Cloning and Functional Analysis of Cytosolic Ascorbate Peroxidase *(APX)* Isolated from *Arachis hypogaea* in a Model Plant Tobacco

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CERTIFICATE

The research work embodied in this thesis entitled "**Cloning and Functional Analysis of Cytocylic Ascorbate Peroxidase (*APX*) Isolated from *Arachis hypogaea* in a Model Plant Tobacco**" has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or in full for any other degree or diploma of any University.

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'Whoever could make two ears of corn or two blades of grass to grow upon a spot of ground where only one grew before, would deserve better of mankind, and do more essential service to his country, than the whole race of politicians put together.'
Jonathan Swift (1667–1745)

This thesis is dedicated to my loving Family & Friends

Abbreviations:

AsA	Ascorbate
APX	Ascorbate peroxidase
ABA	Abscisic acid
BAP	6- Benzyleaminopurine
^{0}C	Degree Celsius
cAPX	Cytosolic ascorbate peroxidase
CaMV	Cauliflower mosaic virus
cDNA	Complimentary DNA
DHA	Dehydroascorbate
DNA	Deoxyribonuclic acid
CI	Chloroform isoamyl alcohol
CAT	Catalase
CTAB	Cetyl trimethyle ammonium bromide
DDW	Double distilled water
EDTA	Ethylene diamine tetra acetic acid
FW	fresh weight
GR	Glutathione reductase
GPX	Glutathione peroxidase
GSH	Reduced glutathione
GSSH	Oxidised glutathoine
g	Gram
hr	Hour
HCL	Hydrochloric acid
Kb	Kilo basepair
kDa	Kilo Dalton
LB	Luria Bertani medium
M	Molar
MDAsA	Monodehydroascorbate
MDAR	Monodehydroascorbate reductase
mM	Millimolar
mg	Milligram
min	Minute
ml	Milliliter

MS	Murashige and skooge medium
$MSB_1N_{0.1}$	MS medium with BAP(1mg/l) and NAA (0.1 mg/l)
MSO	MS medium wihout any hormones
µg	Microgram
µl	Microliter
µM	Micromole
NAA	Nepthaleneacetic acid
OD	Optical density
PCI	Phenol-chloroform-isoamyl alcohol
Rboh	Respiratory burst oxidase
rpm	Revolution per minute
RT	Room temperature
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TAE	Tris-acetate EDTA buffer

CONTENTS

Table of Contents

INTRODUCTION

The plants are best suited to grow in their natural habitat. The slightest change in the climate may lead to alteration in the physiology and yield of crop plants. Moreover, the global climate is changing faster than predicted. The world scenario available for climate change shows an increase in aridity for the semi-arid regions of the globe and the Mediterranean region in near future (Intergovernmental panel on climate change, 2007). The growth in population in near future will also lead to overexploitation of water resources for domestic and agriculture purpose, leading to increased constraints to plant growth and survival and therefore, decrease in crop yield potential (Chaves et al. 2003, 2003; Passioura et al. 2007). Apart from the extreme environmental conditions faced, the plants are also affected by various pathogens that limit the crop yield. Thus all these factors that alter the optimum condition, for a plant to grow lead to stress. Hence stress is defined as "any deviation from the optimum condition". Larcher (1987) defined plant stress as "a state in which increasing demands made upon a plant lead to an initial destabilization of functions, followed by normalization and improved resistance. If the limits of tolerance are exceeded and the adaptive capacity is overtaxed, permanent damage or even death may result"

The factors that attribute to stress may be abiotic or biotic. Abiotic stress includes environmental factors, such as drought, salinity, cold and heat. It causes adverse effects on the growth of plants and the productivity of crops. Abiotic stress is the primary cause of crop loss worldwide, reducing average yield for most major crop plants by more than 50%. The most important abiotic factor that has contributed to the plant evolution is availability of water. Water stress encompasses both drought and salt stress. Drought and salinity are becoming particularly widespread in many regions, and may cause serious salinization of more than 50% of all arable lands by the year 2050 (Ashraf et al. 1994). It is estimated that >6% of the world's land and 30% of the world's irrigated area already suffer from salinity problems (Unesco Water Portal 2007). To keep pace with the increasing world population, agricultural lands are being expanded to arid and semi-arid regions. The intensive irrigation in this region will lead to secondary salinization due to changes in hydrologic balance which includes inflow of water to the land, water used by crops and water retained by the soil. Salinity has been recognized to be a major constrain by the Food and Agriculture Organization, accounting for 3% of the total potential land on earth. In India, approximately 7% of the potential arable land is rendered uncultivable due to over accumulation of salt (www.fao.org/ 2005). According to FAO report 2005, out of US$ 1.3 trillion annual food production capacity worldwide, the biotic stresses

account for 31–42 percent loss (amounting to (US$500 billion), with an additional 6–20 percent (amounting to US$120 billion) lost post harvest to insects and to fungal and bacterial rots. Another 6–20 percent (US$ 120 billion) is estimated to be lost due to abiotic stress.

An ideal approach for avoiding the drought induced by inadequate rainfall is to utilize water reserves to provide supplementary crop irrigation. If sufficient water reserves are not available, their availability can be increased by the appropriate introduction of water conservation, water engineering and agronomic technologies (Tal 2006). However at any stage, ideas and improvements reach their limits. Therefore, some alternative methods must be explored to cope up with increasing stress factors that limit crop yield. Other solutions to increase the performance of plants under drought conditions involve selection, breeding or genetic-engineering approaches. Recent advances in plant genomics have led to the identification of a vast number of potentially beneficial water-stress-related genes, as well as technologies for gene overexpression or silencing. Moreover, these genes can be introduced into crop plants under the control of appropriate promoters and are transmitted to subsequent generations (Delmer et al. 2005; Ma and Bohnert 2007).

The potential to improve plant productivity relies largely on newly developed DNA biotechnologies and molecular markers, as well as genomics. These techniques enable the selection of successful genotypes, better isolation, characterization and cloning of favourable traits and the creation of transgenic plants of importance to agriculture (Altman et al. 1998; 1999). Thus conventional crop breeding combined with modern day transgenic approaches will improve and increase crop production in the near future.

The transgenic approach includes the expression of unique genes from various sources, as well as integration of specific promoters and transcription factors for enhanced and controlled gene expression. The non-transgenic approach includes genomics-assisted gene discovery, marker-assisted selection and efficient mutations.

To develop better food crops for the future, the knowledge of plant physiology and plant responses to various stresses is essential. It is well known that the biotic and abiotic stresses are both accompanied by production of reactive oxygen species leading to oxidative stress. Reactive oxygen species include superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$). The ROS are a product of altered chloroplast and mitochondrial metabolism during stress. Several recent studies have demonstrated that ROS also function as important signalling molecule when present in

lower concentration and are involved in the control of processes such as pathogen defence, hormonal signalling, stress response, and plant growth and development (Torres et al. 2002; Foreman et al. 2003; Kwak et al. 2003). However, ROS are also toxic molecules capable of injuring or even killing plant cells. Therefore their level in cells needs to be tightly regulated (Neill et al. 2002; Mittler et al. 2004).

The ROS cause oxidative damage to different cellular components including membrane lipids, proteins and nucleic acids (Haliwell et al. 1986). A balance exists in cells between the ROS generation and scavenging (Dutilleul et al. 2003). The ROS are scavenged by both enzymatic and non-enzymatic antioxidant pathways. Thus a "redox homeostasis" is maintained inside the cell. Plants use low molecular mass antioxidants such as ascorbic acid and reduced glutathione and employ a diverse array of enzymes such as superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidases (APX), glutathione S-transferases (GST) and glutathione peroxidases (GPX) to scavenge ROS. The APX is thought to play the most essential role in scavenging ROS and protecting cells against these toxic effects in higher plants, algae, *Euglena* and other organism (Ishikawa et al. 2003; Panchuk et al. 2005; Sano et al. 2001; Teixeira et al. 2006). Among the different isoenzymes of APX, the cytosolic APX is found to have more general stress protective function (Davletova et al. 2005; Fourcroy et al. 2004).

Attempts have been made earlier to develop stress tolerant plants by over-expressing different isoenzymes of APX. *Arabidopsis* Peroxisomal Ascorbate Peroxidase (*AtAPX*) gene has been expressed in tobacco for protection against oxidative stress (Jing Wang et al. 1999). Peroxisomal *APX* gene (*PpAPX*) from *Populus tomentosa* has been cloned and shown to be involved in scavenging toxic oxygen species (Lu et al. 2009). It has also been reported that over-expression of cytosolic ascorbate peroxidase (*cAPX*) from *Arabidopsis thaliana* in tobacco chloroplasts enhances tolerance of transgenic plants to salt stress and water deficit (Badawi et al. 2003). Transgenic tomato (*Lycopersicon esculentum*) over-expressing *cAPX* from *Pisum sativum* has also been shown to exhibit enhanced tolerance to UV-B and heat stress (Wang et al. 2006).

The effect of overexpressing APX in conjunction with other antioxidant genes has also been investigated. Lim et al (2007) showed that transgenic sweet potato plants that express both Copper Zinc Superoxide Dismutase *(CuZnSOD)* and *APX* in chloroplasts exhibited enhanced tolerance to methyl viologen-mediated oxidative stress and chilling. Enhanced tolerance to reactive oxygen species in tobacco transformed simultaneously with *SOD, APX* and Dehydroascorbate (*DHA*) has also been observed (Lee et al. 2007).

The present work was carried out to study the effect of overexpression of cytosolic ascorbate peroxidase (*APX*) (accession number EF165068) isolated from the salt tolerant cell lines of *Arachis hypogaea* in the model plant tobacco.

The objectives of the study are listed below:

1. Sub-cloning of the cytosolic *APX* cDNA isolated from *Arachis hypogaea* in a binary vector pCAMBIA 1301 under the control of a strong constitutive promoter.
2. To transform model plant *Nicotiana tabacum* with c*APX* cDNA-binary vector construct.
3. Molecular analysis of T_0 putative transgenic plants.
4. Physiological analysis of T_0 transgenic plants.

REVIEW OF LITERATURE

Environmental stress is considered to be primarily a response to the physical features of the environment. Extrinsic stress that results from changes in abiotic factors such as temperature, climatic factors and chemical components, either naturally occurring or manmade, is regarded as the most important stress agent. In addition, biotic stress such as bacterial, fungal, algal and viral diseases also cause stress in plants. Therefore on the basis of the factors causing stress, it can be classified as abiotic or biotic.

Abiotic stress can be attributed to the following factors:

- Drought
- Salinity
- Temperature (heat, cold)
- Light(high light, darkness, UV)
- Heavy metals stress

Biotic stress can be attributed to following agents:

- Bacterial pathogen
- Fungi
- Plant virus
- Weeds
- Pests
- Other necrotrophic agents

Plants are sessile organisms which are constantly challenged by these abiotic and biotic stresses. Thus, it is important to devise specific strategies to make stress-tolerant plants. Evolution has provided plants with genome specific characters which qualify the individuals of plant populations like hygrophytes, mesophytes, xerophytes and halophytes to perform optimally in their respective environments. The mechanisms of adaptation or the way plants tolerate various stresses are well documented in the literature and form an important branch of study in plant sciences (Hanson et al. 1986; Hamlyn et al. 1988). All the available knowledge can be used to develop strategies to minimise crop loss and meet the increasing demand of food. Therefore understanding the molecular mechanism and deciphering the stress response pathways will pave the path for developing stress tolerant plants in future.

2.1 Abiotic Stress

Environmental factors such as water availability, salinity and temperature are the most important factors which when altered from normal conditions contribute to abiotic stress and limit the growth and productivity of crops. The abiotic stress affects metabolic activity inhibits cell division and down-regulates photosynthesis leading to altered morphology and physiology. Particular environmental conditions may affect specific mechanisms, e.g. drought and salinity lead to identical physiological effect that is increased cellular osmotic concentration or "osmotic stress" resulting in disruption of homeostasis and ion distribution in cells (Zhu et al. 2002). Low temperature severely hampers reproductive development. Exposure of rice plants to chilling temperature at anthesis (floral opening stage) lead to male sterility (Imin et al. 2006; Mamun et al. 2006). More extreme cold stress mainly results in disruption of membrane integrity and solute leakage, leading to severe cellular dehydration and osmotic imbalance (Thomashow et al. 1999).

The production of reactive oxygen species (ROS) is enhanced when plants are exposed to drought, salinity and low temperature stresses. Moreover, the generation of ROS is a common phenomenon irrespective of the type of stress faced by plants. The ROS are generated in light reactions of photosynthesis when photosystem I (PSI) and II (PSII) are excited inducing water splitting and electron transport and the excited electrons exceed the requirements of normal metabolism. The excess electrons reduce oxygen, generating superoxide (O_2^-). Such over excitation of the system is characteristic of stress conditions. The ROS are produced in organelles with high oxidizing metabolic activity like chloroplasts, mitochondria and microbodies. The light reactions occur in the thylakoid membranes which are susceptible to damage (Moller et al. 2007; Takahashi 2008). The ROS cause peroxidation and de-esterification of membrane lipids, and also lead to protein denaturation as well as other forms of photo-oxidative damage (Bowler et al. 1992). The physiological and biochemical changes in plants under particular stress conditions are related to altered gene expression. Onset of stress is initially perceived by cell surface receptors or sensors, which in turn activate cytoplasmic Ca^{2++} and protein signalling pathways. This leads to stress-responsive gene expression and physiological changes (Bressan et al. 1998; Xiong et al. 2002). There is an accumulation of abscisic acid (ABA), which plays an important role in abiotic stress signalling and transduction pathways, mediating many responses (Wasilewska et al. 2008). Abiotic stress also alters gene expression and protein turnover. Abiotic stresses in general, through regulation of

both gene expression and protein turnover, alter the abundance of many transcripts and proteins (Seki et al. 2002; Wong et al. 2006; Yan et al. 2006; Jiang et al. 2007). Thus the transcriptional and post-transcriptional regulation plays an essential role in the adaptation of cellular functions to environmental stresses. In contrast to plant resistance to biotic stresses, which is mostly dependent on monogenic traits, the genetically complex responses to abiotic stresses are multigenic and thus more difficult to control and engineer. Plant engineering strategies for abiotic stress tolerance (Wang et al. 2003) rely on the expression of genes that are involved in signalling and regulatory pathways (Seki et al. 2003; Shinozaki et al. 2003) or genes that encode proteins conferring stress tolerance (Wang et al. 2004) or enzymes present in pathways leading to the synthesis of functional and structural metabolites changes (Rontein et al. 2002; Park et al. 2004). The transcript level of some genes encoding proteins with antioxidant functions are also upregulated during stress response. These include glutathione reductase (GR) and ascorbate peroxidase (APX), whose higher transcript levels during recovery from water stress may play a role in protecting the cellular machinery against photo-oxidation by ROS (Ratnayaka et al. 2003).

