Chapter 2

Review of Literature
Chapter 2 Review of Literature

Fundamentally, plants require energy (light), water, carbon and mineral nutrients for growth. Abiotic stress is defined any of these environmental conditions that reduce growth and yield below or beyond optimum levels. One of the earliest metabolic responses to abiotic stresses is the inhibition of growth and protein synthesis (Good and Zaplachinski, 1994; Vincent et al., 2007; Liu et al., 2010) and an increase in protein folding and processing. Energy metabolism is affected as the stress becomes more severe (e.g. sugars, lipids and photosynthesis) (Cramer et al., 2007; Kilian et al., 2007). Thus, there are gradual and complex changes in metabolism in response to stress. The plant molecular responses to abiotic stresses involve interactions and crosstalk with many molecular pathways (Takahashi et al., 2004). Systems biology approaches have been used to elucidate some of the key regulatory pathways in plant responses to abiotic stress.

One of the earliest signals in many abiotic stresses involve ROS and reactive nitrogen species (RNS), which modify enzyme activity and gene regulation (Molassiotis and Fotopoulos 2011; Mittler et al., 2011). ROS signalling in response to abiotic stresses and its interactions with hormones has been thoroughly reviewed recently (Mittler et al., 2011). ROS and RNS form a coordinated network that regulates many plant responses to the environment; there are a large number of studies on the oxidative effects of ROS on plant responses to abiotic stress, but only a few studies have documented the nitrosamine effects of RNS (Molassiotis and Fotopoulos, 2011).

Phytotoxic amounts of metals are occasionally found in soils under natural conditions, but they originate more frequently from industrial and agricultural activities. Non-ferrous metal industry, mining, waste disposal, pesticides, fertilizers or metal-contaminated sludge are important sources of metal dispersion in the terrestrial and aquatic environment. Pollution of the environment by heavy metals gains much interest in reference to their effects on plants. However, some resistant organisms have been studied and are shown to be of considerable value in the remediation of soils that are heavily contaminated with heavy metals (Zhu et al., 1999b). The presence of such heavy metals inhibits plant growth, limiting the
application of phytoremediation (Goldsborough, 1998). Stunted growth, chlorosis and necrosis, leaf epinasty and red-brownish discoloration are visible symptoms of severe metal phytotoxicity (Woolhouse, 1983; Wallnofer and Engelhardt, 1984). At the metabolic level, enzyme capacity can be substantially inhibited. Metals may also interfere with other cellular components; for examples, bio membranes. These interactions trigger secondary responses, involving enzymes, which either protect membranes against oxidative damage, or might partially bypass metal sensitive reactions. Since some metals (e.g. Zn and Cd) preferentially accumulate in the chloroplast (Ernst, 1980; Van Assche and Clijsters, 1986), further research on metal effects in the leaf seems obvious. However, data on enzyme induction indicates that other metals (e.g. Cu) might act mainly at the root level; therefore, interactions with membranes from roots (and other organs) should also be taken into consideration.

