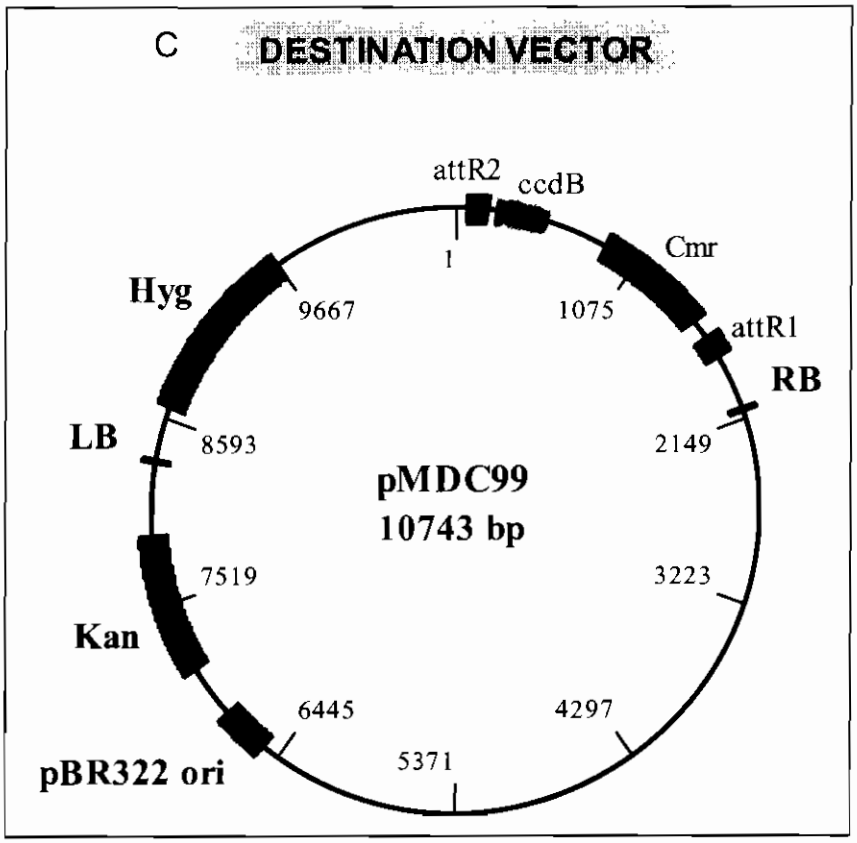


Figure 3.2 Vectors used for gateway cloning. **(A)** Vector diagram of Entry vector 1 (EV1). **(B)** Vector diagram of Entry vector 2 (EV2). **(C)** Vector diagram of destination vector pMDC99.

Table 3.2 Composition of LB media.

Component	g/100 ml
Tryptone	1
Yeast extract	0.5
NaCl	1
The above components were mixed and dissolved in 80ml distilled water, pH was adjusted to 7.0, and volume was made upto 100 ml. For preparation of solid medium 1.5% agar was added.	

Table 3.3 Composition of YEM media.

Component	g/100 ml
Yeast Extract	0.04
Mannitol,	1.00
NaCl	0.01
$MgSO_4.7H_2O$	0.02
K_2HPO_4	0.05
The above components were mixed and dissolved in 80ml distilled water, pH was adjusted to 7.0, and volume was made upto 100 ml. For preparation of solid medium 1.5% agar was added.	

Table 3.4 Concentrations of the antibiotics used in this study.

Antibiotic	Concentration
Kanamycin	50 µg/ml
Streptomycin	50 µg/ml
Chloramphenicol	50 µg/ml
Rifampicin	15 µg/ml
Ampicillin	100 µg/ml
Hygromycin	50 µg/ml
Cefotaxime	250 µg/ml
Carbenicillin	250 µg/ml

3.7 Preparation of Bacterial Competent Cells and Transformation

The *Escherichia coli* and *Agrobacterium* competent cells were prepared according to a procedure of Hanahan (Hanahan and Meselson, 1991) and Hofgen (Hofgen and Willmitzer, 1988) respectively. The competent cells can be used for many standard molecular biology applications.

3.7.1 Preparation of *E. coli* Competent Cells

A single colony of freshly streaked DH5α cells was inoculated in 5 ml of LB broth and grown overnight at 37 °C. Overnight grown culture was inoculated and grown till A_{600} ~0.5. The culture was incubated on ice for 10-15 min. All further steps were

carried out under sterile conditions. The culture was divided into two pre-chilled 50 ml centrifuge tubes and centrifuged at 5000 rpm for 10 min at 4 °C. To the pellet, 1.5 ml of chilled 100 mM $CaCl_2$ was added and centrifuged at 5000 rpm for 5 min at 4°C. The pellet was resuspended in 12.5 ml of $CaCl_2$ and incubated on ice for 1 h. Cells were pelleted by centrifugation at 4000 rpm for 5 min at 4°C. To the pellet, 6 ml of 100 mM $CaCl_2$ was added and incubated on ice for 30 min. The cells were centrifuged at 4000 rpm for 5 min at 4°C and the pellet re-suspended in 3 ml of 13 % glycerol (1.3 ml glycerol + 5 ml 100 mM $CaCl_2$ + volume made to 10 ml with sterile water). The cells were pooled in one tube, mixed gently and divided into small aliquots of 100 - 200 µl each. Aliquots were frozen in liquid nitrogen immediately and stored in -70°C.

3.7.2 Transformation of *E. coli* Cells

Competent cells (DH5α or BL21) were thawed on ice. Ligation mix or plasmid DNA (0.2-1 µg) to be transformed was added to the cells, mixed well with pipette and incubated on ice for 30 min. The cells were subjected to heat pulse at 42 °C for 90 sec and immediately chilled on ice. Cells were diluted 10 fold with liquid LB and grown at 37°C, 200 rpm for 60 min for cell revival. Cells were then pelleted by centrifugation at 5000 rpm for 5 min, resuspended in 100-200 µl volume of LB and plated on LB plates with appropriate antibiotic selection. The plates were incubated in a 37°C incubator for 12-14 h.

3.7.3 Preparation of *Agrobacterium* Electro-competent Cells

A starter culture of *A. tumefaciens* strain was raised in 5 ml liquid YEM medium containing rifampicin (15 mg/L). *Agrobacterium cells* were cultured for 48 h at 28 °C on an orbital shaker maintained at 220 rpm. 1 ml of starter culture was inoculated into 100 ml of fresh YEM medium containing rifampicin (15 mg/L) and the culture was allowed to grow to log phase ($O.D_{600}$ 0.4 - 0.6). The log phase culture was chilled on ice for 30 min and centrifuged at 5000 rpm for 15 min at 4 °C. The pellet was suspended in equal volumes of ice-cold sterile double distilled water and centrifuged at 5000 rpm for 5 min at 4 °C. The pellet was resuspended in equal volume of 0.1 mM HEPES (pH 7.0) and incubated on ice for 1 h before centrifuging at 5000 rpm for 5 min at 4 °C. The obtained bacterial pellet was suspended in equal volume of 0.1mM HEPES (pH 7.0) and 10% ice-cold glycerol and centrifuged at 7000 rpm for 10 min at 4 °C. Finally the pellet was suspended in 300 µl of 10% glycerol and aliquots of 40 µl were made and stored at -70 °C till further use.

3.7.4 Electro-transformation of *Agrobacterium*

Transformation was initiated by adding 10 ng of plasmid DNA to the frozen *Agrobacterium* competent cells. The suspension was carefully mixed with a pipette tip and transferred to a pre-chilled 2 mm cuvette. Electroporation was carried out at 2.5 KV, 25 µF capacitance and 201 Ω resistance for nearly 5 ms. For all the successive electroporations, a pulse time of 5.25 ms was recorded. Immediately after electroporation, 1 ml of fresh LB medium was added and the bacterial suspension was incubated at 28 °C with shaking for 2-3 h. After 2-3 h of incubation, 50 µl of the transformed cells were then plated on LB semi-solid plates with respective antibiotics for selection of transformed clones, and the plates were incubated at 28 °C for 48 h. Single colonies were picked up from the plate and incubated for plasmid mini preparation.

3.8 Nucleic Acid Isolation

3.8.1 Small-Scale Isolation and Purification of Plasmid DNA

Bacterial cell culture (5ml) grown overnight was harvested by centrifugation at maximum speed (13,000 rpm) for 30s. Pellet was re-suspended in 100 µl of ice-cold solution I (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) and vortexed vigorously. The cells were lysed by adding 200 µl freshly prepared solution II (200 mM NaOH, 1% SDS) and gently mixed by inversion. To this, 150 µl of chilled solution III (3.0 M potassium acetate, pH 5.5) was added, mixed and incubated on ice for 3-5 min. Supernatant was collected by centrifugation at maximum speed for 5 min at 4 °C and extracted once with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). DNA was precipitated by adding 0.7 volumes of isopropanol and pellet recovered by centrifugation at 12, 000 rpm for 5 min. Plasmid pellet was washed with 70% ethanol, air-dried and dissolved in TE buffer.

3.8.2 Isolation of Plasmid DNA by Midi Preparation

Plasmid DNA was isolated by alkaline lysis method as described by Sambrook *et al.*, (1989) with modifications. Bacterial cells 100 ml were grown overnight in LB medium with either 100 µg/ml ampicillin or 50 µg/ml kanamycin depending upon the selection marker of the vector. Cells were then harvested by centrifugation at 5000 rpm for 10 min at 4°C. The pellet was re-suspended in 10 ml of solution I (50 mM

glucose, 25 mM Tris-HCl, pH 8.0 and 10 mM EDTA). Subsequently, 20 ml of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added, mixed by inversion and incubated at room temperature for 10 min. 15 ml of ice cold solution III (prepared by adding 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of H_2O) was added, mixed thoroughly and incubated on ice for 10 min. The mixture was centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was filtered through two layers of mira cloth into a fresh tube. Plasmid DNA was precipitated by adding 0.6 volumes of iso-propanol and incubated at room temperature for 30 min. DNA was recovered by centrifugation at 10,000 rpm for 15 min. The DNA pellet was air-dried and dissolved in 5 ml of TE. To this 0.96 g ammonium acetate was added (final concentration of 2.5 M), mixed and incubated at -20 °C for 30 min. Supernatant was collected after centrifugation at 10,000 rpm at 4 °C for 10 min and DNA was precipitated by adding $1/10^{th}$ volume of 3 M NaOAc, 2.5 volumes of chilled ethanol and incubated at -70 °C for 1 h. After centrifugation at 12,000 rpm for 15 min at 4 °C, DNA pellet was dissolved in 4 ml of H_2O and treated with RNase A (20 µg/ml) at 37 °C for 30 min. After the RNase A treatment, phenol-chloroform extraction was performed and DNA was re precipitated with ethanol and sodium acetate as described above and eventually at -70 °C for 1h. After centrifugation at 12,000 rpm for 15 min at 4 °C, DNA pellet was dissolved in 2 ml of water and OD was taken to measure the concentration.

3.8.3 Isolation of Plant Genomic DNA

Rice genomic DNA was isolated from young leaves following the cetyl-trimethyl-ammonium bromide (CTAB) protocol described by Doyle and Dickson (1987). Briefly, 1g of tissue was frozen in liquid nitrogen, ground into a powder and resuspended in preheated CTAB extraction buffer (4% CTAB, 1.4 M NaCl, 100 mM Tris–HCl (pH 8.0), and 20 mM EDTA) plus 2% β-mercaptoethanol. Approximately, 10 ml of preheated extraction buffer was added to 1g of finely powdered plant tissue and the suspension was incubated at 65 °C for 30 min. The lysate was centrifuged at 12000 rpm for 20 min at room temperature. An equal volume of a tris-phenol and Chloroform: Isoamyl alcohol (24:1) was added and mixed properly. The aqueous phase was separated by centrifugation at 12000 rpm for 5 min at room temperature. The extraction step was repeated till a clear inter phase was obtained following which

DNA was precipitated by addition of 2 volumes of 100% ethanol to the aqueous phase. The pellet obtained by centrifugation was air-dried and resuspended in Tris EDTA (pH 8.0) or milli Q water.

3.8.4 Isolation of Total RNA

Total RNA was isolated according to the protocol described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Liquid nitrogen frozen plant tissue (5 g) was ground to a fine powder in liquid nitrogen using a mortar and pestle. The powdered tissue was mixed with 5 ml of denaturation buffer (4 M guanidium thiocyanate in 42 mM sodium citrate, 0.83% N-lauryl sarcosine and 0.2 mM β-mercaptoethanol), mixed thoroughly with intermittent vortex till a clear suspension was obtained. The lysate was then centrifuged at 10,000 rpm for 10 min at 4 °C and the aqueous phase was collected in a fresh tube. Then Phenol: chloroform: isoamyl alcohol extraction step was repeated three to four times till a clear interface was obtained. After that, a single step of chloroform: isoamyl alcohol (24:1) extraction was done. To the aqueous phase, an equal volume of isopropanol was added, mixed and kept for 30 min at -20 °C. RNA was then pelleted down by centrifugation at 10,000 rpm for 15 min at 4 °C followed by 70% ethanol wash, air-dried, dissolved in 4 ml DEPC treated water. After dissolving the pellet, 1 ml of 10 N LiCl was added to a final concentration of 2 N and kept overnight at 4 °C. RNA was pelleted down at 10,000 rpm for 10 min (supernatant containing genomic DNA and carbohydrate was discarded). Pellet was air-dried and dissolved in 3 ml of DEPC treated water. RNA was re-precipitated by adding 1/10 volume of 3 M sodium acetate and 2.5 volume of ethanol and incubated overnight at -20 °C. RNA was collected by centrifugation, washed with 70% ethanol and stored in 80% ethanol at -80 °C till further use.

3.8.4.1 Integrity Test of Total RNA

The integrity of the prepared RNA to be used for subsequent cDNA synthesis was confirmed by checking for the presence of 28S rRNA and 18S rRNA on agarose gel electrophoresis. Total RNA (2 µg) was mixed with appropriate amount of RNA loading buffer and incubated at 65 °C in water bath for 15 min followed by cooling in ice for 5 min. This was mixed with 2 µl of RNA loading dye and loaded onto 1% formaldehyde agarose gel made in 1X MOPS buffer. The gel was run at 5 volt/cm for 45 min in electrophoresis unit (Bio-Rad, USA). After completion of electrophoresis the gel was taken in an RNAse free container and washed thoroughly with DEPC

water and stained with ethidium bromide (10µg/ml) The gel was photographed using gel documentation system (Alphaimager EP, Innotech).

3.8.5 Isolation of mRNA from Total RNA

The mRNA was isolated by magnetic separation after annealing with biotinylated oligo-dT primer and immobilizing it onto streptavidin-linked paramagnetic beads. Approximately 250 µg of total RNA was dissolved in DEPC treated water in micro centrifuge tube in a final 600 µl reaction volume to that add 6 µl of 1 M Tris, pH 7.5 (final conc. 10 mM), 100 µl of 3 M KCl (final conc. 0.5 M) and 1 µg of 20mer biotinylated oligo-dT was added to it. RNA was heated to 65 °C for 5 min to remove all secondary structures followed by 10 min incubation at 37 °C for primer annealing. Meanwhile 100 µl of Streptavidin magnetic beads suspension (from Roche) was washed twice with 10 mM Tris pH 7.5 containing 0.5 M KCl (made with DEPC water). Washed streptavidin magnetic beads suspension was added to RNA oligo-dT containing solution and kept at room temperature for 10 min with gentle intermediate mixingin order to prevent the beads from settling. mRNA along with streptavidin beads was pulled out with the help of magnetic separator (Promega). RNA solution (without mRNA) was removed and streptavidin beads (attached to mRNA) were washed two to three times with 500 µl of 10 mM Tris containing 0.5 M KCl to wash out any contaminating unbound RNA adhered to the bead surface. Washed beads were suspended in 100 µl of DEPC treated water and incubated for 5 min at 65 °C in water bath to denature the hybridization between oligo-dT and mRNA. Streptavidin beads were quickly separated out with magnetic separator and solution containing mRNA was collected. This process was repeated twice. Solution containing mRNA was given a short spin to remove any residual trace of beads and precipitated by adding 1/10 volume of 3 M sodium acetate and 2.5 volume of ethanol and incubated overnight at -20°C. The mRNA was collected after centrifugation, washed with 70% ethanol and dissolved in appropriate amount of DEPC water for cDNA synthesis.

3.8.6 First Strand cDNA Synthesis

mRNA (5 µg) was taken and first strand cDNA synthesis was carried out using Invitrogen Superscript III First Strand cDNA Synthesis Kit according to the manufacturer's instructions. After the first strand synthesis the reaction was terminated by heat inactivation at 70 °C for 15 min followed by addition of 4 units of

RNase H (Invitrogen) to remove mRNA. cDNA was purified from excess primer using Qiagen PCR purification kit according to manufacturer's protocol.

3.9 Spectrophotometric Estimation of Nucleic Acids

Quantity and quality of nucleic acids in solution was determined by measuring the absorbance at 260 and 280 nm. The absorbance at 260 nm in a 1-cm quartz cuvette of a 50µg/ml solution of double stranded DNA, 40µg/ml solution of single stranded RNA and 33 µg/ml of single stranded oligonucleotides or primers is equal to 1. Purity of nucleic acid solution was checked by taking the A_{260}/A_{280} ratio.

3.10 DNA restriction analysis

Restriction digestion of the plasmid DNA was carried out using 1.0 µg of plasmid DNA, 5 µl of appropriate 10 X restriction enzyme buffer, 10 Units of restriction enzyme in a 50 µl reaction. The reaction was incubated at 37 °C (or at an appropriate temperature according to the restriction enzyme used) for 1 h and the digestion pattern was analyzed on 1% agarose gel. The band of interest was cut and the fragment is eluted with Qiagen gel purification kit.

3.11 Ligation

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. The ligation reaction was carried out in a total reaction volume of 10 µl containing 50 ng of restriction digested vector DNA, known amount of fragment DNA, 1 µl 10 X ligase buffer, 1 µl (400U) of T4 DNA ligase and sterile double distilled water to make up the volume. The reaction was incubated overnight at 16 °C. After completion of the reaction, an aliquot of 5 µl was used for transformation.

3.12 DNA Purification

3.12.1 Gel Extraction

To the cut pieces of agarose gel containing the desired DNA fragment, 3 volumes of QG buffer, as supplied with Qiaquick gel extraction kit, (Qiagen GmbH, Hilden,

Germany) was added and dissolved by heating at 50 °C for 10 min. The mixture was loaded onto Qiaquick spin column and spun briefly. The flow through was discarded and 0.5 ml of buffer QG was added to QIAquick column and centrifuged for 1 min (This step removes all traces of agarose). Then the column was washed twice with wash buffer PE. The purified DNA fragment was eluted with 50 µl of 10 mM Tris-Cl, 1 mM EDTA pH 8.0.

3.12.2 PCR Purification

For PCR purification, PCR product was mixed with 5 volumes of PB buffer, supplied with Qiagen PCR purification columns and the mixture was loaded onto Qiaquick spin column and spun briefly. The flow through was discarded and the column was washed twice with wash buffer PE. The purified DNA fragment was eluted with 50 µl of 10 mM Tris-Cl, 1 mM EDTA pH 8.0.

3.13 Polymerase Chain Reaction (PCR)

Rapid amplification of the DNA fragments was done using Taq DNA polymerase and a set of convergent primers. 150 ng of forward and reverse primers along with 200 µM of each dNTP and 2.5U of Taq DNA polymerase was used for PCR. The reaction condition for PCR included denaturation (94 °C) for 1 min, primer annealing (55 °C) for 1 min and extension (72 °C) for 1 min per kb of expected product for 30 cycles in 50 µl reaction volume. An aliquot from the mix was run on 1% agarose gel to check the amplification.

3.14 Colony PCR

Putative recombinant clones were identified by colony PCR. Cells from a single colony were suspended in 50 µl of water (after being streaked on a master plate) and boiled for 10 min. The mixture was centrifuged at 15000g for one min and 15 µl of the supernatant was used as template for PCR using a combination of specific forward and reverse primers.

3.15 Cloning and Sequencing of PCR Fragments

The selected DNA fragment(s) that were generated by the nested PCR-amplified products were gel-purified using Qiagen gel extraction columns (Cat. No. 28104) and cloned into a TA cloning vector as per the manufacturer's instructions (Invitrogen, USA). Either the recombinant plasmid and/or the purified PCR products were sequenced using a cycle-sequencing protocol.

3.16 Isolation of Full Length Genes Encoding Enzymes of SOD-AsA-GSH Pathway

The nucleotide sequences of EST clones of the genes *PgSOD*, *PgAPX*, *PgGR*, *PgDHAR* and *PgMDHAR* were retrieved by sequence homology search from the *Pennisetum glaucum* EST database (www.ncbi.nlm.nih.gov, gene bank accession numbers CD724312 - CD726805, Mishra *et al.* 2007). The full length cDNA sequences of the genes were isolated using RACE PCR (unpublished data).The full length genes were amplified using the following set of primers (Table 3.5):

Table 3.5 List of primers used for full length amplification of genes of interest.

S. No	Name of the Primer	Primer Sequence
1	SOD Forward SOD Reverse	5'ATGGTGAAGGCTGTTGCTGTG-3' 5'TCAGCCCTGAAGTCCAATG -3'
2	APX Forward APX Reverse	5'ATGGCGAAGTGCTACCCGAC -3' 5' TTATGCATCAGCGAACCCCAG -3'
3	GR Forward GR Reverse	5'-ATGGCGAGGAAGATGCTCGTC-3' 5'-TTACAAGCTTGTCTTTGGCT-3'
4	DHAR Forward DHAR Reverse	5'-ATGGCCGTGGAGGTGTGCGTGAA-3' 5'-TTACGCGTTCACCTTGGGTGCC-3'
5	MDHAR Forward MDHAR Reverse	5'-TGGCGAGTGAGAAGCACTTCA-3' 5'-TCAGATCTTGCTGGCGAACTG-3'

3.17 Cloning of *PgSOD*, *PgAPX*, *PgGR*, *PgDHAR* and *PgMDHAR* Full Length Genes into pET-28a Expression Vector

For the over-expression of His-tagged proteins, *PgSOD*, *PgAPX*, *PgGR*, *PgMDHAR* and *PgDHAR* were cloned into pET 28a vector (Novagen, USA) at *Nde* I and *BamH I* restriction sites (Table 3.6). The complete coding sequence of the genes were amplified by PCR (150 ng of each primer, 200µM dNTPs, 2.5 U Taq DNA

polymerase and 1x Taq buffer in a 50 µl reaction; 94 °C-1 min; 55 °C-1 min and 72 °C-1 min 30 s; 30 cycles) using total cDNA as template. The amplified products of PgSOD, PgAPX, PgDHAR, PgMDHAR and PgGR was gel purified, digested with the respective restriction enzymes and ligated on to appropriate restriction sites of pET-28a vector. The ligated products were transformed into *E. coli* BL21 (DE3) cells and selected on kanamycin LB agar plate. The clones were examined or screened for the expression of protein of interest with a His-tag at its N-terminal end.

Table 3.6 List of primers used for cloning of SOD, APX, GR, DHAR and MDHAR genes in pET-28a expression vector.

S. No	Name of the Primer	Primer Sequence
1	SOD Forward SOD Reverse	5'-CGAGTCCATATGGTGAAGGCTGTTGCTGTG-3' 5'-CTAGGATCCTCAGCCCTGAAGTCCAATG -3'
2	APX Forward APX Reverse	5'-CGAGTCCATATGGCGAAGTGCTACCCGAC -3' 5'-CTAGGATCCTTATGCATCAGCGAACCCCAG -3'
3	GR Forward GR Reverse	5'-CGAGTCCATATGGCGAGGAAGATGCTCGTC-3' 5'-CTGCTAGGATCCTTACAAGCTTGTCTTTGGCT-3'
4	DHAR forward DHAR reverse	5'-ATTACCATATGGCCGTGGAGGTGTGCGTGAA-3' 5'-ATAAGGATCCTTACGCGTTCACCTTGGGTGCC-3'
5	MDHAR forward MDHAR Reverse	5'-AACGGATCCATGGCGAGTGAGAAGCACTTCA-3' 5'-ACA AAGCTTTCAGATCTTGCTGGCGAACTG-3'

3.18 Purification of His-tagged Recombinant Proteins in Native Condition

The *E. coli* BL21 (DE3) harboring the recombinant pET-28a (+) plasmid, was grown in LB medium to OD_{600} 0.4 and induced with 1 mM IPTG for 3 h at 37 °C. Cells were harvested by centrifugation. Bacterial cell pellet was re suspended in sonication buffer (50 mM Na_2PO_4 [pH. 8.0], 300 mM NaCl, 0.5% Triton X 100, 1 mM each of PMSF and benzamidine). Further processing of the protein was done at 4 °C. Cells were lysed by sonication for 15 min in 30 s pulse with one min gap in between. Cell pellet and clear supernatant were separated by high speed (13,000 rpm, in SS-34 rotor) centrifugation in oakridge tubes at 4°C. An aliquot of pellet and supernatant was taken and analyzed by SDS-PAGE to determine the amount of recombinant protein in soluble (supernatant) or insoluble (pellet) fractions. The recombinant proteins (except PgMDHAR) were present mainly in the soluble fraction and therefore purified from soluble fraction by Ni-NTA affinity chromatography. Supernatant containing the soluble fraction of the recombinant protein was incubated with Ni-NTA agarose (pre-

equilibrated with sonication buffer) for 30 min in presence of 10 mM of imidazole and loaded onto a column. The column was washed with 15-20 volumes of wash buffer containing 50 mM Na_2PO_4 pH. 8.0, 300 mM NaCl, 0.5% Triton X 100, 1 mM each of PMSF and benzamidine, 30 mM of imidazole and 10 mM β-mercaptoethanol and 10% glycerol. The wash fractions were collected in aliquots of 5 ml each. Recombinant proteins were eluted with one step elution from 200 mM to 350 mM imidazole in a buffer containing 50mM Na_2PO_4 pH 8.0, 300 mM NaCl, 5 mM DTT, protease inhibitors mix and 10% glycerol. Purified protein was dialyzed against storage buffer (100mM Na_2PO_4 pH. 8.0, 100 mM NaCl and 30% glycerol) and stored in –80 °C until further use.

3.19 Polyacrylamide Gel Electrophoresis of Proteins

Polyacrylamide gel electrophoresis (PAGE) was performed according to the protocol of Laemmli (1970). Gels were prepared and run either in the absence (native) or presence of SDS (denaturing). The composition of the solutions for preparation of gel is given in table Table 3.7. Protein samples were prepared by mixing with equal volume of 2X sample buffer (50 mM Tris-HCl pH 6.8, 4% SDS (for denaturing gel), 20% glycerol, 4% β-mercaptoethanol (for denaturing gel), 0.05% bromophenol blue). Samples were kept in boiling water for 5 min and loaded on the gel for denaturing condition and loaded without boiling for native condition. Gels were run at 50 V till the proteins was stacked properly and thereafter gels were run at a constant voltage of 100 V. Gels were either electro blotted onto the nitrocellulose membrane (Hybond-C, Amersham, England) or stained with 0.25% Coomassie blue R-250, 50% methanol and 10% acetic acid. The amount of acrylamide solution and buffers required for preparing various percentage of running and stacking polyacrylamide gel is given in Table 3.8 and 3.9.

Table 3.7 Composition of the solutions required for the preparation of PAGE gel.

1	**Acrylamide-bisacrylamide Solution** (30%)		
i	Acrylamide	29.2 g	Final volume was made to 100 ml. Filtered through Whatman No.1 filter paper and stored at 4°C in dark brown bottle.
ii	Bis acrylamide	0.8 g	
iii	Distilled water	70 ml	
2	**1.5 % APS (Ammonium per sulfate)**		
i	APS	150 mg	Total volume was made to 10 ml with distilled water, prepared freshly.
3	**TEMED (N, N, N', N'-tetramethyl ethylene diamine)**		
i	TEMED was undiluted from the bottle. Stored at cool, dry place and protected from light.		
4	**4X Resolving Gel Buffer** (1.5 M Tris-HCl, pH 8.8)		
i	Tris base	18.2 g	Final volume made to 100 ml, filtered and stored at 4 °C.
ii	Distilled water	80 ml	
5	**4X Stacking Gel Buffer** (1 M Tris – HCl, pH 6.8)		
i	Tris	6 g	Final volume made to 100 ml, filtered and stored at 4 °C.
ii	Distilled water	80 ml	
6	**2X Stock Sample Buffer** (0.125 M Tris–HCl, 20% glycerol, 0.05% bromophenol blue, pH 6.8)		
i	0.5 M Tris–HCl buffer (pH 6.8)	2.4 ml	Final volume made to 10 ml and stored at room temperature.
ii	Glycerol	2.0 ml	
iii	0.5% bromophenol blue	1.0 ml	
7	**1X Electrode Buffer** (0.025 M Tris, 0.192 M glycine, pH 8.3)		
i	Tris	3 g	Total volume made to 1000 ml
ii	Glycine	14 g	

Table 3.8 Composition of various resolving gels for Tris-Glycine SDS-PAGE. All units are expressed in ml.

S. No	Resolving Gel	5 ml	10 ml	15 ml	20 ml	25 ml
1	8%					
i	H_2O	2.3	4.6	6.9	9.3	11.5
ii	30% bis acrylamide	1.3	2.7	4.0	5.3	6.7
iii	4X Resolving Gel Buffer	1.3	2.5	3.8	5.0	6.3
iv	10%SDS	0.05	0.1	0.15	0.2	0.25
v	10%APS	0.05	0.1	0.15	0.2	0.25
vi	TEMED	0.003	0.006	0.009	0.012	0.015
2	10%					
i	H_2O	1.9	4.0	5.9	7.9	9.9
ii	30% bis acrylamide	1.7	3.3	5.0	6.7	8.3
iii	4X Resolving Gel Buffer	1.3	2.5	3.8	5.0	6.3
iv	10%SDS	0.05	0.1	0.15	0.2	0.25
v	10%APS	0.05	0.1	0.15	0.2	0.25
vi	TEMED	0.002	0.004	0.006	0.008	0.01
3	12%					
i	H_2O	1.6	3.3	4.9	6.6	8.2
ii	30% bis acrylamide	2.0	4.0	6.0	8.0	10.0
iii	4X Resolving Gel Buffer	1.3	2.5	3.8	5.0	6.3
iv	10%SDS	0.05	0.1	0.15	0.2	0.25
v	10%APS	0.05	0.1	0.15	0.2	0.25
vi	TEMED	0.02	0.004	0.006	0.008	0.01

Table 3.9 Composition of stacking gel for Tris-Glycine SDS-PAGE. All units are expressed in ml.

5%	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml
H_2O	0.68	1.4	2.1	2.7	3.4	4.1
30% bis acrylamide	0.17	0.33	0.5	0.67	0.83	1.0
4X Stacking Gel Buffer	0.13	0.25	0.38	0.5	0.63	0.75
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06
10% APS	0.01	0.02	0.03	0.04	0.05	0.06
TEMED	0.001	0.002	0.003	0.004	0.005	0.006

3.20 In-gel Assay for Antioxidant Enzymes

The activity of the ROS scavenging enzymes was studied using PAGE under non-reduced, non-denatured conditions at 4 °C according to the method suggested by Laemmli (Laemmli, 1970). Specific conditions were maintained for keeping native protein intact.

3.20.1 SOD

For SOD isoenzymes separation (Rucinska *et al.*, 1999) native PAGE was performed using 5% stacking and 11% resolving at 4°C and constant voltage (70 V). 50 µg of protein was loaded. After completing the run, gel was soaked in 2.45 mM NBT for 20 min followed by immersion for 15 min in a solution containing 28 mM TEMED, 3

μM riboflavin and 50 mM potassium phosphate at pH 7.8. The gel was then placed on dry white tray and illuminated for 5 to 15 min. During illumination gel became uniformly blue except at positions containing SOD. Illumination was discontinued when maximum contrast between the achromatic zones and the general blue colour had been achieved. The gel was then photographed.

3.20.2 APX

Native PAGE was performed according to the method of Mittler and Zilinskas (Mittler and Zilinskas, 1993) using 4% stacking and 10% resolving gel. Electrode buffer containing 2 mM ascorbate was prepared and the gel was pre run for 30 min before the samples were loaded. 50 μg of protein was loaded in each well After electrophoresis, the gel was immersed for 30 min in 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM ascorbic acid with a change of the solution every 10 min. The gel was soaked in 50 mM sodium phosphate buffer containing 4 mM ascorbic acid and 2 mM H_2O_2 for an additional 20 min before a brief wash in 50 mM sodium phosphate buffer. Finally, the gel was incubated in 50 mM sodium phosphate buffer, (pH 7.8), 100 mM TEMED and 1 mM NBT until the gel turned uniformly blue except at positions exhibiting APX activity. Upon achieving the best contrast of colourless APX bands the reaction was stopped by rinsing the gel in water.

3.20.3 GR

For measuring GR activity, native PAGE was performed according to the method described by Smith *et al.* (Smith *et al.*, 1988) using 5% stacking and 10% resolving gel at 4 °C and at constant voltage (70 V). 50 μg of protein was loaded in each well. After electrophoresis, the gel was incubated in phosphete buffer (pH 8) containing 2 mM DTNB for 30 min. GR activity staining was performed by incubating the gel in sodium phosphate buffer (pH 8) containing 1.5 mM oxidized glutathione (GSSG) and 0.5 mM NADPH until the appearance of yellow coloured bands. The yellow coloured bands showing the activity of GR were photographed immediately.

3.20.4 DHAR

In-gel enzyme activity staining was done following the method of De Gara *et al.* (De Gara *et al.*, 1994). Native PAGE was performed using 5% stacking and 7.5% separating gels at 4 °C and constant voltage (70 V). After electrophoresis, the gels were incubated under agitation for 50 min in 100 mM sodium phosphate buffer (pH

6.4) containing 2 mM DHA and 4 mM GSH. Gels were then washed with distilled water and stained for DHAR activity by incubating for 15 min in a solution of 0.1 N HCl containing 0.1%(w/v) ferrocyanide and 0.1% ferrichloride (w/v). Proteins representing DHAR activity were observed as dark blue bands on a light blue background the latter due to non-enzymatic AsA formation occurring in the reaction between DHA and GSH.

3.21 Isolation of RA8 Promoter from *Oryza sativa*

Two specific primers with restriction sites were designed as per the available RA8 promoter genomic sequence (Jeon *et al.*, 1999). *Kpn* I and *Nco* I restriction sites were incorporated respectively, in the forward and the reverse primers (Table 3.10). The complete RA8 promoter was amplified by PCR (150 ng of each primer, 200µM dNTPs, 2.5 units Taq DNA polymerase and 1X Taq buffer in a 50 µl reaction; 94 °C 1 min; 55 °C 1 min and 72 °C 1 min; 30 cycles) using *Oryza sativa* genomic DNA as template. The isolated promoter was digested with *Kpn* I and *Nco* I restriction enzyme and cloned in to entry vector 1 (Fig. 3.2A).

Table 3.10 List of primers used for amplifying RA8 promoter.

S. No	Name of the Primer	Primer Sequence
1	pRA8 Forward	5'-TCGGTACCACATTCAGAATCATCTCCAGC-3'
2	pRA8 Reverse	5'-GACCATGGGAGGAGCTGGAAGGAGAAGA-3'

3.22 Isolation and Modification of *T7RNA Polymerase* Gene

The *T7RNA polymerase* gene sequence was retrieved from the www.ncbi.nlm.nih.gov website. The full-length cDNA amplification T7 RNA polymerase gene was performed by using gene specific primers (Table 3.11). The complete T7 RNA polymerase gene was amplified by PCR (150 ng of each primer, 200µM dNTPs, 2.5 U Taq DNA polymerase and 1X Taq buffer in a 50 µl reaction; 94 °C-1 min; 55 °C-1 min and 72 °C-1 min 30 sec; 30 cycles) using the phage DNA as template. After PCR amplification, the full-length inserts were PCR purified and cloned in TA cloning vector.