2.1.1 Drought and salinity stress

Drought and salinity are the major limitations in crop productivity world-wide. One third of the earth's surface is classified as arid or semi-arid and most of the humid regions, wherein much of the food crop is produced, the crops are subjected to periods of severe drought. Moreover, nearly 40 percent of the world land surface can be categorised as having potential salinity problems (Boyer et al. 1982). Drought and floods occur in all continents. During the period 1991-2000, floods affected on an average 140 million people annually, while famine caused by drought affected nearly 42 million and accounted for most deaths (42%) caused by natural disasters (www.waterforfood.org). Drought and salinity induce common metabolic effect, that is, decreased water activity inside the cell. Once stress sets in, various biochemical and physiological mechanisms are switched on in order to protect major processes such as cell respiration, photosynthetic activity and nutrient transport. Also stress tolerance responses are induced to preserve cell organelles and tissue structure such as meristems (Kramer 1983).

2.1.2 Salinity stress

Salinity is the major environmental factor limiting plant growth and productivity (Allakhverdiev et al. 2000). About 22% of the world agricultural land is saline (FAO 2004). The detrimental effects of high salinity on plants can be observed at the whole-plant level as the death of plants and/or decrease in productivity. High salt stress leads to altered nutrient uptake, especially of ions such as K^+ and Ca^{++}, accumulation of toxic ions, especially Na^+, osmotic stress and oxidative stress. The major component of most saline soils is NaCl, therefore the term salinity refers to stress caused by high levels of NaCl. Salt stress differs from the low water potential imposed by soil drying or a high molecular weight solute. In case of saline stress the major factor causing long-term injury is the ionic imbalance and toxicity caused by excess Na^+ rather than the effects of salt on water potential (Huh et al. 2002; Munns et al. 2002). Studies report that rapid responses to salt (responses that occur within a few hours of application of salt) often resemble responses to low water potential (drought stress) imposed using non-ionic solutes. However, long term responses that occur over a time frame of days to weeks are more salt specific (Munns et al. 2002). The evidence of this is provided by the isolation of several salt overly sensitive (SOS) mutants that are hypersensitive to salt but not to non-ionic osmotic stress. These mutants were unable to regulate a number of ion transport processes and genes specifically involved in tolerance of salt stress (Gong et al. 2001; Shi et al. 2002; Wu et al. 1996; Zhu 2000; Zhu et al. 1998).

2.1.3 Mechanism of salt tolerance

Plants have developed a network of biochemical and molecular mechanisms to cope with salt stress. Biochemical pathways lead to products and processes that improve salt tolerance. The effects are either additive or help plants in a cooperative manner to tolerate stress (Iyengar and Reddy 1996). Biochemical strategies include (i) selective accumulation or exclusion of ions (ii) control of ion uptake by roots and transport into leaves (iii) compartmentalization of ions at the cellular and whole-plant levels (iv) synthesis of compatible solutes (v) change in photosynthetic pathway (vi) alteration in membrane structure (vii) induction of antioxidative enzymes and (viii) induction of plant hormones. Salt tolerance mechanisms are either low-complexity or high-complexity mechanisms. Low-complexity mechanisms appear to involve changes in many biochemical pathways. High-complexity mechanisms involve changes that protect major processes such as photosynthesis and respiration, water use efficiency, and those that

preserve such important features as cytoskeleton, cell wall, or plasma membrane–cell wall interactions (Botella et al. 1994). These also include chromosome and chromatin structure changes like DNA methylation, polyploidization, amplification of specific sequences, or DNA elimination (Walbot et al. 1985). It is believed that for the protection of higher-order processes, low-complexity mechanisms are induced co-ordinately (Bohnert et al. 1995).

2.1.4 Drought stress

In a simplified way drought can be defined as a period below normal precipitation that limits plant productivity in a natural or agricultural system (Boyer et al. 1982; Kramer et al. 1995). In the field, drought can be caused by a number of stress factors including temperature, light and nutrient stresses. However, the stress component that defines drought is a decrease in the availability of soil water or sub-optimal availability of water for unrestricted plant growth and transpiration.

On the basis of the extent of drought, it can be permanent, seasonal or random (Kramer et al. 1983). Permanent drought is present in desert areas wherein lack of rainfall is very common whereas seasonal drought occurs when either winter or summer are regularly dry. Finally random drought occurs in humid regions subjected to occasional variation in amount and distribution of rainfall. In addition, another type of drought to be considered is non-apparent drought. It is observed in hot summer days when high temperature or wind conditions induce an increase in transpiration rate that exceeds water absorption rate by roots. Consequently, despite adequate soil water content, plants can show drought symptoms (Sanchez-Diaz et al. 1993).

2.1.5 Plant responses to drought stress

The responses of plants to drought stress are highly complex. Some plants have the ability to survive harsh conditions while others adapt themselves to the changes. Plants have different strategies to avoid the deleterious effects of drought and these can be classified as follows:

a) Drought escapes: Those plants which can avoid drought like the annuals which complete their life cycle exclusively during the period of the year when soil water is available. Drought avoidance is the ability of the plant to maintain high tissue water potential under drought conditions. Drought avoidance is usually achieved through morphological changes in the plant, such as reduced stomatal conductance, decreased

leaf area, development of extensive root system and increased root/shoot ratios like in xerophytes (Levitt et al. 1980).

b) Drought tolerance: These plants can not avoid water stress and consequently develop a number of adaptation mechanisms aimed to increase drought tolerance. Drought tolerance is a plant's ability to maintain its normal functions even at low tissue water potential. Drought tolerance is achieved by cell and tissue-specific physiological, biochemical, and molecular mechanisms, which include specific gene expression and accumulation of specific proteins under drought stress.

Both morphological and anatomical strategies are developed by plants to resist dehydration, such as deep and branched root systems or presence of thick cuticle as in cactus leaves and stems. All the strategies play an important role in drought tolerance by either increasing the water input by absorption or by reducing water output by transpiration (Kramer et al. 1983). In addition to these strategies, the tolerance of plants to drought is also mediated by metabolic responses. Both drought and salinity induce osmotic stress and have similar biochemical responses that include the accumulation of compatible osmolytes; increase of reactive oxygen species (ROS) and scavenging enzyme levels (Zhu et al. 1997).

2.2 Reactive Oxygen Species (ROS)

What are ROS ?

ROS are free radicals produced as by-products of oxidation–reduction (REDOX) reactions. ROS when present in low concentration are known to act as important signalling molecules. For instance, in plants, ROS are used to facilitate an array of essential biological processes such as programmed cell death (PCD), development, gravitropism and hormone signalling in response to stress (Apel & Hirt 2004; Foyer & Noctor 2005; Gechev et al. 2006). Reactive oxygen species also called active oxygen species (AOS) or reactive oxygen intermediates (ROI) are the result of the partial reduction of atmospheric O_2. There are basically four forms of cellular ROS, singlet oxygen ($^1O_2$), superoxide radical ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$). Each of these has a characteristic half-life and an oxidizing potential, H_2O_2 being the most stable. ROS can be extremely reactive, especially the singlet oxygen and the hydroxyl radical. Unlike atmospheric oxygen, they can oxidize multiple cellular components like proteins, lipids, DNA and RNA (Mittler et al. 2002).

2.2.1 Production of ROS

Plants are natural producers of ROS under normal conditions and continuously produce ROS. The production of singlet oxygen and the hydroxyl radical is regulated and kept at minimum levels (Foyer et al. 1994; Jakob et al. 1996). On the other hand superoxide and H_2O_2 are synthesized at very high rates under normal conditions (Noctor et al. 1998). One of the major cellular sites responsible for ROS production is the chloroplasts (Foyer et al. 1998). During photosynthesis, energy from the sunlight is captured and transferred to two light-harvesting complexes (photosystem II and photosystem I) in the chloroplast thylakoidal membranes. A succession of redox reactions occur within the electron transport chain in light until electrons finally reach CO_2 in the dark reaction. However, it is not uncommon that through this path other final acceptors of electrons are used, namely oxygen (Baker 1991). Singlet oxygen can be formed by energy transfer from triplet excited state chlorophyll to O_2 (Asada et al. 1987). On the other hand, the thylakoidal electron transport components on the PSI side such as the Fe-S centres and the reduced thioredoxin are auto-oxidable resulting in the reduction of O_2 (the Mehler reaction) thus forming superoxide and H_2O_2 (Asada et al. 1987; Mehler et al. 1951). It has been estimated that approximately 10% of the photosynthetic electrons flow through the Mehler reaction. This leakage of electrons to O_2 with the generation of ROS is in fact favourable to the electron transport chain since it balances the electron carriers thus making them more efficient (Noctor 1998). During photosynthesis there is a different pathway, called photorespiration that can also generate ROS. Infact, rubisco, the enzyme that catalyses the carboxylation of ribulose-1,5-bisphosphate (RuBP) during carbon assimilation, can also use O_2 to oxygenate ribulose-1,5-bisphosphate. This reaction yields glycolate which is transported from chloroplasts to peroxisomes where it is oxidized by glycolate oxidase, thus generating H_2O_2 (Winglar, 2000). Apart from this the mitochondrial electron transport chain is also responsible for ROS generation under normal conditions, although to a lesser extent than chloroplasts and peroxisomes in light (Foyer 2003). It has been estimated that approximately 1–2% of the consumed oxygen, respired by plant mitochondria, is used to form superoxide (Puntarulo 1988). This ROS production is likely to occur mainly in complexes I and III of the mitochondrial electron transport chain (Moller 2001).

2.3 Production of ROS is Common to Different Environmental Stresses

2.3.1 Salinity induced oxidative stress

Apart from the ROS generated under drought stress, a secondary aspect of salinity stress in plants is the stress induced production of reactive oxygen species (ROS). Salt stress indirectly imposes a water deficit condition on plants and causes osmotic effects on a wide variety of metabolic activities (Greenway and Munns et al. 1980; Cheeseman et al. 1988). This water deficit leads to the formation of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) (Halliwell and Gutteridge et al. 1985), and singlet oxygen ($^1O_2$) (Elstner et al. 1987). Onset of stress leads to change in cellular physiology and metabolism in chloroplasts and mitochondria leading to generation of ROS. These species cause oxidative damage to different cellular components including membrane lipids, proteins and nucleic acids (Halliwel et al. 1996). The alleviation of this oxidative damage is imperative for the survival of plants under salt stress. Plants use low molecular mass antioxidants such as ascorbic acid and reduced glutathione and employ a diverse array of enzymes such as superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidases (APX), glutathione S-transferases (GST) and glutathione peroxidases (GPX) to scavenge ROS.

2.3.2 Drought induced oxidative stress

The first plant organ to detect a limitation of water supply is the root system. It has been shown that besides water and minerals, roots also send signals to the leaves through the xylem sap, and the phytohormone abscisic acid is considered to be one of the major root-to-shoot stress signal (Jiang et al. 2007). When the stress signal reaches the leaves, it triggers stomatal closure and the plant shifts to a water-saving strategy. Hence, by adjusting stomatal opening, plants are able to control water loss by reducing the transpiration flux, but they are at the same time limiting the entrance of carbon dioxide (CO_2). This will have direct and indirect effects on the reduction of net photosynthesis and on the overall production of ROS by plants under drought stress (Mittler et al. 2002). There are many studies that have reported on increased ROS accumulation and oxidative stress under drought stress (Sgherri et al. 1996; Boo et al. 1999). In fact, under drought stress, ROS production is enhanced through multiple ways. For instance, the limitation of CO_2 fixation will reduce $NADP^+$ regeneration through the Calvin cycle, hence leading to over-reduction of the photosynthetic electron transport chain. Under drought stress, during the process of photosynthesis there is a higher leakage of electrons to O_2 by the

Mehler reaction (Smirnoff 1993). It was estimated that in drought stressed wheat, the leakage of photosynthetic electrons to the Mehler reaction was increased by approximately 50% as compared to unstressed wheat (Biehler et al. 1996). An increase in thylakoid membrane electron leakage to O_2 under drought stress was also seen in sunflower (Sgherri et al. 1996). However, it is difficult to distinguish the part of ROS generated by the Mehler reaction and that generated by photorespiration. In fact, under drought stress the photorespiratory pathway is also enhanced, especially when RuBP oxygenation is maximal due to limitation on CO_2 fixation (Noctor et al. 2002). The increased oxidative stress during photorespiration in response to drought stress has been well documented by Noctor and co-workers. They have estimated that photorespiration is likely to account for over 70% of total H_2O_2 production under drought stress conditions. But the chloroplast is a robust cellular compartment towards ROS because of the different scavenging enzymes and metabolites present (Asada et al. 1999; Noctor et al. 2002; Asada et al. 2006). However, under drought stress one of the real problems is the production of the hydroxyl radical in the thylakoids through "iron-catalysed" reduction of H_2O_2 by both superoxide dismutase (SOD) and ascorbate. The hydroxyl radical is the ROS which has the shortest half-life (~1 millisecond) but it also has an extremely strong oxidizing potential reacting with almost every biological molecule (Dat et al. 2000). Furthermore, there is no enzymatic reaction known to eliminate the highly reactive hydroxyl radical (Vranova et al. 2002; Yakota et al. 2002) and its accumulation will inevitably lead to harmful reactions which damage the thylakoidal membranes and the photosynthetic apparatus.

2.3.3 Temperature induced oxidative stress

The generation of ROS has also been observed when plants are exposed to low temperature conditions. Under low temperature, chloroplasts are the main source of ROS production. This production is due to decrease in CO_2 fixation in the Calvin-Benson cycle (Yamazaki et al. 2003). Photon utilization for CO_2 fixation decreases as a result, leading to over-reduction of electron transport chain and hence over-production of NADPH and H^+. This may result in increased formation of ROS in the electron transport chain and subsequent damage to the photosynthetic system (Yamazaki et al. 2003).