Cadmium (Cd) exists in many forms in natural sources throughout the world. The global problem concerning the ecological contamination caused by Cd is serious and is one of the major contributors of abiotic stress to plants (Ona et al., 2006). Heavy metals like Cd pass into the environment through crops and affect the crop yield and quality. Metal accumulates in plasmatic compartments of cells, such as cytosol and chloroplast stroma leading to direct and immediate impairment of metabolism and indirect activation of signalling pathways (Brune et al., 1995). In roots and leaves of bush bean, Cd ions are mostly bound by pectic sites and histidine groups of the cell wall (Leita et al., 1996). However, the importance of these mechanisms may vary in accordance with the concentration of Cd supplied, the species involved, the exposure time etc. (Sanita di Toppi and Gabrielli, 1999). Wagner (1993) has estimated that non-polluted soil solutions contain Cd concentrations ranging from 0.04 to 0.32 mM. Soil solutions, which have a Cd concentration varying from 0.32 to about 1 mM can be regarded as pollutent to a moderate level (Sanita di Toppi and Gabrielli, 1999). Toxic levels in soils with respect to plant growth are reported as 3-8 mg Kg\(^{-1}\) Cd (Kabata-Pendias and Pendias, 1992). The relationship between metal toxicity and cellular redox imbalances leading to oxidative stress in plants has been studied intensely over the past decades (Sharma and Dietz, 2008). Increased ROS leads to the production of oxidative stress (Sreedevi et al., 2008), Whereas, under normal growth conditions, the production of ROS in cells is low (240 \(\mu\)M O\(_2^{-}\) and a
steady state level of 0.5 μM H₂O₂ in chloroplasts (Polle, 2001), many stresses that disrupt the cellular homeostasis of cells enhance the production of ROS (240-720 μM s⁻¹ O₂ and steady state level of 0.5-15 μM H₂O₂) (Polle, 2001). ROS can be extremely harmful to organisms at high concentrations. ROS can oxidize proteins, lipids, pigments and nucleic acids, often leading to alterations in cell structure and mutagenesis causing lipid peroxidation, membrane damage, inactivation of enzymes, thus affecting cell viability (Foyer et al., 1997).

Toxic levels of Cu occur naturally in some soils whereas others may contain high levels of Cu as a result of the anthropogenic release of heavy metals into the environment through mining, smelting, manufacturing, and agriculture and waste disposal technologies. At concentrations above those required for optimal growth Cu has shown to inhibit growth and to interfere with important cellular processes such as photosynthesis and respiration (Marschner, 1995; Prasad and Strzalka, 1999). Plants grown in the presence of high levels of Cu normally show reduced biomass and chlorotic symptoms. A lower content of chlorophyll and alterations of chloroplast structure and thylakoid membrane composition has been found in leaves under such growth conditions (Baszynski et al., 1988; Lidon and Henriques, 1991; Ciscato et al., 1997; Päätsikkä et al., 1998; Quartacci et al., 2000). In particular, degradation of grana stacking and stroma lamellae, increase in the number and size of plastoglobuli and appearance of intra thylakoidal inclusions have been observed. It has been proposed that Cu interferes with the biosynthesis of the photosynthetic machinery modifying the pigment and protein composition of photosynthetic membranes (Lidon and Henriques, 1991; Maksymiec et al., 1994). Furthermore, lipid peroxidations, decreases of lipid content and changes in fatty acid composition of thylakoid membranes were observed (Sandmann and Böger, 1980; Luna et al., 1994; Maksymiec et al., 1994). As a consequence of such modifications, alteration of PSII membrane fluidity has been found (Quartacci et al., 2000). On the other hand, the decrease of the photochemical activity caused by Cu is accompanied in vivo by an alteration of the structure and composition of the thylakoid membranes, which can influence the formation and function of the photosystems (Baszynski et al., 1988, Ouzounidou et al., 1992, Lidon et al., 1993). Baszynski et al (1988) have
proposed that those processes induced by Cu could involve either the destruction of the oxygen-evolving complex polypeptide composition or the interaction with ions necessary for proper functioning of the complex such as Mn, Ca and Cl.

It is well known that transition metals like Cu catalyze the formation of hydroxyl radicals (OH) from the non-enzymatic chemical reaction between superoxide (O$_2$-) and H$_2$O$_2$ (Haber-Weiss reaction) (Halliwell and Gutteridge, 1984). Hence, the presence of excess Cu can cause oxidative stress in plants and subsequently increase the antioxidant responses due to increased production of highly toxic oxygen free radicals. Accordingly, it has been observed that excess Cu in plants lead to oxidative stress inducing changes in the activity and content of some components of the antioxidative pathways (i.e., ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), superoxide dismutases (SODs), guaiacol peroxidase) (De Vos et al., 1992; Luna et al., 1994; Stohs and Bagchi, 1995; Navari-Izzo et al., 1998; Gupta et al., 1998; Drazkiewicz et al., 2003; Wang et al., 2004). Plant responses to abiotic stresses are dynamic and complex (Skirycz and Inze, 2010; Cramer, 2010). The plant responses to stress are dependent on the tissue or organ affected by the stress. For example, transcriptional responses to stress are tissue or cell specific in roots and are quite different depending on the stress involved (Dimmeny et al., 2008). In addition, the level and duration of stress (acute vs chronic) can have a significant effect on the complexity of the response (Tattersall et al., 2007; Pinheiro and Chaves, 2011).