Table 3.11 List of primers used for amplifying T7 RNA polymerase gene

S. No	Name of the Primer	Primer Sequence
1	T7 RNA Pol Forward	5'-ATGAACACGATTAACATCGCTAA-3
2	T7 RNA Pol Reverse	5'-GATCTGGATTTTAGTACTGGATTTTG-3'

The *T7 RNA polymerase* gene was modified by adding a 35S poly A sequence at the 3' end and SV40 large T antigen Nuclear localisation signal (NLS) sequence at the 5'end. The 35S poly A was amplified from the 35S hygromycin cassette of pGreen Vector and cloned adjacent to *T7 RNA polymerase* gene at *Eco* RI and *Not* I restriction site available downstream of the gene .The 45 bp oligo of SV40 large T antigen NLS was commercially synthesized, and cloned at *Nco* I site available upstream of the gene. The modified *T7 RNA polymerase gene* was sub-cloned at *Nco* I and *Not* I restriction site of EV-1vector containing the RA8 promoter.

3.23 Cloning, Expression and Purification of *T7 RNA polymerase*

Two oligonucleotides were synthesized corresponding to the amino-terminal (5'-AACGGATCCATGGCTCCGCCCAAGAAAAAGCGA-3') and carboxyl-terminal (5'-TATGCGGCCGCCGCGAACGCGAAGTCCGACTCTA-3') regions of the T7 RNA polymerase open reading frame with *Bam* HI and *Not* I restriction sites incorporated respectively into the forward and the reverse primers. The complete coding sequence of the genes was amplified by PCR (150 ng of each primer, 200 µM dNTPs, 2.5 U Taq DNA polymerase and 1X Taq buffer in a 50 µl reaction; 94 °C -1 min; 55 °C -1 min and 72 °C -3 min; 30 cycles) using isolated full length *T7 RNA polymerase* amplicon as a template. The amplified product was digested with *Bam* HI and *Not* I restriction enzyme and cloned into pET-28a vector. The recombinant vector was transformed into protein expression host *E. coli* Rosetta 2 (DE3) pLysS. The T7 RNA polymerase protein was expressed and purified following the protocol as described earlier.

3.24 Preparation of T7 Promoter Regulated Gene Cassettes

To express *PgSOD, PgAPX, PgGR, PgDHAR* and *PgMDHAR* genes under a T7 promoter in rice plant system, the individual cassettes were prepared for each gene having a T7 promoter and a 5' ribulose bisphosphate carboxylase small chain (Rbc S) UTR at the upstream of the gene and a 3' *Rbc S* UTR followed by T7 terminator at the

downstream of the gene. All genes were cloned in a modified pET-14b expression vector (Fig. 3.2B), in which 5'and 3' UTRs of rice *Rbc S* gene was already cloned between T7 promoter and T7 terminator. For ensuring the cytoplasmic localisation of PgMDHAR, the potential peroxisomal targeting sequence (SKI) was deleted. This was performed by designing the reverse primer from the region of the gene upstream of the C-terminal peroxisomal targeting sequence and amplifying the gene using the new set of primers. The *PgSOD, PgAPX, PgGR, PgDHAR* genes were cloned in *Nde* I-*Bam* HI restriction sites of pET-14b vector. A schematic representation of the prepared cassette has been shown in Fig. 3.3.

Figure 3.3 Schematic representation of gene cassette.

3.25 Gateway Cloning

The *in vitro* pyramiding of the genes was done by multi-round gateway technology using LR clonase enzyme. The Gateway technology is a flexible and universal cloning approach based on λ phage site-specific recombination which facilitates the integration of lambda into the *E. coli* chromosome and the switch between the lytic and lysogenic pathways (Ptashne, 1992). Lambda recombination occurs between site-specific attachment (att) sites: attB on the *E. coli* chromosome and attP on the lambda chromosome. The att sites serve as the binding site for recombination proteins and have been well-characterized (Weisberg and Landy, 1983). Lambda recombination is catalyzed by a mixture of enzymes that bind to att sites, bring together the target sites, cleave them, and covalently attach the DNA. Recombination occurs following two pairs of strand exchanges and ligation of the DNAs in a novel form.

The Gateway® Technology uses the lambda recombination system to facilitate transfer of heterologous DNA sequences (flanked by modified att sites) between vectors (Hartley *et al.*, 2000). The LR Reaction facilitates recombination of an attL substrate (entry clone) with an attR substrate (destination vector) to create an attB-containing expression clone. This reaction is catalyzed by LR Clonase™ enzyme mix.

The two basic steps of Gateway cloning technology are:

i. The initial insertion of a DNA fragment into a plasmid (Entry vectors, Fig 3.2A and B) with two flanking recombination sequences called "att L 1" and "att L 2", to develop "Entry clones".

ii. The second step involves the transfer of the gene cassette in the Gateway Entry clone into any Gateway Destination vector (a plasmid that contains Gateway "att R" recombination sequences, Fig. 3.2B) using proprietary enzyme mix, "LR Clonase".

3.25.1 Sub-cloning of Gene Cassettes in Entry Vectors

Two primers were designed from the nucleotide sequence of pET-14b vector backbone for all gene cassettes; the forward primer (5'-CCGGATATAGTTCCTCCTTTCAGC-3') was designed from the region upstream of the T7 promoter whereas the reverse primer (5'-ATATAGGCGCCAGCAACCGCA C-3') was designed from the region downstream of T7 terminator. To clone gene cassettes into entry vectors (Figure 3.2) different restriction sites were introduced in primer for each cassette (Table 3.12). The cassette of each gene was amplified by PCR (150 ng of each primer, 200µM dNTPs, 2.5 U Taq DNA polymerase and 1X Taq buffer in a 50 µl reaction; 94 °C-1 min; 55 °C-1 min and 72 °C-2 min 30 sec; 30 cycles) by using recombinant pET-14b vector harbouring the gene cassettes as template. The *PgSOD* cassette was cloned into entry vector 2 (EV-2-PL34R12-CmR-ccdb) at *EcoR* V and *Hind* III restriction site, *PgAPX* cassette was cloned at *Hind* III and *Cla* I restriction sites of EV-2-PgSOD vector, *PgGR* cassette was cloned to EV-2 in *Apa* I and *Xba* I site, *PgMDHAR* was cloned in *Spe* I and *Xba* I restriction site of EV-1 and *PgDHAR* was cloned in *Xba* I and *Not* I of the EV1–PgMDHAR vector (Table 3.12).

Table 3.12 Details of restriction sites flanking different cassettes cloned in EV1 and EV2.

S. No	Cassette	Genes	Vectors	Flanking Enzymes
1	Cassette 1	*PgMDHAR and PgDHAR*	EV1	*Spe* I/*Not* I
2	Cassette 2	*PgGR*	EV2	*Apa* I/*Xba* I
3	Cassette 3	*RA8-T7 RNA polymerase*	EV1	*Kpn* I/*Sac* I
4	Cassette 4	*PgSOD-PgAPX*	EV2	*EcoR* V/*Cla* I
5	Cassette 5	*bar*	*EV1*(-Amp)	*EcoR* I/*Hind* III

3.25.2 Construction of EV1^{-Amp}

The ampicillin resistance gene in EV1 is flanked by *Bsp* HI restriction site. Therefore, for deleting the ampicillin resistance gene, the vector was digested with *Bsp* HI and then circularised using a ligase.

3.25.3 Pyramiding Of Gene Cassettes and Promoter Cassette in Plant Transformation Vector by Multiple Rounds of LR Recombination Reactions

All the genes in the entry vector were stacked into plant transformation vector-pMDC99 through LR clonase recombination reactions following the instructions given in the manufacturer's manual (Invitrogen, Carlsbad, CA). Multiple rounds of LR reactions were carried out between the pMDC99 vector and entry vectors. The LR reactions between the destination vector (pMDC99) and the entry vectors (EV-1 and EV2) were carried out at 25 °C for 19 h. This LR reaction was terminated by adding 1 µl proteinase K. In case of recombination between pMDC99and EV1, an aliquot (2µl) of the LR product was transformed into *E .coli* DH5α cells using heat shock and selected on LB agar plates supplemented with 50µg/ml kanamycin and 50µg/ml ampicillin and in case of recombination between pMDC99and EV2, 2µl aliquot was transformed into *E .coli* DB3.1 cells and selected on LB agar plate supplemented with 50µg/ml kanamycin and 50µg/ml chloramphenicol. The colonies so obtained were screened for the presence of inserts using screening primers listed in the Table 3.13.

Table 3.13 List of primers used for screening positive clones.

S. No	Name of the Primer	Primer Sequence
1	SOD Forward SOD Reverse	5'-TGCTGTGCTTGCTAGCAGTGAGGG-3' 5'-TCAGCCCTGAAGTCCAATGATCCC-3'
2	APX Forward APX Reverse	5'-GCGAAGTGCTACCCGACCGTCAG -3' 5'-AGTTCAGAGAGCCTGAGGTGGGCC -3'
3	GR Forward GR Reverse	5'-CCCCATCAGCTCTGATTGGCAAGG-3' 5'-CACCTGCAGCTTGCAAGTTCAGCC-3'
4	DHAR forward DHAR reverse	5'-CCGTGGAGGTGTGCGTGAAGGC-3' 5'-CTTGGGTGCCCATCCTGCAATCAG-3'
5	MDHAR forward MDHAR Reverse	5'-TCAGAATGCTGCAAGGCTCCCAGG-3' 5'-CATCGCCAATGGCGTATACTCCAGG-3'
6	T7-pol Forward T7-pol Forward	5'-AGCGTTTAGCTCGCGAACAGTTGG-3' 5'-ACGGCTTAGGAGGAACTACGCAAGG-3

The first round of LR recombination consisted of the recombination reaction between EV1-PgMDHAR-PgDHAR recombinant vector and pMDC99 vector. EV1 containing DHAR and MDHAR cassette and pMDC99 vector were mixed in 2:1 ratio in terms of nanomoles in presence of LR clonase enzyme mix, incubated at 16 °C for 19 h. and transformed in DH5α strain of *E. coli*. The colonies so obtained were screened for the presence of *PgDHAR* and *PgMDHAR* genes by colony PCR using gene specific primers (Table 3.13, Primer No. 4 and 5). The supercoiled recombinant plasmid (pMDC99-PgMDHAR-PgDHAR) was isolated from the positive colonies. The second round of LR reaction constituted the recombination between EV2-PgGR recombinant vector and the pMDC99 vector harbouring *PgMDHAR* and *PgDHAR* genes. Similarly T7RNA polymerase-RA8, PgSOD-PgAPX and bar cassettes were assembled in the destination vector, pMDC99 during the third, fourth and fifth rounds of LR cloning. The recombinant plant transformation vector was mobilised into *Agrobacterium* (EHA105) via electroporation and the transformed cells were selected on YEM agar plate supplemented with 50 µg/ml kanamycin, 50 µg/ml chloramphenicol and 15 µg/ml rifampicin. The positive colonies were screened for the presence of all the genes by PCR using primers listed in Table 3.13.

3.26 Development of Rice Transgenic through *Agrobacterium* Mediated Transformation of Rice Embryogenic Calli

For the development of rice transgenic we followed the rice transformation protocol described by Nishimura (Nishimura *et al.*, 2006) and Hiei and Komari (Hiei and Komari, 2008). The composition of the different media used is given in Table 3.14.

Table 3.14 Composition of different media used in rice transformation.

1	**MS Medium**		
i	MS salt (Duchefa)	4.4 g	Final pH was adjusted to 5.8; total volume was made up to 1000 ml with distilled water and 0.03% phytagel was added.
ii	Caesin hydrolysate	0.3 g	
iii	Proline	0.5 g	
iv	Maltose	30 g	
v	Distilled water	800 ml	
2	**MS Basal Medium**		
i	MS salt (Duchefa)	4.4 g	Final pH was adjusted to 5.8 and total volume was made to 500 ml with distilled water.
ii	Sucrose	30 g	
iii	Distilled water	400 ml	
3	**Co-cultivation Medium**		
i	MS salt (Duchefa)	4.4 g	After adjusting final pH to 5.2, total volume was made up to 1000 ml with distilled water and 0.03% phytagel was added.
ii	Maltose	30 g	
iii	Caesin hydrolysate	0.3 g	
iv	Glucose	10 g	
v	Distilled water	800 ml	
4	**Regeneration Medium**		
i	MS salt (Duchefa)	4.4 g	After adjusting final pH to 5.8, total volume was made up to 1000 ml with distilled water and 0.05% phytagel was added.
ii	Sucrose	30 g	
iii	Caesin hydrolysate	0.3 g	
iv	Distilled water	800 ml	

3.26.1 Sterilization of Seeds

Mature seeds of rice were dehusked and surface sterilized in 70% alcohol for 1min and rinsed thrice with double distilled water, sterilized in 0.1% mercuric chloride for 7 min with vigorous shaking, followed by five rinses in sterile water. The seeds were dried on Whatman sheet No. 1, and then plated on callus induction medium with half of the embryo in contact with the medium. Approximately, 15 sterilized seeds per plate were placed on the surface of MS medium (Table 3.14) supplemented with 2.5 mg/l 2, 4-D (2, 4-di-chloro phenoxy acetic acid) and the plates were sealed with parafilm.

3.26.2 Callus Induction

Seeds were allowed to induce callus under continuous dark condition at 25±2 °C for three weeks. Embryonic calli from three weak old seeds were dissected with scalpel, and then transferred into fresh plates containing MS media supplemented with 2.5 mg/l 2, 4-D. The plates were incubated in dark for another 6 d prior to co-cultivation.

3.26.3 *Agrobacterium* Mediated Rice Transformation

Single colony of *Agrobacterium* (EHA105) was inoculated in 5 ml of YEM broth with appropriate antibiotics such as 50 mg/l chloramphenicol, 15 mg/l rifampicin, and 50 mg/l kanamycin. About 1% of this primary culture was inoculated in a 250 ml flask containing 100 ml YEM broth. The culture was then grown at 28 °C till $0.D_{600}$ reached up to 0.5-0.6. The cells were pelleted down by centrifugation at 5000 rpm for 10 min at 4 °C. The pellet was suspended in 100 ml of MS basal liquid media with 100 µM acetosyringone, which was added to the media 15 min prior to the co-cultivation. The embryonic calli (~100) were immersed in the bacterial suspension for 10 min. The embryonic calli were then dried between folds of sterile filter paper and cultured on plates containing co-cultivation media (Table 3.14). The plates were incubated in dark for 3 d.

3.26.4 Selection and Regeneration

The co-cultivated calli were then transferred to plates containing MS medium with 50 mg/l hygromycin and 250 mg/l cefotaxime. The plates were incubated under continuous dark conditions at 25±2 °C for 14 d; the resistant calli was sub cultured for an additional 14 d in the same medium. The hygromycin resistant calli, which survived after two rounds of selection were transferred to regeneration medium (Table 3.14) containing 3 mg/l 6-Benzyl amino purine (BAP), 1 mg/l zeatin, 0.5 mg/l α-naphthalene acetic acid (NAA), 50 mg/l hygromycin and 250 mg/l cefotaxime. The regenerated rice shoots were separated and transferred to fresh tubes containing MS basal media. For rooting the explants were transferred to solid MS medium supplemented with 250 mg/l cefotaxime. After rooting the plants are transferred to the autoclaved soil for hardening in green house. These plants were used for further transgenic analysis.

3.27 Screening of Transgenics on the Basis of Herbicide Resistance

Transgenic plants were screened for the expression of the *bar* gene by using Basta (Bayer Crop Science Ltd.), a commercial formulation of glufosinate ammonium salt. The herbicide Basta (10 mg/l) was painted on leaf tissues. The plants were observed over a one week period to determine resistance or susceptibility to the herbicide.

3.28 Detection of Genomic Integration of Transgenes by PCR

Five sets of transgene-specific primers were designed to detect the genomic integration of transgene cassette by PCR. Nucleic acid sequence of full length genes was aligned with genomic sequence of the endogenous gene and primers were designed from the non-conserved regions. The reaction mixture was set to 50 µl and placed in a thermo cycler (Bio-Rad) using an initial denaturing time of 5 min at 94 °C, followed by 35 cycles of denaturation for 1 min at 94 °C, annealing at 55 °C for 1 min, extension for 1 min at 72 °C and final extension for 6 min at 72 °C. The amplified products were separated and visualized on 1.0 % agarose gels stained with ethidium bromide (1µg/ml). The primers used for transgene detection are listed in Table 3.15.

Table 3.15 List of the primers used for screening of transgene cassettes in transgenic plants.

S. No	Name of the primer	Primer sequences
1	hpt Forward	5'- ATGAAAAAGCCTGAACTCACC -3
2	hpt Reverse	5'- CTATTTCTTTGCCCTCGGAC 3'
3	RA8 Forward	5'- CATCGTAATTCACTTACCGACC-3
4	T7 RNA pol Reverse	5'-GCTACGGCTTCCGGCTTGATT-3
5	SOD Forward	5'-AGAGGGGGATGGCCCCACTACT-3'
6	APX Forward	5'-CCGTCTCGCGTGGCACTCGGCG-3'
7	GR Forward	5'-AGAATTTGAGGATTCAAAGAAT-3'
8	DHAR Forward	5'-TCGGCGACTGCCCGTTCTCCCA-3'
9	MDHAR Forward	5'-AGAGAAAGGTATTGAGCTGATC-3'
10	T7 promoter	5'- TAATACGACTCACTATAGGG -3'

3.29 Southern Hybridization

In order to confirm the presence of the number of copies in transgenic plants, the genomic DNA was extracted from 5 g of fresh leaves of transgenic and non-transgenic plants by CTAB method. 10 µg of genomic DNA was digested with 60 U of *Sac* II for overnight by incubating at 37 °C to digest DNA completely. The digested samples were run on 0.8% agarose gel in 1X TBE, overnight at 30 V. In order to improve the transfer efficiency, DNA in agarose gel was treated with 0.2 N HCl for depurination by gentle agitation, until the blue colour of bromophenol blue dye in gel loading buffer turned yellow. The acid solution was decanted and the depurinated DNA in the gel was treated with denaturation buffer (to cleave the DNA at depurinated site) (Table 3.16) by gentle agitation for 1 h until the dye revived the original blue colour. This was followed by soaking the gel in neutralisation buffer

(Table 3.16) for 1 h on gentle shaking the denatured DNA was transferred from agarose gel to nylon membrane (Hybond N^+, Amersham Pharmacia) by conventional capillary transfer using 3 mm Whatman paper wick in presence of 20X SSC (Table 3.16) overnight. After the transfer was complete, the membrane was washed in 2X SSC for 5 min twice. The DNA was then cross-linked to membrane by exposure to short wave length ultraviolet light (Amersham UV-cross linker). Thereafter, the membrane was either stored dry at 4 °C or used immediately to proceed for pre-hybridisation.

The Nylon membrane with cross linked DNA was incubated with 40 ml of pre-warmed (65 °C) pre-hybridisation buffer (Table 3.16) for 2 h at 65 °C in a rotating cylinder. The 1000 bp *hpt* gene fragment was labelled with αP^{32} dCTP using random labelling kit (Fermentas) following manufacturer's instruction. The probe mixture was denatured and mixed with a minimal volume of pre-warmed pre-hybridisation solution (15ml), added to the membrane and incubated overnight at 65 °C. The membrane was washed once with pre-warmed 3X SSC + 0.1% SDS (low stringency wash) at 65 °C for 30 min followed by a second wash with 1X SSC + 0.1% SDS at 65 °C for 30 min and a high stringency final wash with twice with 0.2X SSC + 0.1% SDS for 15 min at 65 °C and twice for 15 min at 65 °C. Membrane was removed and successfully washed briefly in low stringency buffer (3X SSC, 0.1% SDS) at room temperature. After low stringency wash, the membrane was treated with pre-warmed high stringency buffer (0.5X SSC, 0.1% SDS) twice for 15 min, each at 65 °C. The high stringency buffer was pre-warmed to 65 °C before use. After low and high stringency wash to reduce the back ground, the membrane was washed once in 2X SSC for 5 min. The membrane was sealed inside a transparent plastic bag and exposed to X-ray film (Kodak) for 24-48 h at -70 °C with an intensifying screen to obtain an autoradiograph image.

Table 3.16 Composition of the buffers and solutions used for Southern Hybridisation.

1	**10X TBE**		
i	Tris	107.8 g	Total volume was made up to 1000 ml with distilled water, and pH was adjusted to 8.2.
ii	EDTA	8.41 g	
iii	Boric acid	559 g	
iv	Distilled water	600 ml	
2	**Denaturation Solution**		
i	NaCl(1 M)	29.22 g	Total volume was made to 500 ml with distilled water.
ii	NaOH (0.5 N)	10g	
iii	Distilled water	400 ml	
3	**Neutralisation Solution**		
i	NaCl	43.83 g	The final pH was adjusted to 7 with concentrated HCl and the final volume was made up to 500ml.
ii	Tris	30.28 g	
iii	Distilled water	350 ml	
4	**20X SSC**		
i	NaCl	175.3 g	The final pH was adjusted to 7 with concentrated HCl and the final volume was made up to 1000ml.
ii	Na citrate	88.2 g	
iii	Distilled water	800 ml	
5	**Pre-hybridisation Buffer**		
i	2 M Na_2HPO_4 (pH 7.2)	25 ml	Final volume was made to 100 ml.
ii	10% SDS	70 ml	
iii	0.5 M EDTA	0.2 ml	
iv	Distilled water	4.8 ml	

3.30 Detection of Transgenic Integration Loci in the Transgenic Genome by Locus Finding PCR (LF-PCR)

Locus finding PCR (LF-PCR) is an affinity-based genome walking method developed to determine the transgene flanking sequences of plants transformed by *A. tumefaciens*. LF PCR includes a primary PCR by a degenerated primer and transfer DNA (T-DNA)-specific primer, a nested PCR, and a method of enriching the desired amplicons by using a biotin-tagged primer that is complementary to the T-DNA. This enrichment technique separates the single strands of desired amplicons from the off-target amplicons, reducing the template complexity by several orders of magnitude (Thirulogachandar *et al.*, 2011). Various steps of LF PCR are schematically represented in Fig. 3.4.

Two forward primers, named genome walkers (GW 1 and 2), were designed to be used in parallel with a cassette specific primer (CSP) to improve the chance of amplifying the desired location in the primary PCR. Both the GWs have four bases in

random combinations of A, T, G, and C at their 3′ end, and upstream to these bases are four degenerate bases. The remaining 18 or 19 bases on the 5′ end are common to both primers, which is the sequence of GW-5. Three CSPs with an average length of 20–22 bp are designed downstream to the left border (LB) of pMDC99 T-DNA. Apart from these primers, one more reverse primer, the capture primer (CP), was also designed downstream to the LB but upstream to the three reverse CSPs with 29 bp in length. This primer is biotinylated at the 5′ end and is used in the purification of desired amplicons from the primary PCR product. All primer sequences and their locations in the T-DNA are given in table 3.17 and figure 3.5, respectively.

Table 3.17 List of primers used for LF PCR.

S. No	Name of the primer	Primer Sequence
1	GW1	AAGCTGCTCCGTAGGGTANNN
2	GW2	CTGAAGCTGCTCCGTAGGGTA
3	CSP1	GTATTGTGGTGTAACAAATT
4	CSP2 2	ATTTCGATGATGCAGCTTGGG
5	CSP3 3	TCTGGACCGATGGCTGTGTAG
6	CSP4 4	CGTCCGAGGGCAAAGAAATAG
7	CP	GCTTAGACAACTTAATAACACATTGCGGA

The different steps of LF PCR are explained as follows:

3.30.1 Primary PCR

Primary (random-priming) PCR was performed using CSP-1 and GW (1/2) in the following 50 µl reaction: 1X Taq buffer, 200 µM of dNTPs, 5 U of Taq DNA polymerase, 150 ng of CSP-1, 150 ng of GW (1/2), and 300 ng of genomic DNA. The reaction mixture was heated to 94 °C for 4 min and cycled 30 times with the following program: 94 °C for 1 min, 40 °C for 1 min, and 72 °C for 1 or 2 min. Then the final extension was given at 72 °C for 10 min. The reaction was chilled on ice, and 10 µl of PCR product was analysed in 1.2% agarose gel.

3.30.2 Purification of Desired Amplicons from Primary PCR Product

The remaining 40 µl of primary PCR product was purified with the help of biotinylated CP, which binds specifically to its complementary strand of the positive amplicons. The purification was done by the following program: 5 pmol of CP was added to the 40 µl of primary PCR products and mixed by gentle pipetting. Then the reaction mixture was heated to 94 °C for 4 min and 55 °C for 2 min in a PCR machine

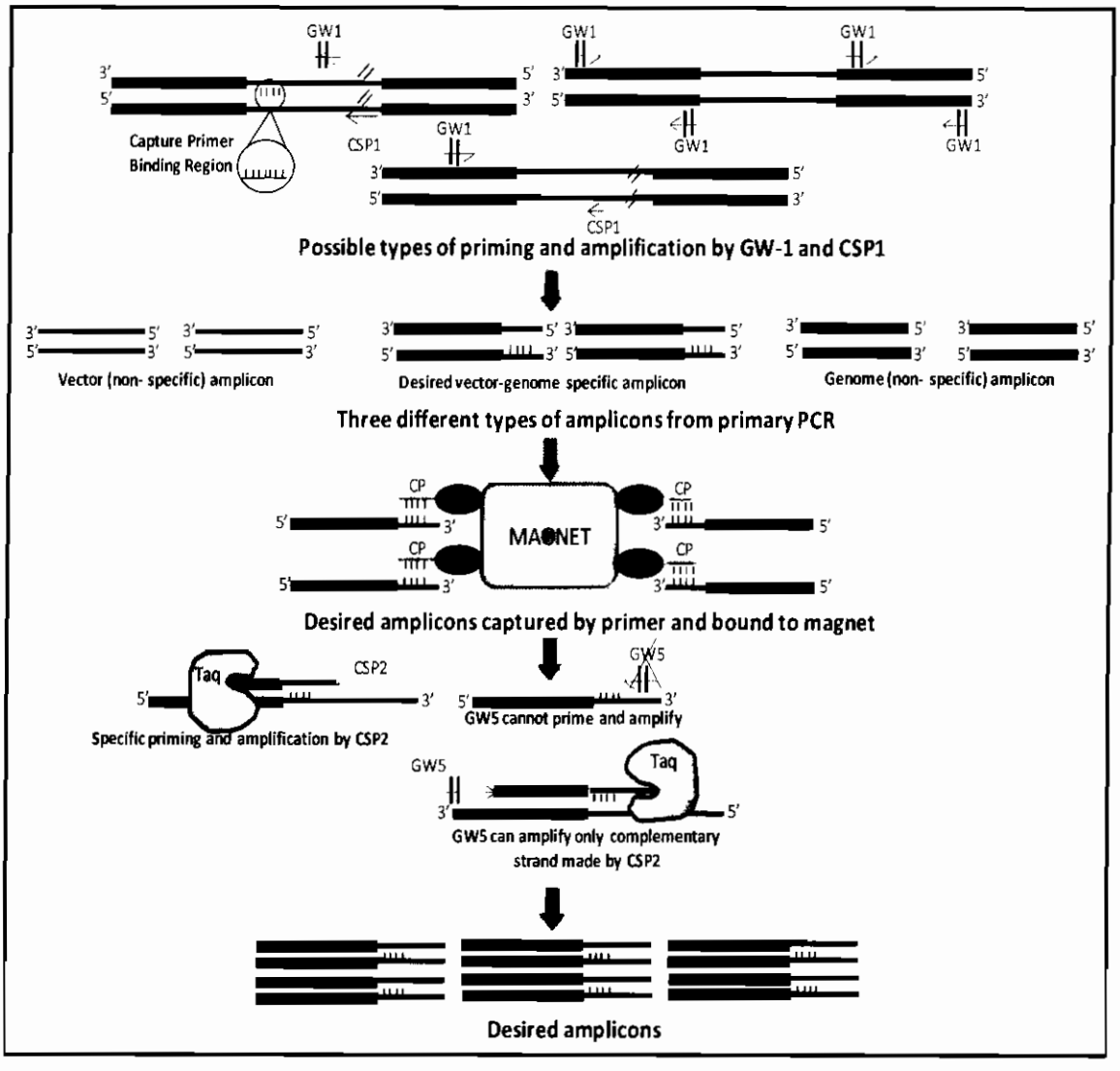


Figure 3.4 Diagrammatic representation of LF PCR. Step 1 random PCR showing the possible types of priming and amplification by involving the GW primers and CSP-1. Step 2 shows the different types of vector-specific, genome-specific, and desired vector/genome-specific amplicons. In step 3 template enrichment, CP (arrows having a circle in their ends) binds to the single strand of desired amplicons having its binding region. The bound primer–DNA complex is separated from the pool of PCR products by a magnet. Step **4** nested PCR shows the CSP-2 priming and making of a double strand from the captured single strands, GW-5 cannot prime the single strands. It can only amplify the complementary strand made by CSP-2, which suppresses the robust non-specific amplicons. The final step 5 shows the desired amplicons amplified in the nested PCR.

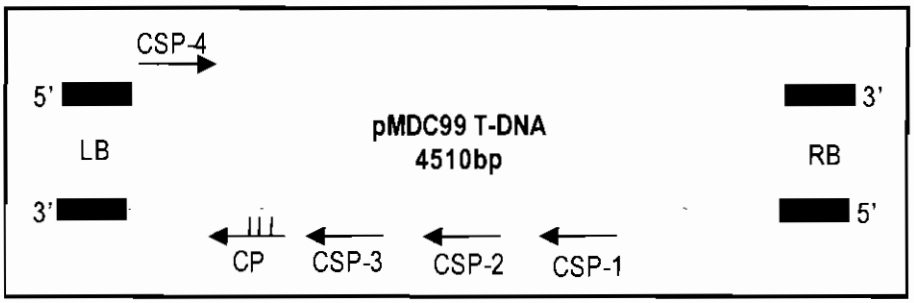


Figure 3.5 Location of primers in T-DNA. Schematic representation showing the 4510 bp T-DNA region of pMDC99 plasmid. The CSP reverse primers (1, 2, and 3) are designed near the LB. The CP is located between CSP-3 and LB, whereas the CSP-4 forward primer is positioned before CP.

(MyCycler). Immediately after, 60 µl of prewashed streptavidin beads (Roche, Germany) was added to the reaction mixture, mixed by gentle pipetting, and then kept in a magnetic stand (MagneSphere, Promega) to separate the unbound PCR products. Bound beads were washed with 60 µl of TEN-100 buffer (10 mM Tris–HCl, 1 mM EDTA, and 100 mM NaCl, pH 7.5) twice, and finally 40 µl of Milli Q water was added and mixed by pipetting. Then the tube was heated to 72 °C for 1 min and immediately transferred to the magnetic stand, from which a clear 40 µl solution was taken and transferred to a new PCR tube for further analysis.

3.30.3 Amplification of Transgene Integration Locus Using Nested PCR

Nested PCR was performed with an enriched PCR product as template and CSP-2 and GW-5 as primers. The reaction was set up in the following 50-µl reaction: 1X Taq buffer, 200 µM of dNTPs, 5 U of Taq DNA polymerase, 150 ng of CSP-2, 150 ng of GW-5, and 10 µl of purified primary PCR product. The reaction mixture was heated to 94 °C for 4 min and cycled 30 times with the following program: 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min. Then the final extension was given at 72 °C for 10 min (MyCycler, Bio-Rad). After the PCR, the tubes were chilled on ice. Half of the PCR products were used for cloning, and the other half were subjected to purification and then sequencing.

PCR products (taken for cloning) were separated on a 1.2% agarose gel, and the respective DNA fragments were excised and purified using a QIAquick Gel Extraction Kit (Qiagen, USA). The purified DNA fragments were cloned into a T/A pCR4-TOPO vector (Invitrogen, USA) and transformed into chemically competent DH5α *E. coli* cells. Transformants were selected on LB plates having 50 µg/ml kanamycin and incubated at 37 °C for 15 h to get individual colonies. Positive colonies were identified by colony PCR using vector-specific M13 primers and then were inoculated and multiplied in the LB broth for 15 h with 50 µg/ml kanamycin. Plasmids were isolated by a QIAprep Spin Miniprep Kit (Qiagen) and sequenced by M13 primers.

3.31 Selection of Seeds for Hygromycin Resistance

The seeds wild type and T_2 transgenic lines were germinated in sterile condition. Around 10 germinating seeds were inoculated in half MS medium with and without

hygromycin (30mg/l) in a glass jar. The glass jars were kept at 28 °C under in a cycle of 12 h dark and 12 h light to select seedlings for hygromycin resistance.

3.32 Chlorophenol Red Assay

The accumulation of ammonium in non-transformed plants in the presence of herbicide, basta, was determined by the chlorophenol red (CR) method as described by Kramer *et al.* (Kramer *et al.*, 1993). Putatively transgenic seeds and wild type seeds were grown on MS medium, in sterile test tubes containing 10 ml MS medium with 3 mg/l Basta and 50 mg/l chlorophenol red (CR) solidified with 0.03% phytagel. The pH of the medium was adjusted to 6.0, a pH at which the medium is a deep red colour. The change of colour to yellow was evaluated after 10-15 d.

3.33 Semi-quantitative RT-PCR Analysis of Transgene Expression

The mRNA was isolated from the DNAse treated total RNA. mRNA (2 µg) was used to synthesize first-strand cDNA with an oligo (dT) primer in a 20 µl reaction volume using SuperScript III (Invitrogen). Semi quantitative RT-PCR was performed using this cDNA as template. In order to analyze the transcription of transgene, forward primer and the reverse primer were designed to give an amplicon of around 500 bp. The gene specific forward and/or reverse primers for, *PgSOD, PgAPX, PgMDHAR, PgDHAR, PgGR*, 3'Rbc S UTR and tubulin were used (Table 3.18). Equal amount of cDNA as template in each PCR reaction was ensured by using house-keeping gene tubulin as the standard in PCR. The first-strand cDNA was diluted 5 times and 5 µl of it was used for PCR amplification in a reaction volume of 50 µl. The following PCR conditions were used: initial denaturation was performed at 94 °C for 2 min, followed by 30 cycles each of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 5 min. The PCR amplicons products were resolved by electrophoresis using 1% agarose gels.

Table 3.18 List of the primers used for semi-quantitative RT-PCR for transcriptional analysis of transgenes.

Gene	Forward Primer (5'-3')		Reverse Primer (5'-3')
PgSOD	TGCTGTGCTTGCTAGCAGTGAGGG	RBCS–3'UTR	AACAAGGCTATATATGACGAT
PgAPX	GAGGAGTTCCCCATCCTCTCG		
PgGR	TGTATGGGCCGTGGGTGATGT		
PgMDHR	CATTGACTACTCTCTTCAAA		
PgDHAR	ACCTACGAGATGAAGCTCGTC		
Tubulin	GGCTTGTGTCTCAGGTTATCTCATC	Tubulin	CATGGAGGATGGCTCGAAGG
RA8	CATGGCTCTCATGGTGGTTC		GTTCTCGTCGGCGTTCTGTA

3.34 Detection of Pollen Viability by Staining with KI/I_2 Solution

The pollen viability was detected according to the method described by Gunawardena *et al.* (Gunawardena *et al.*, 2003). Mature pollens were collected just before anthesis and stained with KI/I_2 (2% KI and 0.5% I_2) solution. Pollens in the anthers were released on to a slide in a drop of KI/I_2 and were observed and photographed under a light microscope.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Global warming and its associated adverse environmental conditions, such as drought, salinity, extreme temperature and oxidative stress are the primary cause of crop loss worldwide. The adverse environmental fluctuations are known to affect various stages of plant development, the sexual reproductive phase being the most vulnerable. The susceptibility of the reproductive phase of plants to environmental stresses results in geographical displacement of plants to different habitats for successful completion of their life cycle. However, in domesticated agricultural cropping systems the existing genetic variability for adaptive reproductive fitness is narrow due to continuous selection for the quantity and quality of grains. Moreover, different crops differ in their adaptability to various stress conditions. Crop plants like rice, wheat and barley are known to be very susceptible to the abiotic stress conditions. However, *Pennisetum glaucum* (L.), one of the widely cultivated crop plants in the semi-arid regions of Africa and India, can grow under very harsh environments such as drought, salinity and high temperature conditions where other crops are difficult to grow (Basavaraj *et al.*, 2010).