2.3.4 Heavy metal induced oxidative stress

It has been observed that on exposure to heavy metal stress, reactive oxygen species, such as $^{\cdot}O_2^-$, H_2O_2, and $^{\cdot}OH$, which bear strong oxidizing activities, are commonly generated and can attack all types of biomolecules (Wojtaszeket al. 1997). In fact, these oxygen species represent intermediates emerging during the successive reduction of O_2 to H_2O. Therefore, exposure of plants to certain heavy metal ions shifts the balance of free radical metabolism towards an accumulation of H_2O_2. In the presence of redox active transition metals such as Cu^+ and Fe_2^+, H_2O_2 can be converted to the highly reactive $^{\cdot}OH$ molecule in a metal-catalyzed reaction via the Fenton reaction (Wojtaszeket al. 1997). The oxidized metal ions undergo a re-reduction in a subsequent reaction with superoxide radicals ($^{\cdot}O_2^-$). An alternative mechanism of $^{\cdot}OH$ formation directly from H_2O_2 and $^{\cdot}O_2^-$ is the metal-independent Haber–Weiss reaction (Wojtaszeket al. 1997). The Hg^{2+} ions inhibit the activities of antioxidative enzymes especially of glutathione reductase, and also raise a transient depletion of GSH (Schutzendubel 2002). The Fenton reaction (1) and Haber-Weiss reaction (2) are represented as follows:

(1) Fenton reaction: (Wojtaszek 1997)

$$H_2O_2 + Fe^{2+}/Cu^+ \longrightarrow {}^{\cdot}OH + OH^- + Fe^{3+}/Cu^{2+}$$

$$^{\cdot}O_2^- + Fe^{3+}/Cu^{2+} \longrightarrow Fe^{2+}/Cu^+ + O_2$$

(2) Haber–Weiss reaction: (Wojtaszek 1997)

$$H_2O_2 + {}^{\cdot}O_2^- \longrightarrow OH + OH^- + O_2$$

2.3.5 ROS generation under ozone and UV-light

In an interesting study it was shown that ozone (O_3) induces an oxidative burst that has similarities to the pathogen-induced oxidative burst (Overmeyer et al. 2003). In *Arabidopsis*, ozone exposure drives both the spatial and the temporal progression of ROS signalling, commencing with the elevation of ROS levels in guard-cells, chloroplasts and membranes, spreading to neighbouring cells (Joo et al. 2005). Heat and ultraviolet light (UV-B, 280-320 nm) stimulate the production and accumulation of toxic ROS, which results in lipid peroxidation and membrane injury (Mackernesset al. 2000; Davidson et al. 1996; He et al. 2002; Jiang and Huang 2001; Sairam et al. 2000). Since the production of reactive oxygen species is observed to be a common phenomenon on exposure of

plants to biotic or abiotic stress, there seems to be a cross-talk between the different stresses experienced by plants (Fig. 1)

2.3.6 Pathogen/herbivore-induced oxidative stress

The ROS are produced not only under abiotic stress but also induced by biotic factors and accumulate in plants as a defence response to pathogen attack. This has been very well documented and described as oxidative burst (Wojtaszek et al. 1997). The ROS that have been detected in plant pathogen interactions are $^{\cdot}O_2^-$, $^{\cdot}O_2H$, H_2O_2, and $^{\cdot}OH$ (Scheel 2002). Wounding caused by insect feeding has been shown to result in the production of ROS in the damaged tissue (Gatehouse et al. 2002; Kessler et al. 2002). As shown for soybean, herbivory by the insect *Helicoverpa tea* induced a shift in the oxidative status of the plant causing an increase in $^{\cdot}O_2^-$ and $^{\cdot}OH$ radical formation (Bi et al. 1995). In a more direct way, insect salivary gland-derived enzymes such as the H_2O_2 generating glucose oxidase might contribute to the increase in the concentration of ROS at the site of herbivore attack (Felton et al. 1999).

2.4 Scavenging of ROS in Plant Cells

2.4.1 Important detoxifying enzymes

Plants have a diverse array of enzymes for ROS scavenging (Apse et al. 2002). The antioxidant enzymatic system includes SOD which can be found in various cell compartments and catalyses the disproportionation of two $^{\cdot}O_2^-$ radicals to H_2O_2 and O_2 (Scandalios et al. 1993; Khosravinejad et al. 2008). The SOD is found in cell organelles, like chloroplasts, mitochondria and peroxisomes (Scandalias et al. 1990; Beyer et al. 1991; Bowler et al. 1994). The H_2O_2 is eliminated by various antioxidant enzymes such as catalases (CAT) (Kono and Fridivich 1983; Scandalios 1993; Khosravinejad et al. 2008) and peroxidases (POX) (Jablonski and Anderson 1982; De Gara et al. 2003; Khosravinejad et al. 2008), which convert H_2O_2 to water. Other enzymes that are very important in ROS scavenging system and function in ascorbate-glutathione and xanthophyll cycles are, glutathione reductase (GR), monodehydro ascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Yoshimura et al. 2000). There seems to exist a balance between the activities of these enzymes and production of ROS so that the steady state of the superoxide radicals and hydrogen peroxide is maintained (Bowler et al. 2000).

2.4.2 Enzymatic components of the ROS-scavenging pathways of plants

Different developmental or environmental signals affect the ROS signalling network and perturb ROS homeostasis in a compartment-specific or even cell-specific manner. The ROS are perceived by different ROS sensors and activate cellular responses (e.g. pathogen or stress defence). The ROS-scavenging pathways are also responsible for maintaining a low steady-state level of ROS which plant uses as signalling molecules to control more specialized processes such as plant growth, development, defence, and hormonal signalling. Modulation of ROS levels might also involve a positive feedback loop between ROS perception and ROS production (shown by dashed line Fig. 2). The ROS scavenging enzymes together with the low molecular mass hydrophilic antioxidants such as ascorbic acid and reduced glutathione provide efficient machinery for ROS detoxifying $^{\cdot}O_2^-$ and H_2O_2. The antioxidants, ascorbic acid and glutathione are maintained within the cell in their reduced state by a set of enzymes capable of using NAD(P)H to regenerate oxidized glutathione or ascorbic acid (e.g. monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase). In addition to dehydroascorbate reductases and glutathione reductase indicated, many other enzymes in plants can catalyze the reduction of dehydroascorbic acid with different efficiencies (Chew et al. 2003). Moreover, monodehydroascorbate radicals can be reduced back into ascorbic acid via ferredoxin using electrons diverted from the photosynthetic apparatus in the water–water cycle in chloroplasts (Asada et al. 1999). Apart from this, some class III peroxidases are also involved in H_2O_2 scavenging and are present in cytosol, vacuoles, apoplast and cell wall (Hirago et al. 2001; Tognolli et al. 2002).

2.4.3 Cellular localization and co-ordination of the ROS scavenging pathways of plants

The ROS-producing and the ROS-scavenging pathways determine the intensity, duration and localization of the ROS signals. Various scavenging enzymes encoded by the ROS network can be found in almost every subcellular compartment. In addition, usually more than one enzymatic scavenging activity per a particular ROS can be found in each of the different compartments e.g. GPXs, PrxRs and APXs are present in the cytosol and chloroplast, while APXs and CATs in peroxisomes (middle left Fig. 3a). The water–water cycle detoxifies O_2^- and H_2O_2, and alternative oxidase (AOX) reduces the production rate of O_2 in thylakoids (top left in Fig. 3b).

Table: 1 The key enzymes and antioxidants involved in scavenging of ROI in the plant cells are shown.

Scavenging of ROS:

Enzymes	Localization	Free radical
Superoxide Dismutase (SOD)	Chl, Cyt, Mit, Per, Apo	O_2^-
Ascorbate peroxidase (APX)	Chl, Cyt, Mit, Per, Apo	H_2O_2
Catalase (CAT)	Peroxisome	H_2O_2
Glutathione peroxidase (GPX)	Cytoplasm	H_2O_2, ROOH
Class III Peroxidases	CW, Cyt, Vac	H_2O_2
Thioredoxin Peroxidases	Chl, Cyt, Mit	H_2O_2
Alternative peroxidase (AOX)	Chl, Mit	O_2

Antioxidants	Localization	Free radicle
Ascorbic acid	Chl, Cyt, Mit, Per, Apo	H_2O_2, O_2^-
Glutathione	Chl, Cyt, Mit, Per, Apo	H_2O_2
α-tocopherol	Membranes	ROOH, $^1O_2$
Carotenoids	Chl	O_2

(Abbreviations: Apo, apoplast; Chl, chloroplast; CW, cell wall; Cyt, cytosol; Mit, mitochondria; $^1O_2$, singlet oxygen; Per, peroxisome; Vac, vacuole) (Adopted from Mittler et al. 2004).

ROS that escape this cycle and/or are produced in the stroma undergo detoxification by SOD and by the stromal ascorbate–glutathione cycle. The peroxiredoxin (PrxR) and glutathione peroxidase (GPX) are also involved in H_2O_2 removal from the stroma (top right in Fig. 3c). The ROS produced in peroxisomes during photorespiration, fatty acid oxidation or other reactions are decomposed by superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (middle right in Fig. 3d). The SOD and other components of the ascorbate–glutathione cycle are also present in mitochondria (bottom right in Fig. 3e). In addition, AOX prevents oxidative damage in mitochondria. The balance between SOD and the different H_2O_2 scavenging enzymes in cells is considered to be crucial in determining the steady-state level of $^{\cdot}O_2^-$ and H_2O_2. This balance, together with the sequestering of metal ions by ferritin and other metal-binding proteins,

prevents the formation of the highly toxic ·OH radical via the metal-dependent Haber–Weiss reaction or the Fenton reaction (Jonak et al. 2004). In principle, the cytosol contains the same set of enzymes as found in the stroma (bottom left in Fig. 3f) (Mittler et al. 2004).

2.5 Ascorbate Peroxidase (APX)

Since ascorbate peroxidase is the main focus of the present study, various aspects of it are detailed below.

2.5.1 Occurrence

In plants the heme peroxidases have been classified into three categories. The intracellular class I (EC 1.11.1.5/.6/.11), the class II released by fungi (EC 1.11.1.13/.14), and the secreted class III plant peroxidases (EC 1.11.1.7). In plants ascorbate peroxidase (APX E.C. 1.11.1.11) occurs as heme enzyme present both in the cytosol and the chloroplasts. It catalyzes the conversion of H_2O_2 to H_2O using ascorbate as specific electron donor. The occurrence of APX was first shown in *Euglena* (Groden 1979; Shigeoka et al. 1980). However, way back, in 1931 it was shown that plant extracts could reduce ascorbate via reduced glutathione (Szentgyorgi et al. 1931). It was also shown that ascorbate and glutathione were localized in chloroplasts together with the glutathione reductase (Gutteridge et al. 1976). Presence of ascorbate peroxidase has also been shown in mitochondria, apoplast and peroxisomes (Asada et al. 1987; 1999).

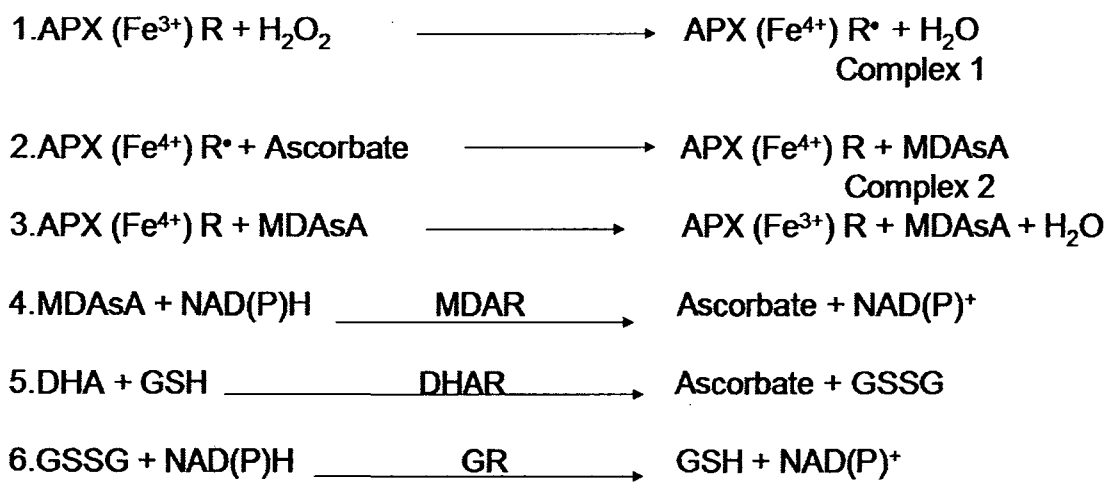
2.5.2 The isoezymes of APX

APX is widely distributed in plants and eukaryotic algae that have acquired the ability to produce ascorbate (AsA). Cytochrome *c* peroxidase and APX from yeast and catalase-peroxidase from cyanobacteria belong to the Class I category of plant-type heme peroxidases indicating that these enzymes developed from the same ancestral gene (Welinder et al. 1992). Genome analysis in higher plants has shown that APX belongs to a multigenic family. So far, nine genes have been isolated from *Arabidopsis*, eight from rice and seven from tomato (Chew et al. 2003; Najami et al. 2008). The APX family has been further classified on the basis of localization within the cell into three subfamilies, chloroplastic, mitochondrial and microbody isoenzymes (Shigeoka et al. 2002). All the isoenzymes of APX have a very high affinity for ascorbate (AsA) as electron donor. Chloroplastic APX (*chl APX*) and mitochondrial APX (*mit APX*) can oxidise only AsA (Yoshimura et al. 1998, Asada et al. 1999, Leonardis et al. 2000). However, cytosolic

APX (cAPX) can also oxidise the artificial electron donar such as guaiacol and pyrogallol (Ishikawa et al. 1989; Asada et al. 1999; Yoshimura et al. 1998). It can not reduce hydroperoxides and its prosthetic group of protoporphyrin is inhibited by cyanides and azides (Shigeoka, et al. 1980; Chen et al. 1989). In the absence of AsA, APX is very unstable. The thylakoid APX is a monomer whereas the cytosolic APX exists as a dimer consisting of identical subunits with molecular masses of 28 kDa each (Mittler et al. 1991; Miyake et al. 1993).