To avoid the build up of excess metal levels in the cytosol, plant cells adopt various strategies which also prevent the onset of toxicity symptoms. In general, the ability of plants to tolerate and accumulate heavy metals is recognized in a variety of ways, such as immobilization at the level of cell wall, exclusion through the action of plasma membrane, chelation and compartmentalization in vacuoles (Hall, 2002). It appears likely that specific mechanisms are employed for specific metals in particular species. Further control of metal homeostasis and redox status inside plant cells are carried out by defense mechanisms, such as Cd complexation by metal binding peptides like metallothioneins, phytochelatins (Hall, 2002; Ma et al.,
and transport processes. Defense system of the plant to ROS constitutes oxygen free radical species detoxifying enzymes like SOD, CAT, APX, GPX, GR and other antioxidant compounds such as GSH, ascorbate, α-tocopherol, carotenoids and other compounds capable of quenching ROS (Demirevska-Kepova et al., 2006; Liu et al., 2009). Other biologically active molecules which indicate stress are heat shock proteins, amino acids, polyamines, phytoalexins and carbohydrates. Each of these antioxidant enzyme or compound has physiological function under non-stressed conditions, but their activity or quantity is increased under oxidative stress.

Among amino acids in plants, free proline accumulates in response to the imposition of a wide range of biotic and abiotic stresses. Proline accumulation serves as a biomarker to stress. It is supposed to participate in the reconstruction of chlorophyll, activates the Krebs cycle and constitutes an energy source (Ramon et al., 2003). It is also an important part of structural proteins and enzymes and participates in repair processes. Most attempts to account for the phenomenon have focused on the ability of proline to mediate osmotic adjustment, stabilize sub-cellular structures and scavenge free radicals (Hare and Cress, 1997). Proline accumulation may reduce stress-induced cellular acidification or prime oxidative respiration to provide energy needed for recovery. High levels of proline synthesis during stress may maintain NAD(P)⁺/NAD(P)H ratios at values compatible with metabolism under normal conditions. Free proline has been reported to accumulate in a range of plants in response to a wide range of environmental stresses including heavy metals (Alia and Saradhi, 1991; Alia et al., 1993; Abdel-Latif, 2008)

Thiol groups, present in plant cells are a major non-enzymatic scavenger of ROS being itself less susceptible to attack by oxidants. Of the non-enzymatic constituents GSH is the most soluble antioxidant. GSH, a disulphide reductant which protect thiol groups of enzymes and reacts with O₂, H₂O₂ and OH⁻ radicals. Together with ascorbate and antioxidative enzymes, it plays a pivotal role in protecting the plants from the ROS (John et al., 2007; Arya et al., 2008). The ability of GSH to participate in the redox regulation of cellular processes is presumed to be largely dependent upon its concentration. GSH levels in plant tissues are known to change under metal stress (Koricheva et al., 1997). Not only elevated levels of GSH are
correlated with environmental stress tolerance, but also enhance GSH synthesis seems to be an intrinsic response of plants to stress (May et al., 1998). GSH is a substrate for glutathione S-transferases, enabling neutralization of potentially toxic xenobiotics as well as, GSH is the precursor for phytochelatins (Marrs, 1996). Non-protein thiols (NPTs), which contain high percentage of Cys sulfhydryl residues in plants, play a pivotal role in heavy metal detoxification (Zhu et al., 1999). The reduced form of glutathione (g-Glu-Cys-Gly, GSH) is one of the most important components of NPT metabolism. The accumulation of non protein thiols upon Cd supply also corresponded to the production of phytochelatins (Vögeli-Lange and Wagner, 1990). Ascorbate-glutathione cycle, a major H$_2$O$_2$ scavenging pathway operates both in chloroplast as well as in the cytosol (Zhang J and Kirkham, 1996).