All the abiotic stresses are known to culminate into the production of oxidative stress in plants at the cellular level. In an attempt to improve the tolerance of plants against the various abiotic stresses, a large number of gene products that play putative roles in plant adaptation to oxidative stresses have been identified so far. Physiological and genetic evidence clearly indicates that the ROS scavenging systems of plants are important components of the stress protective mechanisms. Superoxide dismutase (SOD) and the ascorbate-glutathione (AsA-GSH) pathway are an integral part of the network of reactions involved in the detoxification of ROS.

In this study, the genes involved in ascorbate-glutathione pathway were isolated from *Pennisetum* and the entire pathway was reconstituted by *in vitro* gene pyramiding. As the male reproductive development of crop plants is the most sensitive stage to abiotic stress, the entire pathway was placed under the control of an anther specific promoter and transformed into rice plant to widen its adaptability to harsh environmental conditions in

the reproductive stage. The regulated over-expression of the entire ascorbate-glutathione pathway in rice anther tissue can be an effective strategy to minimize the oxidative damage in the anthers under adverse environmental conditions.

The results of the present study have been discussed in two parts. Part-I deals with the cloning and characterization of the genes encoding SOD and the AsA-GSH pathway from *P. glaucum* while part-II deals with the transgenic over-expression of the whole pathway in the anthers for combating environmental stress induced oxidative damage in the male reproductive part of rice plant.

PART I

Cloning and Characterization of SOD-Ascorbate-Glutathione (AsA-GSH) Pathway Encoding Genes from *Pennisetum glaucum*

PART I - Cloning and Characterization of SOD-Ascorbate-Glutathione (AsA-GSH) Pathway Encoding Genes from *Pennisetum glaucum*

SOD and the AsA-GSH pathway exist as important components of the anti-oxidant machinery present in plants to mitigate the deleterious effects of oxidative stress. SOD forms the first line of defense against the toxic ROS and catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide (H_2O_2). The H_2O_2 thus formed, is detoxified by the AsA-GSH pathway. The AsA-GSH pathway is constituted by ascorbate, glutathione and four anti-oxidant enzymes namely, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR).

Although all plants are equipped with the defense machinery to deal with the abiotic stress, few species are able to thrive better under unfavorable circumstances. This differential sensitivity of tolerant and susceptible plants may be attributed to the varying pattern of expression of various anti-oxidant genes resulting in differential anti-oxidant response. Being a stress-tolerant crop, *P. glaucum* is considered to be equipped with better ROS-detoxification machinery. Therefore, genes encoding the SOD-AsA-GSH pathway were isolated and characterized from *P. glaucum* for subsequent over-expression in crop plants.

4.1 Isolation and Sequence Analysis of SOD-AsA-GSH Pathway Encoding Genes from *P. glaucum*

The genes encoding the enzymes of SOD-AsA-GSH pathway i.e. superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) were amplified from the stress adapted crop plant, *P. glaucum* by PCR as mentioned in materials and methods (Section 3.16). The PCR amplified DNA fragments were cloned and sequenced; the sequence analysis of each of the genes is described below.

4.1.1 *Pennisetum* CuZn Superoxide Dismutase (PgCuZnSOD)

The nucleotide sequence of the open reading frame (ORF) encoding for PgCuZnSOD is 459 bp long (Fig. 4.1A), encoding a protein of 152 amino acids with an apparent molecular weight of 15 kDa and an estimated isoelectric point (pI) of 5.76. PgCuZnSOD has a high degree of homology with CuZnSODs isolated from other plant species and exhibits overall 96-83% amino acid sequence identity with CuZnSOD isolated from rice (*Oryza sativa*, OsCuZnSOD; **P28756**), maize (*Zea mays*, ZmCuZnSOD; **P23345**), wheat (*Triticum aestivum*, TaCuZnSOD; **ACO90194**), tobacco (*Nicotiana tabaccum*, NtCuZnSOD; **P27082**) and Arabidopsis (*Arabidopsis thaliana*, AtCuZnSOD; **P24704**).

A 4-element fingerprint, CUZNDISMTSE that provides a signature for the CuZnSODs, is present in the PgCuZnSOD protein sequence. The fingerprint consists of four characteristic motifs: motif 1 and 3 contain the conserved residues (H-45, H-47, H-62 and H-113) involved in binding copper ligand. Motif 2 harbors the histidine (H-79) and aspartate (D-82) that binds the Zn^{2+} ion (Fig. 4.1B). The analysis of PgCuZnSOD sequence and its comparison with other characterized CuZnSODs proteins revealed that the two cysteine residues, at position 56 and 145 form a unique disulphide bridge and are evolutionarily conserved. These two residues are presumed to contribute substantially to the stability of the tertiary structure of CuZnSODs (Pilon *et al.*, 2010). The multiple alignments of different CuZnSOD proteins showed strict conservation of all the Cu-Zn ligand co-ordinating amino acids (Fig. 4.2).

PgCuZnSOD was predicted to be localized in cytoplasm as it lacks any signal peptide. The phylogenetic analysis of PgCuZnSOD with various sub-cellular isoforms of Arabidopsis, rice and maize CuZnSODs revealed that PgCuZnSOD clusters along with the cytoplasmic isoforms of CuZnSOD which further validates its cytoplasmic nature (Fig. 4.3).

4.1.2 *Pennisetum* Ascorbate Peroxidase (PgAPX)

The full length cDNA coding for PgAPX is 753 bp long (Fig. 4.4A) and encodes a protein of 250 amino acids with an apparent molecular weight of 27.5 kDa and estimated pI of 5.62. The deduced amino acid sequence of PgAPX was found to share highest similarity with APX isolated from barley (*Hordeum vulgare*, HvAPX; **CAA06996**) followed by rice (*Oryza sativa*, OsAPX; **Q10N21**), maize (*Zea mays*,

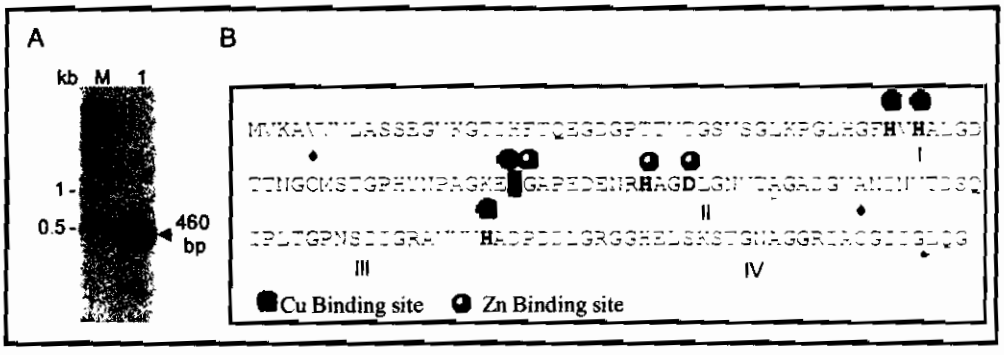


Figure 4.1 Isolation and sequence analysis of PgCuZnSOD. **(A)** Agarose gel depicting amplification of *PgCuZnSOD*. Lane 1 denotes the 459 bp amplicon of *PgCuZnSOD*. M denotes 1 kb DNA ladder. **(B)** Schematic representation of characteristic motifs and ligand binding sites of PgCuZnSOD protein. Arrows indicate the four CuZnSOD fingerprints (I-IV) as analyzed by PRINTS whereas coloured spheres represent Cu and Zn binding sites. The residues involved in binding Cu and Zn are shaded.

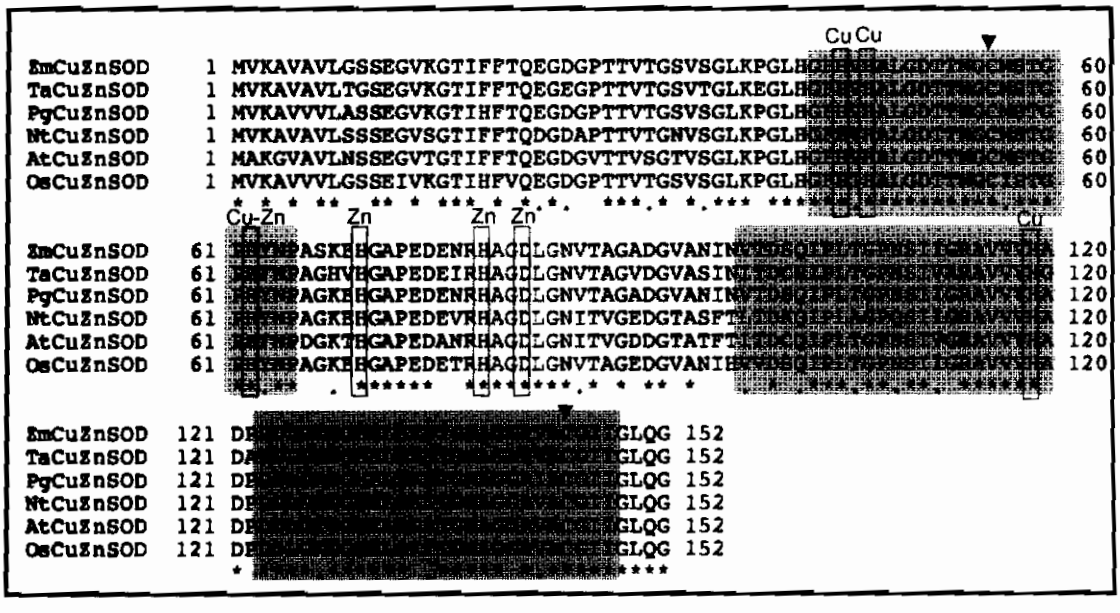


Figure 4.2 Multiple alignments of deduced amino acid sequences of CuZnSODs from *Arabidopsis thaliana* (AtCuZnSOD), *Nicotiana tabacum* (NtCuZnSOD), *Oryza sativa* (OsCuZnSOD), *Triticum aestivum* (TaCuZnSOD), *Zea mays* (ZmCuZnSOD) and *Pennisetum glaucum* (PgCuZnSOD) depicting conservation of the ligand (Cu and Zn) binding sites. Different motifs of CUZNDISMTASE fingerprint are shaded. Arrows indicate the conserved cysteine residues involved in imparting stability to the tertiary structure of the protein.

ZmAPX; **CAA84406**), tobacco (*Nicotiana tabacum*, NtAPX; **AAA86689**), Arabidopsis (*Arabidopsis thaliana*, AtAPX; **NP_001077481**) and tomato (*Solanum lycopersicum*, SlAPX; **AAX84654**).

An ASPEROXIDASE fingerprint is present in PgAPX sequence. ASPEROXIDASE is a 10-element fingerprint that provides a signature for all APXs (Fig. 4.4B). Peroxidases, and among them APXs, are proteins containing the heme prosthetic group in which iron plays an important role in the catalytic site. So, all the APXs have a characteristic heme-binding site. Like other APX proteins, PgAPX was found to contain a core catalytic region with two typical functional domains; the active site domain, with a conserved histidine (H-42) that acts as an acid-base catalyst in the reaction between H_2O_2 and the enzyme, and the other heme-binding domain. The amino acid residues like R-38, N-71, Q-66 and D-208, which are responsible for binding the ligand-heme in most APXs, were found to be conserved in PgAPX. Additionally, W-179 together with H-163 and D-208 which are the participants in hydrogen bonding network were also present in PgAPX. Ascorbate binds to the active site of APX proteins by four hydrogen bonds with lysine and arginine residues (Sharp and Raven, 2004). These residues are highly conserved in all the APX sequences including PgAPX (Fig. 4.5).

The analysis of PgAPX protein sequence using sub-cellular localization prediction tools (Target P and pSORT) showed that the protein was cytosolic. PgAPX lacks any N-terminus organelle-specific targeting region or C-terminus trans-membrane region which is found in organelle specific or membrane bound isoforms of APX. Moreover, the phylogenetic analysis of PgAPX with various sub-cellular isoforms of Arabidopsis and rice revealed that PgAPX clusters along with the cytoplasmic isoforms, which further supports its cytoplasmic nature (Fig. 4.6).

4.1.3 *Pennisetum* Monodehydroascorbate Reductase (PgMDHAR)

The full length *PgMDHAR* cDNA consists of an ORF of 1308 bp (Fig. 4.7A) encoding a protein of 435 amino acids with an apparent molecular weight and pI of 47 kDa and 5.39, respectively. The ClustalW alignment of PgMDHAR with the characterized MDHAR sequences revealed that PgMDHAR exhibits maximum sequence similarity of 97% with MDHAR isolated from rice (*Oryza sativa, OsMDHAR,*; **BAA77214**) followed by turnip (*Brassica rapa*, BrMDHAR;

AAK72107), 85-87% similarity to MDHAR from grape (*Vitis vinifera*, VvMDHAR; **ABQ41114**), sweet potato (*Ipomea batata*, IbMDHAR; **ABO33631**), alfalfa (*Medicago sativa*, MsMDHAR; **AEX20344**), apple (*Malus domesticus*, MdMDHAR; **ACN88682**) and cucumber (*Cucumis sativus*, CsMDHAR; **Q42711**). PgMDHAR shares 84% sequence similarity with MDHAR isolated from Arabidopsis (*Arabidopsis thaliana*, AtMDHAR; **NP_568125**) and 63% sequence similarity with maize cytosolic MDHAR (*Zea mays*, ZmMDHAR; **AFW73040**).

The domain analysis of the protein revealed the presence of two overlapping domains: Pyr_redox (Pfam ID: Pfam00070) and Pyr_redox 2 (Pfam ID: Pfam07992). PgMDHAR was found to possess a FAD-dependent pyridine nucleotide reductase (FADPNR) signature. FADPNR is a 5-element fingerprint that provides a signature for the FAD-dependent pyridine nucleotide reductase family. Motifs 1 and 5 contain conserved residues (motif 1; KYVILGGGVAAGYAAREF, motif 5; TSVPGVYAIGD) involved in the binding of FAD flavin moiety. Additionally, residues I-37 to A-42 (IISKEA) participate in FAD binding. The residues K-166 to L-183 (KAVVVGGGYIGLELSAAL) and M-192 to E-196 (MVFPE) are known to be involved in the binding of NAD(P)H (Murthy and Zilinskas, 1994; Sano and Asada, 1994) (Fig. 4.7B). The multiple alignment of different MDHAR proteins revealed that these motifs were highly conserved (Fig. 4.8).

The sequence of PgMDHAR contains a peroxisomal targeting sequence, serine-lysine-isoleucine (SKI), at the C terminus. The phylogenetic analysis of the protein with various isoforms of MDHAR from rice and Arabidopsis revealed that PgMDHAR clustered with the peroxisomal isoforms, suggesting its peroxisomal localization (Fig. 4.9).

4.1.4 *Pennisetum* Dehydroascorbate Reductase (PgDHAR)

The nucleotide sequence of the ORF encoding for PgDHAR is 648 bp (Fig. 4.10A). Further *in silico* sequence analysis showed that the ORF encodes for a protein of 215 amino acids. Based on the cDNA sequence data, the predicted molecular mass and pI of PgDHAR protein was found to be 23.5 kDa and 4.98, respectively. The deduced PgDHAR amino acid sequence showed 80% similarity with rice DHAR (*Oryza sativa*, OsDHAR; **AAV44199**) amino acid sequence. PgDHAR showed second highest similarity (79%) with wheat DHAR (*Triticum aestivum*, TaDHAR;

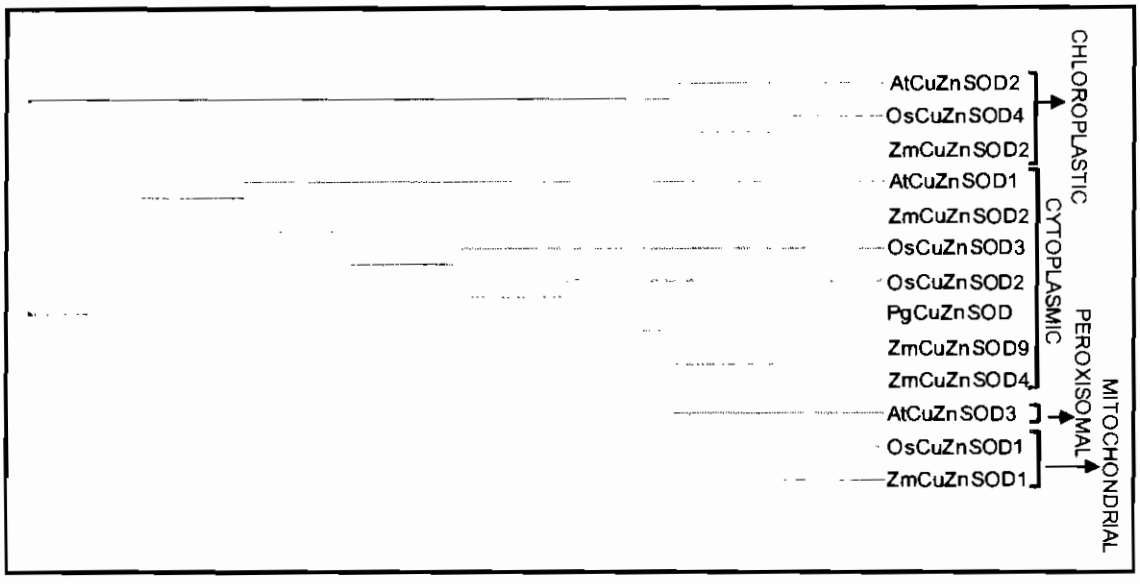


Figure 4.3 Phylogenetic tree and putative sub-cellular localization of CuZnSODs. The deduced amino acid sequence of PgCuZnSOD was compared with different sub-cellular isoforms of CuZnSODs of *Arabidopsis thaliana* (AtCuZnSOD1, NP_172360.1; AtCuZnSOD2, NP_565666.1; AtCuZnSOD3, NP_197311.1), *Oryza sativa* (OsCuZnSOD1, LOC_Os03g11960; OsCUZNSOD2, LOC_Os03g22810; OsCuZnSOD3, LOC_Os07g46990; OsCuZnSOD4, LOC_Os08g44770) and *Zea mays* (ZmCuZnSOD2, NP_001105335.1; ZmCuZnSOD9, NP_001105423.1; ZmCuZnSOD4, NP_001105704.1; ZmCuZnSOD1, NP_001105742.1; ZmCuZnSOD2, NP_001104871.1).

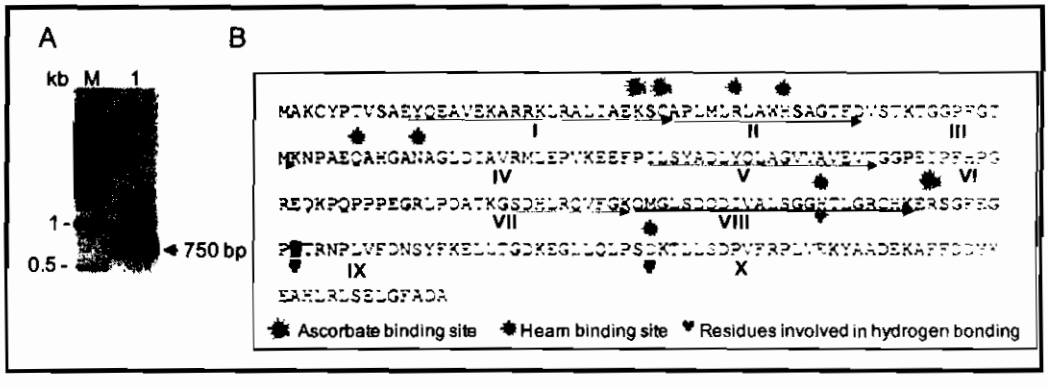


Figure 4.4 Isolation and sequence analysis of PgAPX. **(A)** Agarose gel showing amplification of *PgAPX*. Lane 1 depicts 750 bp amplicon of *PgAPX*. M represents 1 kb DNA ladder. **(B)** Schematic representation of characteristic motifs and ligand binding sites of PgAPX protein. Arrows indicate the ten ASPEROXIDASE fingerprint motifs (I-X) as analyzed by PRINTS whereas the colored spheres indicate the ascorbate and heme binding sites. Functionally important residues involved in binding ascorbate (K-30, C-32 and R-172) are shaded by green colour whereas the amino acid residues involved in binding heme (R-38, H-42, Q-66, N-71 and H-163) are highlighted by yellow colour. Blue spheres indicate residues taking part in hydrogen bonding.

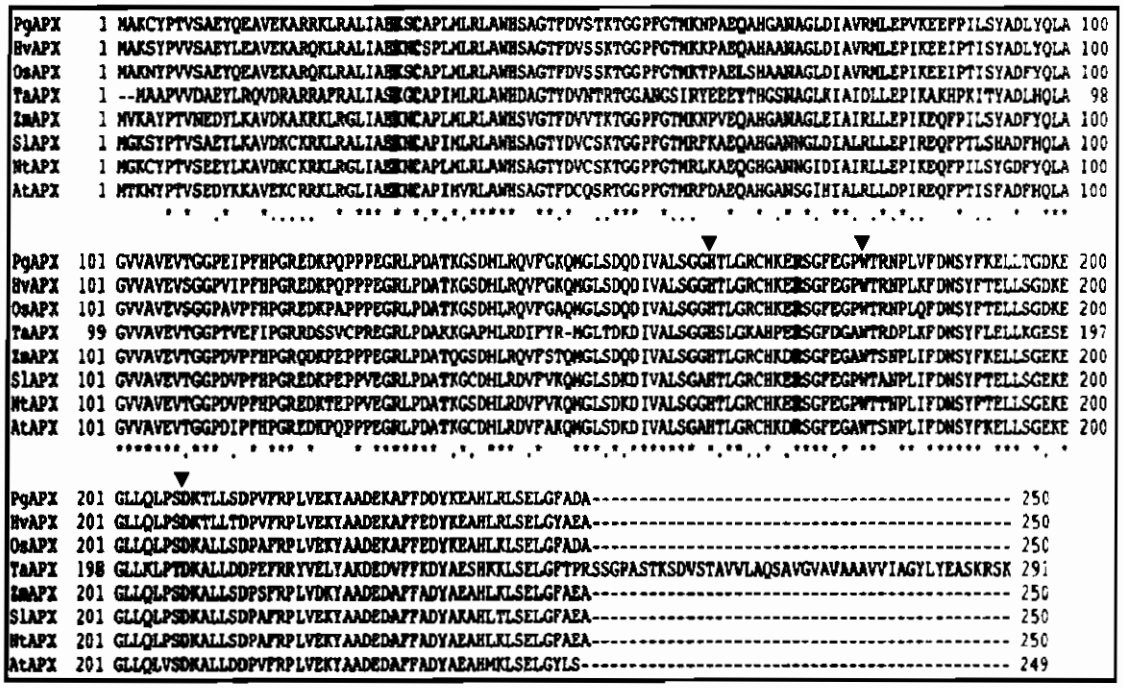


Figure 4.5 Multiple alignments of deduced amino acid sequences of APX proteins from *Arabidopsis thaliana* (AtAPX), *Hordeum vulgare* (HvAPX), *Nicotiana tabaccum* (NtAPX), *Oryza sativa* (OsAPX). *Pennisetum glaucum* (PgAPX) *Solanum lycopersicum* (SlAPX), *Triticum aestivum* (TaAPX) and *Zea mays* (ZmAPX). The conserved residues (K-30, C-32 and R-172) involved in ascorbate binding are shaded in red whereas the residues involved in binding heme (R-38, H-42, Q-66, N-71 and H-163) are shaded in blue. The residues involved in hydrogen bonding (H-163, W-179 and D-178) are indicated by arrow.

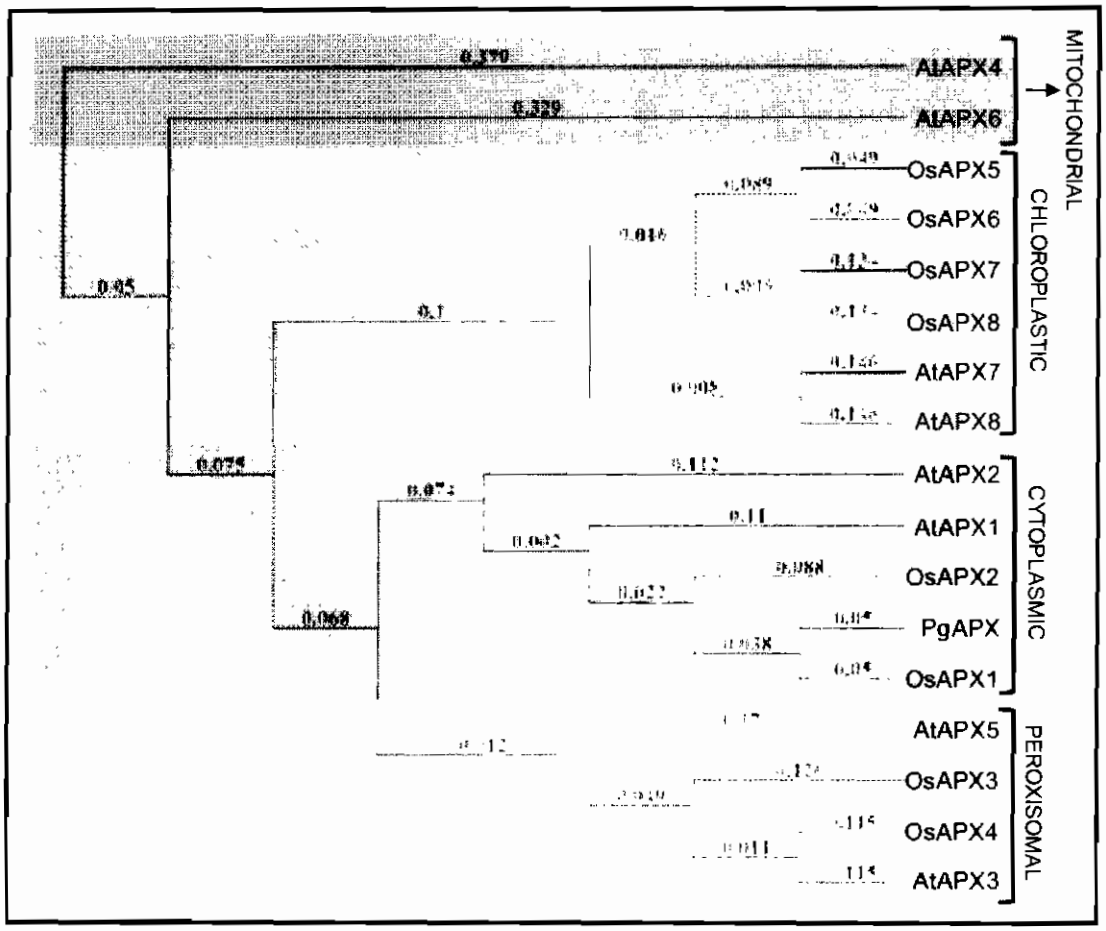


Figure 4.6 Phylogenetic tree and putative sub-cellular localization of APX proteins. Deduced amino acid sequence of PgAPX was compared with different sub-cellular APX isoforms of *Arabidopsis thaliana* (AtAPX1, NP_001077481.1; AtAPX2, NP_187575.2; AtAPX3, NP_195226.1; AtAPX4, NP_192640.1, AtAPX5, NP_195321.1; AtAPX6, NP_194958.2; AtAPX7, NP_177873.1; AtAPX8, NP_192579.1) and *Oryza sativa* (OsAPX1, LOC_Os03g17690; OsAPX2, LOC_Os07g49400; OsAPX3, LOC_Os04g14680; OsAPX4, LOC_Os08g43560; OsAPX5; LOC_Os12g07830; OsAPX6, LOC_Os12g07820; OsAPX7, LOC_Os04g35520; OsAPX8, LOC_Os02g34810).

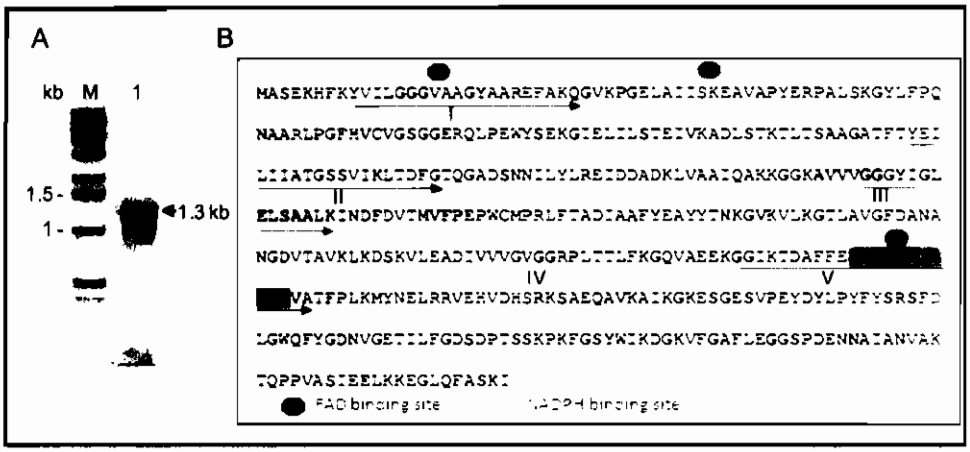


Figure 4.7 Isolation and sequence analysis of PgMDHAR. **(A)** Agarose gel depicting amplification of *PgMDHAR* gene. Lane 1 denotes 1300 bp amplicon of *PgMDHAR*. M indicates 1 kb DNA marker. **(B)** Schematic representation of characteristic motifs and ligand binding sites of PgMDHAR protein. Arrows indicate the five FADPNR fingerprint motifs (I-V) as analyzed by PRINTS whereas colored hexagons denote ligand binding sites.

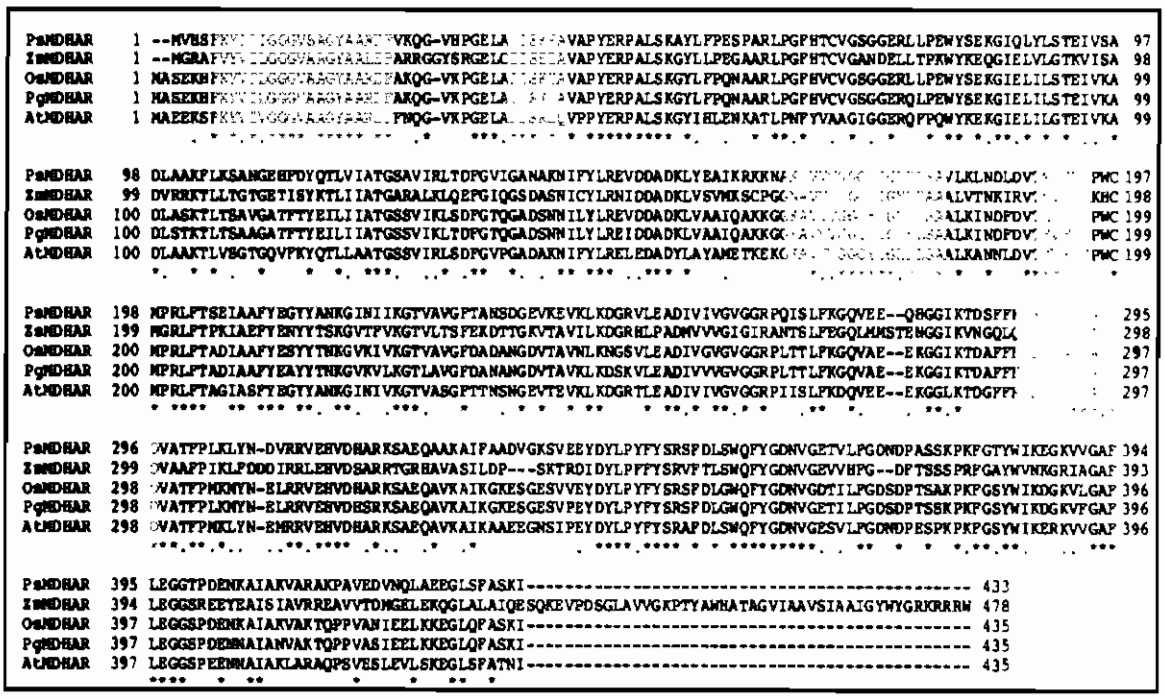


Figure 4.8 Multiple alignments of deduced amino acid sequences of MDHAR proteins from *Arabidopsis thaliana* (AtMDHAR), *Glycine max* (GmMDHAR), *Hordeum vulgare* (HvMDHAR), *Oryza sativa* (OsMDHAR), *Pennisetum glaucum* (PgMDHAR), *Pisum sativum* (PsMDHAR) and *Zea mays* (ZmMDHAR). Conserved motifs involved in FAD binding are shaded in yellow whereas the motifs involved in binding NADPH are shaded in green.

AAL71854) protein followed by 76% similarity with potato (*Solanum tuberosum*, StDHAR; **ABX26128**) and tomato (*Solanum lycopersicum*, SlDHAR; **AAY47048**) DHARs, 75% similarity with Arabidopsis (*Arabidopsis thaliana*, AtDHAR1; **AAM65005**) and tobacco (*Nicotiana tabacum*, NtDHAR; **AAL71857**) DHAR proteins and 56% similarity with DHAR isolated from maize (*Zea mays*, ZmMDHAR; **AFW76801**).

The PgDHAR sequence was further analysed for the presence of conserved motifs by CD-search (NCBI) (Marchler-Bauer *et al.*, 2013). DHARs have been identified as plant specific members of glutathione-s-transferase (GST) superfamily (Jakobsson *et al.*, 1999). PgDHAR was found to contain the two characteristic domains of GST proteins: GST-family N-terminal domain (cd00570) spanning amino acid residues 1 to 80 and GST-family C terminal α helical domain (cl02776)spanning amino acid residues 90 to 215 (Fig. 4.10B). The above two domains are connected by a short stretch of linker region. The C-terminal domain of PgDHAR forms approximately two-thirds of the protein and is less conserved than the N-terminal domain. The differences in the C-terminal domains of GST family of proteins are thought to be responsible for differences in substrate specificity between the different GST classes (Wilce and Parker, 1994). The PgDHAR sequence consists of conserved catalytically active cysteine residues at position 6 and 20 (Fig. 4.11). Unlike most other GSTs, DHARs have an active site cysteine instead of serine/tyrosine which forms a mixed disulfide with GSH as part of the catalytic mechanism (Dixon *et al.*, 2002).

The sub-cellular localization of PgDHAR protein as predicted from tools like Target P and pSORT revealed the protein to be cytoplasmic. The protein sequence lacked any targeting peptide and the phylogenetic analysis of PgDHAR showed that the protein clusters with the cytoplasmic isoforms of DHAR. These evidences suggest that the PgDHAR protein is apparently localized in the cytoplasm (Fig. 4.12).