2.5.3 Mode of action

The activity of APX in plants generally increases along with activities of all the other ROS-scavenging enzymes, such as catalases, superoxide dismutase, and GSH reductase, in response to environmental stress (Shigeoka et al. 2002). The isoenzymes of APX are involved in a multistep reaction leading to the formation of water from hydrogen peroxide.



(Abbreviations: APX, ascorbate peroxidase; MDAsA, monodehydroascorbate; MDAR, monodehydroascorbate reductase; DHA, dehydroascorbate; GSH, reduced glutathione; GSSG, oxidised glutathione; DHAR, dehydroascorbate reductase; GR, glutathione reductase) (Adapted from Wojtaszek 1997).

First, the heme iron is oxidized to oxyferryl and an organic compound R. This may be a heme ring or an amino acid side chain. The substrate ascorbate then donates an electron and converts the reactive oxyferryl state to ferric (Fe^{3+}) state. Plant APX utilises ascorbic acid as electron donor to reduce H_2O_2. In this reaction monodehydroascorbate (MDAsA) is generated which disproportionates into reduced ascorbate (AsA) and dehydroascorbate (DHA).The enzyme DHA reductase (DHAR) uses the reducing equivalent NAD(P)H and oxidized GSH is formed. The APX along with the AsA-GSH cycle helps in the detoxification of H_2O_2 (Asada 1992; Asada 1997). The cycle closes with glutathione reductase (GR) converting GSSG back into GSH with the reducing agent NAD(P)H. The mode of action is defined below.

2.5.4 Distribution of APX in plants

APX is widely distributed in plants and eukaryotic algae. In contrast to higher plants, in algae the number and distribution of APX proteins are quite limited. *Euglena gracilis* lacks catalase and contains APX and enzymes related to the ascorbate redox cycle solely in the cytosol (Shigeoka et al. 2002; Ishikawa et al. 1996). Two cytosolic APX (cAPX) isoenzymes have been identified in the unicellular red algae *Galdieria partita* and *G. sulphuraria* (Sano et al. 2001; Oesterhelt et al. 2008). Halotolerant *chlamydomonas* contains one APX gene, the translated product of which is predicted to be located in the chloroplast stroma (Takeda et al. 2000). The limited number and subcellular localization of APX isoenzymes in these eukaryotic algae suggest a cellular metabolism of H_2O_2 different from that in higher plants. It is apparent that some parastic protozoa, such as *Trypanosome cruze* and *Leishmania major*, also have a functional APX, but the location in the endoplasmic reticulum is very different from that of plant APXs (Wilkinson et al. 2002; Adak et al. 2005). Protozoa APXs, including *Euglena* APX, form a novel category, because when the phylogenetic tree using protein sequences of APX is constructed, it reveals a clear divergence between plants and protozoa. APX is present as a family of isoenzymes in higher plants. Unlike guaiacol peroxidase (GP), which is distributed in the vacuoles, cell wall and cytosol and not in organelles? APX isoenzymes are distributed widely in most of the organelles, like stroma (stromal or *sAPX),* thylakoid membrane (*t-APX*), cytosol *(c-APX)* and mitochondria *(mit-APX)* (Chen et al; 1990; Miyake et al; 1993; Yamaguchi et al. 1995; Yamaguchi et al. 1995; Bunkelmann et al. 1996; Jimenez et al. 1997; Ishikawa et al. 1996; Ishikawa et al. 1998 Leonardis et al. 2000). *APX* gene has been isolated from many plants like sweet potato (*Ipomoea batana*) (Park 2004),

tomato (*Lycopersicum esculentum*) (Zou et al. 2005), maize (*Zea mays*), potato (*solanum tuberosum*) (Leonardis et al. 2000), and *Nicotiana tabacum* (Orver and Ellis, 1995). In higher plants, two or more cytosolic *APX* genes have been reported suggesting a multigenic family of these enzymes (Santos et al. 1996; Jespersen et al. 1997). From spinach, two cytosolic *APX, c-APX1* and *c-APX2* have been cloned and characterized (Ishikawa et al. 1995). Also in soybean (Caldwell et al. 1998) and *Arabidopsis* (Jespersen 1997), *cAPX* has been reported to belong to a multigenic family.

2.5.5 Effect of abiotic stress on APX

Environmental stresses like drought, salt, wounding, pathogen, temperature and paraquat have been shown to affect the level of APX isoenzymes (Tanaka et al. 1985; Mittler et al. 1994; Prasad et al. 1994; Conklin et al. 1995; Yoshimura et al. 2000). Along with APX, the activity of other antioxidant enzymes like SOD and CAT also increases during stress. This suggests that there is a correlation between their scavenging mechanisms. In maize, the drought resistant variety was shown to have higher activity of APX (Pastori et al. 1992). Salt stress tolerance was demonstrated in correlation with increased activity of antioxidants in *Gossypium hirsutum* (Dionisio et al. 1998). A novel mutant, *pst1* designated for photoautotrophic salt tolerant mutant of *Arabidopsis*, growing on NaCl containing medium showed higher activity of APX and SOD (Tsugana et al. 1991). *Pst1* mutants have been found to be salt sensitive. In pea, the activity of cytosolic APX increased four times during drought stress and increased to fifteen times on withdrawing the stress (Mittler and zilinskas, 1994). This result suggested the regulation of APX at post transcriptional level. During stress there is an enhancement in the normal production of ROS in plants. The APX enzyme being an important H_2O_2 scavenger shows an increase in the constitutive expression level of its different isoenzymes. In spinach, several stresses have been applied to analyze the expression of various isoenzymes of APX and it was found that in response to stress, the cytosolic *APX* gene showed an increased expression, whereas the chloroplastic *APX (chlAPX)* and microbody *APX (mAPX)* were expressed at constitutive level during normal and stressed condition for H_2O_2 detoxification (Yoshimura et al. 2000). The upregulation of antioxidant enzymes that are involved in the detoxification of ROS under salinity stress is correlated. An increase of APX and SOD at both transcriptional and translational level during salt stress has been also reported (Bueno et al. 1998). It has also been reported that the transgenic tobacco plants overexpressing Cu-ZnSOD show a three fold increase in the transcript

level of cytosolic APX as well (Sengupta et al. 1993). Increased tolerance to salt stress has been shown by over expressing cytosolic APX in tobacco plants (Torsethaugen et al. 1997). The above literature indicates that antioxidant enzymes are involved in removal of the excess ROS produced in stress and their overexpression in heterologous plant systems would enable the transgenic plants to survive in stress. Based on this literature survey an investigation on the effect of overexpression of *APX* gene cloned from salt tolerant cell line of *Arachis hypogaea* (Shrivastava 2007) in a model plant *Nicotiana tabacum* has been carried out.

MATERIALS & METHODS

3.1 Plant Material

The seeds of *Nicotiana tabacum* var. *Xanthium* were available in the laboratory.

3.2 Vectors

The pGEMT vector containing *Arachis hypogaea APX* cDNA cloned in the laboratory from salt tolerant cell lines was available. The basic vectors pRT101 and pCAMBIA 1301 (pC1301) were also available in the lab.

3.3 Chemicals and Reagents

The enzymes and chemicals used for DNA manipulations were purchased from New England Biolabs, Fermentas, and Promega. Megaprime DNA labeling system was from Amersham Biosciences. Positively charged nylon membrane was from Pall Life Sciences. Culture media components were obtained from HiMedia (India). Radioisotopes were purchased from Board of Radiation and Isotope Technology, JONAKI, Hyderabad. The oligonucleotides and other reagents were obtained from Sigma Chemical Corp. (USA).

3.4 Bacterial Strains and Culture Conditions

Escherichia coli strain $DH_5\alpha$ was used for the routine maintenance and propagation of plasmids. Cultures of DH5α were initiated from 200 µl aliquot of glycerol stock stored at -80°C and grown on 10ml of Luria Bertani (LB) liquid medium supplemented with ampicillin (100 mg/l) maintained in shaker at 37°C and 220 rpm in 10ml culture tube.

Agrobacterium tumefaciens strain GV 3101 was grown in Yeast Extract Broth (YEB) medium supplemented with kanamycin (50 mg/l), when required. The cultures were maintained at 28°C in culture flask (10ml) kept on a shaker (220 rpm).

3.5 Composition of Culture Media

Tobacco seeds were germinated in Murashige and Skoog (MS) medium (1962). This was supplemented with various hormones as and when needed. All the media were

prepared in double distilled water and were autoclaved at 15lb/in^2 pressure and 121 ^{0}C for 18 min.

3.5.1 Murashige and Skoog (MS) medium

Composition of MS medium

(A) Major stock (20x)

NH_4NO_3	16.5 gm
KNO_3	19.0 gm
$CaCl_2$	4.4 gm
$MgSO_4$	3.7 gm
KH_2PO_4	1.7 gm

Dissolved each component separately in distilled water and mixed. The final volume was made up to 500 ml with double distilled water. From this stock 50 ml/l was used in the culture medium.

(B) Minor stock (1000x)

KI	0.45 gm
H_3BO_3	3.10 gm
$MnSO_4.4H_2O$	8.45 gm
$ZnSO_4.7H_2O$	4.30 gm
$Na_2MoO_4.2H_2O$	0.125 gm
$CuSO_4.5H_2O$	0.0125 gm
$CoCl_2.6H_2O$	0.0125 gm

Dissolved each component in double distilled water and the final volume was made up to 500 ml with double distilled water. From this stock 1 ml/l was used in the culture medium.

(C) Fe.EDTA stock (200x)

Na_2EDTA	0.746 gm
$FeSO_4.7H_2O$	0.556 gm

Dissolved each chemical separately and mixed them with slight heating. Final volume was made up to 100 ml. From this stock 5 ml/l was used for the culture medium.

(D) Vitamin stock (1000x)

Nicotinic acid	0.05 gm
Pyridoxine	0.05 gm
Thiamine-HCl	0.01 gm
Glycine	0.02 gm

Dissolved each of the vitamins in double distilled water and made the volume to 100 ml. From this stock, 1 ml/l was used in the culture medium.

(E) Myo-inositol

100 mg of myoinositol was added in 1 litre of the medium.

(F) 6-benzyl amino purine (BAP)

A 0.1 mg/ml stock was made by dissolving 25 mg of BAP in minimum amount of 0.1N NaOH and making the final volume to 25 ml with distilled water. From this stock, 1 mg/l was used in the culture medium.

(G) Naphthalene acetic acid (NAA)

A 0.1 mg/ml stock was made by dissolving 25 mg of NAA in minimum amount of 0.1N NaOH and making the final volume to 25 ml with double distilled water. From this stock, 0.1 mg/l was used in the culture medium.

Yeast Extract Broth (YEB) Medium - for 100ml

Yeast extract	0.1 g/l
Beef extract	0.5 g/l
Peptone	0.5 g/l
Sucrose	0.5 g/l

$MgSO_4$ 0.048 g/ l

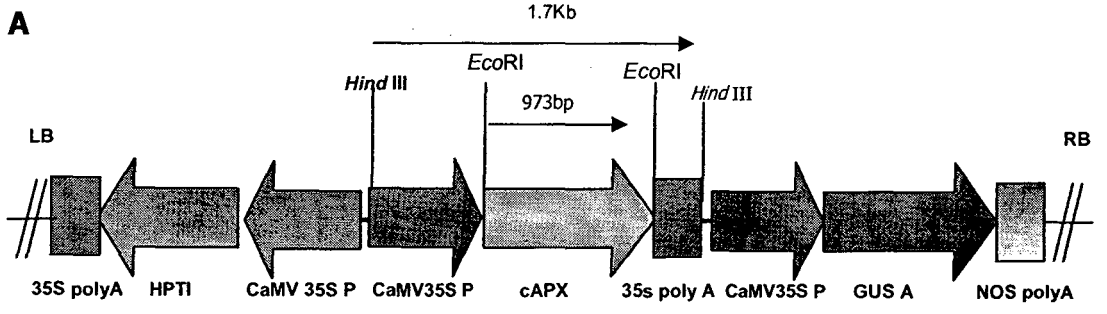
The above components were weighed as per the volume required and dissolved in double distilled water. The pH was adjusted to 7.0 and the final volume was made up to 100ml with double distilled water.

3.6 Construction of plant transformation vector- *pCAM-APX*

The cytosolic ascorbate peroxidase cDNA (EF165068) was isolated from salt tolerant cell lines of *Arachis hypogaea* (Shrivastava 2007). The cDNA cloned in pGEMT vector was available in the lab. The *Arachis hypogaea APX* cDNA cloned in pGEMT vector was released from this vector by digestion with restriction enzymes *Eco*RI and cloned at the *Eco*RI site in the pRT101 vector in between CaMV 35S promoter and polyA terminator sequences. The entire cassette having the CaMV 35S promoter, *APX* cDNA, and terminator was released from pRT101 vector by digestion with *Hind*III and cloned at the *Hind*III site in the multiple cloning site of pCAMBIA 1301 vector to give rise to *pCAM-APX*. The pCAMBIA 1301 has hygromycin as plant selection marker and kanamycin as bacterial selection marker. The presence of the cassette in the construct was confirmed by restriction digestion with *Hind*III as well as by PCR amplification of *APX* sequence by using the following primers:

Forward primer: 5'- AAGAACGTCAAGCCATG -3'

Reverse primer: 5'- AACTTCTTGCATTCATCACT -3'



Schematic diagram of the construct used

3.7 Transformation of *Agrobacterium tumefaciens*

Binary vector containing the c*APX* cDNA, referred to as pCAM-APX was transferred into *Agrobacterium* by the freeze-thaw method. For the preparation of competent cells, *A. tumefaciens* (GV 3101) was grown in 50 ml of YEB medium at 28°C with vigorous

shaking until the OD at 600 nm reached 0.5. The culture was chilled on ice and centrifuged at 3000xg for 10 min at 4°C. The pellet was resuspended in 50 ml of 20 mM $CaCl_2$, incubated on ice for 10 min and again pelleted down. This step was repeated with 25 ml of 50 mM $CaCl_2$. Finally, the pellet was resuspended in 10 ml of 50 mM $CaCl_2$, aliquoted in prechilled microcentrifuge tubes, and stored at -80°C. Transformation of *A. tumefaciens* was done by incubating the competent cells with 1 µg of DNA on ice for 30 min followed by freezing in liquid nitrogen. Heat shock was given at 37°C for 5 min followed by 2 min of incubation on ice. Thereafter, 1 ml YEB medium was added and the tubes were incubated at 28°C for 4-6 h with gentle shaking. Cells were plated on YEB agar plate supplemented with 50 mg/l kanamycin and incubated at 28°C. Colonies appeared after 2-3 days. The presence of the gene construct was confirmed by colony PCR and restriction digestion of the purified recombinant plasmid.