Besides these constituents, the removal of ROS generated via heavy metals is regulated by antioxidant enzymes such as Superoxide dismutases (SOD, EC 1.15.1.1), Catalase (CAT, EC1.111.1.6), Peroxidases like Guaiacol peroxidase (GPOD, EC 1.111.1.7), and Ascorbate peroxidase (APX, EC1.11.1.11), Glutathione reductase (GR, EC 1.6.4.2) and other associated glutathione metabolism enzymes (Mittler 2002; Razinge et al. 2007). The importance of antioxidant enzymes in preventing oxidative stress by scavenging ROS and thus preventing cellular damage is generally emphasized (Dawes, 2000). The activities of antioxidative enzymes are inducible by oxidative stress and represent a general plant response to adverse conditions (Foyer et al., 1997). But the direction and size of the response vary with plant species and tissues analyzed and the kind and intensity of the stress treatment (Schützendübel and Polle, 2002).

SOD the first metalloenzyme in the ROS detoxification process, coverts O$_2^{-}$ radicals to H$_2$O$_2$ and O$_2$ (Polle, 2001). The accumulation of H$_2$O$_2$, a strong oxidant is prevented in the cell either by CAT or by the ascorbate-glutathione cycle where APX reduces it to H$_2$O so that the accumulation of O$_2^{-}$ and H$_2$O$_2$ is effectively prevented (Liu et al., 2002; Parida et al., 2004). In the ascorbate glutathione cycle, the enzymatic action of APX reduces H$_2$O$_2$ using ascorbate as an electron donor. Ascorbate is a small, water-soluble antioxidant molecule, used as substrate for APX which catalyzes H$_2$O$_2$ detoxification.
Detoxification reactions must involve right balance between the formation and detoxification of ROS. Increased activities of these enzymes have been reported in plants exposed to metals (Chaoui et al., 1997; Koricheva et al., 1997). The interaction of metal and antioxidative systems in animals and plant have been studied (Zikic et al., 1997; Sandalio et al., 2001; (Ferreira et al., 2002). Metal treatment affects the activities of antioxidative enzymes but sometime contrasting results have been reported. For example, in leaves of Cd exposed Helianthus annuus (L.) plants; the activities of ascorbate-glutathione related defense enzymes were decreased (Gallego et al., 1996). Roots and leaves of Phaseolus vulgaris (L.) add author contain elevated APX activities after metal exposure (Chaoui et al., 1997). Thus understanding the biochemical detoxification strategies that plants adopt against oxidative stress induced by accumulated metal ions is a key to manipulate heavy metal tolerance in plants (Markovska et al., 2009).

Heme oxygenase (1.14.99.3) is a universal and active enzyme present in animals as well as in plant systems. It catabolizes free heme (Iron protoporphyrin IX) to Fe$^{2+}$, carbon monoxide (CO) and biliverdin (Shekhawat and Verma, 2010). The ubiquitous expressions of the heme oxygenase gene in the majority of living organism hint that HO may have evolutionary enzymatic role in living system. Substrate of heme oxygenase enzyme is molecule of heme which is highly conserved and exists in biological system as a stable prosthetic group for hemoproteins, which act as carriers, electron transporters, heme-based gas sensors and catalysts of biodegradation or biosynthesis. Besides these important roles they can be cellular messengers as well. This enzyme has originally been identified as degrader of heme in rat and was characterized as a distinct protein entity in photosynthetic organism and higher plants. Heme oxygenase activity has been described in algae, cyanobacteria, red algae and cryptophytes. The enzymatic properties of algal heme oxygenase are different from those of animals (Troxler et al., 1979) and little bit different from higher plants. In higher plant, HO synthesizes phytocrome chromophore since biliverdin IX acts as precursor for phytocrome chromophore synthesis (Elich et al., 1989) and for protection of cells against oxidative stress (Balestrasse et al., 2005; Shekhawat and Verma, 2010) as well as attenuation of inhibition of seed germination and salt stress alleviation (Liu et al., 2007). Its role
has been explored in developmental pathways as stomata closure (Yu et al., 2007) and in leghemoglobin metabolism (Baudouin et al., 2004). Due to environmental unpredictable and rapid changes plants are facing different stresses. For that plants have strong inbuilt defense mechanism against abiotic stress through antioxidant network containing catalase, peroxidase and superoxide dismutase. Hemeoxygenase has been recently shown to be an active part of this complex (Balestrasse et al., 2005). Its major role has been established in animal system as second messenger (Maines, 1997) and as an antioxidant (Vogt et al., 1995). Hemeoxygenase is responsible for the physiological breakdown of heme into equimolecular amounts of biliverdin, carbon monoxide, and iron. Three isoforms (HO1, HO2, and HO3) have been identified. HO1 is ubiquitous and its mRNA and activity can be increased several-fold by heme, other metalloporphyrins, transition metals, and stimuli that induce cellular stress. HO1 is recognized as a major heat shock/stress response protein.