4.1.5 *Pennisetum* Glutathione Reductase (PgGR)

The nucleotide sequence of the ORF encoding for PgGR was found to be 1494 bp long (Fig. 4.13A). The ORF encodes a protein of 497 amino acids having an apparent molecular weight of 53.5 kDa and pI of 6.25. The sequence homology search of PgGR protein showed that it shares maximum homology with GR isolated from rice (*Oryza sativa*, OsGR; **BAA11214**), barley (*Hordeum vulgare*, HvGR;

BAF80309) and wheat (*Triticum aestivum*, TaGR; **AAQ64632**), exhibiting 94% similarity. PgGR showed 85% similarity with Arabidopsis GR (*Arabidopsis thaliana*, AtGR; **AEE76867**), 83% similarity with pea GR (*Pisum sativum*, PsGR; **Q43621**) and 60% similarity with GR protein isolated from tobacco (*Nicotiana tabacum*, NtGR; **CAA53925**).

The protein sequence of PgGR was further analyzed for the presence of conserved motifs. PgGR was found to contain a PNDRDTASEI fingerprint. PNDRDTASEI is a 9-element fingerprint that provides a signature for the pyridine nucleotide-disulphide reductase (PNDR) class I family. Among the nine functional motifs of PNDRDTASEI fingerprint identified in the PgGR protein sequence, motif 2 contains two Cys residues (C-70 and C-75) involved in the redox-active disulphide bond, motifs 1 and 4 contain the conserved glycine residues (G-29, G-34, G-33, G-221 and G-207-209) and correspond to the ADP binding site for FAD and NAD(P), respectively. Motif 6 encodes the binding site for the FAD flavin moiety; and motif 9 contains a conserved histidine-glutamate diad (Fig. 4.13B). PgGR contains a highly conserved GGGYIA fingerprint motif in the NADPH binding domain. The two cysteines of the GR redox center(C-70 and C-75) are also highly conserved (Fig. 4.14).

PgGR was predicted to be localized in the cytoplasm by the sub-cellular targeting prediction tools (Target P and pSORT). The phylogenetic tree of PgGR protein with various sub-cellular isoforms of GR from Arabidopsis and pea that depicts PgGR being placed in a single cluster along with the cytoplasmic isoforms of GR, further confirms its cytoplasmic localization (Fig. 4.15).

4.2 Expression of *Pennisetum* AsA-GSH Pathway Encoding Genes in *E. coli* and Verification of Recombinant Proteins for Their Enzymatic Activity

Before proceeding for the construction of the gene cassettes for plant transformation, we needed to confirm that *PgSOD*, *PgAPX*, *PgMDHAR*, *PgDHAR* and *PgGR* coded for functional proteins. For this we chose to express the proteins in bacterial system and check the functional activity of the purified proteins.

Each gene encoding for SOD-AsA-GSH pathway enzymes was cloned separately into *Nde* I and *Bam*H I sites of pET-28a expression vector. *E. coli* was utilized as

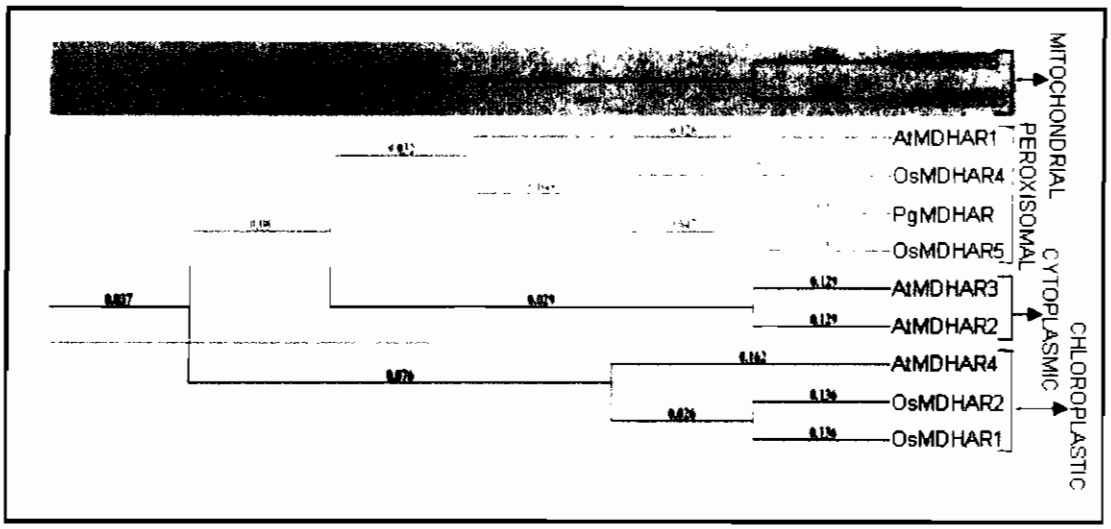


Figure 4.9 Phylogenetic tree and putative sub-cellular localization of MDHAR proteins. The deduced amino acid sequence of PgMDHAR was compared with the different sub-cellular isoforms of MDHAR from *Arabidopsis thaliana* (AtMDHAR1, NP_190856.1; AtMDHAR2, NP_568125.1; AtMDHAR3, NP_001118607.1; AtMDHAR4, NP_189420.1; AtMDHAR6, NP_849839.1) and *Oryza sativa* (OsMDHAR1, LOC_Os02g47790; OsMDHAR2, LOC_Os02g47800; OsMDHAR3, LOC_Os08g05570; OsMDHAR4, LOC_Os08g4434; OsMDHAR5, LOC_Os09g39380).

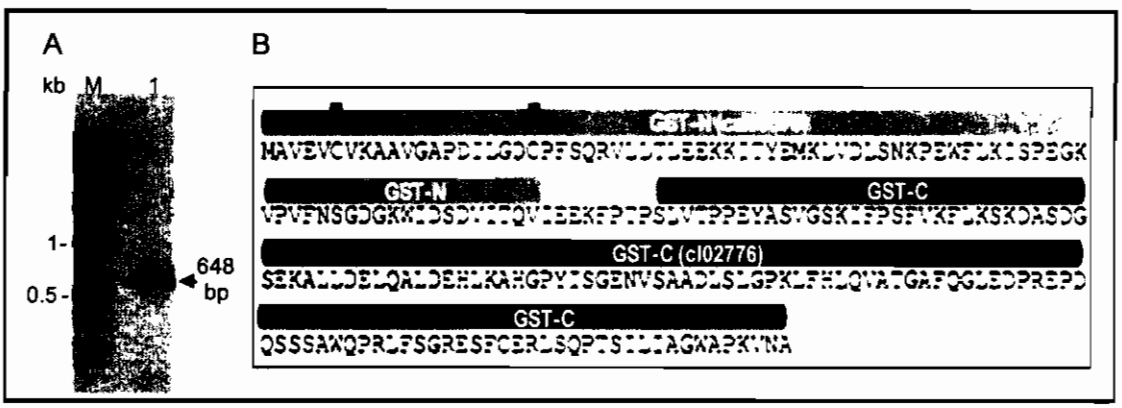


Figure 4.10 Isolation and sequence analysis of PgDHAR **(A)** Agarose gel depicting amplification of *PgDHAR*. Lane 1 denotes 648 bp amplicon of *PgDHAR*. **M** indicates 1 kb DNA ladder. **(B)** Schematic representation of the characteristic domains of PgDHAR. PgDHAR protein comprises of two domains- GST-N (green) and GST-C terminal (red) domain. Arrows represent the conserved cysteine residues characteristic of DHAR proteins.

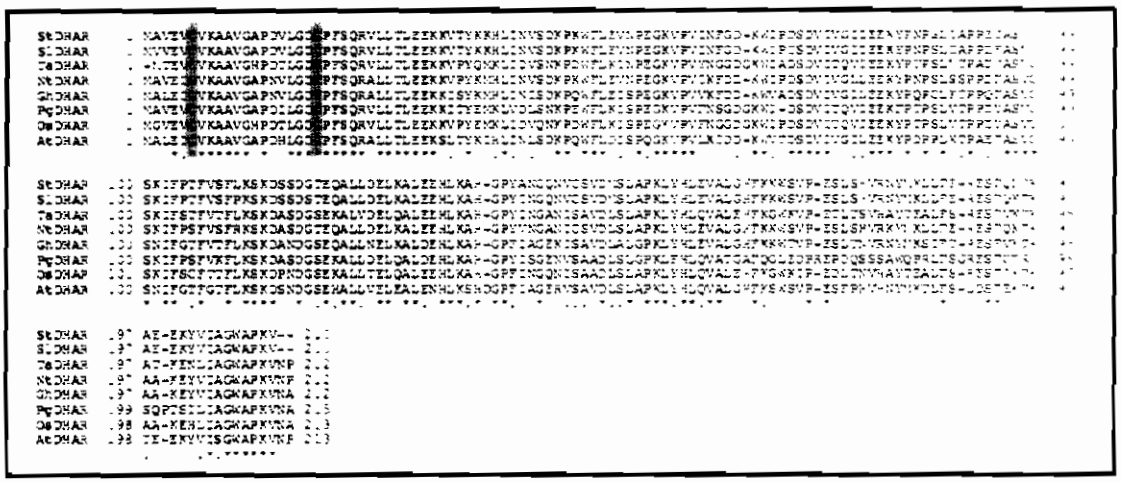


Figure 4.11 Multiple alignments of deduced amino acid sequences of DHAR proteins from *Arabidopsis thaliana* (AtDHAR1), *Gossypium hirsutum* (GhDHAR), *Nicotiana tabacum* (NtDHAR), *Oryza sativa* (OsMDHAR), *Pennisetum glaucum* (PgMDHAR), *Solanum lycopersicum* (SlDHAR), *Solanum tuberosum* (StDHAR), *Triticum aestivum* (TaDHAR) and *Zea mays* (ZmDHAR). The alignment shows higher degree of conservation in the N-terminal domain as compared to the C-terminal domain (conserved residues are shown by asterisks). The highly conserved cysteine residues present in the active site are highlighted in red.

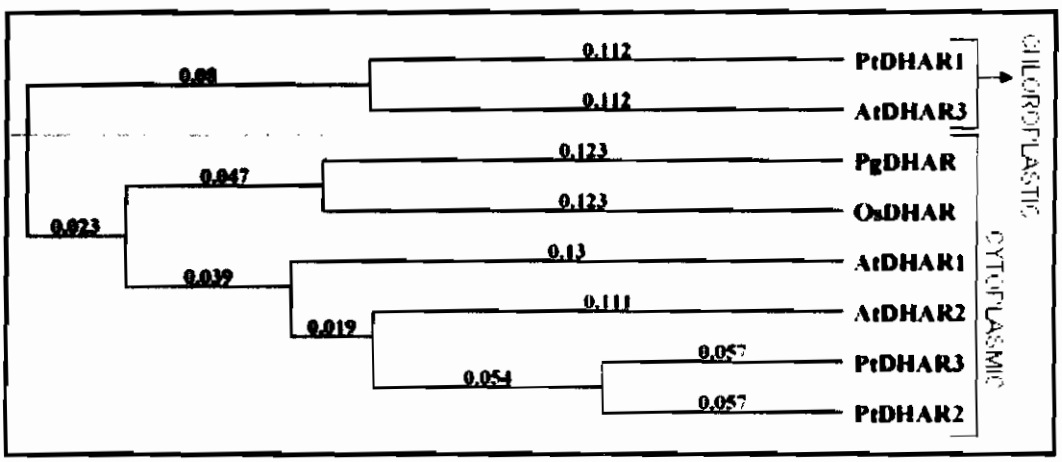


Figure 4.12 Phylogenetic tree and putative sub-cellular localization of DHAR proteins. The deduced amino acid sequence of PgMDHAR was compared with the different sub-cellular DHAR isoforms of *Arabidopsis thaliana* (AtDHAR1, NP_173387.1; AtDHAR2, NP_177662.1; AtDHAR3, NP_568336.1), and *Oryza sativa* (OsDHAR, LOC_Os02g47790; and *Populus trichocarpa* (PtDHAR1, ADB11343.1; PtDHAR2, ADB11344.1; PtDHAR3 ADB11345.1).

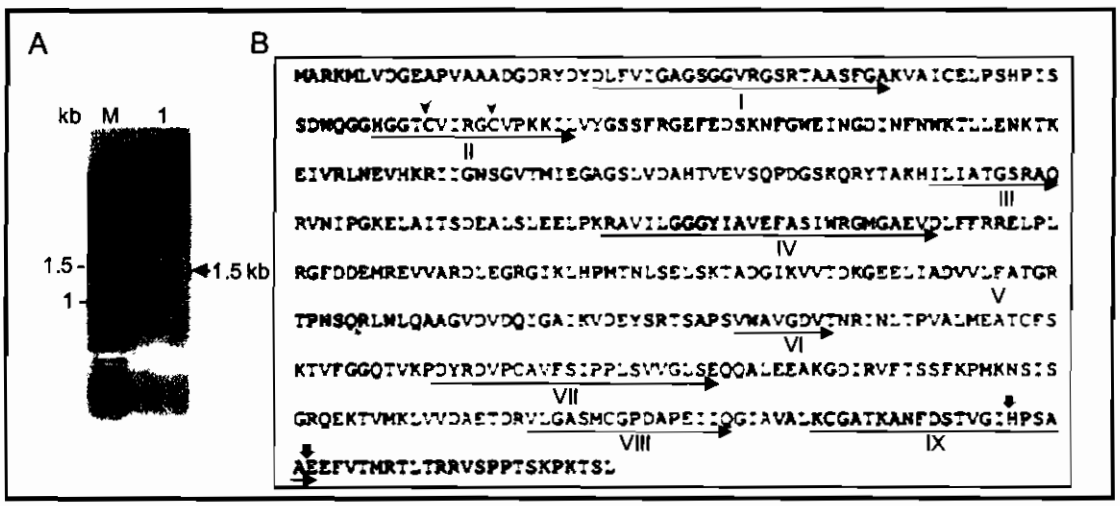


Figure 4.13 Isolation and sequence analysis of PgGR. **(A)** Agarose gel depicting amplification of *PgGR*. Lane 1 denotes ~1.5 kb amplicon of *PgGR*. M indicates 1 kb DNA ladder. **(B)** Schematic representation of the characteristic domains of PgGR. The horizontal arrows indicate the nine PNRDTASEI fingerprint motifs (I-X). Region highlighted in yellow represents the GR redox center with the two cysteine residues (indicated by blue vertical arrows). Region highlighted in green represents the conserved glycine residues involved in NADPH binding. Red vertical arrows indicate the His-Glu diad. The amino acid residues of FAD-binding domain, NADPH binding domain and interface region are indicated in red, blue and purple colours, respectively.

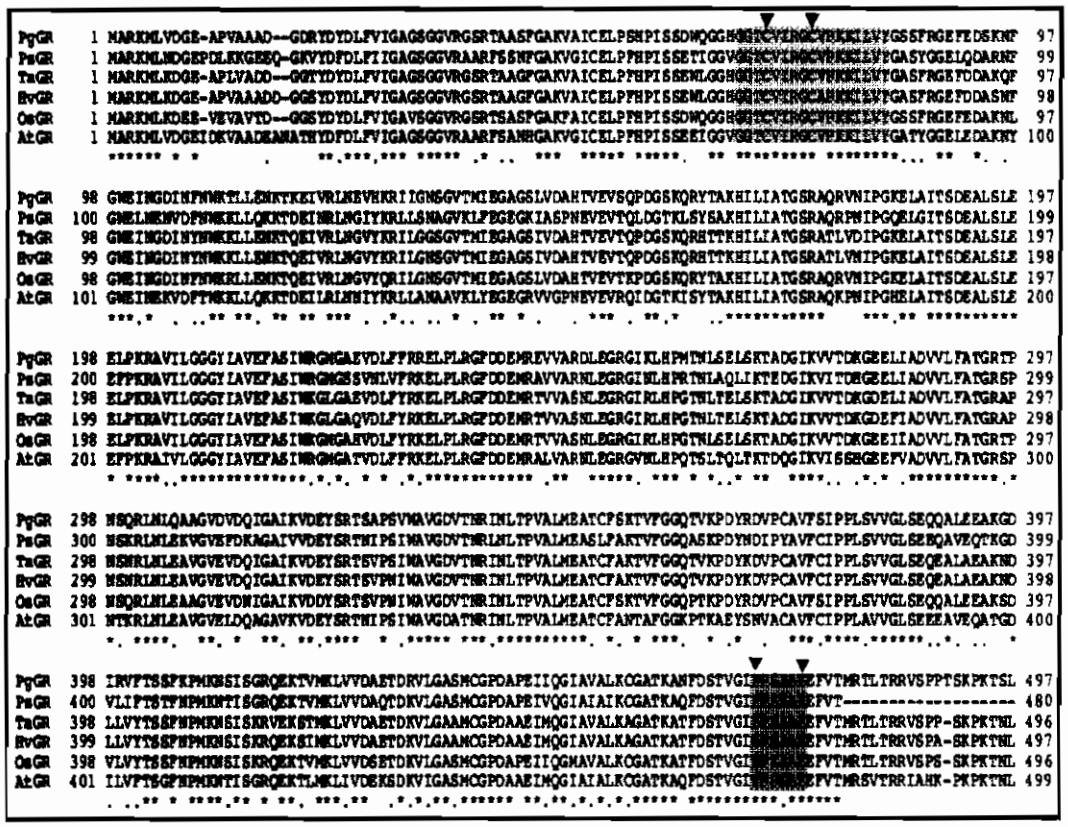


Figure 4.14 Multiple alignments of deduced amino acid sequences of GR proteins from *Arabidopsis thaliana* (AtGR), *Nicotiana tabacum* (NtGR), *Hordeum vulgare* (HvGR), *Oryza sativa* (OsGR), *Pennisetum glaucum* (PgMDHAR), *Pisum sativum* (PsGR), *Triticum aestivum* (TaGR) and *Zea mays* (ZmDHAR). The conserved cysteine residues in GR redox center (shaded in red) and the His-Glu diad are marked by blue and red arrows, respectively. The highly conserved G rich motif is highlighted by green colour.

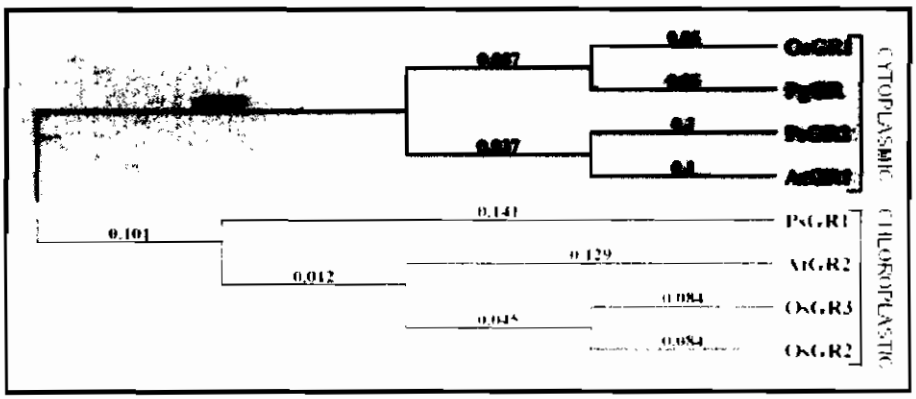


Figure 4.15 Phylogenetic tree and putative sub-cellular localization of GR proteins. The deduced amino acid sequence of PgGR was compared with the different sub-cellular GR isoforms from *Arabidopsis thaliana (*AtGR1, NP_001030756.2; AtGR2, NP_191026.1), *Oryza sativa* (OsGR1, LOC_Os02g56850; OsGR2, LOC_Os03g06740; OsGR3, LOC_Os10g28000) and *Pisum sativum* (PsGR1, P27456.1; PsGR2, Q43621.1).

heterologous expression system to express the recombinant polypeptides of SOD-AsA-GSH pathway with additional hexa histidine tag at the N-terminus. The recombinant polypeptides were purified to near homogeneity on a Ni–NTA column chromatography and assayed for their activity as described below.

4.2.1 Recombinant PgSOD

The *E. coli* BL21 (DE3) cells transformed with recombinant pET28a-SOD construct expressed an approximately 18 kDa recombinant PgSOD (Fig. 4.16). Majority of the recombinant protein was partitioned in the soluble fraction of the *E. coli* lysate. The recombinant protein was purified to near homogeneity from clarified *E. coli* lysate by Ni-NTA chromatography (Fig. 4.16B; lane 3). The recombinant PgSOD activity was assayed by an in-gel activity assay based on its ability to compete with nitroblue-tetrazolium (NBT) for superoxide anions generated by the xanthine-xanthine oxidase system, which in turn results in the inhibition of reduction of NBT (Beauchamp and Fridovich, 1971) (Fig. 4.16C; lane 1). The recombinant PgSOD was found to be relatively thermostable and showed optimum activity at pH 7.6.

4.2.2 Recombinant PgAPX

The *E. coli* BL21 (DE3) cells transformed with recombinant pET28a-APX construct expressed an approximately 29 kDa recombinant PgAPX protein (Fig. 4.17) with majority of the recombinant protein being partitioned in the soluble fraction of the *E. coli* lysate. The recombinant protein was purified to near homogeneity from clarified *E. coli* lysate by Ni-NTA chromatography (Fig. 4.17B; lane 3). The APX activity was detected by in-gel activity assay (Fig. 4.17C; lane 1). The recombinant PgAPX protein preferred ascorbate as reducing substrate and showed maximum activity at pH 6.6.

4.2.3 Recombinant PgMDHAR

The *E. coli* BL21 (DE3) cells transformed with recombinant pET28a-MDHAR construct expressed an approximately 48 kDa recombinant PgMDHAR protein (Fig. 4.18). The recombinant protein was purified to near homogeneity from clarified *E. coli* lysate by Ni-NTA chromatography (Fig. 4.18B; lane 3). The purificd protein was found to exhibit MDHAR activity.

4.2.4 Recombinant PgDHAR

The *E. coli* BL21 (DE3) cells transformed with recombinant pET28a-DHAR construct expressed an approximately 26 kDa recombinant protein (Fig. 4.19). Majority of the recombinant protein was partitioned in the soluble fraction of the *E. coli* lysate. The recombinant protein was purified to near homogeneity from clarified *E. coli* lysate by Ni-NTA chromatography (Fig. 4.19B; lane 3). The purified His-tagged recombinant protein showed DHAR activity as indicated by the in-gel activity assay (Fig. 4.19C; lane 2).

4.2.5 Recombinant PgGR

The *E. coli* BL21 (DE3) cells transformed with recombinant pET28a-GR construct expressed an approximately 55 kDa recombinant protein (Fig. 4.20). Majority of the recombinant protein was partitioned in the soluble fraction of the *E. coli* lysate. The recombinant protein was purified to near homogeneity from clarified *E. coli* lysate by Ni-NTA chromatography (Fig. 4.20B; lane 3).The activity of the recombinant protein was assayed by in-gel activity assay and the protein was found to be enzymatically active (Fig. 4.20C; lane 1).

Thus, all the genes isolated in this study encoded for enzymatically active polypeptides and hence could be used for transformation and subsequent over-expression in crop plants for increasing their resistance against oxidative stress.

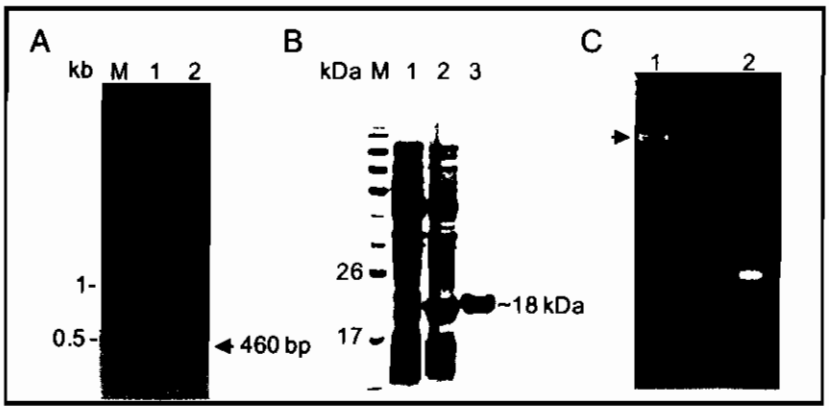


Figure 4.16 Expression of recombinant PgSOD protein. **(A)** Agarose gel denoting the cloning of *PgSOD* in pET-28a vector. Lane M: 1 kb DNA ladder, Lane 1: un-digested pET-28a-PgSOD vector, Lane 2: double digestion of pET-28A veetor harboring *PgSOD* gene using *Nde* I and *Bam* HI. **(B)** SDS-PAGE profile of the expressed PgSOD protein from bacterial cells. Lane M: Pre-stained protein marker, Lanes 1 and 2 indicate the un-induced and induced eulture pellets of reeombinant clone, Lane 3 shows the Ni-NTA column purified recombinant PgSOD protein. **(C)** Representative in-gel SOD activity assay. SOD activity is indicated by the appearance of achromatic bands (indicated by arrow). Lane 1: purified PgSOD protein, Lane 2: crude protein isolated from *P. glaucum* leaves.

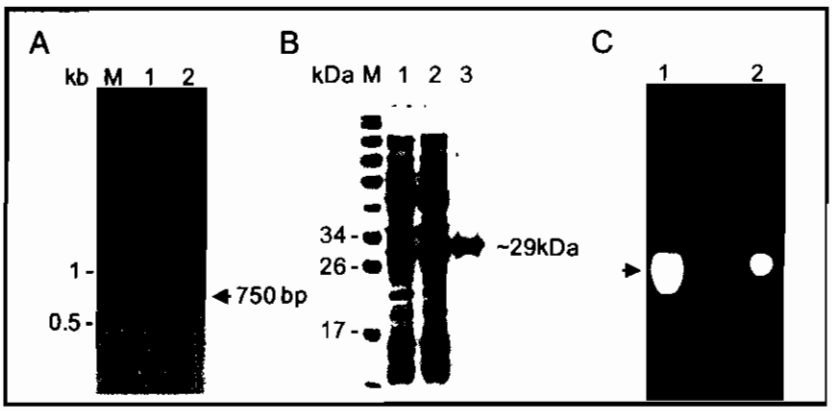


Figure 4.17 Expression of recombinant PgAPX protein. **(A)** Agarosc gel denoting the cloning of *PgAPX* in pET-28a vector. Lane M: 1 kb DNA ladder, Lane 1: un-digested pET-28a-PgAPX vector, Lane 2: double digestion of pET-28A vector harboring *PgAPX* gene using *Nde* I and *Bam* HI. **(B)** SDS-PAGE profile of the expressed PgAPX protein from bacterial cells. Lane M: Pre-stained protein marker, Lanes 1 and 2 indicate the un-induced and induced culture pellets of recombinant clone. Lane 3 shows the Ni-NTA column purified recombinant PgAPX protein. **(C)** Representative in-gel APX activity assay. The APX activity was observed as an achromatic band (indicated by arrow) on a purple-blue background. Lane 1: purified PgAPX protein, Lane 2: crude protein isolated from *P. glaucum* leaves.

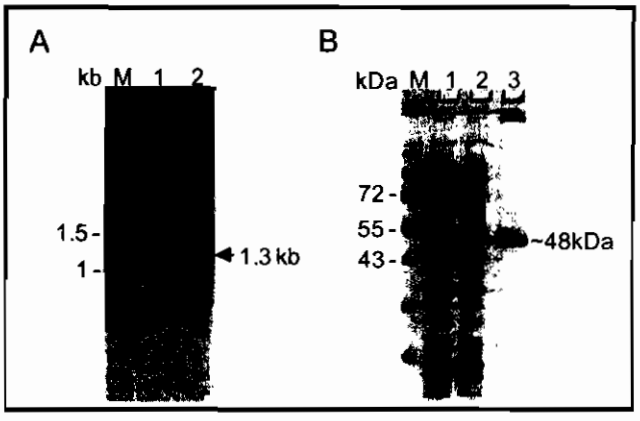


Figure 4.18 Expression of recombinant PgMDHAR protein. **(A)** Agarose gel denoting the cloning of *PgMDHAR* in pET-28a vector. Lane M: 1 kb DNA ladder, Lane 1: un-digested pET-28a-PgMDHAR vector, Lane 2: double digestion of pET-28A vector harboring *PgMDHAR* gene using *Bam* HI and *Hind* III. **(B)** SDS-PAGE profile of the expressed PgMDHAR protein from bacterial cells. Lane M: Pre-stained protein marker, Lanes 1 and 2 indicate the un-induced and induced culture pellets of recombinant clone, Lane 3 shows the Ni-NTA column purified recombinant PgMDHAR protein.

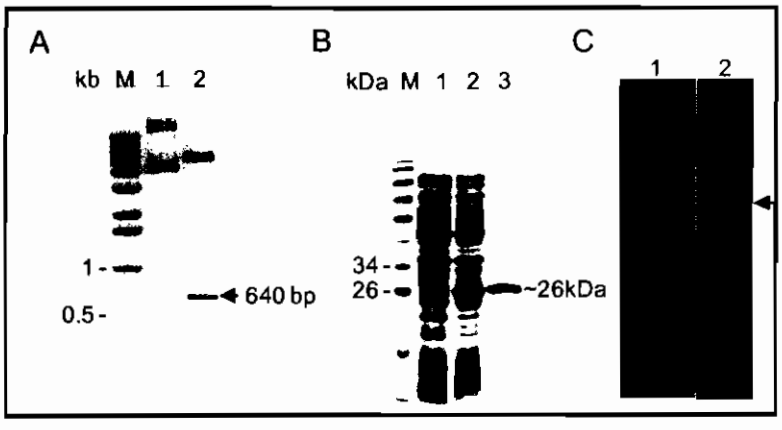


Figure 4.19 Expression of recombinant PgDHAR protein. **(A)** Agarose gel denoting the cloning of *PgDHAR* in pET-28a vector. Lane M: 1 kb DNA ladder, Lane 1: un-digested pET-28a-PgDHAR vector, Lane 2: double digestion of pET-28A vector harboring *PgDHAR* using *Nde* I and *Bam* HI. **(B)** SDS-PAGE profile of the expressed PgDHAR protein from bacterial cells. Lane M: Pre-stained protein marker, Lanes I and 2 indicate the un-induced and induced culture pellets of recombinant clone, Lane 3 shows the Ni-NTA column purified recombinant PgDHAR protein. **(C)** Native PAGE showing in-gel aetivity of DHAR protein. Native PAGE gel depicting in-gel DHAR activity of crude protein isolated from *P. glaucum* leaves is indicated by I whereas 2 represents native PAGE gel showing the activity of recombinant PgDHAR protein.

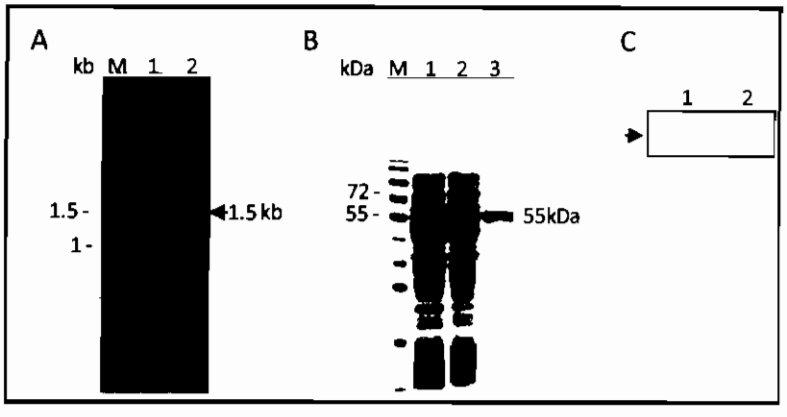


Figure 4.20 Expression of recombinant PgGR protein. **(A)** Agarose gel denoting the cloning of *PgGR* in pET-28a vector. Lane M: 1 kb DNA ladder; Lane 1: un-digested pET-28a-PgGR vector, Lane 2: double digestion of pET-28A vector harboring *PgGR* gene using *Nde* I and *Bam* HI. **(B)** SDS-PAGE profile of the expressed PgGR protein from bacterial cells. Lane M: Pre-stained protein marker, Lanes 1 and 2 indicate the un-induced and induced culture pellets of recombinant clone, Lane 3 shows the Ni-NTA column purified recombinant PgGR protein. **(C)** Representative in-gel GR activity assay. GR activity is indicated by the appearance of yellow colour bands (indicated by arrow). Lane 1: purified PgGR protein, Lane 2: crude protein isolated from *P. glaucum* leaves.

PART II

Transgenic Over-expression of SOD-Ascorbate-Glutathione Pathway Encoding Genes for Combating Environmental Stress Induced Oxidative Damage in Rice Anthers

PART-II Transgenic Over-expression of SOD-Ascorbate-Glutathione Pathway Encoding Genes for Combating Environmental Stress Induced Oxidative Damage in Rice Anthers

The ability to manipulate the levels of SOD and the enzymes of the AsA-GSH pathway using gene transfer technology has provided insights into their roles in conferring tolerance to plants under abiotic stress conditions (Table 2.4). However, in some cases, it was found that the over-expression of a single anti-oxidant enzyme did not provide protection against oxidative or abiotic stresses (Tepperman and Dunsmuir, 1990; Pitcher *et al.*, 1991; Torsethaugen *et al.*, 1997). The over-expression of two or more enzymes has yielded better results when compared with over-expression of single enzymes in a number of reports (Kwon *et al.*, 2002; Lee *et al.*, 2007). The possible explanation of this observation lies in the fact that the different enzymes of a pathway always work in a co-ordinated manner and limitation of any one enzyme ultimately slows down the entire pathway. Transgenic tobacco plants over-expressing both CuZnSOD and APX showed enhanced tolerance to oxidative stress (Kwon *et al.*, 2002). This double transgenic plant when transformed with DHAR gene exhibited further enhanced oxidative stress tolerance (Lee *et al.*, 2007) suggesting that the over-expression of the entire AsA-GSH pathway encoding genes might be far better strategy rather than over-expressing any single gene of this pathway.

Several approaches, such as co-transformation (Chen *et al.*, 1998; Zhu *et al.*, 2008), re-transformation (Li *et al.*, 2003), multi-gene linking and sexual crossing between plants carrying separate transgenes (Zhao *et al.*, 2003), have been used for the delivery of multiple genes into plants. Each approach has its specific limitations. For example, genetic crossing between transgenic plants and sequential re-transformation are very time-consuming and require the use of different selectable marker genes whereas the efficiency of co-transformation with multiple plasmids decreases progressively with increasing plasmid number. In case of co-transformation with multiple plasmids, the inserted copy numbers and the relative arrangement among transgenes cannot be controlled. In addition, multiple plasmids co-transferred by biolistics are found to be integrated at high copy number into a

few chromosome loci, which is not desired for expression of transgenes (Chen *et al.*, 1998; Gelvin, 1998; Maqbool and Christou, 1999). Transformation with linked transgenes in single vectors is a conventional and reliable approach. However, the lack of unique restriction cloning sites as well as the relatively low efficiency for ligation of inserts into larger vectors become the technical problems when three or more genes are sub-cloned into a transformation vector by using existing cloning methods. The stacking of multiple expression cassettes onto a single binary plasmid sometimes has a great advantage over the use of the other approaches mentioned above (Dafny-Yelin and Tzfira, 2007). The homing endonuclease-based pRCS/pAUX and pSAT vector systems (Goderis *et al.*, 2002; Tzfira *et al.*, 2005), Cre/loxP recombination (Lin *et al.*, 2003), MultiSite Gateway (Karimi *et al.*, 2007), and Multi-Round Gateway technology (Chen *et al.*, 2006b) have been specially developed in order to assemble multiple genes.

The Gateway technology is a flexible and universal cloning approach based on λ phage site-specific recombination (Papagiannis *et al.*, 2007). This method is more convenient than other methods because it does not involve DNA digestion or ligation. Once captured in a gateway-compatible plasmid 'entry vector' (EV), any transgene expression cassette flanked by recombination sites can be recombined into a variety of plant transformation vectors (destination vectors) that possess compatible recombination sites (Earley *et al.*, 2006). Thus, *in vitro* pyramiding of desired gene combinations on to a single T-DNA region by gateway technology appears to be one of the most attractive methods of multi-gene manipulation in plants. This method has several advantages including the linking of the desired gene combinations together during subsequent generations.