3.8 Transformation of tobacco (Horsch et al. 1985)

- Young leaves from i*n vitro* grown tobacco plants (20-25 days old) were taken and their mid-rib portion was removed.
- Leaf discs (1 cm^2) were cut out from the leaf with a scalpel and co-infected with the tertiary culture of *Agrobacterium tumefaciens* at a final O.D 0.3 at 600 nm for 20 min.
- The co-infected leaf discs were co-cultivated in MS medium supplemented with BAP (1 mg/l) and NAA (0.1 mg/l). This medium is referred to as $MSB_1N_{0.1}$.
- The leaf discs were kept in dark for two days after which the explants were washed several times with sterile distilled water containing cefotaxime (400 mg/l) and plated on $MSB_1N_{0.1}$ containing hygromycin (20 mg/l) and cefotaxime (400 mg/l).
- Single shoots from a cluster of shoots from regenerating leaf discs were separated and placed on MS basal medium containing hygromycin and cefotaxime, for rooting.
- The plantlets were subcultured at a regular interval of 15-20 days for 2-3 cycles.
- Agar was removed from the roots of the plantlets by washing in sterile distilled water. The plants were transferred to small pots (12 cm) containing autoclaved

agropeat and covered with plastic bags for 2-3 days after which the bags were removed and pots transferred to the glass house.

- The plants were watered with Hoagland solution while being maintained in pots.

3.9 Composition of Hoagland solution

		Stock conc.	Working conc.
Solution I -	KH_2PO_4	1M	1mM
Solution II -	$MgSO_4.7H_2O$	1M	2 mM
Solution III -	$Ca(NO_3)_2$	1M	5 mM
Solution IV -	Trace elements		
	H_3BO_3	0.05%	50 ppm
	$MnSO_4.7H_2O$	0.05%	50 ppm
	$ZnSO_4.7H_2O$	0.005%	5 ppm
	$CuSO_4$	0.002%	2 ppm
	$Na_2MOO_4.2H_2O$	0.002%	2 ppm
Solution V -	Fe citrate	0.5%	500 ppm

3.10 Molecular analysis

(A) DNA extraction (CTAB Method, Rogers et al. 1996)

Reagents:

2 X CTAB buffer (100 ml)

2 % CTAB (Cetyl trimethyl ammonium bromide) (w/v)

100 mM Tris, pH 8.0

20 mM EDTA, pH 8.0

1.4 M NaCl

200 µl β-mercaptoethanol

Chloroform: Isoamylalcohol(24:1)

1 M Tris (pH 8.0): 50 ml

Dissolved 6.05 gm of Tris base in 30 ml of water. The pH was adjusted to 8.0 with concentrated HCl and final volume was made up to 50 ml with double distilled water. The solution was autoclaved and kept for use.

5 M EDTA (pH 8.0) : 50 ml

Added 9.3 gm of EDTA (di-sodium).$2H_2O$ to 35 ml water. Dissolved 0.5 gm of NaOH in 1 ml of water, and cooled at room temperature. After cooling this was added to EDTA solution to get pH 8.0. EDTA could be dissolved in the solution only when pH was brought very close to 8.0. Final adjustment of pH was made when temperature of the solution reached room temperature. Final volume was then made up to 50 ml, autoclaved and stored at room temperature.

3.10.1 DNA Isolation

- 1 gram of plant material was weighed and frozen in liquid nitrogen.
- The frozen tissue was ground in presence of liquid nitrogen using mortar and pestle to a fine powder.
- CTAB buffer warmed at 65°C was added to the crushed powder in the ratio 1:5.
- Mixing was done by inversion and equal volume (700µl) of chloroform : isoamylalcohol (24 : 1) was added.
- Thorough mixing by inversion formed an emulsion. This was centrifuged for 15 min in a microfuge at 13,000 rpm. The aqueous phase was pipetted out with a cut yellow tip and transferred into fresh tubes.
- Again equal volume of chloroform:isoamyl alcohol was added and centrifuged at 13,000 rpm for 15 min. The aqueous phase was transferred to fresh tube.
- 0.6 volume of isopropanol was added to the aqueous phase and incubated at room temperature for 20 min.
- Centrifugation was done for 15 min at 10,000 rpm and the supernatant was discarded.
- The DNA pellet was washed using 70% ethanol and again centrifuged at 13,000 rpm for 5 min at 4°C
- The supernatant was discarded and the pellet was dried in a Speed vac. This pellet was dissolved in 50 µl of TE buffer.

3.10.2 Polymerase chain reaction (PCR)

The putative transformants were screened by the polymerase chain reaction (PCR) for the presence of the APX gene.

Reagents:

1. PCR buffer (with 15 mM $MgCl_2$) - 10 X
2. dNTPs (dGTP, dCTP, dTTP, dATP) - 10 mM each
3. *Taq* polymerase - 3 U / µl

4. Primers

   Forward primer – 5' AAGAACGTCAAGCCATG 3'

   Reverse primer – 5' AACTTCTTGCATTCATCACT 3'

Master Mix: (kept on ice)

Reagent	Stock	Final Concentration
PCR buffer (with 15 mM $MgCl_2$)	10 X	1 X (1.5 mM $MgCl_2$)
dNTPs (dGTP, dTTP, dCTP, dATP)	10 mM	120 µM

Primers

Forward primer	10 pmole/ µl	2.5 pmole/reaction
Reverse primer	10 pmole/ µl	2.5 pmole/reaction
Taq polymerase	3 U/ µl	1 U/ reaction

100 ng of plant DNA from transformed and untransformed control plants was added to the PCR master mix respectively. Final volume was made up to 25µl with double distilled water.

The amplification reaction was carried out using a thermal cycler under the following PCR conditions:

Step 1: Denaturation 94°C: 5 min

Step 2: Denaturation 94°C: 30 sec

Step 3: Annealing 53°C: 30 sec

Step 4: Extension 72°C: 1 min

Step 5: Final extension 72° C: 10 min

Hold at 4°C: store the sample for further use.

The steps 2, 3, and 4 were repeated 35 times (35 cycle).

The amplified products thus obtained were separated by electrophoresis on a 0.8 % agarose gel in 1X TAE buffer and detected under UV fluorescence with ethidium bromide.

3.10.3 Southern blotting

Genomic DNA was digested overnight with *Eco*RI, at 37°C temperature overnight to confirm the presence of the insert. The digested DNA was electrophoresed on a 0.8% agarose gel and visualized by ethidium bromide staining. The gel was pictured using the gel documentation system. The DNA from the gel was transferred to a nylon membrane 0.45μm (Pall Life Science) using the capillary transfer method as described in Sambrook et al. (1989). Briefly, the gel was treated with depurination solution (0.2 N HCl), denaturation solution (1.5 M NaCl, 0.5 N NaOH), and neutralizing solution (1 M Tris-HCl, pH 7.4, 1.5 M NaCl) for 20 min, 45 min, and 45 min respectively. The DNA was transferred to the nylon membrane by capillary transfer in 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) overnight and cross-linked by UV irradiation at 254 nm for 2 min. The membrane was pre-hybridized for 2 h at 65°C in a buffer containing 0.5 M sodium phosphate buffer, pH 7.2, 1 mM EDTA, and 7% SDS. Thereafter, denatured radiolabeled probe synthesised from *APX* cDNA was added to the pre-hybridization solution. After incubation for 16-18 h at 62°C, the membrane was washed sequentially in 3X SSC, 0.1% SDS; 0.5X SSC, 0.1% SDS; 0.2X SSC; 0.1% SDS. The membrane was then air dried and placed in phosphoimager (IP) cassette for exposure.

3.11 Physiological Analysis

3.11.1 Leaf disc senescence assay

Stress tolerance tests were performed by exposing equal size leaf discs isolated from control and transgenic plants to sodium chloride (200mM, 400mM & 600mM) and mannitol (200mM, 400mM & 600mM). Sodium chloride was used to test salinity

tolerance as Na^+ and Cl^- ions mostly contributes to salinity stress.Mannitol was used for drought stress as it is an important osmolyte. The leaf discs floated on water were taken as control. The leaf discs were floated for three days (72 hours). The leaf disc assay was done as described by Fan et al (1997). The treatment was carried out under continuous white light at 25 ± 27^0C. The effects of these treatments were assessed by observing phenotypic changes or comparative bleaching in transgenic *vs.* the control.

3.11.2 Chlorophyll estimation

Chlorophyll content in the leaves of transgenic and control plants was estimated according to the procedure of Arnon (1949). The leaves were homogenized thoroughly in 5 ml of 80% acetone and centrifuged at 3,500 rpm for 10 minutes. The supernatant was retained and absorbance was recorded at 663 and 645 nm. The chlorophyll amount was calculated using the formula:

$$\text{Chl (a+b)} = (8.02 \times A_{663} + 20.2 \times A_{645}) \, V/W$$

Where V is the volume of extract in ml and

W is the weight of leaf tissue in gram

Chlorophyll content was expressed in µg/g fresh weight.

RESULTS

4.1 Sub-cloning of *APX* cDNA in Binary Vector pCAMBIA1301.

The cytosolic ascorbate peroxidase cDNA isolated from *Arachis hypogaea*, having accession number EF165608, cloned in pGEMT vector was available in the lab. The cDNA was subcloned in the vector pRT101, where the gene was driven by cauliflower mosaic virus promoter (CaMV 35S) and *poly* A terminator sequences at the ends. The CaMV 35S is a very strong constitutive promoter well proven for driving the expression of transgenes to very high level in different transgenic plant systems. The cytosolic *APX* cDNA was released from pGEMT vector by digestion with *Eco*RI. The digested product was separated in 0.8% agarose gel by electrophoresis. A fragment (~ 1.0 kb) representing *APX* cDNA and a backbone representing the vector (~ 3.0 kb) were observed in gel (Fig. 4). The vector pRT101 having CaMV 35S promoter was also digested with *Eco*RI to get compatible ends. The *APX* cDNA was then ligated to pRT101 at *Eco*RI site between CaMV 35S promoter and terminator sequences. The orientation of the *APX* cDNA was checked by digesting the pRT101 vector with *Bam*HI. An expected band of 650 bp was observed representing the correct orientation with the promoter (Fig. 5). This promoter-transgene-terminator expression cassette was then excised out from the PRT101 vector by single digestion using *Hind*III restriction endonuclease and was ligated at the *Hind*III site in the multiple cloning region of the binary vector pCAMBIA1301. Colony PCR was performed using gene specific primers to select the colonies transformed with pCAMBIA1301-*APX* cassette. Colonies 1, 2 and 3 in lane 1, 2 and 3 respectively gave an amplicon of 973 bp (Fig. 6). The resultant construct was subjected to restriction digestion with *Eco*RI and *Hind*III. Expected fall out of 973 bp (for *APX* cDNA) on digestion with *Eco*RI and ~1.7 kb (promoter-transgene-terminator cassette) on digestion with *Hind*III were observed respectively (Fig. 7).

4.2 Mobilization of the Gene Construct *pCAM-APX* in *Agrobacterium*.

The construct *pCAM-APX* (Fig. 8) was mobilized in *Agrobacterium tumefaciens* strain GV3101. The transformed colonies of *Agrobacterium* having the *APX* gene were selected on YEB medium containing kanamycin (50 mg/l). The presence of the gene was checked by PCR amplification using gene specific primers. An expected amplicon of ~1.0 kb was observed in lanes 1, 2,3,4,5 and 6 corresponding to the colonies 19,20,21,22,23 and 24 respectively (Fig. 9).

4.3 Transformation of Tobacco with the Gene Construct *pCAM-APX*.

The *Agrobacterium tumefaciens* harbouring the gene *cAPX* was used for transformation of the model plant tobacco var. Xanthium using leaf disc method (Horsch et al. 1985). The transformed leaf discs (Fig. 10a) were selected on MS $B_1N_{0.1}$ medium containing hygromycin (20 mg/l) as the plant selection marker and cefotaxime (400 mg/l). The transformed leaf discs selected on a plate with (20 mg/l) hygromycin and (400 mg/l) cefotaxime regenerated slowly as compared to the untransformed control on $MSB_1N_{0.1}$ without any selection (Fig. 10b). The untransformed control leaf discs grown on selection medium with hygromycin (20 mg/l) were unable to survive and died (Fig. 10c).

4.4 Recovery of Putative Transgenic Plantlets

Around 50-60 single shoots regenerating from each of the transformed leaf discs were transferred to MS basal medium containing hygromycin (20 mg/l) and cefotaxime (400mg/l) in sterile culture bottles for rooting. Total 30 shoots recovered and developed to plantlets. Rooting was observed after 15-20 days of transfer (Fig. 11).

4.5 Hardening of Plantlets

The plantlets were taken out of the culture bottles and roots washed with sterile water. Total 20 plantlets were transferred to plastic sterile pots containing autoclaved agropeat and covered with plastic bags for three days for hardening. The plants were kept in growth room maintained at 25-26°C and 8 hr light/16 hr dark photoperiod (Fig. 12). After two weeks, these plants were transferred to soil in earthen pots which were maintained in the glass house (Fig. 13).

4.6 Molecular Analysis

4.6.1 Transgene amplification using gene specific primers

Genomic DNA was isolated from the leaves of putative transgenic tobacco plants growing on selection medium and untransformed control plants growing without selection. The PCR amplification of the genomic DNA was carried out with *APX* gene specific primers. An expected band of 973 bp corresponding to the *APX* gene (in lane marked +ve) was obtained in the case of putative transgenic plantlets shown in lane 1 to 10 while it was absent in the case of untransformed control plantlets in lane marked (UC) (Fig. 14). Total ten independent putative T0 transgenic plants were PCR positive.