In this study, all the genes of the SOD-AsA-GSH pathway namely, superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) were stacked on a single T-DNA region by multi-round gateway cloning system. The multi-round gateway cloning strategy (as described in Materials and Methods-Section 3.25) involves the assembly of many genes through a series of recombination steps while alternating between two different entry vectors, EV1 and EV2. These two entry vectors differ in the attachment (att) sites; EV1 (pL12R34H-Ap) carries the attachment sites attL1-attL2 and attR3-attR4 while EV2 (pL34R12H-

CmR-ccdB) carries attL3-attL4 and attR1-attR2. Recombination occurs between specific att sites on the interacting DNA molecules. These sites serve as the binding site for recombination proteins (Weisberg and Landy, 1983). Upon lambda integration, recombination occurs between attL and attR sites to give rise to attB and attP sites (Landy, 1989). Thus EV1 and EV2 (containing attL sites) can be used in conjunction with the gateway compatible destination vectors (pMDC99, in the present case) carrying the attR sites. As a prerequisite for the multi-round gateway system, all the components of the SOD-AsA-GSH cassette were cloned in the entry vectors, EV1 and EV2 and subsequently assembled in the T-DNA region of destination vector pMDC99. In order to reconstitute active SOD-AsA-GSH pathway in rice anther tissue with co-ordinated expression of all the five genes, the T7 RNA polymerase based transgene expression system was introduced in the rice plant.

4.3 Design of T7 RNA Polymerase Based Transgene Expression System for Co-ordinated Expression of SOD-AsA-GSH Pathway Encoding Genes in Developing Rice Anthers

The expression levels of the transgene(s) depend upon the promoter sequences and transcription machinery and its associated signal transduction networks. A promoter is the most crucial element in the regulation of transgene expression in an optimal fashion. Constitutive promoters, those that are expressed all the time in all tissues, are presently the primary means used to express transgenes in plants. Metabolic energy waste, negative pleiotropic effects and potential gene escape are some of the disadvantages associated with the use of constitutive promoters. Expression of stacked genes under different plant promoters might lead to the non co-ordinated expression of transgenes, as each promoter will lead to a different degree of expression of transgenes. The co-ordinated expression of the different genes stacked together in a single plasmid is quite difficult to obtain. However, the co-ordinated and high expression of the stacked genes can be achieved by using the T7 RNA polymerase coupled expression system consisting of T7 promoter and T7 RNA polymerase protein. The T7 RNA polymerase system has been used to obtain high and controlled expression of transgenes in a couple of studies (McBride *et al.*, 1994; Chen *et al.*, 2002; Nguyen *et al.*, 2004). Stable plastid transformation of tobacco expressing the T7 RNA polymerase protein with a T7 promoter-β glucuronidase

(GUS) reporter gene construct resulted in expression of GUS mRNA and showed enzyme activity in all tissues (McBride *et al.*, 1994). Nguyen *et al.* have also reported T7 RNA polymerase directed inducible and tissue specific expression of GUS gene in tobacco and rice (Nguyen *et al.*, 2004).

In view of the above mentioned facts, the T7 system was chosen to drive the expression of the SOD-AsA-GSH pathway. The cassettes of each gene involved in SOD-AsA-GSH pathway were designed in such a way that each gene was placed separately under the control of T7 promoter. In this way, the transgene expression would be dependent on the introduced T7 RNA polymerase gene. The expression of T7 RNA polymerase would be regulated by the anther specific RA8 promoter. The overall design of the T7 RNA polymerase based SOD-AsA-GSH pathway expression strategy is represented in Fig. 4.21. This strategy would over-express the active SOD-AsA-GSH pathway enzymes in developing anther tissue to minimize the oxidative damage incurred on the male reproductive organs of rice under the adverse environmental conditions and thereby enhance the reproductive fitness of rice plants. The detailed construction and assembly of different components involved in T7 RNA polymerase coupled SOD-AsA-GSH-glutathione pathway expression strategy is discussed below.

4.4 Construction of T7 Promoter Regulated SOD-AsA-GSH Pathway Encoding Genes Cassettes

For ensuring the co-ordinated expression of all the stacked genes encoding for SOD-AsA-GSH pathway, the genes were placed individually under the control of T7 promoter. T7 promoter is known to be specifically recognized by T7 RNA polymerase protein and has been used for over production of recombinant proteins conventionally in *E. coli* and occasionally in plants (Nguyen *et al.*, 2004; McBride *et al.*, 1994).

The SOD-AsA-GSH pathway genes isolated from *P. glaucum* (PgMDHAR, PgDHAR, PgGR, PgSOD and PgAPX) were individually cloned between the T7 promoter and T7 terminator of pET-14b vector as described in materials and methods (Section 3.24). The putative peroxisomal targeting signal of PgMDHAR was deleted for ensuring the cytoplasmic location of PgMDHAR. The 5' and 3' un-translated

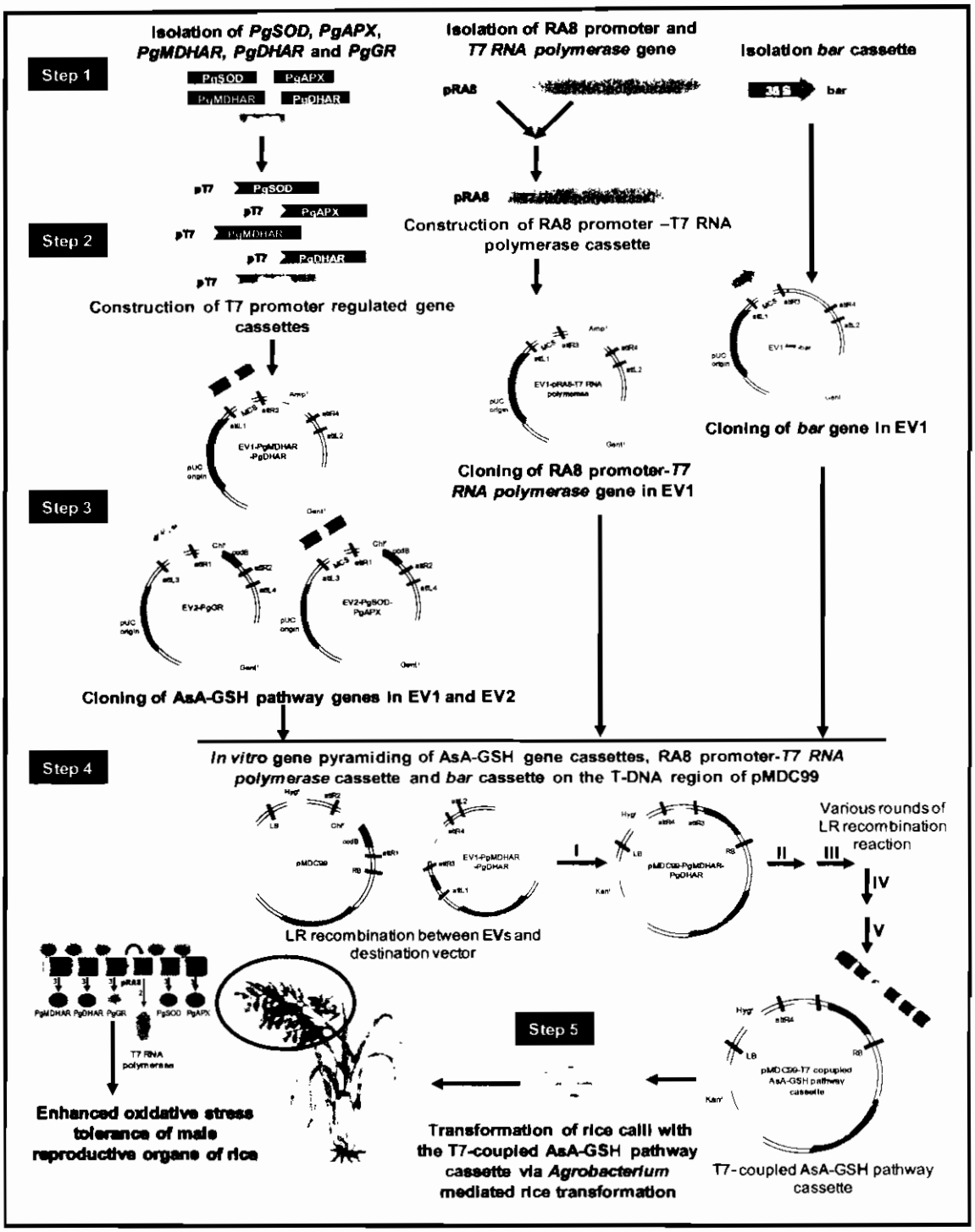


Figure 4.21 Schematic representation of different steps involved in the construction of T7 RNA polymerase coupled SOD-AsA-GSH pathway multi-gene construct. Step 1: Isolation of the component genes and promoter from different sources. Step 2: Construction of the gene cassettes with their promoters and UTRs. Step 3: Preparation of entry clones. Step 4: *In vitro* gene pyramiding of the different components of the SOD-AsA-GSH pathway construct on the T-DNA region of pMDC99 vector by 5 rounds of LR recombination reaction. Step 5: Transformation of rice with the T7 RNA polymerase coupled SOD-AsA-GSH pathway multi-gene construct. The expression of the cassette occurs in anthers where RA8 promoter is active. The expression of T7 RNA polymerase gene is activated by RA8 promoter (1). The T7 RNA polymerase protein thus formed (2) activates T7 promoter and leads to expression of expression of PgMDHAR, PgDHAR,PgGR, PgSOD and PgAPX genes (3) in anthers of rice.

regions (UTRs) of rice ribulose bisphosphate carboxylase small chain (Rbc S) gene were cloned respectively, upstream and downstream of the coding sequence of individual gene. The 5' and 3' UTRs of Rbc S gene have been shown to confer strong translational enhancement (Patel *et al.*, 2004). Thus, each gene cassette consisted of T7 promoter, T7 terminator and the full-length coding sequence of the SOD-AsA-GSH pathway genes flanked by 5' and 3' UTRs of rice Rbc S gene (Fig. 4.22). In this way, all transgenes with their own promoter and terminator would express individually, however, their expression was co-ordinated by introduced T7 RNA polymerase protein.

The size of the gene cassettes of *PgSOD*, *PgAPX* , *PgGR*, *PgDHAR* and *PgMDHAR* were found to be 1130, 1424, 2160, 1380 and 1979 bp, respectively (Fig. 4.22).

4.5 Anther Specific Expression of T7 RNA Polymerase

4.5.1 Cloning and Sequence Analysis of Anther Specific RA8 Promoter

Out of all the anther specific promoters studied so far (Koltunow *et al.*, 1990; Paul *et al.*, 1992; Twell *et al.*, 1990; 1991; Kim *et al.*, 1997; Okada *et al.*, 2000), the promoter for the gene *RA8* was chosen to drive the expression of the SOD-AsA-GSH cassette in anthers. *RA8* is an anther specific gene that is specifically expressed in tapetum, endothecium and connective tissue of the anther. The expression of the gene was found to be developmentally regulated, starting when the microspores are released from the tetrads and reaching to the maximum level at the late vacuolated-pollen stage (Jeon *et al.*, 1999). As discussed earlier, this stage of pollen development has been found to be most sensitive to various abiotic stress conditions. *RA8* gene has also been identified as stress responsive because of the presence a BURP-domain (OsBURP15) in the protein sequence. Many BURP domain-containing proteins have been found to be responsive to stress treatments (Ding *et al.*, 2009; Shao *et al.*, 2011). In order to confirm the tissue specificity of RA8 promoter, the presence of *RA8* transcript was analysed in different tissues by a semi-quantitative RT RCR using cDNA prepared from RNA isolated from leaves, roots, flag leaf, panicle and different parts of the rice panicle. Expression of *RA8* gene was found to be confined to the panicles (Fig. 4.23A). The expression of the gene was further, found to be occurring exclusively in the anthers of rice plant (Fig. 4.23B)

A 1.2 kb region upstream of the coding region of *RA8* gene was amplified from genomic DNA of rice using promoter specific primers (Fig. 4.24A) as listed in table 3.10 and used as RA8 promoter.

In silico analysis of the RA8 promoter region revealed the presence of putative TATA box sequence (TATAAT) at -101/-94. A number of other *cis*-regulatory sequences like CGTCA-motif, GARE motif, AT rich elements were also found in the promoter sequence. The CGTCA-motif, GARE motif are involved in methyl jasmonate and gibberlic acid responsiveness, respectively. It has been reported that gibberellins modulates anther development in rice (Aya *et al.*, 2009) whereas jasmonic acid controls anther dehiscence, filament elongation, and pollen viability (Scott *et al.*, 2004). Several potential regulatory elements that are related to anther specific gene expression were also present (Table 4.1). The position of the motif in the promoter sequence is represented in Fig. 4.24B. The promoter was found to contain elements like TAPNAC motif (TCTGA)), POLLEN1LELAT52 motif (AGAAA), GTGANTG10 motif (GTGA) and the PB core motif (GTGGTT). These motifs have been reported to be present in genes whose expression is confined to anthers. A conserved TCGTGT motif was identified in the TAPNAC promoter and other tapetal expressed promoters (Alvarado *et al.*, 2011). The POLLEN1LELAT52 motif is one of two co-dependent regulatory elements responsible for pollen-specific activation of tomato *LAT52* gene (Bate and Twell, 1998). GTGANTG10 element (GTGA) motif was identified in the promoter of the tobacco late pollen gene *g10* (Rogers *et al.*, 2001) and was also found in the promoter region of anther specific *RTS* gene of rice (Luo *et al.*, 2006). Promoter regions of rice *OSIPA*, *OSIPK*, *OsACE1*, *OsCER1*, *OsMADS58*, *OsPGT1*, *OsERFL1*, and *OsRIP1* genes also share the two cis-elements, GTGANTG10 and POLLEN1LELAT (Gupta *et al.*, 2007; Kato *et al.*, 2010). The PB core motif is involved in modulating the activity of the LAT gene promoters in pollen of tomato (Twell *et al.*, 1991).

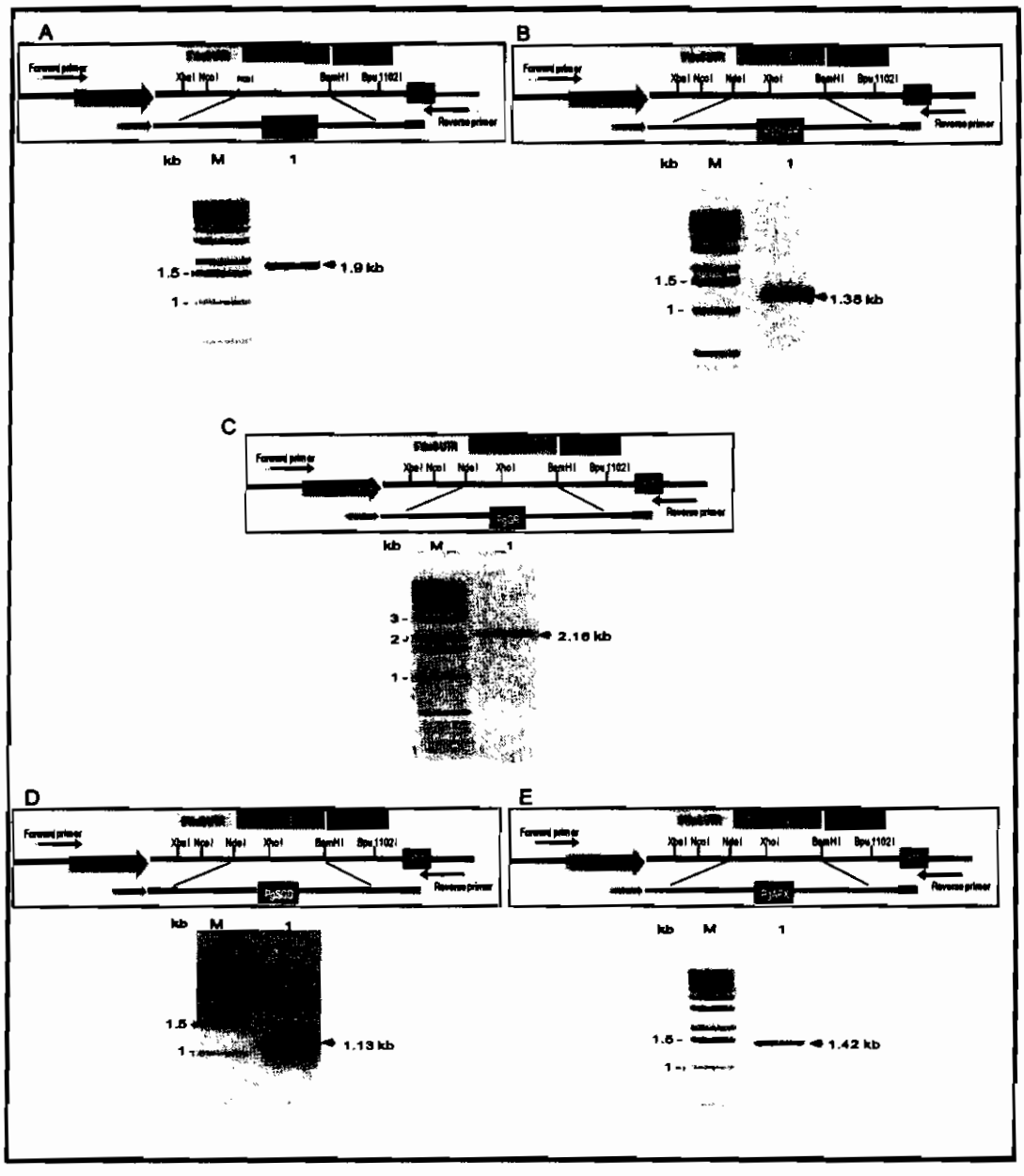


Figure 4.22 Construction of SOD-AsA-GSH gene cassettes **(A)** Construction of gene cassette of PgMDHAR. Upper panel shows schematic representation of pET-14b vector with *PgMDHAR* gene and UTRs. Agarose gel showing amplification of PgMDHAR cassette is shown below. Lane 2 indicates 1979 bp amplicon of *PgMDHAR* gene cassette **(B)** Construction of gene cassette of PgDHAR. Upper panel shows schematic representation of pET-14b vector. Agarose gel showing amplification of PgDHAR cassette is shown below. Lane 2 indicates 1380 bp amplicon of *PgDHAR* gene cassette **(C)** Construction of gene cassette of PgGR. Upper panel shows schematic representation of pET-14b vector with PgGR gene and UTRs. Agarose gel showing amplification of *PgGR* cassette is shown below. Lane 2 indicates 2160 bp amplicon of *PgGR* gene cassette **(D)** Construction of gene cassette of PgSOD. Upper panel shows schematic representation of pET-14b vector with PgSOD gene and UTRs. Agarose gel showing amplification of PgMDHAR cassette is shown below. Lane 2 indicates 1130 bp amplicon of PgSOD gene cassette **(E)** Construction gene cassette of *PgAPX*. Upper panel shows schematic representation of pET-14b vector harboring APX gene, UTRs. Agarose gel showing amplification of PgAPX cassette is shown below. Lane 2 indicates 1420 bp amplicon of *PgAPX* gene cassette. Lane M: 1 kb DNA ladder.

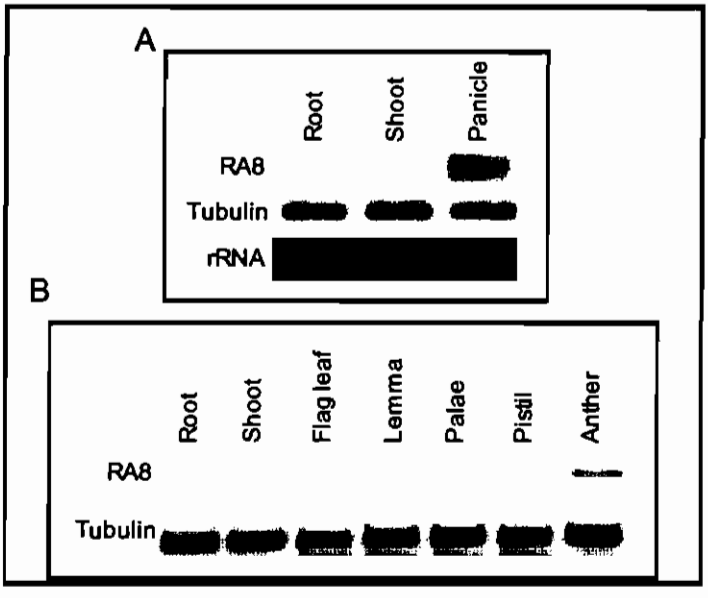


Figure 4.23 Semi-quantitative RT-PCR analysis of RA8 gene expression in various tissues. Panel **A** represents analysis of the expression of RA8 gene in roots, mature leaves (Shoot) and panicles of *O. sativa* cv Swarna plants. Panel **B** shows the expression analysis of RA8 gene in various parts of panicle.

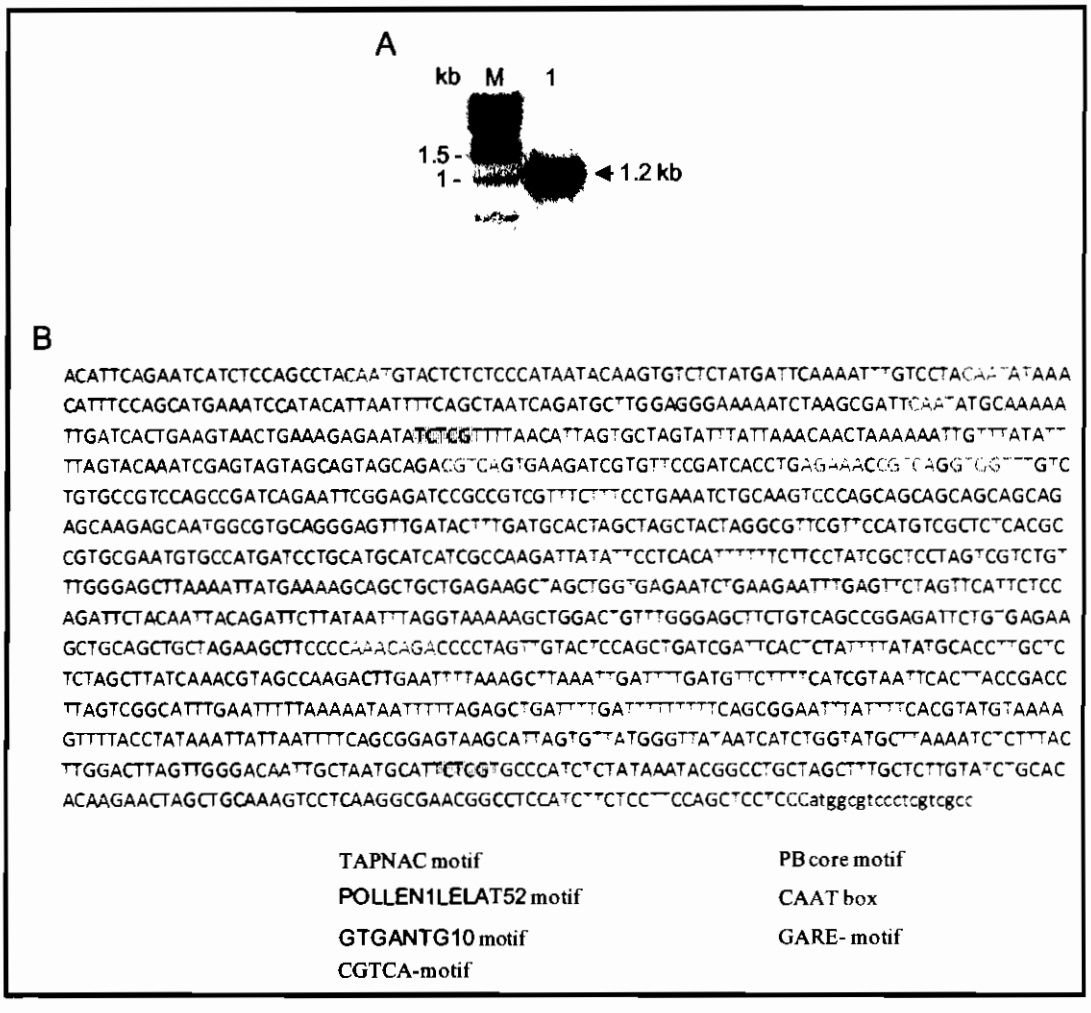


Figure 4.24 Isolation and cloning of anther specific RA8 promoter **(A)** Agarose gel showing amplification of RA8 promoter from rice genome. Lane 1 shows a 1.2 kb amplicon of RA8 promoter. **(B)** Nucleotide sequences and putative cis-acting elements found in RA8 promoter. The different cis acting regulatory elements are shaded in different coloured boxes. The putative TATA box is highlighted in red. The ORF of RA8 gene is shown by small letters.

Table 4.1 Potential *cis*-acting regulatory elements identified in the RA8 promoter

Name of the motif	Sequence	Position from ATG	Details	Reference
TAPNAC	TCGTG	-115/-110, -950/-945	A *cis* regulatory element in the TAPNAC promoter that directs tapetal gene expression.	Alvarado *et al.*, 2011
POLLEN1-LELAT52	AGAAA	-932/-927	One of two co-dependent regulatory elements responsible for pollen-specific activation of tomato *LAT52*gene.	Bate and Twell,1998
GTGANTG10	GTGA	-498/-493,-613/-610, -957/-954	GTGA motif in the promoter of the tobacco late pollen gene *g10*.	Rogers *et al.*, 2001
GARE	AAACAGA	-467/-461	*Cis* regulatory element involved in gibberlic acid responsiveness	Gubler and Jacobsen, 1992; Ogawa *et al.*, 2003
CGTCA	CGTCA	-962/-958, -926/-922	*Cis* regulatory element involved in MeJA responsiveness.	Kim *et al.*, 1993; Rouster *et al.*, 1997
PB core	GTGGTT	-920/-915	Cis-acting element found in *LAT* gene of tomato	Twell *et al.*, 1991
DOFCORE-ZM	AAAG	-48/-45, -237/-234,-372/-367, -539/-535,-638/-634, -1059/-1055	Binds dof proteins and enhances transcription in many promoters	Yanagisawa *et al.*, 1999
TATA box	TATAAT	-101/-94	Core promoter sequence	Lescot *et al.*, 2002, (PlantCARE)
CAAT box	CAAT	-132/-128, -567/-563, -819/-816, -1086/-1083, -1166/-1163	Common cis-acting element in promoter and enhancer regions	Lescot *et al.*, 2002, (PlantCARE)
MBS	TAACTG	-1065/-1060	MYB binding site involved in drought-inducibility	Lescot *et al.*, 2002, (PlantCARE)

4.5.2 Cloning and Expression of T7 RNA polymerase Gene

A 2.7 kb DNA fragment corresponding to *T7 RNA polymerase* gene was PCR amplified using T7 RNA polymerase specific primers (Table 3.11) from T7 phage DNA and cloned in TA cloning vector and sequenced (Fig. 4.25A). The *in silico* translation analysis of the DNA sequence revealed that the ORF codes for a protein with an apparent weight of 97 kDa. The protein sequence was found to contain the conserved domains characteristic of T7 RNA polymerase protein (Sousa *et al.*, 1993). The enzyme consists of an N-terminal DNA recognition and a C-terminal RNA polymerase domain. The N-terminal domain encompasses amino acid residues 1-324 and is involved in promoter recognition and DNA melting via the participation of the AT rich recognition loop and intercalating β-hairpin loop. The RNA polymerase domain of T7 RNA polymerase protein is quite similar to other RNA polymerases in comprising of palm, finger and thumb domains. The highly flexible thumb domain (324-411aa) is known to increase the processivity of the enzyme. The palm domain (412-465 and 785-883aa) contain the active site residues D-537 and D-812 which are involved in catalysis via interaction with the two Mg^{2+} ions. The finger domain comprises of amino acid residues 566-784. This domain consist of the specificity loop which is involved in direct base specific interaction with the major groove of the T7 promoter (Fig. 4.25B).

4.5.2.1 Addition of SV40 Nuclear Localization Signal to T7 RNA polymerase

In eukaryotes, transcription and translation takes place in nucleus and cytoplasm, respectively. For developing T7 RNA polymerase coupled transgene expression system, introduction of necessary eukaryotic features to T7 RNA polymerase coding region was needed. A 225 bp of 35S 3' UTR was added to T7 RNA polymerase coding region (Fig. 4.26B-i) for post-transcriptional processing and subsequent export of poly-adenylated mRNA into the cytoplasm. In order to transcribe the transgenes cloned under T7 promoter, the translated T7 RNA polymerase polypeptide from cytoplasm was required to be targeted to nucleus. Fusion of a nuclear localization signal (NLS) at the N-termini of the proteins has been shown to direct the proteins to nucleus. NLS derived from SV40 Large T-antigen (Dingwall and Laskey, 1991) has been used to facilitate nuclear import of various proteins (Nehaus *et al.*, 1984; van der

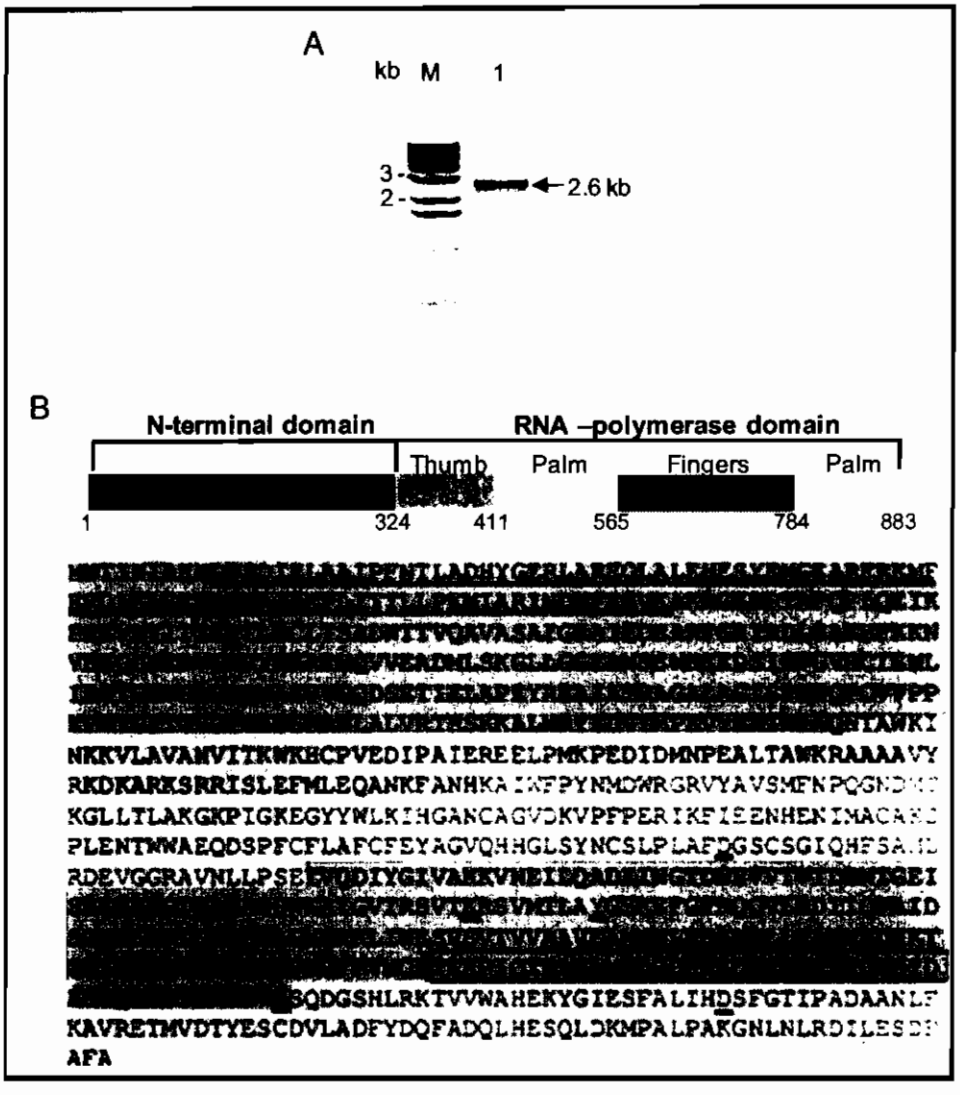


Figure 4.25 Isolation and sequence analysis of T7 RNA polymerase protein. **(A)** Agarose gel denoting amplification of 2.6 kb of *T7 RNA polymerase* gene by PCR using gene specific primers. **(B)** Schematic representation indicating the structural domain of T7 RNA polymerase protein. Amino acid sequence of T7 RNA polymerase protein showing various domains of the protein. The N-terminal domain and. the thumb palm and finger sub-domains of the RNA polymerase domain are shaded in red, blue, yellow and purple colours. The specificity loop and AT rich recognition loop are enclosed in a blue and red boxes respectively. The active site residues are underlined in red.

Krol *et al.*, 1991). The SV40 large T antigen NLS contains a short stretch of basic amino acids (PKKKRKV) that imports the protein into nucleus (Raikhel, 1992).

For directing the nuclear import of T7 RNA polymerase protein, a 45 bp synthetic oligonucleotide sequence coding for N-terminus SV40 NLS polypeptide was added to the 5' end of T7 RNA polymerase coding region. Previously, N-terminus fusion of SV40 NLS signal to T7 RNA polymerase was successfully used for nuclear import and subsequent expression of GUS reporter gene cloned under T7 promoter in transgenic plants (Lassner *et al.*, 1991; Nguyen *et al.*, 2004).

The modified T7 RNA polymerase protein (Fig. 4.26A) was cloned at *Bam*H I-*Not* I restriction site of pET-28a vector (Fig. 4.26B-ii), the recombinant plasmid was transferred into *E. coli* Rossetta (2DE3) pLysS cells to achieve protein expression and purification. The protein profile of the un-induced and induced cells showed over expression of the 97 kDa T7 RNA polymerase in IPTG induced cells (Fig. 4.26B-iii). Before the construction of the gene cassettes, the enzymatic activity of the modified T7 RNA polymerase protein containing the SV 40 NLS signal peptide was verified.

4.6 Isolation and Cloning of *bar* Gene

The bialaphos/Basta resistance (*bar*) gene, which encodes the enzyme phosphinothricin acetyl transferase (PAT), is widely used as a selectable marker for cereal transformation (Christou *et al.*, 1991; Castillo *et al.*, 1994; Altpeter *et al.*, 1996; Brettschneider *et al.*, 1997). The activity of PAT provides the plant with the ability to detoxify phosphinothricin (PPT), the active compound in commercial herbicide formulations of PPT or bialaphos such as Basta (Hoechst, Germany). PPT is a glutamate analogue that irreversibly inhibits glutamine synthetase activity, the key enzyme for ammonium assimilation and the regulation of nitrogen metabolism in plants. The inhibition of glutamine synthetase results in the death of untransformed tissues and plants due to the accumulation of ammonium (Rasco-Gaunt *et al.*, 1999; Tachibana *et al.*, 1986; Wendler *et al.*, 1990).

For effieient screening of the transgenics, *bar* gene was introduced in the T-DNA region. A 1636 bp *bar* cassette containing 35S promoter and *bar* gene was amplified from a plant transformation vector- pMDC123 that carries *bar* gene as a selection marker (Fig. 4.27B-i). The amplified 1.63 kh fragment was cloned in $EV1^{-Amp}$ (EV1

vector in which ampicillin resistance marker gene was removed) at *Eco*R I and *Hind* III restriction sites (Fig. 4.27B-ii and iii).

4.7 *In vitro* Pyramiding of Genes Encoding SOD-AsA-GSH Pathway along with RA8 Promoter-T7 RNA polymerase expression cassette.

4.7.1 Construction of Entry Clones: Cloning of the Genes and Promoter Cassettes in Entry Vectors

The first step of gateway cloning consisted of the preparation of the entry clones i.e., cloning of target genes into gateway-compatible entry vector(s) (EV1 and EV2). The entry vectors consist of a unique pair of attL and attR recombination sites and several restriction sites for the cloning of target genes. EV1 carries the attR3-attR4 recombination sites flanked by a pair of attL1-attL2 sites, while the EV2 carries attR1-attR2 recombination sites flanked by a pair of attL3-attL4 sites . Thus, the entry clones were prepared by cloning *PgMDHAR*, *PgDHAR*, *PgGR*, *PgSOD*, *PgAPX* gene cassettes (containing the respective genes, T7 promoter, 5'and 3' UTRs and T7 terminator) RA8 promoter, *T7 RNA polymerase* gene and bar cassette in the entry vectors by conventional restriction based cloning as described below.

4.7.1.1 Construction of RA8 promoter-T7 RNA polymerase expression cassette: Cloning of of RA8 promoter and T7 RNA polymerase in EV1

For the co-ordinated over-expression of SOD-AsA-GSH pathway genes in rice anthers, the anther specific expression of *T7 RNA polymerase* gene was required. Thus, the first step of the multi-round gateway cloning comprised of cloning of the RA8 promoter and *T7 RNA polymerase* gene in the entry vector EV1.

The 1.2 kb region of RA8 promoter was cloned into *Kpn* I and *Nco* I restriction sites of EV1 (Fig. 4.28). In order to drive the expression of the T7 RNA polymerase protein in the anthers of rice, the 3 kb long modified-*T7 RNA polymerase* gene (containing the SV40 NLS signal and 35S poly A sequence) was cloned downstream of RA8 promoter into *Nco* I and *Sac* I restriction sites of EV1 (Fig. 4.29). Thus, the promoter-polymerase cassette consisting of RA8 promoter and *T7 RNA polymerase gene*, 4.2 kb in size, was integrated between the attL1-attL2 recombination sites of EV1 (4.29A).

4.7.1.2 Cloning of PgMDHAR and PgDHAR Cassettes in EV1

For reducing the number of rounds of stacking both *PgMDHAR* and *PgDHAR* were cloned in EV1 in a sequential manner (Fig. 4.30). The 1479 bp long *PgMDHAR* cassette was cloned into *Spe* I and *Xba* I sites of EV1 (Fig. 4.30B-i and ii). The *PgDHAR* cassette of size 1380 bp was cloned downstream of *PgMDHAR* into *Xba* I and *Not* I sites of EV1 (Fig. 4.30B iii-iv). Thus, the 2.8 kb long, *PgMDHAR-PgDHAR* gene cassette was integrated between the attL1-attL2 recombination sites of EV1.

4.7.1.3 Cloning of *PgGR* Cassette in EV2

Since, the multi-round gateway cloning system involves recombination reactions while alternating between the two entry vectors, some of the gene cassettes of the T7 RNA polymerase coupled SOD-AsA-GSH pathway construct were cloned in EV2. The *PgGR* gene cassette, 2160 bp in length, was cloned into *Spe* I and *Xba* I sites of EV2 (Fig. 4.31B-i and ii). Thus, *PgGR* cassette was inserted between the attL3-attL4 recombination site of EV2 (Fig. 4.31A).

4.7.1.4 Cloning of PgSOD and PgAPX Cassettes in EV2

Similar to the *PgMDHAR-PgDHAR* cassette, the cassette of *PgSOD* and *PgAPX* were sequentially cloned into EV2. The 1424 bp long *PgAPX* cassette was cloned into *Eco* RV and *Hind* III sites of EV2 (Fig. 4.32B-i) and the *PgSOD* cassette of size 1130 bp was cloned downstream of *PgAPX* into *Hind* III and *Cla* I restriction sites of EV2 (Fig. 4.32B-ii and iii). Thus, third gene cassette, 2.5 kb in length, was integrated between the attL3-attL4 recombination sites of EV2 (Fig 4.32).

As already discussed, the *bar* cassette, 1.6 kb in size, was cloned in $EV1^{-Amp}$ between the attL1-attL2 recombination sites (Fig. 4.27).

4.7.2 Pyramiding of the SOD-AsA-GSH Pathway and RA8 Promoter-T7 RNA polymerase Cassettes on Plant Transformation Vector, pMDC99

After the preperation of the entry clones carrying the *SOD*-AsA-GSH pathway gene cassettes, RA8promoter-T7 RNA polymerase expression cassette and the *bar* cassettes, these cassettes were stacked in the destination vector pMDC99 by multi round gateway cloning using LR clonase (as described in Materials and Methods). LR Clonase® (Invitrogen) enzyme contains a a proprietary mix of integrase, integration

host factor and excisionase enzymes. These enzymes catalyze the *in vitro* recombination between an entry clone (containing the genes of interest flanked by attL sites) and a destination vector (containing attR sites). Assembly of the multiple-gene begins with LR recombination between one of the EV(EV1/EV2) and the destination vector (pMDC99). The destination vector pMDC99 is derived from pCAMBIA T-DNA cloning vector which is used for *Agrobacterium*-mediated transformation of a wide range of plant species and contains the hygromycin phosphotransferase plant-selectable marker gene. In this vector, the gateway recombination site attR1-attR2 (for introduction of the gene of interest) is placed towards the right border of the T-DNA.

In the present study, five rounds of LR recombination reactions, as described below, were performed while alternating between EV1 and EV2 to construct the T7 RNA polymerase coupled SOD-AsA-GSH pathway multi-gene construct.

4.7.2.1 First Round of LR Recombination Reaction

The first step of LR cloning involved recombination reaction between the entry clone carrying *PgMDHAR-PgDHAR* cassette (EV1-PgMDHAR-PgDHAR) and destination vector pMDC99 (Fig 4.33). This recombination step resulted in: (i) the transfer of the first gene cassette into the pMDC99; (ii) replacement of the negative selection gene of pMDC99 (ccdB) with the selection marker of EV1; and (iii) the incorporation of a new pair of attR recombination sites (Fig. 4.33A). This resulted in the possibility of a second recombination LR step between the second entry vector (EV2, containing the compatible attL3-attL4 recombination site) and the recombinant pMDC99 which now carried the attR3-attR4 site.

4.7.2.2 Second Round of LR Recombination Reaction

The second step involved stacking of *PgGR* cassette cloned in EV2 to the recombinant pMDC99 containing the *PgMDHAR-PgDHAR* cassette (Fig. 4.34). As described earlier, this recombination step replaced the selection marker of the recombinant pMDC99 (ampicillin resistance gene, Amp^R) with that of EV2 (ccdB and chloramphenicol resistance gene, Chl^R) and led to the insertion of a new pair of recombination site, attR1-attR2 (Fig 4.34A). Thus the second recombination step resulted in the assembly of *PgGR*, *PgMDHAR* and *PgDHAR* gene cassettes.

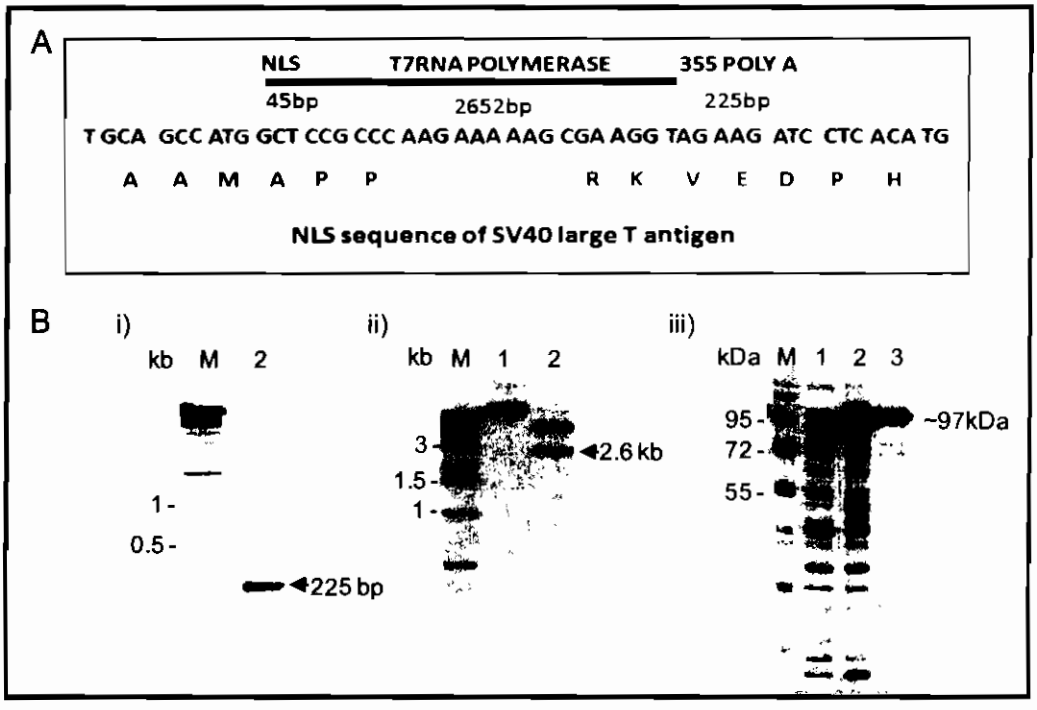


Figure 4.26 Modification and expression of *T7 RNA polymerase.* gene **(A)** Schematic diagram of the modified T7 RNA polymerase gene indicating the positions of SV40 large T-antigen NLS at N-terminal and a 35S poly A at C-terminal end. **(B) (i)** Agarose gel showing PCR amplification of 225 bp 35S poly A sequence. Lane 2: 225 bp amplicon of 35S poly A. **(ii)** Agarose gel indicating confirmation of cloning of T7 RNA polymerase protein in pET-28A vector by restriction digestion, Lane M: 1 kb DNA ladder, Lane 1 double digestion of pET-28A vector harboring T7 RNA polymerase gene using *Bam* HI and *Not* I; Lane 2 :un-digested vector. **(iii)** SDS-PAGE profile of the expressed T7 RNA polymerase protein from bacterial cells. Lane 1: pre-stained protein marker; Lanes 2 and 3 indicate the un-induced and induced culture pellets of recombinant clone; Lane 3 shows the Ni-NTA column purified recombinant protein.

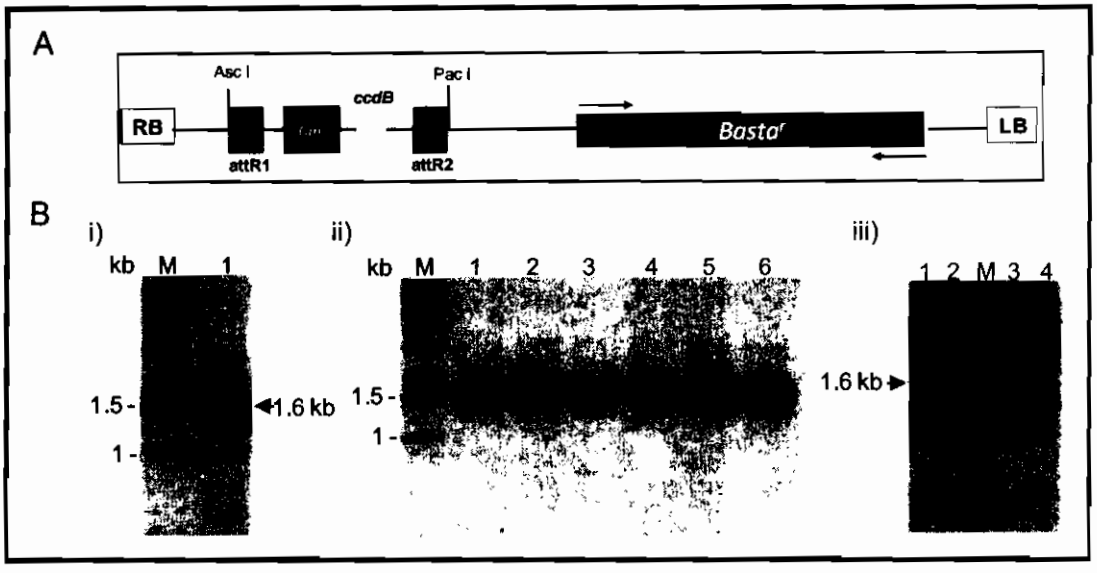


Figure 4.27 Isolation and cloning of bar cassette. **(A)** Schematic representation of pMDC123 vector. **(B)** Agarose gel denoting **(i)** Amplification of ~1.6 kb of bar cassette from pMDC123 vector **(ii)** Confirmation of cloning of bar cassette in EV1 by colony PCR **(iii)** Restriction digestion analysis of bar cassette cloned in EV1 vector; Lanes 1 and 2 denote vector digested with *Eco* RI and *Hind* III while 3 and 4 represent un-digested vector.

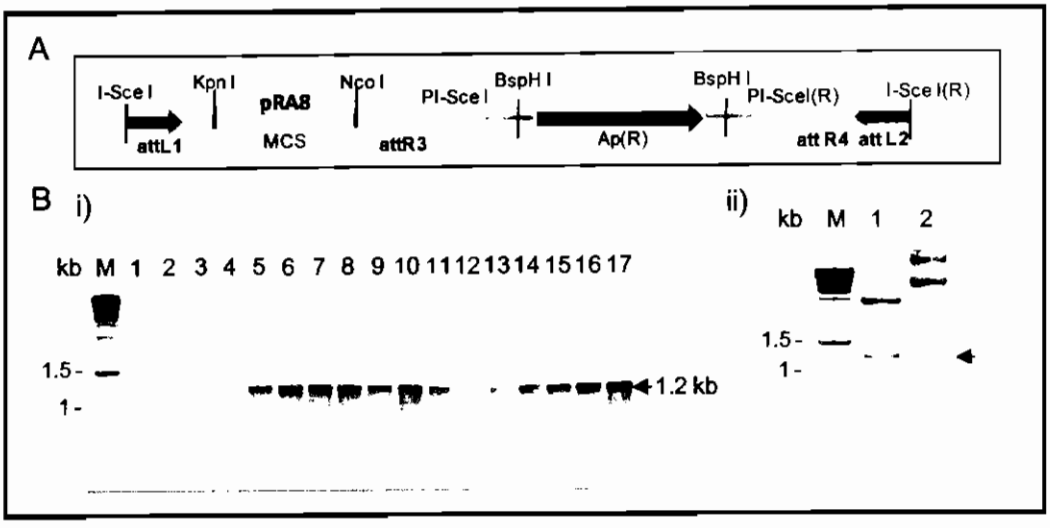


Figure 4.28 Cloning of RA8 promoter in EV1. **(A)** Schematic representation of EV1. **(B)** Agarose gel indicating confirmation of cloning of RA8 promoter (pRA8) in EV1 by **(i)** Colony PCR and **(ii)** Restriction digestion analysis where lane 1 indicates vector digested with *Kpn* I and *Nco* I and lane 2 indicates the un-digested vector, M represents 1 kb DNA ladder.

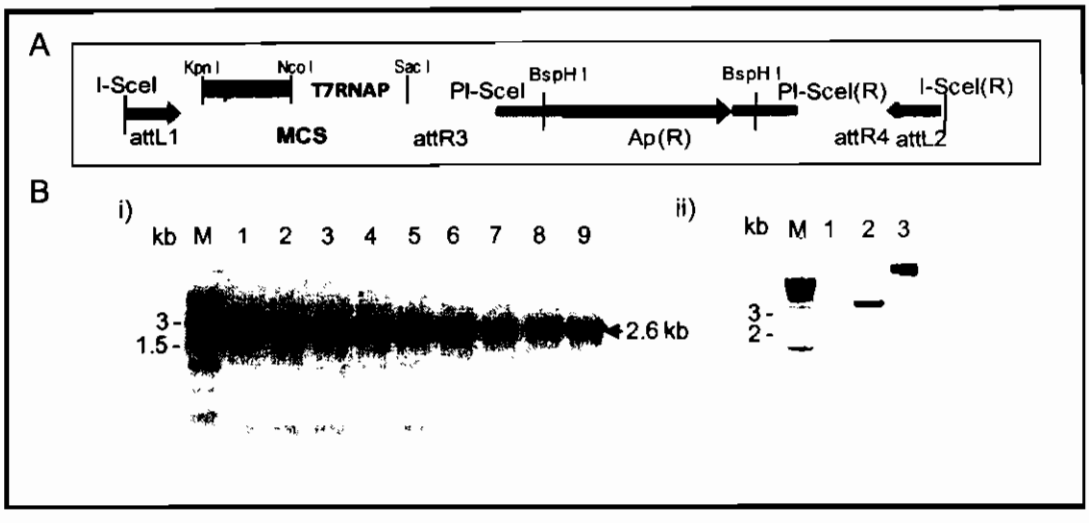


Figure 4.29 Cloning of T7 RNA polymerase gene in EV1 vector harboring RA8 promoter (EV1-RA8). **(A)** Schematic representation of EV1 showing the sites for cloning. **(B)** **(i)** Agarose gel denoting confirmation of cloning of T7 RNA polymerase gene in EV1 by colony PCR using gene specific primers **(ii)** Agarose gel indicating confirmation of cloning of T7 RNA polymerase gene in EV1 vector by restriction digestion. Lane M: 1 kb DNA ladder, Lane 1: double digestion of EV1 vector harboring RA8 promoter and T7 RNA polymerase gene using *Kpn* I and *Sac* I showing ~4 kb fallout corresponding to RA8 promoter and T7 RNA polymerase gene, Lane 2: double digestion of the same vector using *Nco* I and *Sac* I showing ~3 kb fallout corresponding to T7 polymerase gene, Lane 3: un-digested EV1-vector containing RA8 promoter and T7 polymerase gene.

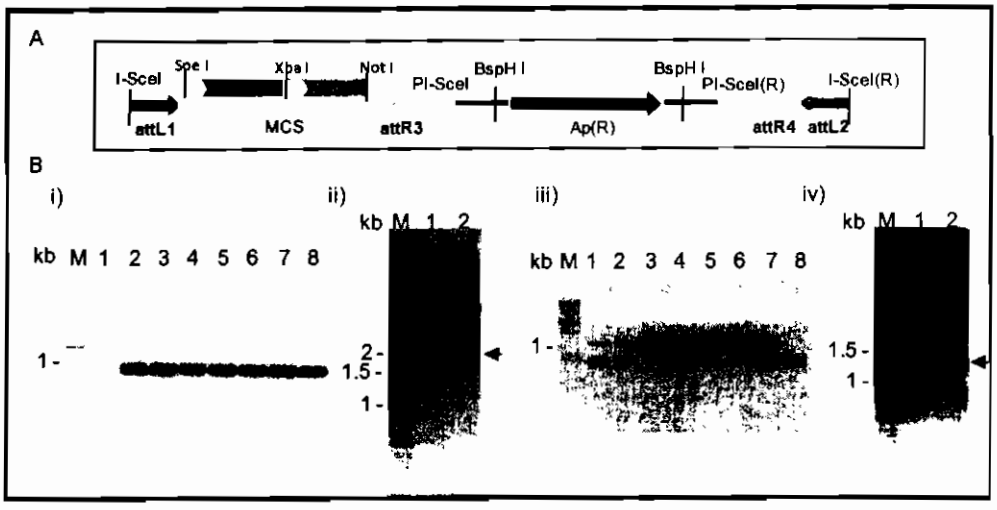


Figure 4.30 Cloning of PgMDHAR and PgDHAR cassettes in EV1. (A) Schematic representation of EV1 vector harboring PgMDHAR and PgDHAR cassette. Yellow arrows indicate the T7 promoter **(B) (i)** Agarose gel denoting confirmation of cloning of PgMDHAR cassette in EV1 by colony PCR using gene specific primers **(ii)** Agarose gel indicating confirmation of cloning of PgMDHAR cassette in EV1 vector by restriction digestion; Lane 2: un-digested vector; Lane 3: double digestion of EV1 vector harboring PgMDHAR cassette using *Spe* I and *Xba* I showing ~1.9 kb fallout corresponding to PgMDHAR cassette. **iii)** Agarose gel indicating confirmation of cloning of PgDHAR cassette in EV1-MDHAR vector by colony PCR using gene specific primers. **(iv)** Agarose gel indicating confirmation of cloning of PgDHAR cassette by restriction digestion. Lane 2: un-digested vector, Lane 3: double digestion of EV1 vector harboring PgDHAR and PgMDHAR cassette using *Xba* I and *Not* I showing ~1.38 kb fallout corresponding to PgDHAR cassette. M indicates 1 kb DNA ladder.

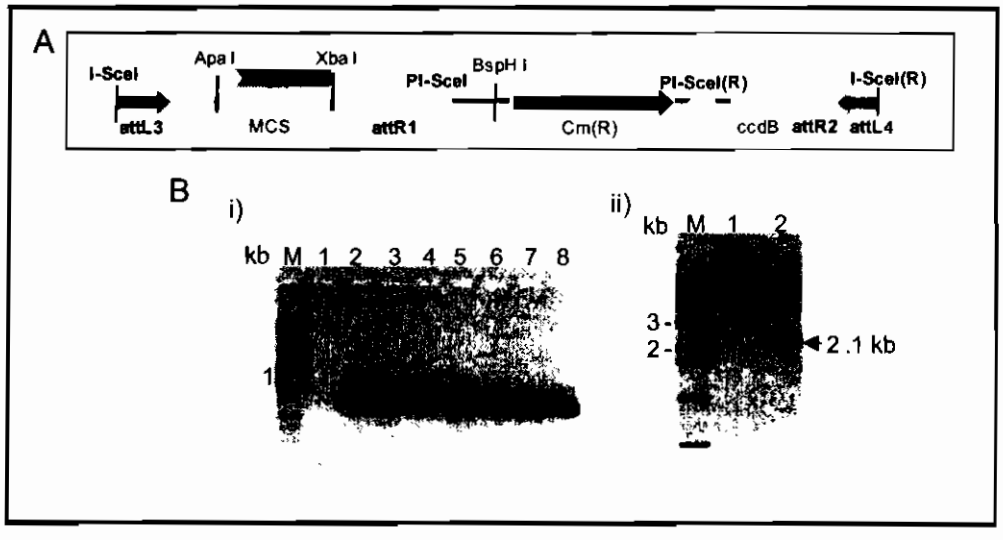


Figure 4.31 Cloning of PgGR in EV2. **(A)** Schematic representation of EV2 vector harboring PgGR cassette. T7 promoter is indicated by yellow coloured arrow **(B) (i)** Agarose gel denoting confirmation of cloning of PgGR in EV2 by colony PCR. using gene specific primers **(ii)** Agarose gel indicating confirmation of cloning of PgGR cassette by restriction digestion. Lane M: 1 kb DNA ladder; Lane 2: un-digested vector; Lane 3: double digestion of EV 2 vector harboring PgGR cassette using *Spe* I and *Xba* I showing ~2.1 kb fallout corresponding to PgGR cassette.

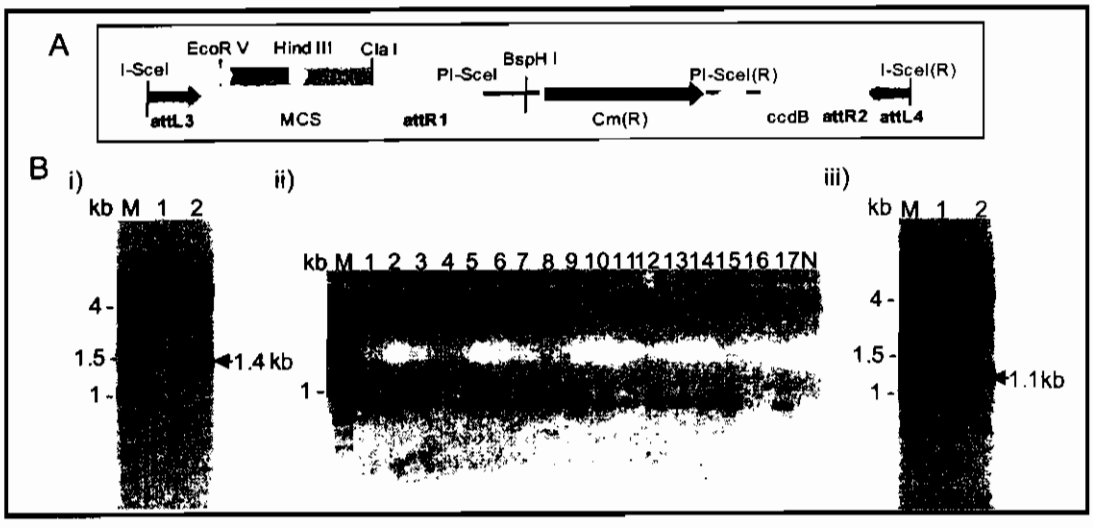


Figure 4.32 Cloning of PgSOD and PgAPX in EV2. **(A)** Schematic representation of EV2 vector carrying PgSOD and PgAPX cassettes. Yellow coloured arrow in the cassette represent T7 promoter. **(B) (i)** Agarose gel indicating confirmation of cloning of PgAPX cassette by restriction digestion. Lane M: 1 kb DNA ladder; Lane 1: un-digested vector, Lane 2: double digestion of EV 2 vector harboring PgAPX cassette using *EcoR* V and *Hind* III showing ~1.4 kb fallout corresponding to PgAPX cassette. Agarose gel denoting confirmation of cloning of PgSOD cassette in EV2 by **(ii)** Colony PCR (Lanes 1-17 denote the number of colonies screened) using SOD specific screening primers. N indicates negative control **iii)** Restriction digestion. Lane M: 1 kb DNA ladder, Lane 2: un-digested vector, Lane 3: double digestion of EV2 vector harboring PgSOD and PgAPX cassette using *Hind* III and *Cla* I showing ~1.1 kb fallout corresponding to PgSOD cassette.

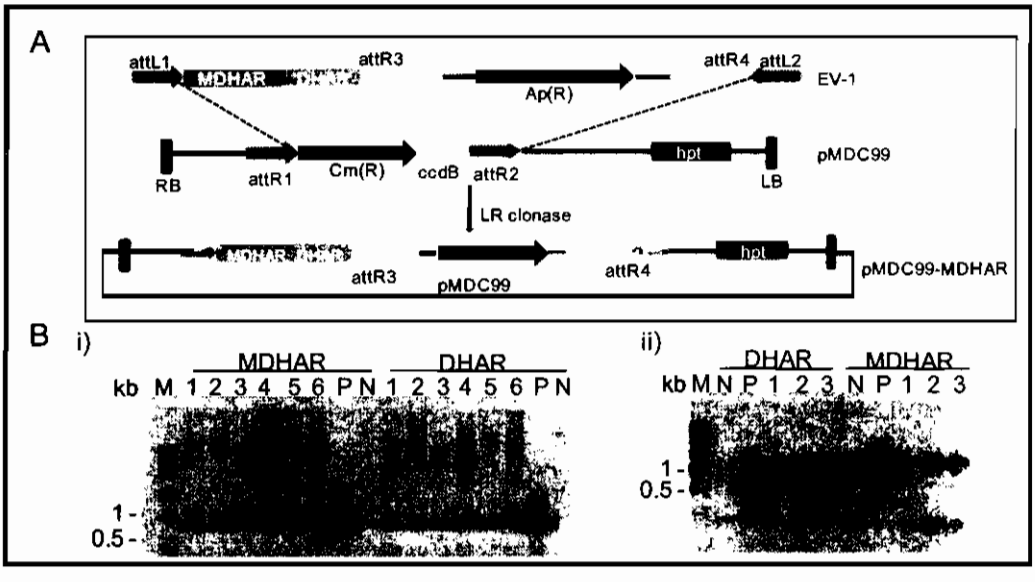


Figure 4.33 First round of gene stacking. **(A)** Schematic representation of the stacking of the first gene cassette containing PgMDHAR and PgDHAR genes in the destination vector pMDC99. **(B)** Agarose gel indicating confirmation of cloning of the first cassette in pMDC99 by **(i)** colony PCR (Lanes 1-6 denote the number of colonies screened) **(ii)** Plasmid-PCR (Lanes 1-3 denote amplification of respective genes from the plasmids isolated from three positive colonies). Lane M denotes 1 kb DNA ladder, Lane P and Lane N denotes positive and negative controls, respectively.

4.7.2.3 Third Round of LR Recombination Reaction

The third round of LR recombination involved the addition of RA8 promoter-T7 RNA polymerase cassette cloned in EV1 to the recombinant pMDC99 which harboured the *PgMDHAR*, *PgDHAR* and *PgGR* genes (Fig. 4.35). This step led to the insertion of a new set of recombination site, attR3-attR4 into the recombinant pMDC99 (Fig 4.35A) thus, making way for another round of recombination reaction.

4.7.2.4 Fourth Round of LR Recombination Reaction

The fourth round of LR recombination involved the recombination between EV2 carrying the *PgSOD-PgAPX* cassette and the recombinant pMDC99 harbouring *PgMDHAR*, *PgDHAR*, *PgGR*, RA8 promoter and *T7 RNA polymerase* gene (Fig. 4.36). This step led to the assembly of *PgSOD* and *PgAPX* genes and insertion of recombination site, attR1-attR2 carrying the ccdB gene into the recombinant pMDC99 (Fig 4.36A).

4.7.2.5 Fifth Round of LR Recombination Reaction

The fifth and final round of recombination reaction was performed to remove the toxic *ccdB* gene introduced in the recombinant pMDC99 as a result of the previous round of recombination. This was achieved by performing a recombination reaction between empty EV1 (carrying no target gene) in which the marker gene (Amp^R) was removed ($EV1^{-Amp}$) (as described in materials and methods) and the recombinant pMDC99. Thus the final construct did not carry any additional selection marker .

In order to facilitate the process of screening of the transgenics, one more construct was made in addition to the above construct in which *bar* gene was also assembled together with the *SOD*-AsA-GSH pathway genes (Fig. 4.37). This was achieved by the recombination reaction between the the entry clone carrying *bar* cassette ($EV1^{-Amp}$ -*bar*) and the recombinant pMDC99 vector carrying the *SOD*-AsA-GSH pathway genes, RA8 promoter and *T7 RNA polymerase* gene. As the selection marker in the entry clone carrying *bar* gene was removed, no additional selection marker was present after this round of recombination. Thus, the T7 RNA polymerase -coupled SOD-AsA-GSH pathway-*bar* construct was made with *PgMDHAR*, *PgDHAR*, *PgGR*, *PgSOD*, *PgAPX* gene cassettes, RA8 promoter-*T7 RNA polymerase* expression cassette and *bar* cassettes inserted in the T-DNA region of pMDC99 (Fig. 4.37A).

4.8 Transformation of *Agrobacterium* with Recombinant pMDC99 Vector Harboring T7 RNA polymerase coupled SOD-AsA-GSH Pathway Construct

The T7 RNA polymerase coupled SOD-AsA-GSH pathway constructs were mobilised into *Agrobacterium* via electroporation. The positive colonies were screened for the presence of all the genes namely, *PgMDHAR*, *PgDHAR*, *PgGR*, *PgSOD*, *PgAPX*, RA8 promoter *T7 RNA polymerase*, *hpt* and *bar* using primers as listed in Table 3.13. The *Agrobacterium* cells harboring the multi-gene construct for several generations and the stability of the construct was checked by checking the amplification of the genes in different generations (Fig. 4.38 and Fig. 4.39). All the genes of the multi-gene construct were found to be stable in *Agrobacterium*.

4.9 *Agrobacterium* Mediated Rice Transformation

The multi-gene cassette harboring the T7 RNA polymerase coupled SOD-AsA-GSH pathway genes and the modified cassette carrying the additional *bar* gene was transformed into IR64 and Swarna cultivars of indica rice, respectively. Owing to the high transformation and regeneration potential, scutellum derived embryogenic calli were used in transformation experiments (Hiei *et al.*, 1994). Approximately 200 embryogenic calli were infected with *A. tumefaciens* strain EHA105 containing the multi-gene constructs. The infected calli was co-cultivated in MS medium supplemented with acetosyringone that was reported not only to induce *vir* genes but also found essential for rice transformation as a signaling compound (Hiei *et al.* 1994; 2006). The culture medium containing hygromycin apparently allowed the transformed calli to grow due to the expression of *hpt* gene (Hiei *et al.*, 1994; Rashid *et al.*, 1996). Hygromycin allowed clear distinction between transformed and non transformed calli. The resistant calli were selected after two rounds of selection pressures. Only few calli proliferated in the selection medium while others turned dark and eventually dried off. Hygromycin resistant calli which survived two rounds of selection was transferred to shoot regeneration medium. Total fourteen putative transgenic plants in case of IR64 and forty putative transgenic plants in case of Swarna were regenerated which were further hardened and transferred to soil pots maintained in green house (Fig. 4.40 and 4.41). The putative transgenic plants were

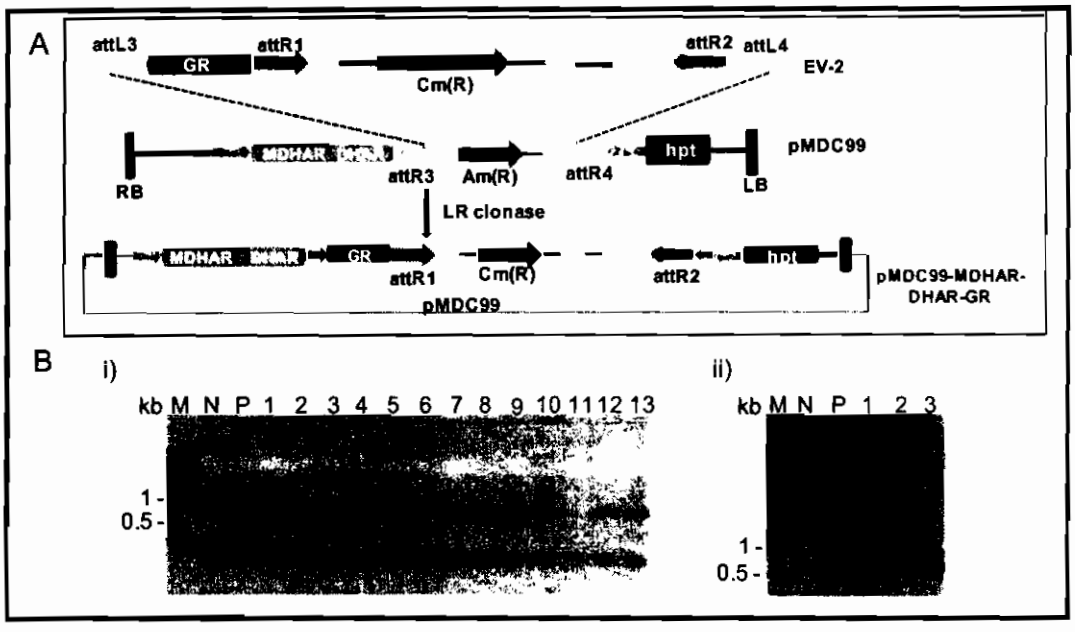


Figure 4.34 Second round of gene stacking. **(A)** Schematic representation of stacking of the second gene cassette containing PgGR gene in the destination vector pMDC99. **(B)** Agarose gel indicating confirmation of cloning of the second cassette in pMDC99 by **(i)** Colony PCR (Lanes 1-13 denote the number of colonies screened) **(ii)** Plasmid-PCR (Lanes 1-3 denote amplification of respective genes from the plasmids isolated from the positive colonies). Lane M denotes 1 kb DNA ladder, Lane P and Lane N denotes positive and negative controls, respectively.