4.6.2 Southern blot analysis of the T0 transgenic plants of *N.tabacum*

The stable integration of *APX gene* cassette in the genome of the PCR positive T0 transgenic plants of *N. tabacum* was confirmed by Southern blot analysis. The genomic DNA of the transgenic and untransformed control plants was completely digested with *Eco*RI to release the *APX* cDNA. This was electrophoresed and blotted on the nylon membrane and probed with *Arachis hypogaea APX* cDNA probe. A 973 bp band (size of the *APX* cDNA) lighted up in the autoradiogram in the lanes marked 1, 2 and 3 of the transgenic lines analyzed, while a similar band was absent from the untransformed control (UC) lane as well as in lanes 4, 5, and 6, thus three transgenic lines showed stable integration of the *APX* gene (Fig. 15). These lines were further used for physiological analysis.

4.7 Physiological Analysis

4.7.1 Leaf disc senescence assay

Leaf discs of equal diameter from fully grown mature leaves of transgenic plants and untransformed control plants were floated on different concentrations (200 mM, 400 mM and 600 mM) of, NaCl and mannitol (200 mM, 400 mM and 600 mM) respectively. Leaf discs floated on distilled water served as the experimental control. Leaf discs from transgenic plants showed delayed senescence compared to leaf discs from untransformed control plants after 72 hours in 400 mM and 600 mM NaCl (Fig. 16a) and mannitol (Fig. 17a). Significant difference in the chlorophyll content in the transgenic *vs.* the untransformed control plants was observed after 72 hours. The chlorophyll content in the control was less on 200 mM, 400 mM and 600 mM concentration of NaCl as compared to transgenic lines (Fig. 16 b). At 600 mM NaCl there was 92% reduction in chlorophyll content in untransformed control as compared to transgenic lines which showed 69% reduction. Similarly the chlorophyll content of the transgenic lines on 200 mM, 400 mM and 600 mM mannitol was more as compared to the control plants (Fig. 17 b) and at 600 mM there was 91% reduction in chlorophyll content in untransformed control as compared to transgenic lines which showed only 66% reduction.

DISCUSSION

The effect of overexpression of cytosolic ascorbate peroxidase (APX) isolated from salt tolerant cell lines of *Arachis hypogaea* in the model plant tobacco has been investigated. Ascorbate peroxidase is a unique H_2O_2 scavenging enzyme that converts H_2O_2 to water (H_2O) using ascorbate as electron donor in cells. In higher plants distinct isoenzymes of APX are localized in chloroplasts, mitochondria, microbodies (glyoxisomes and peroxisomes) and cytosol (Madhusudan et al. 2003). The APX found in organelles scavenges H_2O_2 produced within the organelles where as cytosolic APX eliminates H_2O_2 produced in the cytosol, apoplast or that diffused from organelles to the cytosol (Mittler et al. 1992). The APX enzyme protects cells against H_2O_2 under normal as well as stressful conditions. Increased activity of APX in response to environmental stresses has been reported in different plant species which suggests its possible role in eliminating H_2O_2 from cells under stress (Davis et al. 2001; Bueno et al. 2002). Though the organelle forms of APX are sufficient to scavenge H_2O_2, cytosolic APX has its own importance. It has been reported that in the absence of cytosolic form of APX the whole chloroplastic APX collapses (Davletova et al. 2004). Therefore, in the present study the stress alleviation by overexpression of the cytosolic ascorbate peroxidase was studied in tobacco.

5.1 Overexpression of APX *APX* Gene in Tobacco

The *APX* gene was sub-cloned in plant expression vector pCAMBIA1301 driven by a strong constitutive CaMV35S promoter as it is reported to result in high expression of the gene in almost any type of cell and tissue of the plant at any developmental stage (Fang et al. 1989). Tobacco leaf discs were transformed by *Agrobacterium* mediated transformation which is considered to be the best method for transferring any transgene to plants. The transgenics developed during this study appeared normal and did not show any morphological differences when compared with the untransformed control plants. This is in conformation with the results reported by Young et al (2007) where overexpression of three antioxidant enzymes, copper zinc superoxide dismutase (CuZnSOD), ascorbate peroxidase (APX, EC), and dehydroascorbate (DHA) reductase (DHAR, EC), in the chloroplasts of tobacco plants leads to transgenic plants which were normal in morphology.

5.2 Molecular Analysis

Out of the 20 regenerated plants screened by PCR 10 were found to be PCR positive and gave an amplification of 973 bp with gene specific primers. These were considered as putative transformants as the PCR does not give a confirmation regarding the stable transgene integration and may give misleading result due to *Agrobacterium* contamination. Therefore southern blot analysis was performed which confirmed the stable integration of APX gene.

5.3 Relationship between APX Overexpression and Tolerance to Salinity and Drought Stress

Sodium chloride is known to be the major salt causing salinity stress in soil. NaCl indirectly imposes osmotic stress leading to generation of reactive oxygen species (Halliwell and Gutteridge et al. 1985). This increased tolerance of leaf discs from transgenic plants *vs.* the untransformed control plants observed in this study, to salt stress could be attributed to the overexpression of APX in transgenic plants which helps in scavenging the ROS generated and protect chlorophyll from damage. This result is in agreement with the earlier report where it has been shown that over-expression of cytosolic ascorbate peroxidase (*cAPX*) from *Arabidopsis thaliana* in tobacco chloroplasts enhances tolerance to salt stress (Badawi et al. 2003).

Mannitol being an osmoprotectant when applied extracellularly reduces water potential outside the cell. This situation mimics drought condition and leads to osmotic stress (Zhu et al. 2002). The leaf discs of transgenic plants showed delayed senescence and higher chlorophyll content on exposure to 600 mM mannitol as compared to the leaf discs from untransformed control plants which were bleached. It is possible that the the APX, which is involved in scavenging the excess reactive oxygen species (H_2O_2) produced under osmotic stress conditions may lower the ROS mediated lipid peroxidation and membrane damage, thus protecting the leaf discs from bleaching. This is in accordance with earlier report that transgenic plants over-expressing antioxidant genes such as APX in chloroplasts exhibit less chlorophyll degradation (Gupta et al. 1993; Allen et al. 1997; Kwon et al. 2002; Murgia et al. 2004).

Transgenic plants transformed with various genes have been reported to tolerate different abiotic stresses. Ascorbate Peroxidase (*AtAPX)* gene from *Arabidopsis* has been expressed in tobacco for protection against oxidative stress (Jing Wang et al. 1999). Whereas transgenic tomato (*Lycopersicon esculentum*) over-expressing *cAPX* from

Pisum sativum exhibited enhanced tolerance to UV-B and heat stress (Wang et al. 2006). Transgenic sweet potato plants expressing both copper zinc superoxide dismutase (CuZnSOD) and APX in chloroplasts showed enhanced tolerance to methyl viologen-mediated oxidative stress and chilling (Lim et al. 2007). Increased tolerance to reactive oxygen species in tobacco transformed simultaneously with *SOD, APX* and *DHA* in the chloroplasts has also been observed (Lee et al. 2007). Peroxisomal APX gene (PpAPX) cloned from *Populus tomentosa* has been shown to be involved in scavenging toxic oxygen species (Lu et al. 2009).

Therefore, antioxidant enzymes appear to be critical components in oxidative stress defence mechanisms and APX enzyme might be involved in the detoxification of photoproduced hydrogen peroxide. The function of all the forms of APX thus seems to be to scavenge the H_2O_2 that is continuously generated in cells. The activity of APX has been shown to increase in response to a number of stress conditions (Storozhenko et al. 1998). Therefore, it is proposed that plant stress tolerance can be improved by the enhancement of *in vivo* levels of antioxidant enzymes (Foyer et al. 1994; Allen 1995). The overexpression cytosolic ascorbate peroxidase isolated from *Arachis hypogaea* has been shown to confer Mannitol and NaCl stress tolerance thus mimicking drought stress tolerance. It is envisaged that it might also be involved in the scavenging of reactive oxygen species relieving the plants from oxidative stress.

Further study is necessary to investigate this hypothesis.

SUMMARY & CONCLUSION

The environmental changes and plant pathogens cause stress on plants and limit their growth, development and yield. All these changes affect the physiology and metabolic activity of the plants. One of the common phenomena is the production of reactive oxygen species (ROS) under various stresses (Narusaka 2004). Under normal environmental conditions the reactive oxygen species helps plants in sensing stress and initiates signal transduction pathway to resist stress. However, when ROS production is uncontrolled, as in the case of abiotic or biotic stress, it has deleterious effects and leads to damage of cellular membrane and cellular components, resulting in oxidative stress and cell death (Dat et al. 2000; Mittler et al. 2002).

One of the important enzymes that converts H_2O_2 to water and relieves plant from oxidative stress is APX. Therefore, it was envisaged that by overexpressing APX in plants, it might be possible to develop stress tolerant plants. Based on this the objective of the present investigation was to sub-clone cytosolic ascorbate peroxidase (*cAPX*) isolated from *Arachis hypogaea* in a plant expression vector under strong a constitutive promoter and overexpress it in a model plant tobacco through *Agrobacterium tumefaciens* mediated transformation method in order to study its effect under abiotic stress.

The highlights of the investigation are as follows:

- The cytosolic *APX* cDNA from *Arachis hypogaea* was successfully sub-cloned under the control of a strong constitutive CaMV 35S promoter in a binary vector, pCAMBIA 1301. The vector had *hygromycin phosphotransferase II* (*hptII*) as plant selectable marker gene, and *neomycin phosphotransferase II* (*nptII)* as the bacterial selection gene. The construct was named as *pCAM-APX.*
- *Nicotiana tabacum var. Xanthium* was transformed using *A. tumefaciens* strain GV3101 harbouring *pCAM-APX.*
- The T0 transformed plants were characterized at the molecular level by PCR using gene specific primers, and Southern blot analysis. Total out of 20 putative transgenic plants 10 were PCR positive. Three of the plants were found to be Southern positive with gene specific probe and showed stable integration of the transgene.
- The transgenic plants overexpressing the cytosolic-*APX* gene had similar morphology and growth pattern as compared to the untransformed control plants.

- Retardation of senescence of leaf discs and retention of chlorophyll content by transformed *vs.* the untransformed control plants was recorded. The transformed plants were less bleached as compared to the untransformed control plants which were completely bleached at 600 mM of NaCl and mannitol. The leaf discs of transformed plants were also able to retain more chlorophyll at 600 mM of NaCl and mannitol as compared to untransformed control plants.

Conclusion

The cytosolic form of APX is an important enzyme involved in reactive oxygen species scavenging and is involved in eliminating ROS generated during stress. Overexpression of cytosolic APX in heterologous systems can alleviate ROS mediated damages and help in developing stress tolerant transgenic plants. In this investigation the cytosolic form of APX isolated from *Arachis hypogaea* when overexpressed in tobacco lead to salinity and drought stress tolerance.

Future Prospective

To study the effect of overexpression of cAPX in alleviating other abiotic and biotic stress. The T_1 transgenic plants will be grown in pots maintained in green house. They will be tested for tolerance to various stresses including biotic stress. This study will further strengthen our investigation on the effect of overexpression of cAPX in alleviating stress responses.

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ILLUSTRATIONS

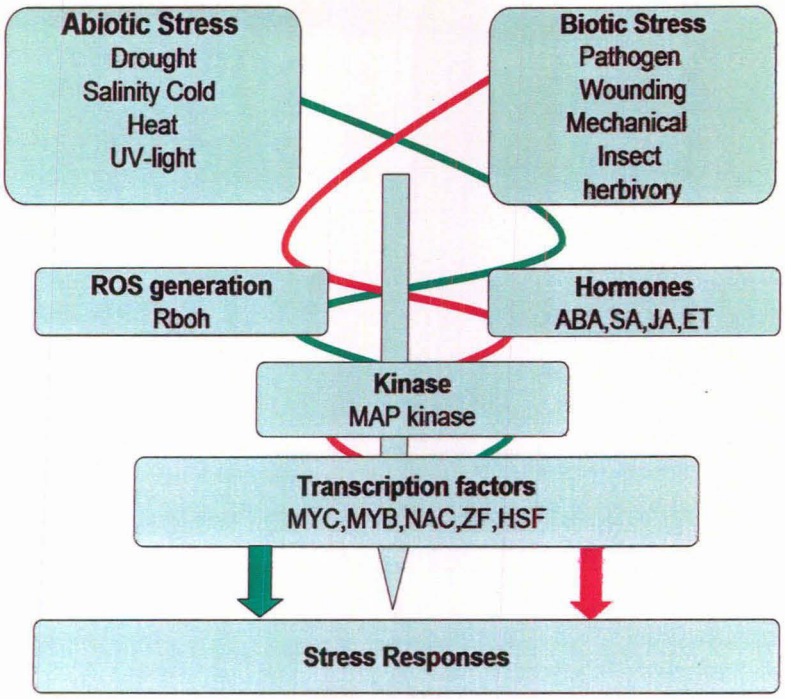


Fig 1: Molecular convergence points between biotic and abiotic stress signalling pathways (Adapted from Fujita et al. 2006)

(Abbreviations: Rboh, respiratory burst oxidase; ABA, abscisic acid; SA, salicylic; JA Jasmonic acid, ET, ethylene).