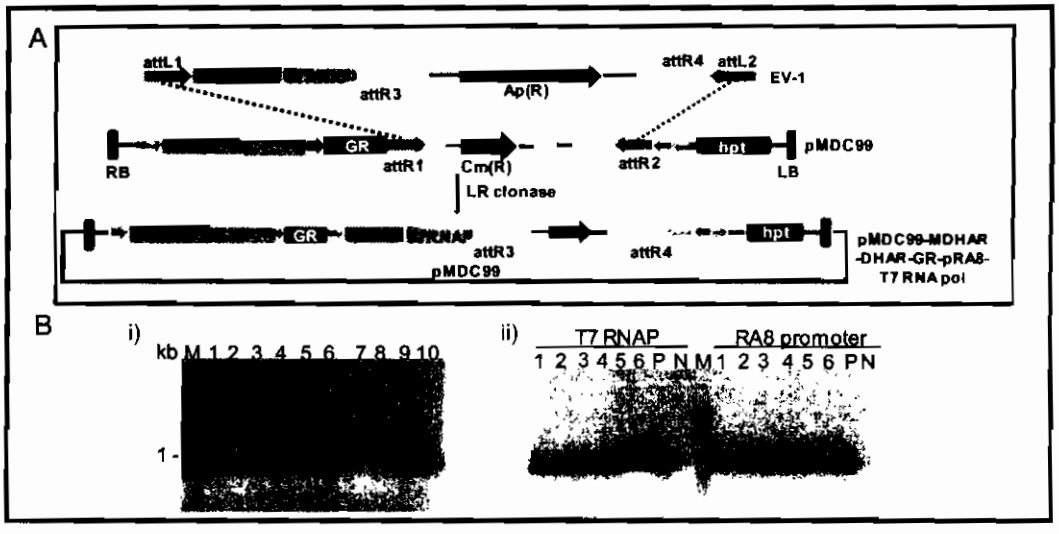


Figure 4.35 Third round of gene stacking. **(A)** Schematic representation of the stacking of the third cassette containing RA8 promoter and T7 RNA polymerase gene in the modified destination vector pMDC99. **(B)** Agarose gel indicating confirmation of cloning of the cassette in pMDC99 by **(i)** Colony PCR (Lanes 1-10 denote the number of colonies screened) **(ii)** Plasmid-PCR (Lanes 1-6 denote amplification of respective genes from the plasmids isolated from the positive colonies). Lane M denotes 1 kb DNA ladder; Lane P and Lane N denotes positive and negative controls, respectively.

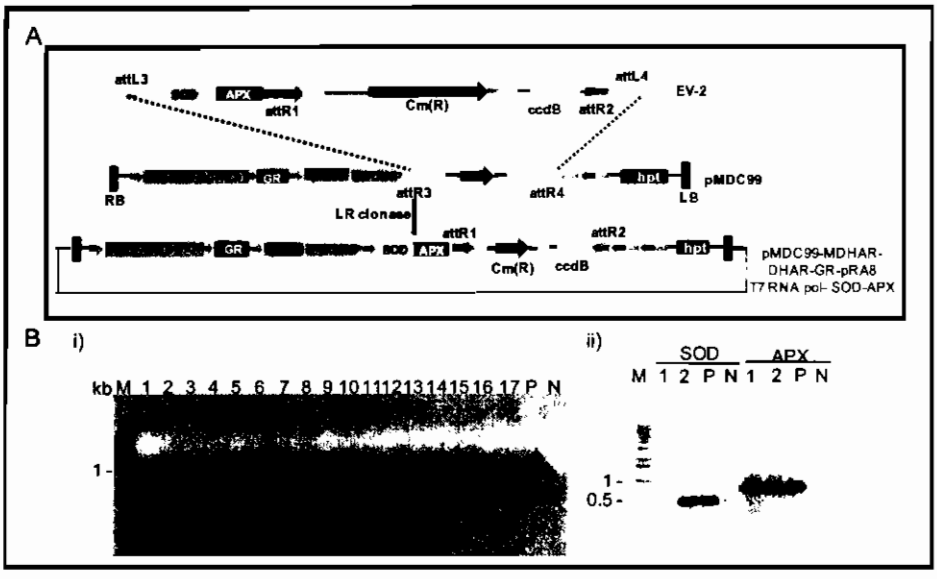


Figure 4.36 Fourth round of gene stacking. **(A)** Schematic representation of the stacking of the fourth cassette containing PgSOD and PgAPX genes in the modified destination vector pMDC99. **(B)** Agarose gel indicating confirmation of cloning of the cassette in pMDC99 by **(i)** colony PCR (Lanes 1-18 denote the number of colonies screened) **(ii)** Plasmid-PCR (Lanes 1 and 2 denote amplification of respective genes from the plasmids isolated from the positive colonies). Lane M denotes 1 kb DNA ladder; Lane P and Lane N denotes positive and negative controls, respectively.

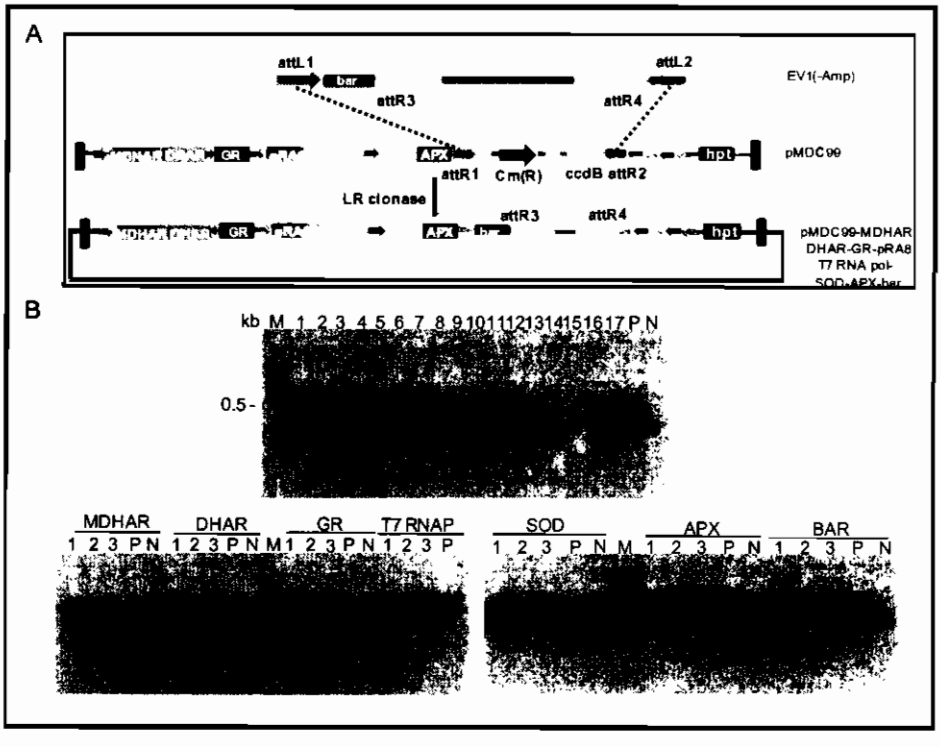


Figure 4.37 Fifth and final round of gene stacking. **(A)** Schematic representation of stacking of bar cassette in the modified destination vector pMDC99. **(B)** Agarose gel indicating confirmation of cloning of bar cassette in pMDC99 by **(i)** Colony PCR (Lanes 1-17 denote the number of colonies screened). **(ii)** Plasmid-PCR (Lanes 1-3 denote amplification of respective genes from the plasmids isolated from the positive colonies). Lane M denotes 1 kb DNA ladder; Lane P and Lane N denotes positive and negative controls, respectively.

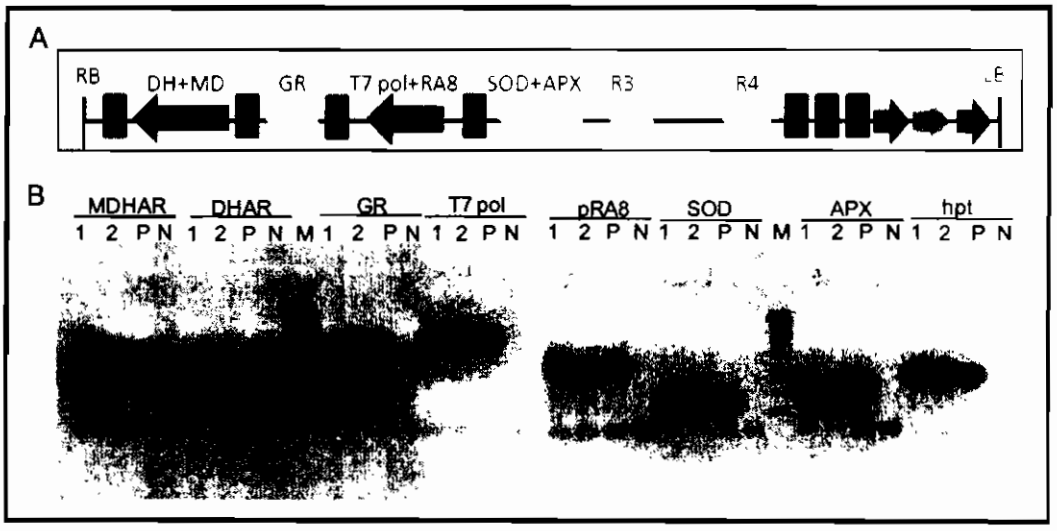


Figure 4.38 The T7 polymerase-coupled SOD-AsA-GSH pathway construct. (A) Schematic representation of the T7 polymerase-coupled SOD-AsA-GSH pathway construct. **(B)** Agarose gel indicating confirmation of stacking of all the genes in pMDC99 by Plasmid-PCR (Lane 1 and 2 denotes amplification of respective genes from the plasmids isolated from the positive colonies). Lane M denotes 1kb DNA ladder; Lane P and Lane N denotes positive and negative controls, respectively.

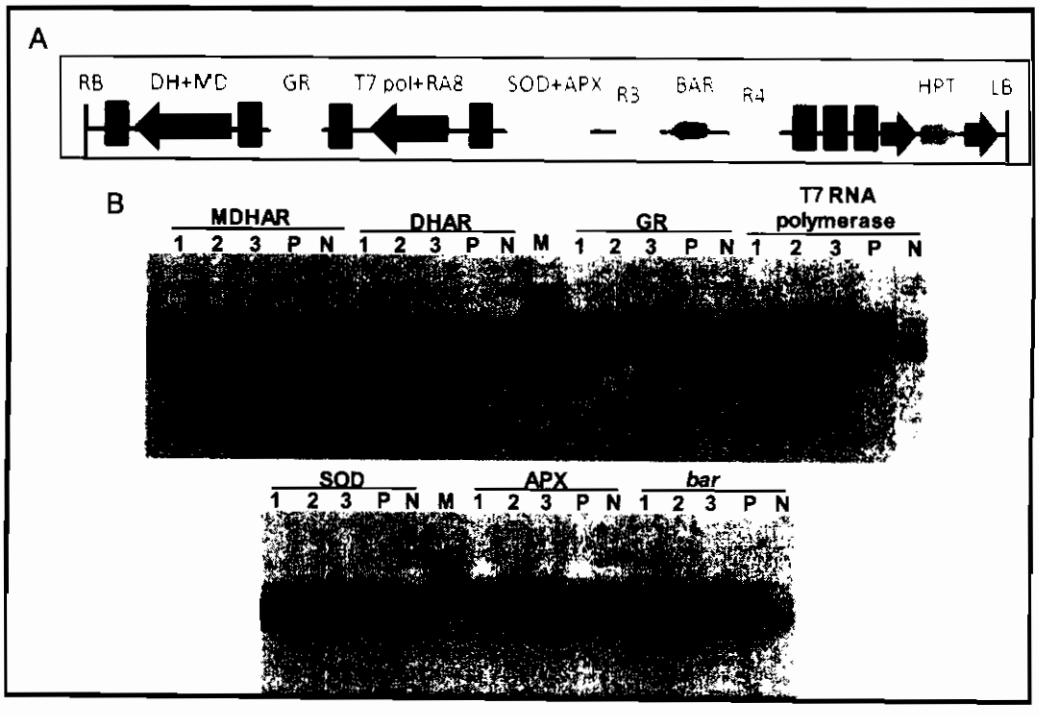


Figure 4.39 The modified T7 polymerase-coupled SOD-AsA-GSH pathway construct with additional bar gene. **(A)** Schematic representation of the T7 polymerase-regulated SOD-AsA-GSH pathway construct with *bar* gene **(B)** Agarose gel indicating confirmation of stacking of all the genes in pMDC99by Plasmid-PCR (Lane 1-3 denotes amplification of respective genes from the plasmids isolated from the positive colonies). Lane M denotes 1 kb DNA ladder; Lane P and Lane N denotes positive and negative controls, respectively.

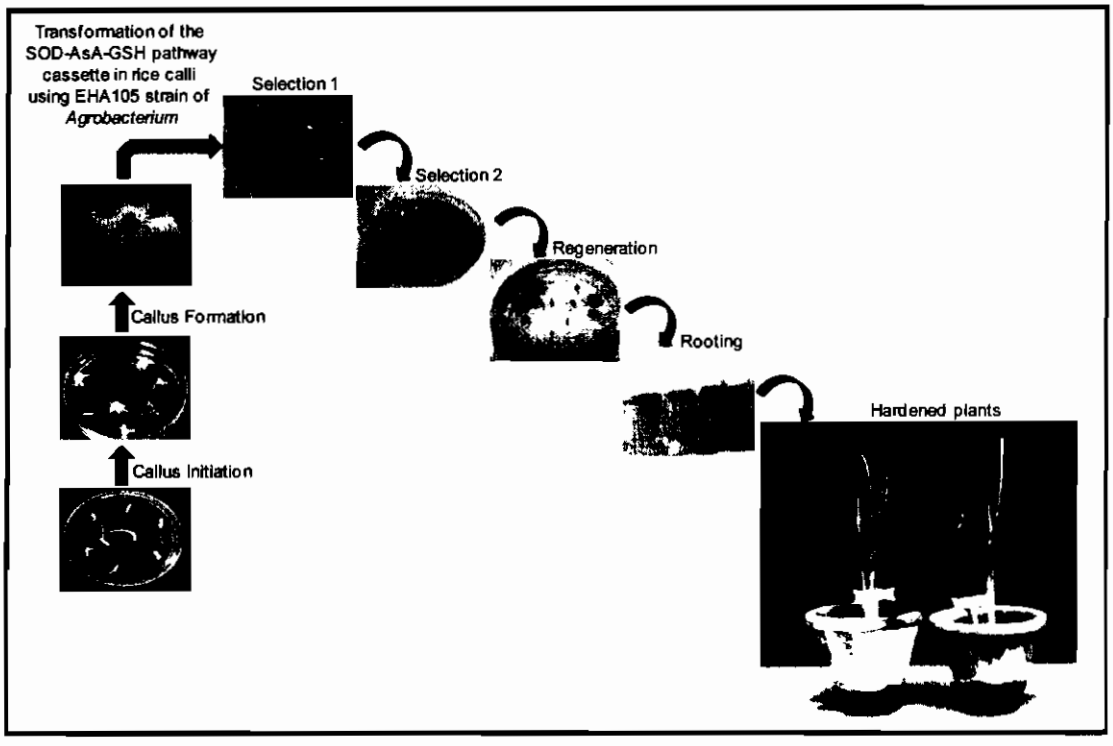


Figure 4.40 *Agrobacterium* mediated transformation of rice (*Oryza sativa* L. cv. IR64) with the T7 RNA polymerase coupled SOD-AsA-GSH pathway construct The different steps involved in transgenic rice regeneration are shown.

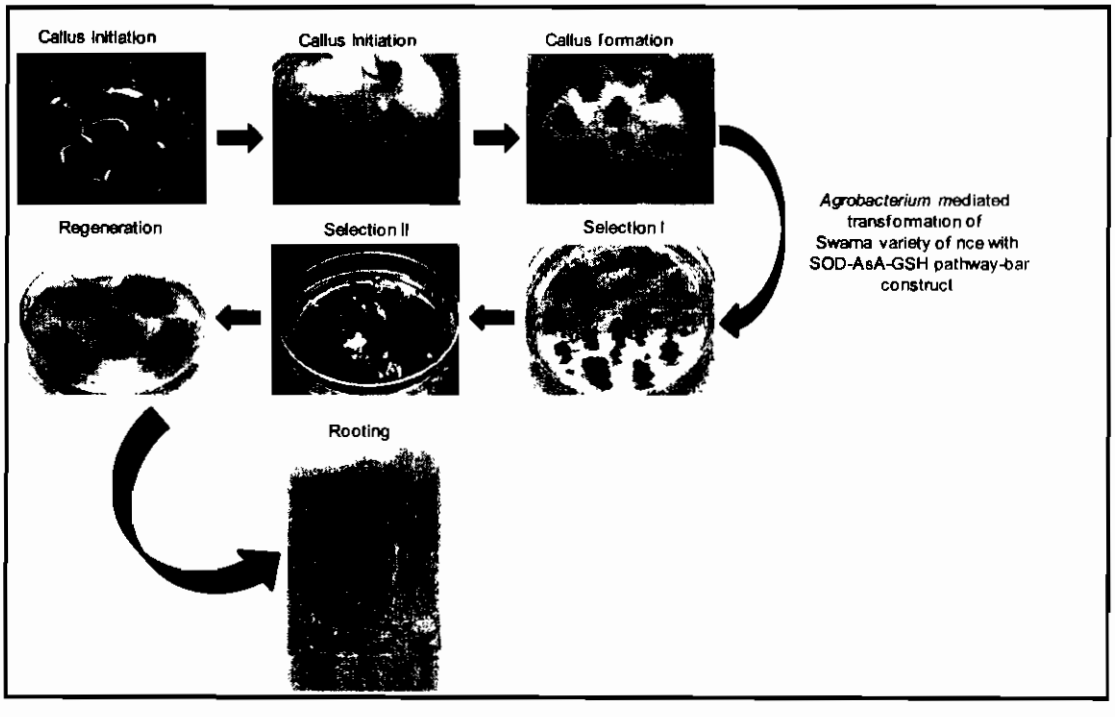


Figure 4.41 *Agrobacterium* mediated transformation of rice (*Oryza sativa* L. cv. Swarna) with the modified T7 RNA polymerase coupled SOD-AsA-GSH pathway-bar construct The different steps involved in transgenic rice regeneration are shown.

subjected to various molecular and physiological analysis.

4.10 Screening and Analysis of the Transgenic Plants

For the identification of independent transgenic lines with the stable integration of all the transgenes, four putative IR64 transgenic plants (I_1, I_2, I_3 and I_4) and thirty putative Swarna transgenic plants were screened by a number of methods as described below.

4.10.1 Screening of Putative Transgenic Plants with Basta

To measure the herbicide tolerance in the transgenic plants, the putative transgenic Swarna lines were tested by spraying the herbicide Basta (Bayer Crop Science) at a concentration of 10 mg/l. The leaves of the wild type (un-transformed) plants showed prominent chlorosis and leaf tip necrosis whereas in the transgenic plants expressing *bar* gene the extent of chlorosis and necrosis was much less (Fig. 4.42).

4.10.2 Confirmation of Putative Transgenic Lines by PCR

Putative transgenic lines were confirmed by PCR using *hpt* specific primers. Out of thirty putative Swarna transgenic plants, twenty four plants (Sw_1, Sw_2, Sw_3, Sw_4, Sw_5, Sw_6, Sw_7, Sw_8, Sw_9, Sw_10, Sw_11, Sw_12, Sw_13, Sw_14, Sw_15, Sw_16, Sw_17, Sw_18, Sw_19, Sw_25 Sw_26, Sw_27, Sw_29 and Sw_30) were tested positive showing the expected amplification of 1000 bp, which was missing in the rest plant including the wild type (Fig. 4.43A-B).

The aforesaid twenty four hygromycin resistant–positive T_0 transgenic plants were further analysed for the presence of transgenes: *T7 RNA polymerase*, *PgSOD*, *PgAPX*, *PgGR*, *PgMDHAR* and *PgDHAR*. Due to extensive homology between the transgenes and the corresponding endogenous genes of rice, gene specific primers were not used for amplification of trangenes. The detection of the transgene was done by using different combinations of forward and reverse primers designed from the promoter, 3'UTRs and non-conserved regions of the transgenes (Table 3.15). The results revealed that some of the transgenics lines (I_1, I_2, Sw_17, Sw_18, Sw_19 and Sw_30) showed loss of one or more transgenes whereas some lines (Sw_10, Sw_11, Sw_16, Sw_25, Sw_26, Sw_27, Sw_29) exhibited complete integration of the cassette (Fig. 4.44). The loss of genes may be attributed to homologous recombination

occurring in plant cells during the integration of T-DNA probably due to repeated elements in the T-DNA. The loss of one or more transgene in *Arabidopsis* transformed with linked multi genes by an *in vivo* DNA assembly method has earlier been reported (Chen *et al.*, 2010). However, the marker gene *hpt* was present in all the transgenics. Both types of transgenics having complete and partial integration were proceeded for further analysis.

4.10.3 Molecular Detection of Genomic Integration of Transgenes

The transgenic nature of rice plants was further confirmed by Southern hybridization analysis using undigested and digested total genomic DNA samples. Genomic DNA digested with a restriction enzyme *Sac* II were loaded for each transgenic line as well as the wild type (non-transformed) plants and hybridized with *hpt* probe. The expected size of the digested fragments are shown in Fig. 4.45 A and B. Representative Southern results are shown in Fig. 4.45C and D for eight transgenic rice plants (I_2, Sw_9, Sw_10, Sw11, Sw_16, Sw_17, Sw_25, and Sw_26) (Fig. 4.45). Hybridization signals corresponding to the *Sac II* fragments of different size were observed in the Southern hybridization analysis. This indicates the integration of transgene copies in the genome of the independently transformed plants. Some of the transformants had single copy integration (I_2, Sw_25, Sw_26) and sometimes a more complex multi-band pattern was observed (Sw_11, Sw_16 and Sw_17) (Fig. 4.45), indicating the occurrence of multiple copy-integrations and possible rearrangements of the transgenes in the genome of the transformants.

4.10.4 Analysis of Transgene Integration Site by Locus Finding PCR

The transgene flanking sequence of the two independent transgenic lines (I_2 and Sw_10) was determined by a method named Locus Finding PCR (LF PCR). LF PCR includes a primary PCR by a degenerated primer and transfer DNA (T-DNA)-specific primer, a nested PCR, and a method of enriching the desired amplicons by using a biotin-tagged primer that is complementary to the T-DNA. The technique has been described in detail in Materials and Methods (Section 3.30). The characteristic amplification of the random-priming PCR (primary PCR) that looks like a DNA fingerprint (Fig. 4.46A) was observed in the primary PCR, which are the non-specific amplicons created by the involved primers. Captured single strands of desired amplicons were subjected to the nested PCR. The transgenic rice plant I_2 and Sw_10

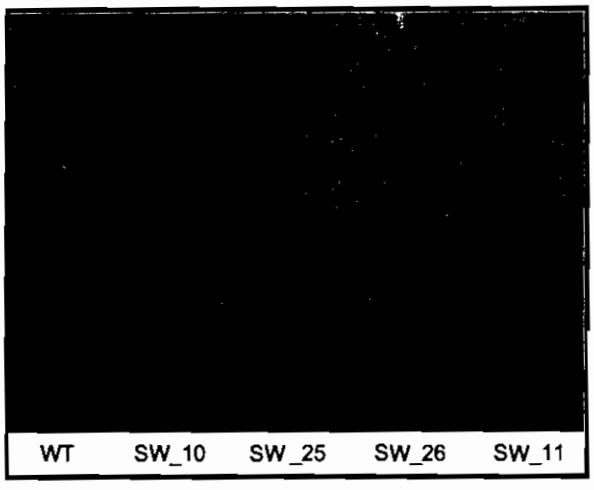


Figure 4.42 Evaluation of herbicide resistance in T_0 transgenic and un-transformed Swarna plants by *in vitro* leaf spray test. The picture was taken at the seventh day after Basta application. Sw_10, Sw_25, Sw_26 and Sw_27 indicate the transgenic plants; WT indicates the untransformed plant.

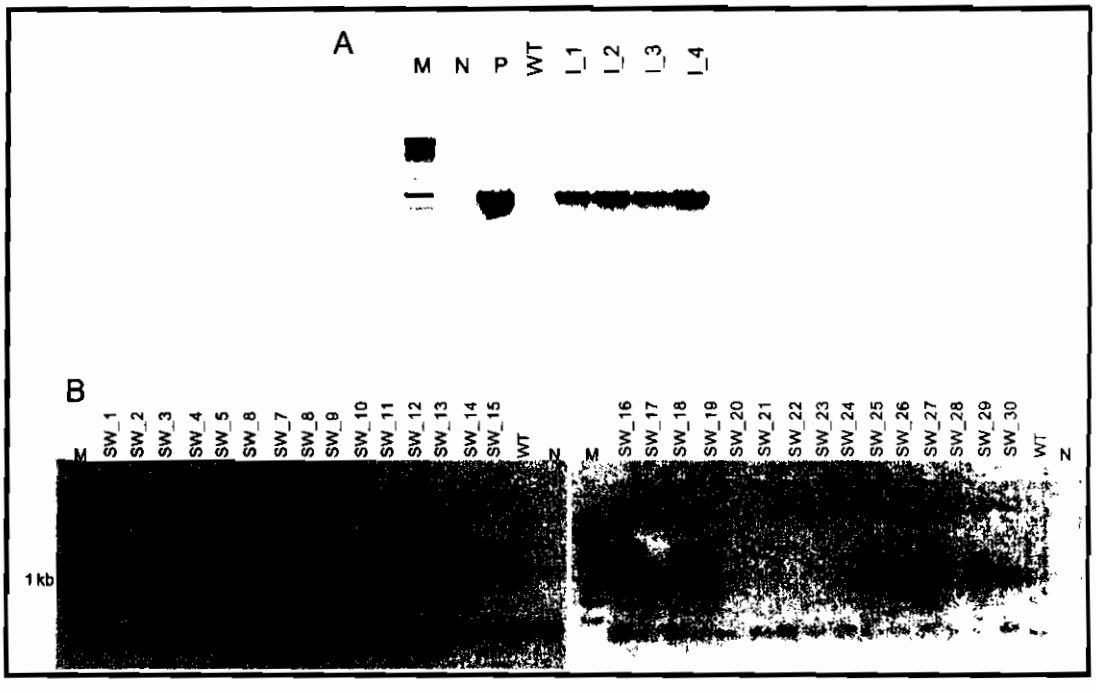


Figure 4.43 Confirmation of integration of *hpt* gene in T_0 transgenic plants. **(A)** Agarose gel showing PCR amplification of the integrated *hpt* selection marker gene by using hpt F hpt R primers in IR64 transgenic plants. **(B)** Agarose gel showing PCR amplification of the hpt genes in Swarna transgenic plants. Lane M denotes 1 kb DNA ladder; Lane P and N denotes positive and negative control respectively and wt denotes Non -transformant control.

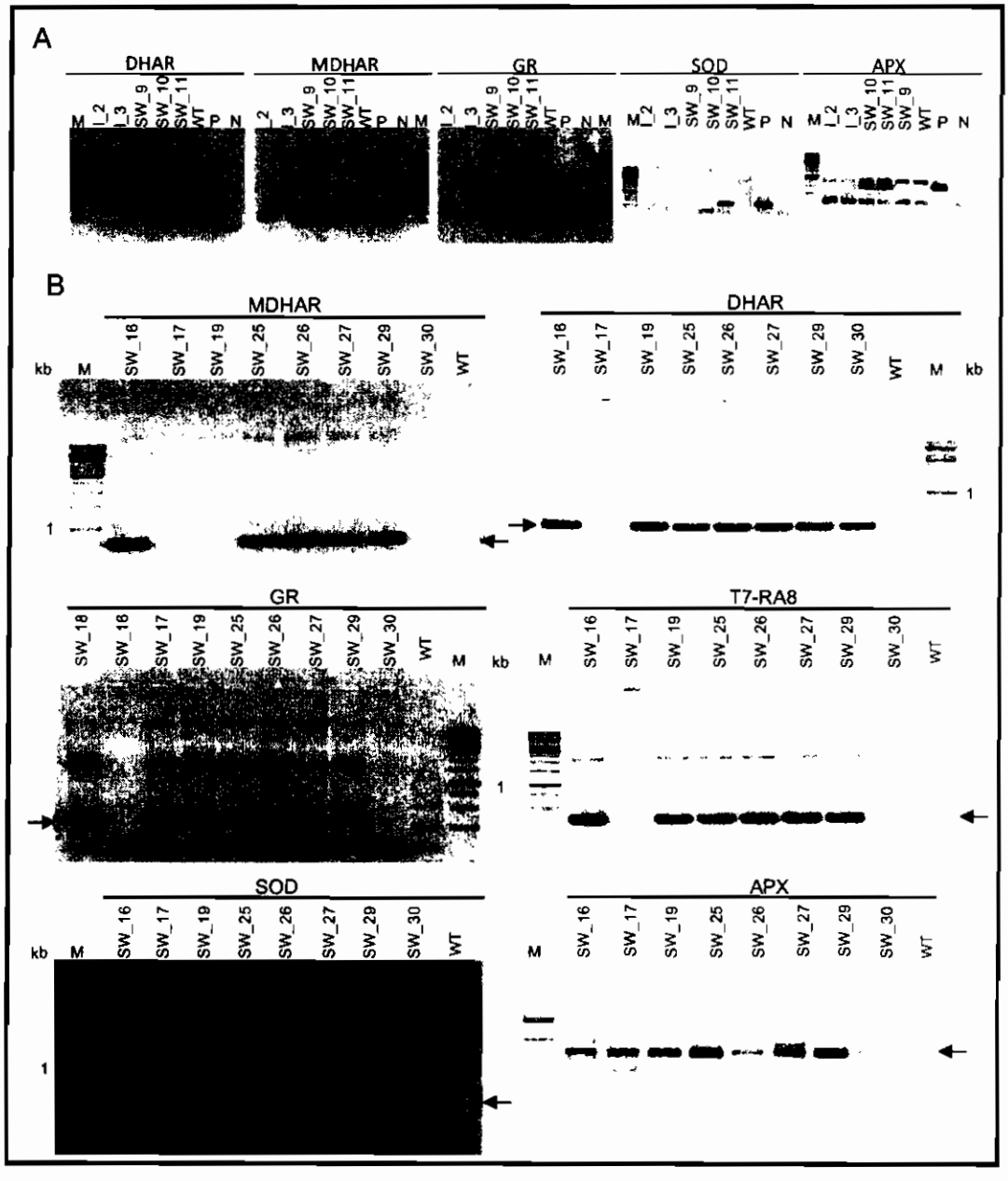


Figure 4.44 PCR analysis showing the presence of different transgenes in the transgenic IR64 and Swarna plants using gene forward and 3'UTR reverse primers **(A)** and T7 forward and gene reverse primers **(B).** Lane M: 1 kb DNA ladder, P: Positive control, N: Negative control, WT: un-transformed plant . I_2, I_3, Sw_9a, Sw_10, Sw_11, Sw_16, Sw_17, Sw_18, Sw_19, Sw_25, Sw_26, Sw_27, Sw_29 and Sw_30 denote the different transgenic lines.

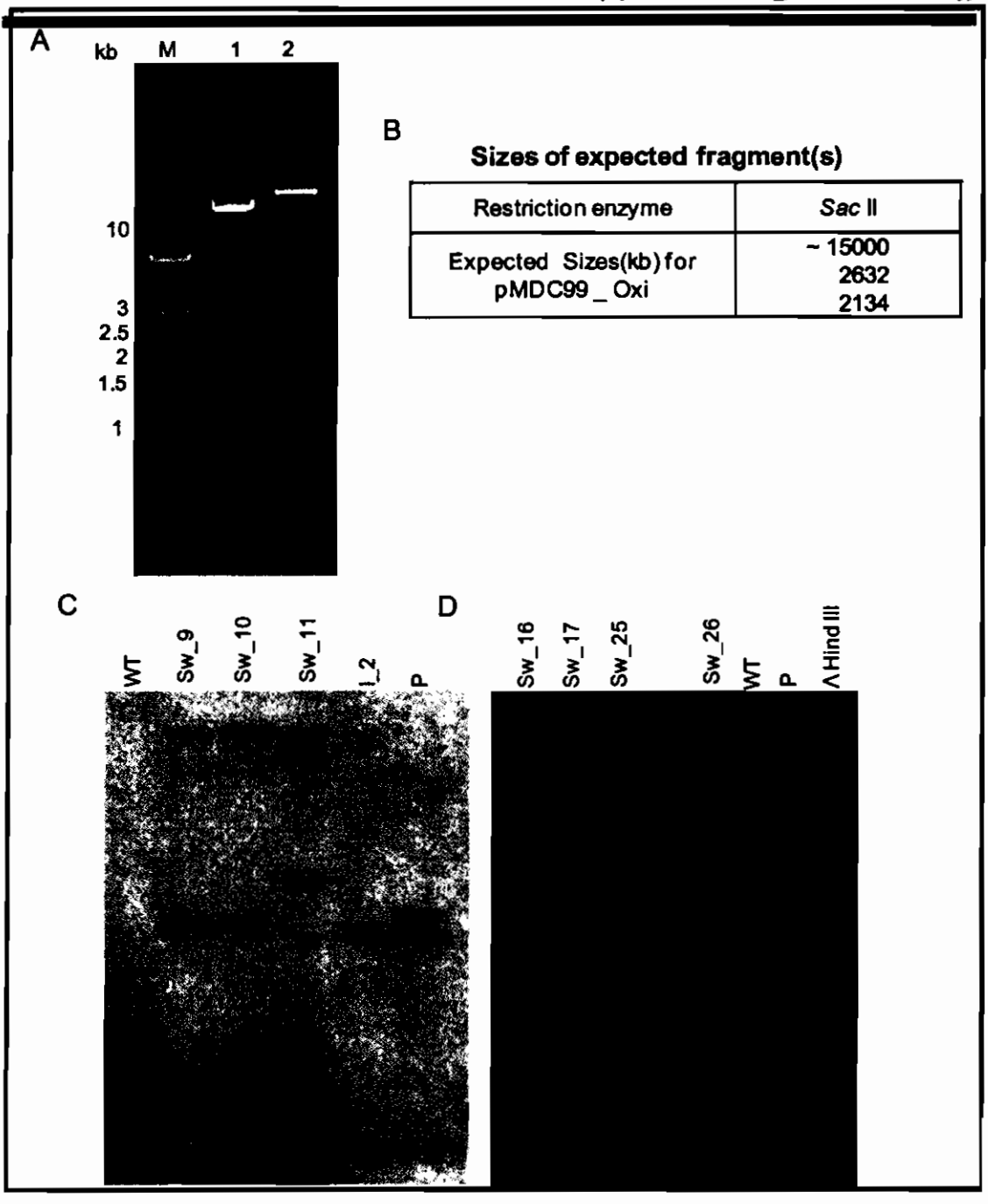


Figure 4.45 Analysis of transgene integration by Southern hybridization **(A)** Agarose gel representing the restriction digestion of recombinant pMDC99 plasmid harboring the T7 RNA polymerase coupled SOD-AsA-GSH pathway construct. Lane 1 represents digestion of the recombinant pMDC99 plasmid harboring the T7 RNA polymerase coupled SOD-AsA-GSH pathway construct using *Sac* II showing the expected fallouts of 15 kb, 2.6 kb and 2.1 kb. Lane 2 indicates the corresponding un-digested vector. Lane M denotes 1 kb DNA ladder **(B)** Size of the expected fallouts after digestion with *Sac* II. **(C)** and **(D)** Southern hybridization of DNA blot containing digested genomic DNA isolated from transgenic Swarna and IR64 lines and hybridized with hph probe. SW_9, SW_10, SW_11, I_2, SW_16, SW_17, SW_25 and SW_26 indicate the different transgenic lines: WT: un-transformed plants serving as control. P indicates plasmid as positive control. λ Hind III denotes λ Hind III DNA ladder.

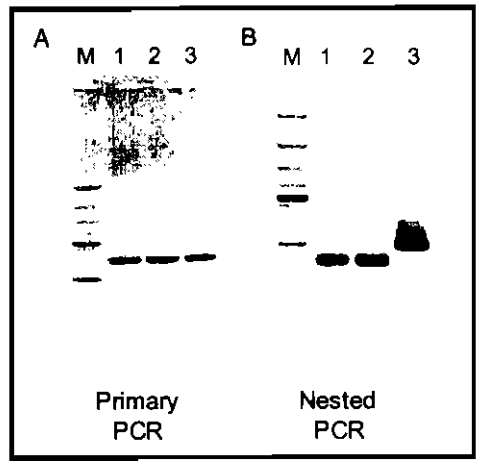


Figure 4.46 Detection of position of integration of the transgenes by Locus Finding PCR. **A)** Agarose gel indicating the amplification pattern of random-priming PCR (Primary PCR). Lanes 1, 2, and 3 are the representative primary (random-priming) PCR of plants I_1, I_2, and Sw_10, respectively, and lane M denotes 500 bp DNA ladder. **(B)** Nested PCR amplification of I_1, I_2, and Sw_10. Lanes 1, 2, and 3 are the amplifications of plants I_1, I_2, and Sw_10, respectively, and lane M denotes 1 kb DNA ladder.

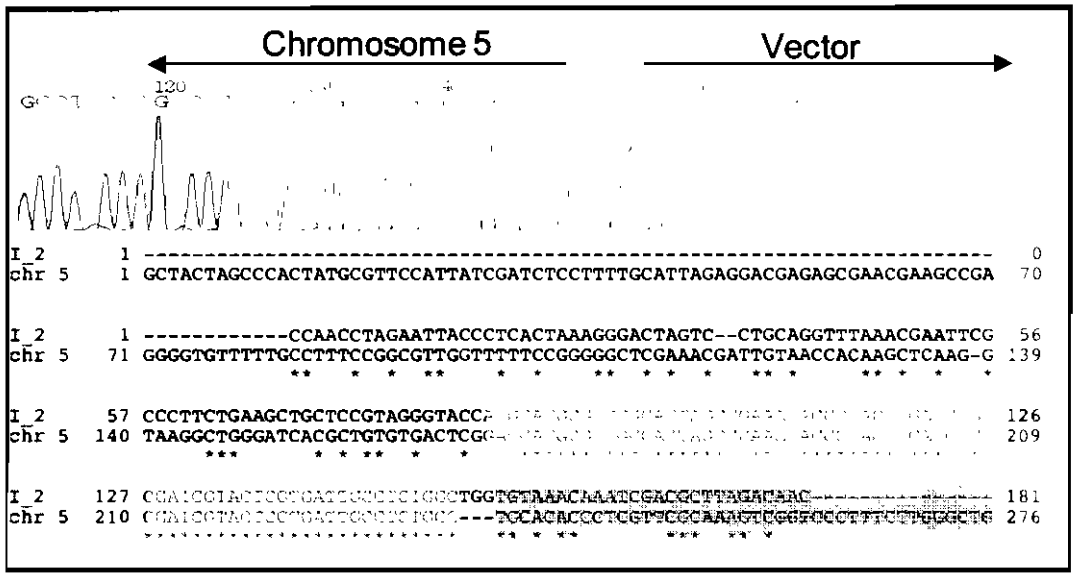


Figure 4.47 Transgene integration locus for transgenic plant, I_2 as determined by by Locus Finding PCR. The upper panel depicts the representative chromatogram of the transgene integration locus showing the junction region of vector and chromosome 5. The alignment of the amplified integration loci and chromosome 5 is shown below wherein the vector sequence is shaded in red and chromosomal sequence is shaded in green.

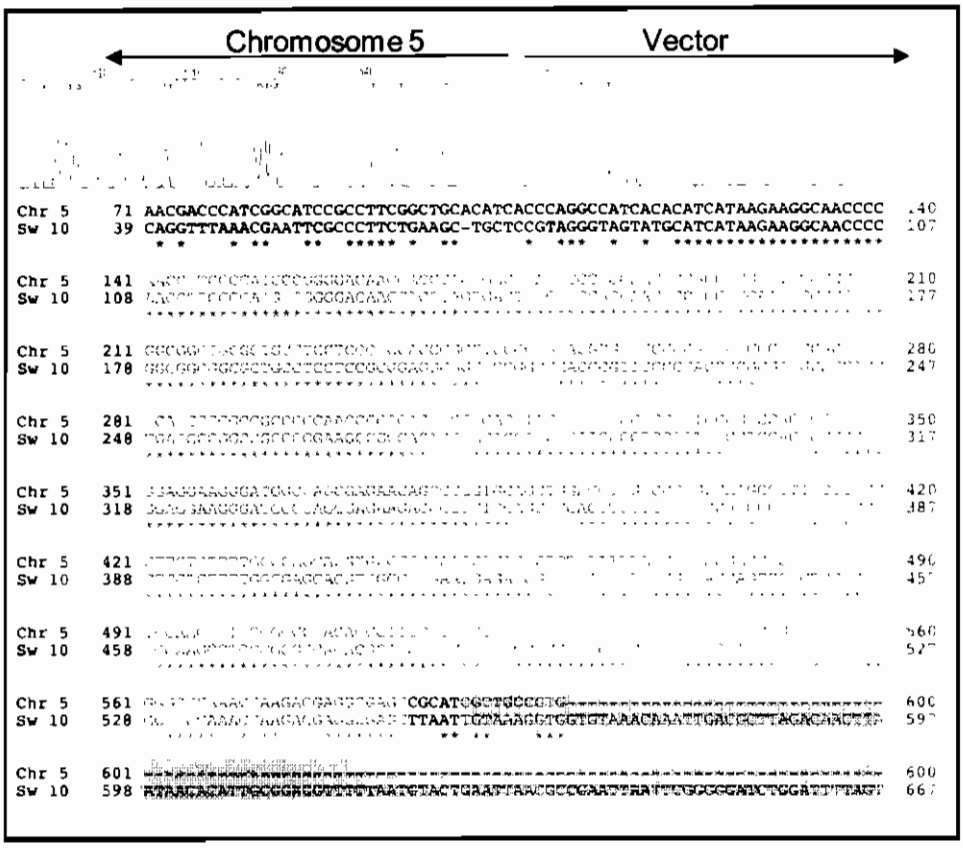


Figure 4.48 Transgene integration locus for transgenic plant, Sw_10 as determined by Locus Finding PCR. The upper panel depicts the representative chromatogram of the transgene integration locus showing the junction region of vector and chromosome 5. The alignment of the amplified integration loci and chromosome 5 is shown below wherein the vector sequence is shaded in red and chromosomal sequence is shaded in green.

TRANSGENIC LINE	CHROMOSOME NO	INTEGRATION POSTION
I_2	5	23576014
Sw_10	5	26634735

Figure 4.49 Transgene integration positions for transgenic plant, I_2 and Sw_10.

amplified ~400 bp and 500 bp bands, respectively, at the end of the nested PCR (Fig. 4.46B). These integration loci amplified in the nested PCR were sequenced. The integration loci of the transgenic plants were identified by performing BLAST N analysis against *O. sativa* (indica cultivar group) whole genome shotgun contigs (Fig. 4.47 and Fig. 4.48). Coincidently, in both the lines, transgene was found to be inserted at Chromosome 5 but at a different position. For line I_2 integration was found to have occurred at 23576014 bp whereas for line Sw_10, the integration occurred at 26634735 bp on chromosome 5 (Fig. 4.49).

4.10.5 Analysis of Transgene Expression

The detection of transgene expression in anthers of the T_0 lines Sw_10 and Sw_11 was carried out by semi-quantitative RT-PCR using primers as listed in Table 3.18. The transcripts corresponding to *PgMDHAR*, *PgDHAR*, *PgGR*, *PgSOD* and *PgAPX* were observed in lines Sw_11 and Sw_10 (Fig. 4.50). The results indicated that all the transgenes in Sw_10 and Sw_11 were expressed at different levels under normal conditions in the anthers. The mechanisms contributing to the expression variation between the different transgenic plants that contain the same gene cassettes are not fully understood. However, it is generally assumed that the position effect from the neighbouring chromosome of insertion site can be the factor responsible for the differential transgene expression (Matzke and Matzke, 1998).

Due to limitation of anther tissue the expression of the transgenes at protein level was not analyzed in anthers of T_0 plants. However, the vegetative parts of the transgenic plants did not show any accumulation of transgenic protein as revealed by the in-gel SOD and APX activity assay performed from proteins extracted from the leaves of transgenic lines Sw_10 and Sw_11 (Fig. 4.51). Thus, there was no leaky expression of the T7 RNA polymerase coupled SOD-AsA-GSH pathway in shoots.

4.10.6 Analysis of Seed Set and Pollen Viability of Transgenic Plants

The T_0 transgenic plants showed normal growth and development and there was no substantial difference in the seed set of transgenic IR64 and Swarna plants and the wild type plants under non-stress conditions (Fig. 4.52 and 4.53). Pollen from non-transgenic plants and transgenic plants was collected and tested for viability. No difference was observed between the transgenic and the non-transgenic plants in terms of the viability of the pollen grains under non-stress conditions.

4.10.7 Analysis of T_1 Generation Transgenic Plants

The growth of T_1 seedlings of Sw_10, Sw_11, Sw_25, Sw_26 and Sw_27 was also found to be similar to the non-transgenic plants (Fig. 4.54).

4.10.7.1 Segregation Analysis of Transgenic Plants

The germination test was performed with seeds of T_0 generation IR64 (I_2, I_3) and Swarna transgenic plants (Sw_10, Sw_11, Sw_25 and Sw_26) in hygromycin supplemented media (Fig. 4.55). Hygromycin at a concentration of 30 mg/l was found to be toxic to the germination of non-transformed control rice seeds of both IR64 and Swarna variety. Hygromycin-resistant and susceptible seedlings were clearly identified within one week. Resistant seeds germinated while the non-transformed (susceptible) seeds did not germinate or died after germination. For majority of cases 70% of the seeds of the transgenic plants were able to germinate on media supplemented with 30 mg/l of hygromycin.

4.10.7.2 Analysis of Expression of *bar* Gene by Chlorophenol Red Assay

T_1 seeds from the transgenic lines Sw_10, Sw_16, Sw_25, Sw_26 and Sw_27 were germinated on MS media with 3mg/l Basta and 50mg/l chlorophenol red. The use of this selective medium allows more efficient screening of the putative transgenic plants since the germination under Basta selection changed pH in the medium from 6.0 to 5.0. Yellow or orange colour indicated bar activity due to acidification of the medium. The colour shift from red to orange–yellow occurred in case of the transgenic lines Sw_9, Sw_10, Sw_11, Sw_25, Sw_26 and Sw_27 within 14-20 d after the germination. In parallel experiment, the non-transformed control plants did not change the colour of the selective medium (Fig. 4.56).

The T_1 transgenic lines exhibiting hygromycin and basta tolerance are being grown in green house for obtaining homozygous lines as well as for the multiplication of seeds. The expression analysis of the transgenes in anthers would be performed in T_1 and subsequent generation plants. The response of the transgenic plants under various stress treatments, particularly during drought and moderate to high temperature stress would be studied as these two stress conditions are the most critical to male reproductive development of rice plants. The panicle development and seed set of transgenic lines will be compared with that of wild type plants under the stress

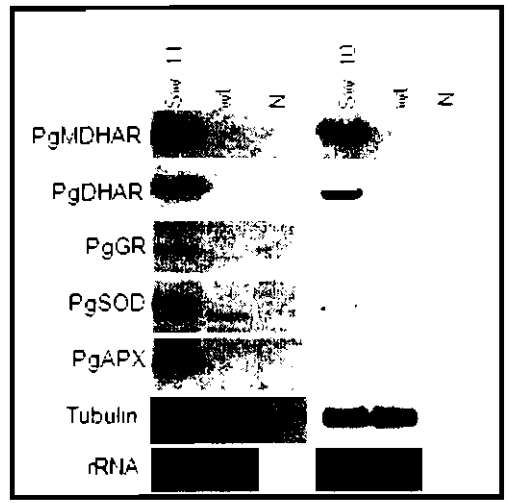


Figure 4.50 Semi-quantitative RT–PCR detection of transcripts of PgMDHAR, PgDHAR, PgGR, PgSOD and PgAPX in panicles of un-transformed (wt) and transgenic lines (Sw_10 and Sw_11). Transcript levels of tubulin expression was used as an internal control in each case. Ethidium bromide-stained rRNAs were used as a loading control (bottom section). N denotes negative control.

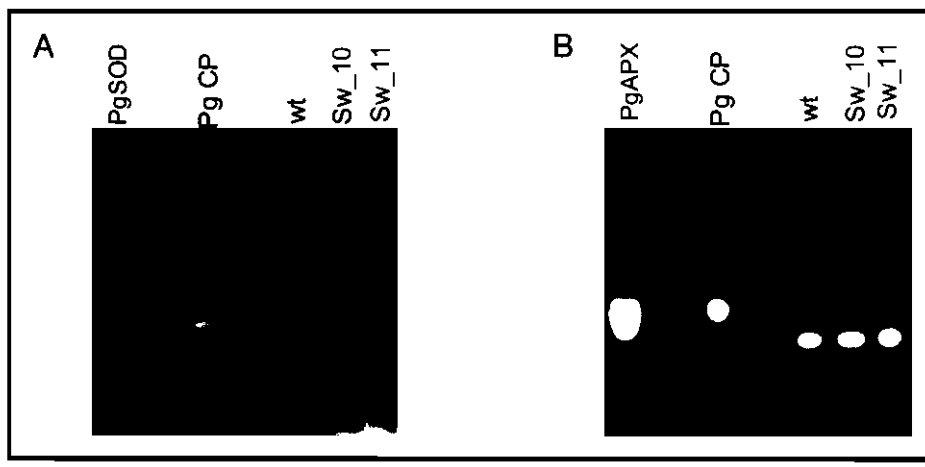


Figure 4.51 Representative in-gel SOD and APX activity of leaves of rice plants. (**A**) Native PAGE gel showing SOD activity (indicated by the appearance of achromatic bands). (**B**) Native PAGE gel stained for APX activity. PgSOD: purified PgSOD protein; PgAPX: purified PgAPX protein; Pg CP: crude protein isolated from *Pennisetum* leaves; wt: crude protein isolated from leaves of non-transformed rice plants; Sw_10: crude protein isolated from leaves of transgenic line Sw_10 plants and Sw_11: crude protein isolated from leaves of transgenic line Sw_11.

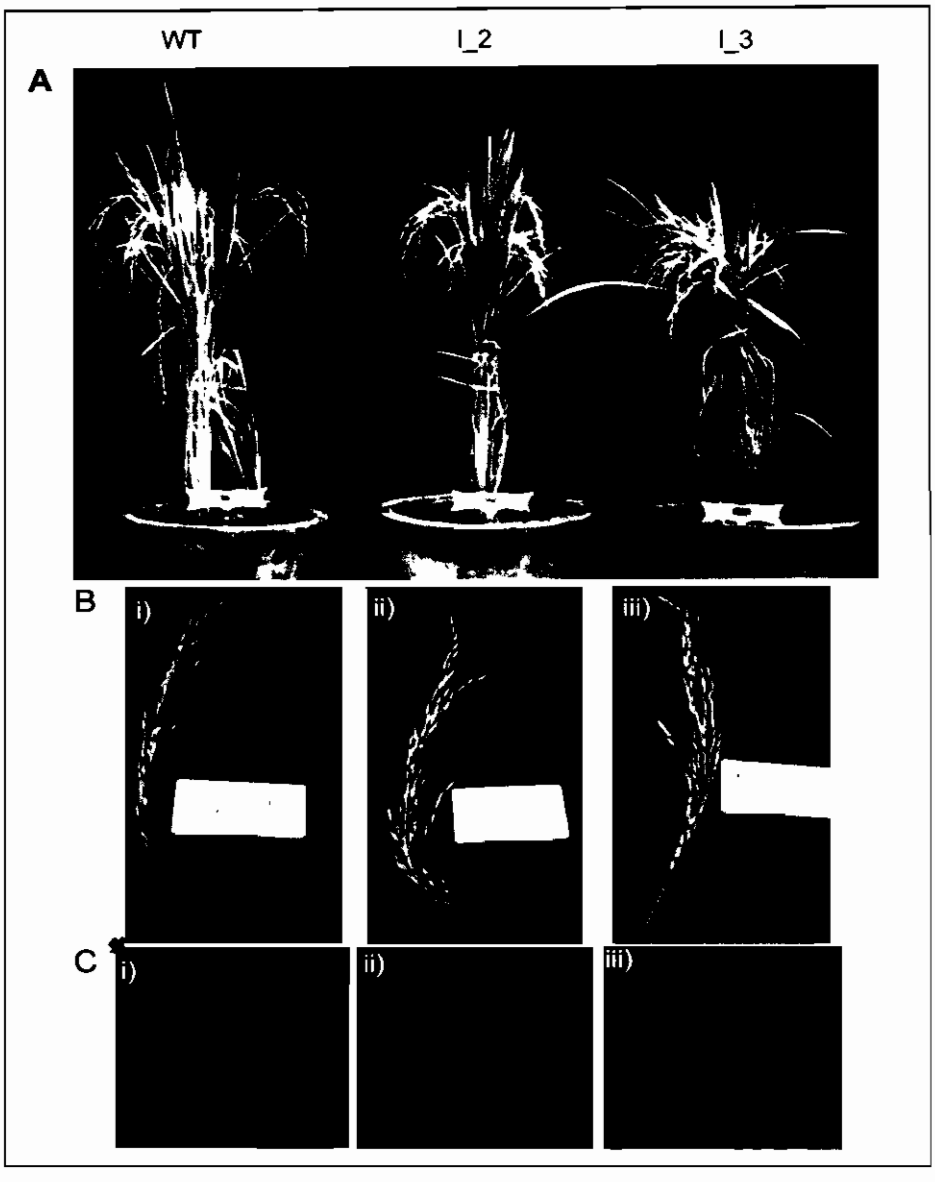


Figure 4.52 Phenotypes and seed set of transgenic plants. **(A)** Different mature phenotypes of IR64 transgenic plants (I_2 and I_3) and untransformed (WT) plants at grain-filling stage. **(B)** Mature panicles of untransformed **(i)** and transgenic plants (ii and iii). **(C)** I_2-KI staining showing pollen viability of transgenic lines and control untransformed plants. **(i)** Control-plant mature pollen grains **(ii)** Mature pollen grains of transgenic line I_2 **(iii)** Mature pollen grains of transgenic line I_3.

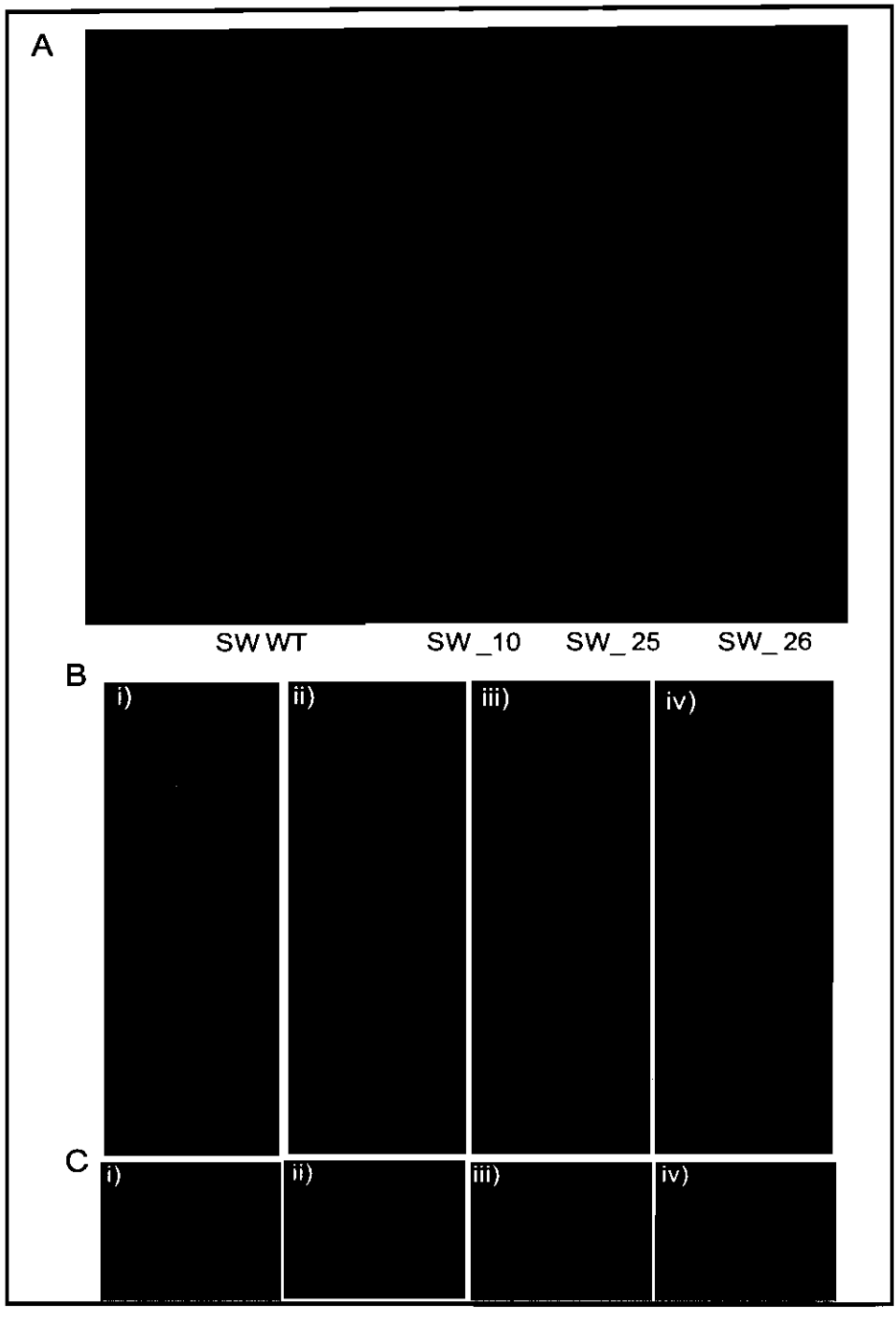


Figure 4.53 Phenotypes and seed set of Swarna transgenic plants. **(A)** Different mature phenotypes of Swarna transgenic plants (Sw_10, Sw_25 and Sw_26) and untransformed (SW_WT) plants at grain-filling stage. **(B)** Mature panicles of untransformed **(i)** and transgenic plants **(ii, iii** and **iv). (C)** I_2-KI staining showing pollen viability of transgenic lines and control untransformed plants. **(i)** Control-plant mature pollen grains. Mature pollen grains of transgenic line Sw_10 **(ii)** Sw_25 **(iii)** and Sw_26 **(iv).**

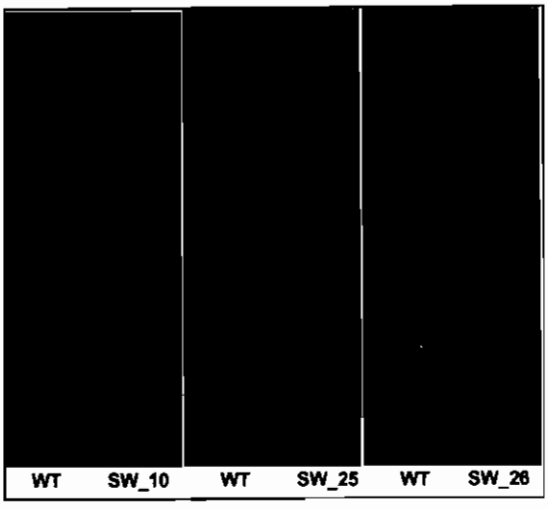


Figure 4.54 Comparison of growth of T_1 seedling sof transgenic lines (Sw_10, Sw_25 and Sw_26) and untransformed control plant (WT).

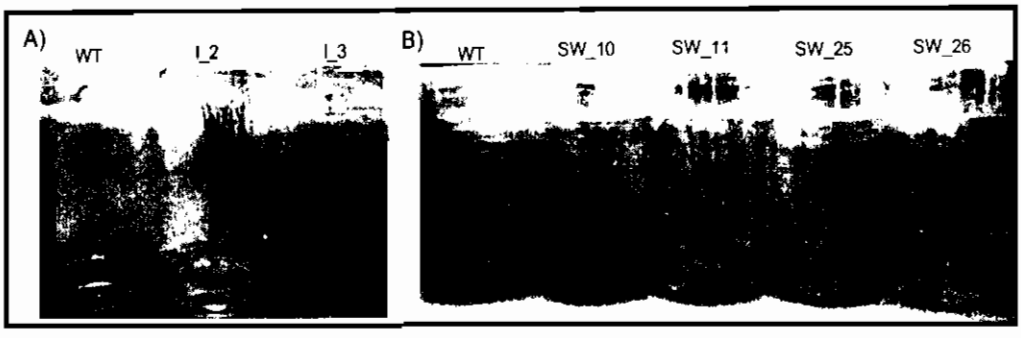


Figure 4.55 Germination of T_1 seeds of IR64 **(A)** and Swarna **(B)** transgenic lines along with untransformed lines (WT) in presence of hygromycin B (30 mg/l).

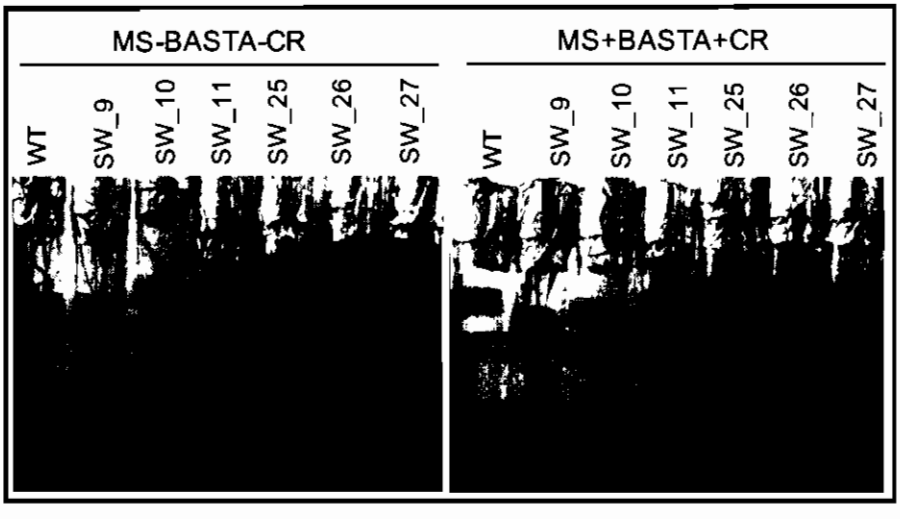


Figure 4.56 Chlorophenol Red Assay. Germination of non-transformed (WT) and T_1 transgenic lines(Sw_9, Sw_10, Sw_11, Sw_25, Sw_26 and Sw_27) on MS media (left panel) and MS media supplemented with Basta and chlorophenol red (right panel).

conditions in order to evaluate the performance of the transgenic plants under different environmental conditions.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSION

The global climate change is known to cast dramatic effects on world agriculture as crop productivity is largely affected by the adverse environmental fluctuations. The global climate change together with the abiotic stresses can be detrimental to any stage of development of plants. However, the sexual reproductive phases are particularly more sensitive to the stress conditions. Moreover, as majority of our food (fruits, vegetables, grains and pulses) is a product of the sexual reproduction of flowering plants, it becomes more important to understand the effect of these stresses on the reproductive development of plants. The various stress conditions like high or low temperature, drought and salinity culminate into the production of reactive oxygen species (ROS) thereby imposing oxidative stress on plants.

Plants have evolved different strategies to minimize the accumulation of excess ROS. These strategies include the avoidance mechanisms such as anatomical adaptation, suppression of photosynthesis and scavenging mechanisms like production of anti-oxidants and anti-oxidative enzymes. Among the various anti-oxidant enzymes produced in the plants, superoxide dismutase (SOD) and the enzymes of ascorbate-glutathione (AsA-GSH) pathway play an important role in detoxifying excess ROS in plant cells. Different plants differ in their capability to scavenge ROS and efficient ROS detoxification is one of the strategies used by stress tolerant plants for combating the various abiotic stresses. Therefore, with an objective of enhancing the tolerance of the reproductive part of plants to the increasing environmental fluctuations, superoxide dismutase along with the enzymes constituting the AsA-GSH pathway of a stress tolerant plant *Pennisetum glaucum* was over-expressed in the anthers of rice plants. All the genes i.e. *PgMDHAR*, *PgDHAR*, *PgGR*, *PgSOD* and *PgAPX* were stacked in a single T-DNA vector by *in vitro* gene pyramiding and in order to achieve the co-ordinated expression of all the component enzymes, T7 RNA polymerase expression system was introduced. The cassette of each gene involved in SOD-AsA-GSH pathway was designed in such a way that each gene was placed separately under the control of T7 promoter. In this way, the

transgene expression would be dependent on the introduced T7 RNA polymerase gene. The expression of the SOD-AsA-GSH pathway was directed to the anthers of rice plant by regulating the expression of T7RNA polymerase gene by the rice anther specific RA8 promoter. The co-ordinated expression of the SOD-AsA-GSH pathway in anthers would help in enhancing the oxidative stress tolerance of anthers finally leading to the improved reproductive fitness of the rice plants.

The results of the present study can be summarized as follows

1. The full length genes coding for CuZn superoxide dismutase (*CuZnSOD*), ascorbate peroxidase (*APX*), monodehydroascorbate reductase (*MDHAR*), dehydroascorbate reductase (*DHAR*) and glutathione reductase (*GR*) were amplified from the genome of a stress tolerant plant *P. glaucum*. The sequence analysis of each of the gene was done and the conserved motifs and residues important for activity were identified. The phylogenetic analysis of the proteins revealed them to be closely related to the corresponding cytosolic proteins of maize and rice, indicating the probable cytosolic nature of these proteins. The full length genes were expressed in heterologous *E. coli* expression system. The recombinant proteins were purified and were found to be enzymatically active.

2. The T7-promoter-SOD-AsA-GSH pathway gene cassettes were constructed. Each individual gene cassette consisted of the T7 promoter, coding sequence of *PgCuZnSOD*, *PgAPX*, *PgMDHAR*, *PgDHAR* and *PgGR* genes, 5'and 3'UTRs derived from rice rbcS protein and T7 terminator.

3. The full length coding sequence corresponding to T7 RNA polymerase protein was isolated from phage genome. The *T7 RNA polymerase* gene was modified by addition of SV40 large T-antigen NLS sequence and 35S poly A tail at the 5' and 3' end respectively, in order to facilitate the efficient expression and nuclear targeting of the T7 RNA polymerase protein. The T7 RNA polymerase protein was heterologously expressed in *E. coli*.

4. To drive the expression of *T7 RNA polymerase* gene in the anthers, the promoter region of a highly tissue specific gene, RA8, was isolated from rice genome. *RA8* gene is reported to be specifically expressed in tapetum, endothecium and connective tissues of anthers. The tissue-specificity of the *RA8* gene was further confirmed and the expression of gene was found to be confined to anthers. The *in silico* analysis of the RA8 promoter sequence revealed the presence of putative cis-acting elements that drive the tapetal gene expression.

5. The RA8 promoter regulated *T7 RNA polymerase* cassette was constructed by cloning the *T7 RNA polymerase* gene downstream to the RA8 promoter.

6. In order to facilitate the efficient screening of the transgenic plants by non invasive method, a 1.63 kb long 35S promoter regulated *bar* gene cassette was cloned and used as an additional marker in the construct. The *bar* gene codes for PAT protein which confers tolerance against the herbicide glufosinate commercially known as Basta.

7. All the above mentioned cassettes i.e., the T7 promoter-SOD-AsA-GSH gene cassettes, the RA8 promoter-*T7 RNA polymerase* cassette and the *bar* cassette were assembled on the T-DNA region of pMDC99 vector. For this, first the individual cassettes were cloned in the gateway compatible entry vectors, EV1 and EV2 and then stacked into pMDC99 vector by *in vitro* gene pyramiding via multi-round gateway technology. Thus, two gene cassettes were constructed that harbored the T7 RNA polymerase coupled SOD-AsA-GSH pathway genes, with one of the cassette carrying the additional *bar* gene.

8. Both the cassettes were mobilized into highly virulent EHA105 strain of *Agrobacterium* via electroporation and the stability of the genes in *Agrobacterium* was verified.

9. The T7 RNA polymerase coupled SOD-AsA-GSH pathway gene cassette with *bar* gene was transformed into Swarna variety of rice while the T7 RNA polymerase coupled SOD-AsA-GSH pathway gene cassette without *bar* gene was

transformed into IR64 variety of rice via *Agrobacterium*-mediated transformation. The putative transgenic plants harboring the modified cassette carrying *bar* gene were screened for glufosinate tolerance by *in vitro* leaf spray test. The transgenic plants were further confirmed by PCR, using gene specific and marker specific primers.

10. The integration of the transgenic cassette into the genome of the transgenic plants was confirmed by Southern hybridization and the position of integration of the T-DNA was identified in two transgenic lines by a novel method called Locus Finding (LF) PCR.

11. The expression of the transgenes was confirmed in the anthers of the transgenic lines by a semi-quantitative RT-PCR analysis. The expression of *hpt* and *bar* gene was confirmed in the T_1 transgenic lines. The expression of the transgenic proteins was not found to be occurring in the leaves showing thereby that there was no leaky expression of the T7 RNA polymerase coupled SOD-AsA-GSH pathway cassette in vegetative parts of the transgenic lines.

12. The growth and development of the T_0 transgenic plants was monitored and the plants were found to show normal growth. The pollen viability tests revealed the pollens to be equally viable as the pollen from non-transformed control plants.

CONCLUSION

We have been successful in obtaining transgenic rice lines exhibiting the integration of five genes thereby proving that *in vitro* gene pyramiding can be an effective strategy for manipulating the genomes of plants for enhancing their abiotic stress tolerance. The T_0 transgenic rice plants over-expressing T7 RNA polymerase coupled SOD-AsA-GSH pathway in anthers were found to exhibit normal growth and development under non-stress conditions. Pollens from transgenic lines were tested for viability and it was found that the introduction and over- expression of the transgene cassette did not impose any negative effect on the pollen development. The transgenic lines are to be analyzed for the

stability of transgene expression in subsequent generations. Further field trials of the transgenic plants, nevertheless, are needed to assess and validate their overall performance under different abiotic stress conditions, particularly high temperature and drought conditions.

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PUBLICATIONS

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1. Thirulogachandar V, **Pandey P**, Vaishnavi CS, Reddy MK (2011) An affinity-based genome walking method to find transgene integration loci in transgenic genome. Anal Biochem 416(2): 196-201

2. Mahanty S, Kaul T, **Pandey P**, Reddy RA, Mallikarjuna G, Reddy CS, Sopory SK, Reddy MK (2012) Biochemical and molecular analyses of copper-zinc superoxide dismutase from a C4 plant *Pennisetum glaucum* reveals an adaptive role in response to oxidative stress. Gene 505(2): 309-17

3. Reddy PS, Reddy GM, **Pandey P**, Chandrasekhar K, Reddy MK. 2012 Cloning and molecular characterization of a gene encoding late embryogenesis abundant protein from *Pennisetum glaucum*: protection against abiotic stresses Mol Biol Rep 39(6): 7163-74