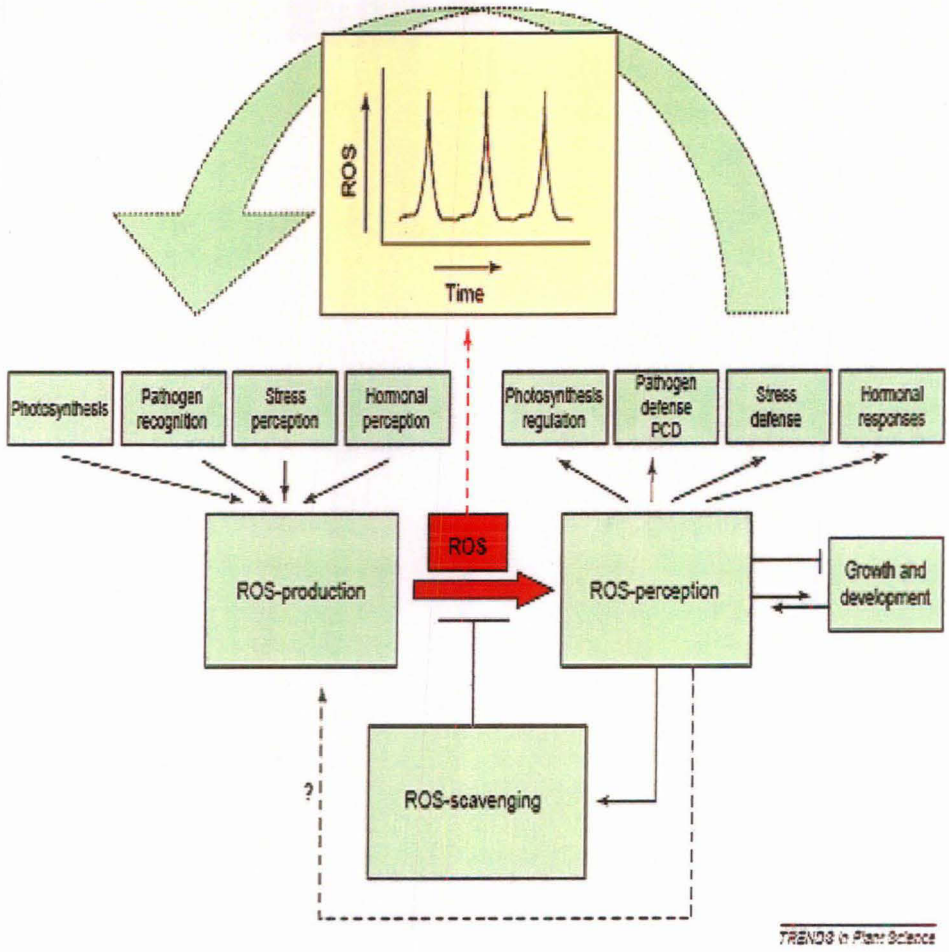


Fig 2: Modulation of reactive oxygen species (ROS) signalling by the reactive oxygen gene network of plants. Different cellular signals (e.g. pathogen recognition or stress perception) result in the enhanced production of ROS in cells by the ROS-producing pathways of the network. ROS are perceived by different ROS sensors and activate cellular responses (e.g. pathogen or stress defence). The intensity, duration and localization of the ROS signals are determined by interplay between the ROS-producing and the ROS-scavenging pathways. The ROS-scavenging pathways are also responsible for maintaining a low steady-state level of ROS on which the different signals can be regulated. Modulation of ROS levels might also involve a positive feedback loop between ROS perception and ROS production (dashed line). (Adapted from Mittler et al. 2004).

(Abbreviations: PCD, programmed cell death)

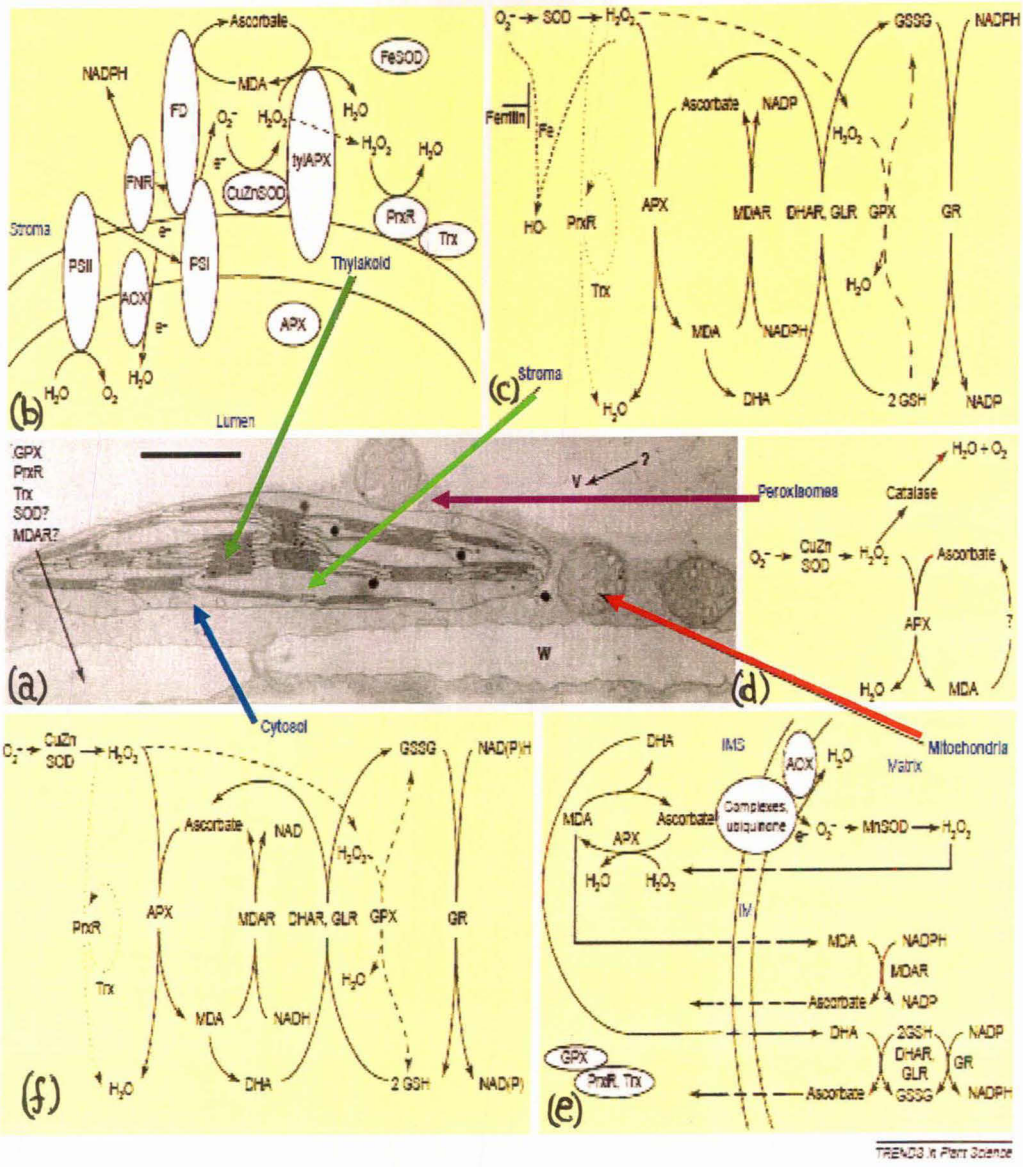


Fig 3: Localization of reactive oxygen species (ROS) scavenging pathways in plant cells. A transmission electron micrograph of a portion of a plant cell is used to demonstrate the relative distribution of ROS scavenging enzymes in different cellular compartments and their physical separation (middle left). (Modified from Mittler et al. 2004).

(Abbreviations: FNR, ferridoxin; GSSG, oxidized glutathione; DHAR, dehydroascorbate reductase; GLR, glutathione redoxin; GPX, glutathione peroxidase; MDA, monodehydroascorbate; DHA, dehydroascorbate; PrxR, peroxiredoxin; Trx, thioredoxin; MDAR, monodehydroascorbate reductase; AOX, alternative oxidase; IM, inner membrane; IMS, inner membrane space; V, vacuole; W, cell wall).

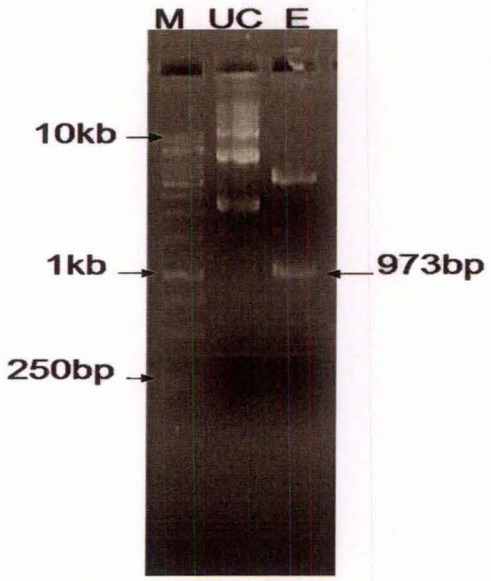


Fig 4: Restriction digestion of pGEMT vector to release *APX* cDNA. The pGEMT was digested with *Eco*RI, lane marked (E) to release *APX* cDNA of size ~1.0 kb. M, 1.0 kb DNA ladder and UC, is uncut pGEMT.

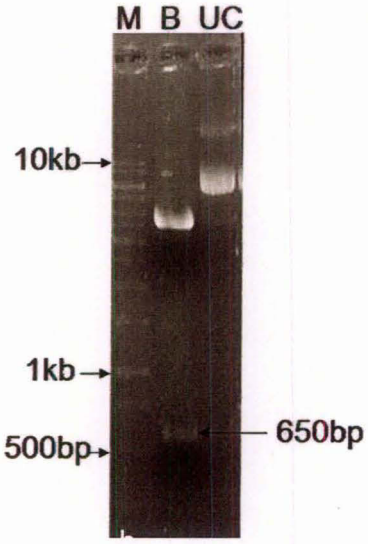


Fig 5: Restriction digestion of pRT101 with *Bam*HI. *APX* cDNA cloned in pRT101 was digested with *Bam*HI to check the correct orientation of *APX* cDNA with the promoter. A band ~650 bp (with correct orientation) was observed in lane marked (B).UC, uncut plasmid.

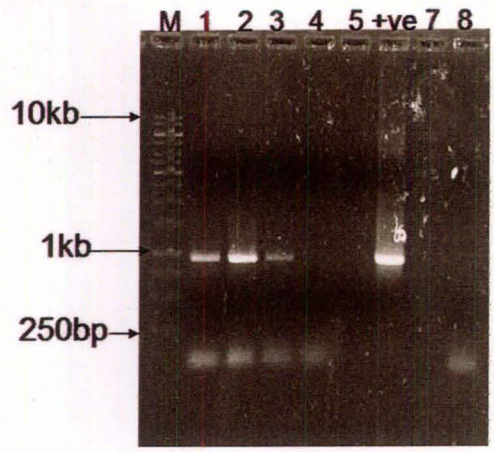


Fig 6: Colony PCR of *E.coli* transformed with pCAM-*APX* construct. Competent cells of *E. coli* were transformed with pCAMBIA1301 having CaMV 35S-*APX* cDNA-35S poly A construct. Colony PCR was done to select positive colonies. An amplicon of ~ 1.0 kb in lane 1, 2 and 3 corresponding to colonies 1, 2 and 3 was obtained respectively. +ve represents the positive colony. Lane 4, 5, 7 and 8 did not show any positive signal.

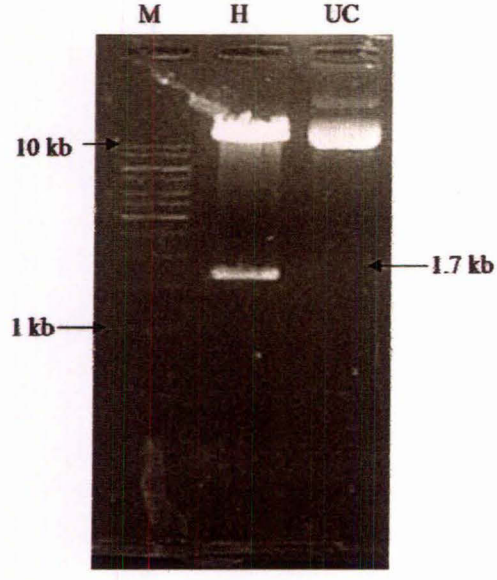


Fig 7: Restriction digestion of pCAMBIA1301 with *Hind*III. The CaMV 35S-*APX* cDNA-35S poly A fragment cloned in pCAMBIA1301 plasmid was digested with *Hind*III. A fallout of ~1.7 kb corresponding to CaMV 35S-*APX* cDNA-35S poly A fragment was observed in lane marked H. The uncut plasmid is represented by UC.

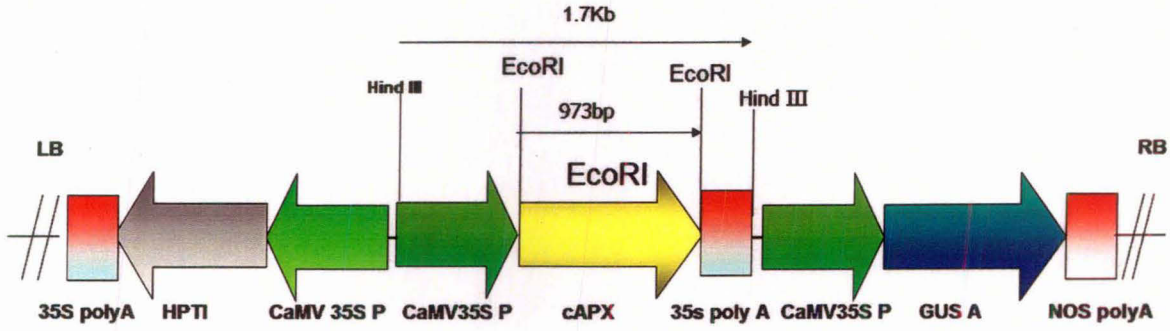


Fig 8: Schematic diagram of the construct having *cAPX* gene in pCAMBIA1301. T-DNA region of the *pCAM-APX* plasmid used for *Nicotiana tabacum* transformation is depicted as 1.7 kb fragment. The CaMV35S-pCAM-*APX*-35S poly A cassette was cloned at the *Hind*III site in the multiple cloning region of pCAMBIA1301.This recombinant plasmid was mobilised in *Agrobacterium tumefaciens* strain GV3101 which was subsequently used for transformation of *N.tabacum.*

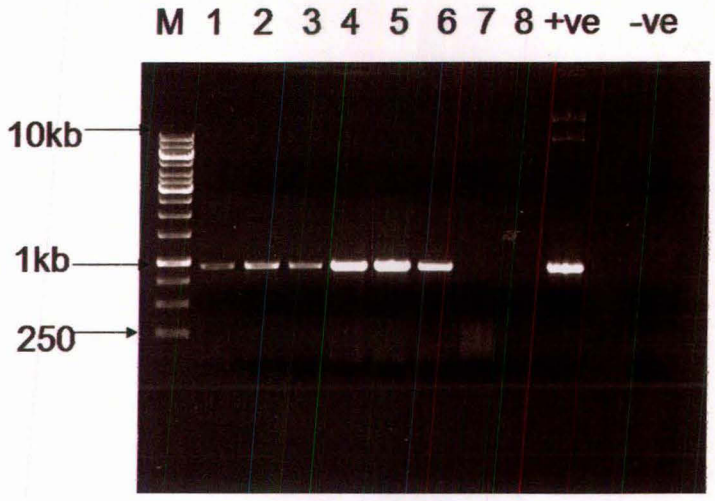


Fig 9: PCR analysis of the transformed *Agrobacterium* colonies mobilized with pCAM-*APX*. The plasmid was isolated from colonies of *A. tumefaciens* mobilized with *pCAM-APX* and subjected to PCR amplification using gene specific primers. A band ~1.0 kb was observed in lanes 1, 2,3,4,5 and 6 corresponding to the colonies 19, 20, 21, 22, 23 and 24 respectively. Lane 7 and 8 corresponding to the colonies 24 and 25 respectively gave no amplicon. Lane +ve represents the positive control (pGEMT + *APX*). Lane -ve shows the negative control.

Fig 10: **Leaf explants of *Nicotiana tabacum* var. *Xanthium* on different media**. (a) Leaf explants transformed with *Agrobacterium* containing the construct pCAM-*APX* were selected on $MSB_1N_{0.1}$ medium supplemented with 20 mg/l hygromycin. (b) Untransformed controls without any selection showing large number of regenerating shoots. (c) Untransformed explants in $MSB_1N_{0.1}$ medium supplemented with 20 mg/l hygromycin show yellowing.

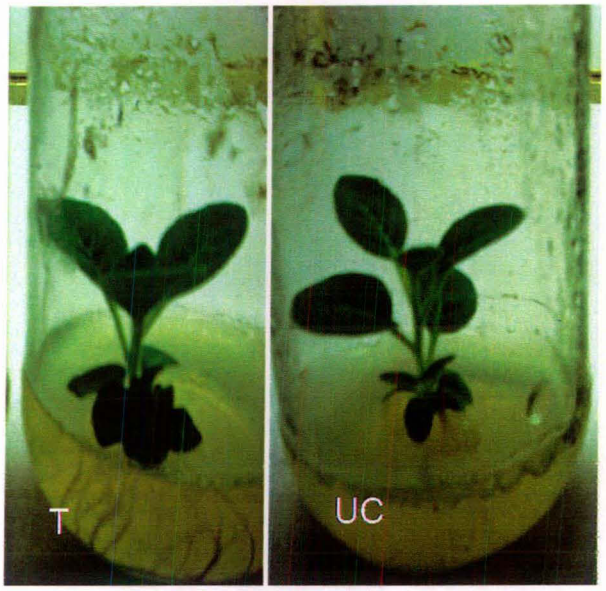


Fig 11: Putative transgenic and untransformed control plantlet growing in rooting medium.

T putative transgenic plantlet growing in MS medium with hygromycin (20 mg/l).
UC untransformed control plantlet growing in MS medium without hygromycin.

Fig 12: Putative transgenic and untransformed control plants in hardening condition.

T putative transgenic plant in agropeat covered with polybag.
UC untransformed control plants in agropeat covered with polybag.

Fig 13: Hardened plants growing in green house.
C untransformed control plants.
T_0 transgenic plant (line 1, L_1)
T_0 transgenic plant (line 2, L_2)

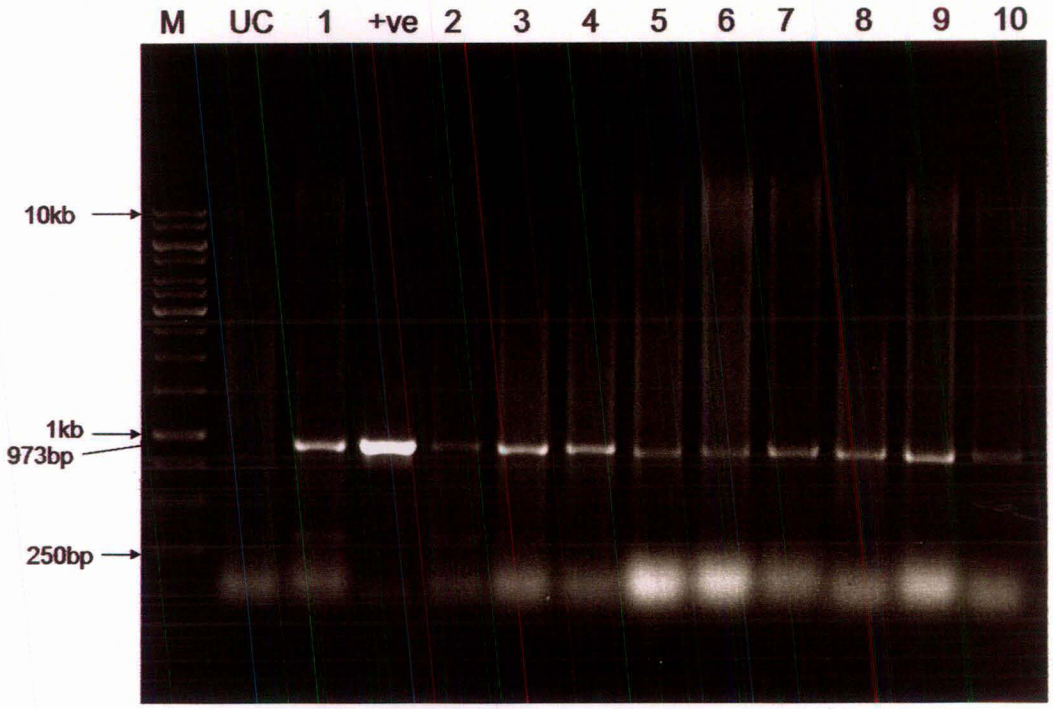


Fig 14: PCR analysis of T_0 transgenic *Nicotiana tabacum* plantlets. Genomic DNA from the T_0 transgenic and untransformed control plantlets was PCR amplified using the c*APX* gene specific primers. A band of 973 bp was amplified in all the putative transgenic plants (lane 1 to 10) while the band was absent in untransformed control plant in lane marked UC. M, 1.0 kb marker; +ve, positive control.

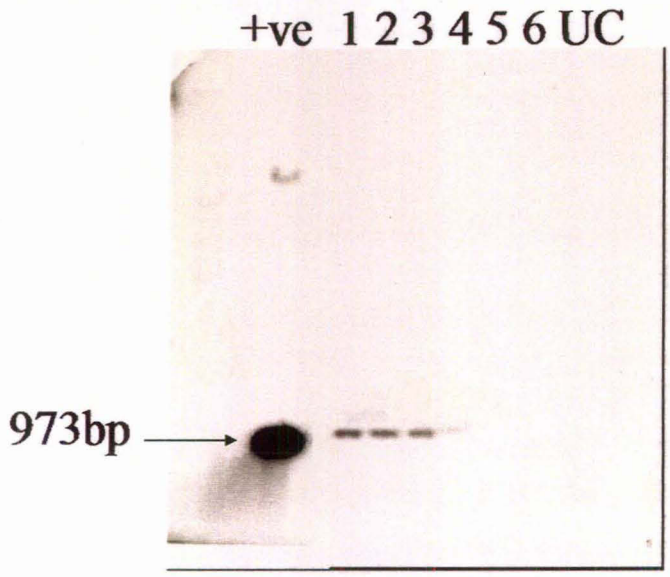
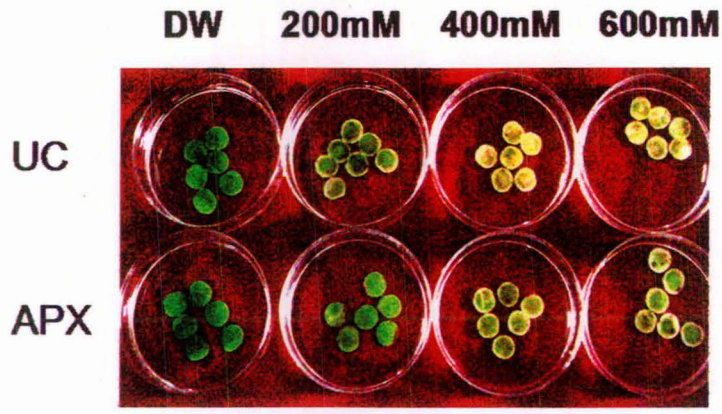
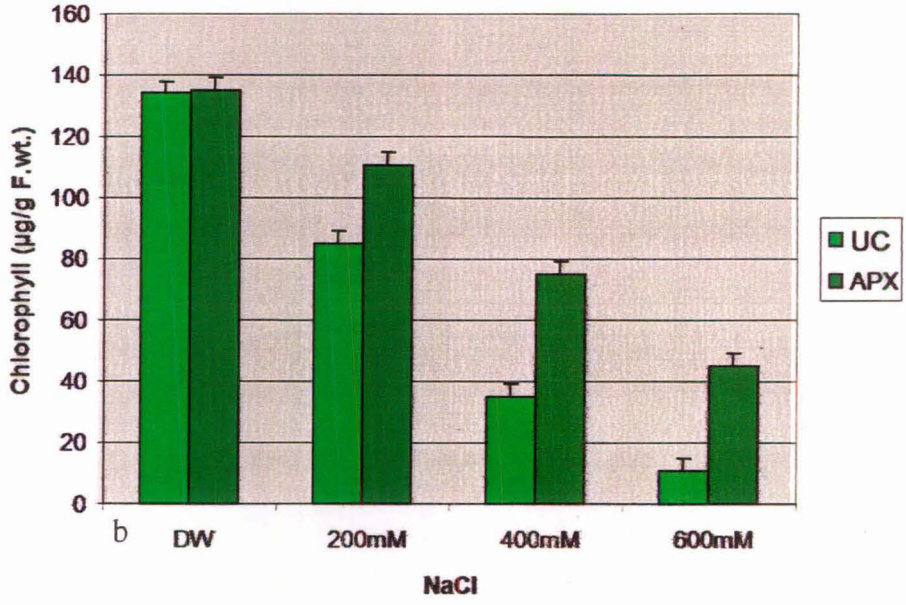


Fig 15: Southern blot analysis of T_0 transgenic *N.tabacum* plants using *APX* cDNA as probe. About 10 µg of the genomic DNA was digested with *Eco*RI, electrophoresed on a 0.8% agarose gel and blotted on to a nylon membrane. The blot was probed with radiolabled *APX* cDNA. M marker, + positive, lane 1, 2 and 3 transgenic lines showing a band ~1.0 kb, UC untransformed control without any band.



a



b

Fig 16: Retardation of salinity induced senescence of leaf discs in transgenic *N. tabacum.*

a) Leaf discs of untransformed control (UC) and transgenic line (APX) floated on 200 mM, 400 mM, and 600 mM NaCl solution. Distilled water served as the experimental control.

b) Chlorophyll content of the leaf discs estimated on the third day of floating.

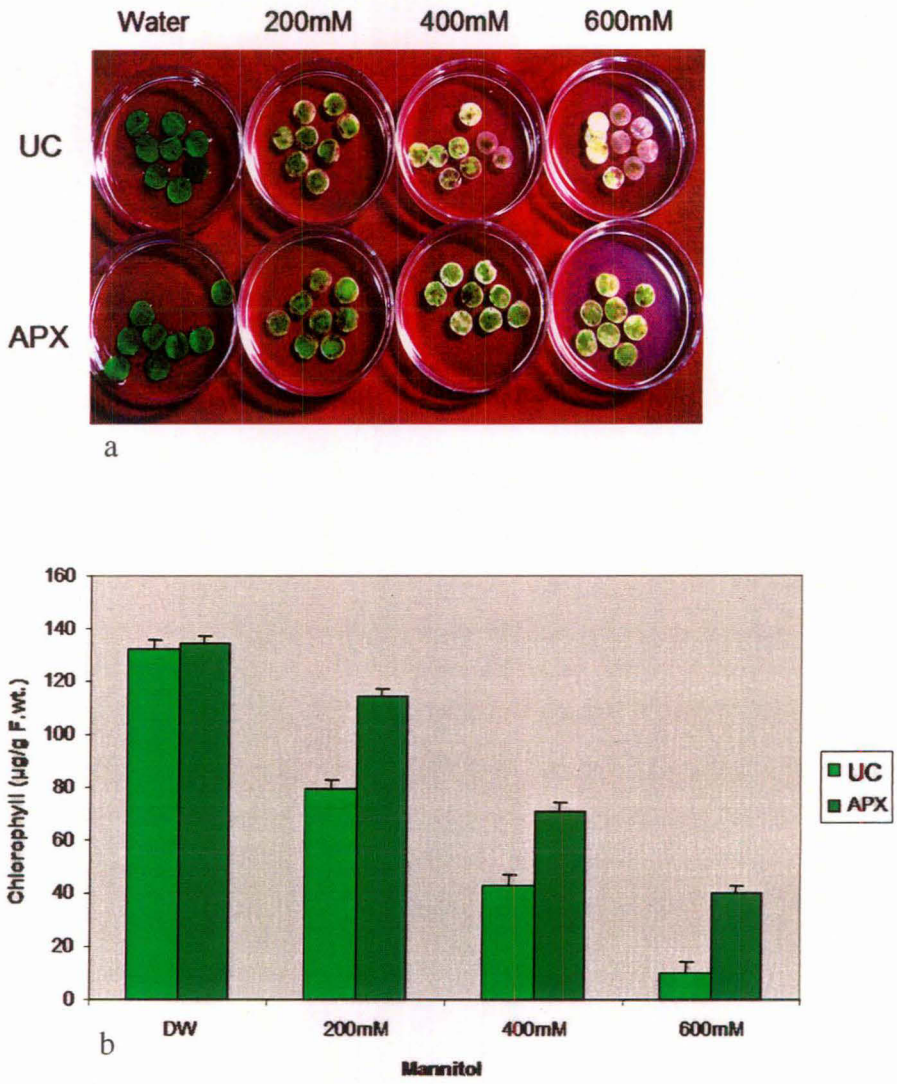


Fig 17: Retardation of drought induced senescence of leaf discs of transgenic *N. tabacum*.

a) Leaf discs of untransformed control (UC) and transgenic line (*APX*) were floated in 200 mM, 400 mM, and 600 mM mannitol for 3 days. Distilled water served as the experimental control.

b) Chlorophyll content of the leaf discs estimated on the third day of floating.