Genes encoding hemeoxygenases have been found in a wide variety of organisms including mammals, higher plants, red algae, cryptophytes, cyanobacteria and pathogenic bacteria (Muramoto et al., 1999). Research on structure of hemeoxygenases suggests that fold of HO is a single compact domain mostly assisting of α-helix. Crystal structures of Rat HO1 (rHO1), Synechocystis HO1 (SynHO1), Pea HO1 (PsHO1) are found to be almost similar to that of human HO1, but difference also exists in structure due to evolution. Crystal structure of mammals HO1 reveals that heme is sandwiched between proximal and distal helices with the d-meso edge (Unno et al., 2007). These two helices serve as a contact sites for heme group. Similarity of crystal structures has been shown among the higher plants hemeoxygenases. Structure of Pea HO1 and Arabidopsis thaliana HO1 (AtHO1) has been compared. In the PsHO1 molecule of ascorbate can be readily accommodated in the intramolecular space at suitable distance to interact with heme. Six amino acid residues: Glu96, Phe120, His207, Ile214, Tyr231 and Ser274 are also suitably placed and interact with ascorbate.

Hemeoxygenase is highly conserved within plant species. Recent study has identified plant HO from Cyanobacteria, Cryptophyceae and Rhodophyceae (red algae), in which HO was involved in the synthesis of the chromophoric part of
photosynthetic antennae (Richaud and Zabulon, 1997). Recently, four HO genes from *Arabidopsis thaliana* have been characterized (Davis et al., 2001; Terry et al., 2002; Emborg et al., 2006; Gisk et al., 2010). Amongst them HO1 has been studied in depth, along with others which are also participating in the heme degradation or phytochrome synthesis pathway (Davis et al., 1999; Muramoto et al., 1999, 2002). *Arabidopsis* HOs can be grouped into two categories based on their amino acid sequence similarity. One subfamily comprises AtHO-1 (or HY1), AtHO-3 and AtHO-4, which contain the HO active site. The other subfamily with only AtHO-2 can be distinguished by the lack of a positionally conserved histidine that is considered as an important ligand for heme binding (Davis et al., 2001; Emborg et al., 2006). Expression of AtHO1 is induced by many intracellular and environmental stimuli, but AtHO2, AtHO3 and AtHO4 show different patterns in expression (Emborg et al., 2006). Recently, much attention has been given to the plant HO1 because it regulates many physiological processes such as root branching (Guo et al., 2008) and iron deficiency (Kong et al., 2010). Expression of HO1 induced by heavy metals has been reported in Soybean (Noriega et al., 2004; Balestrasse et al., 2006).

Close examination of the plant HO sequences reveals that they cluster into two subfamilies to either AtHO1 or AtHO2. The HO1 subfamily included Arabidopsis AtHO1, 3 and 4, Soybean GmHO1 and GmHO3, Tomato LeHO1, Sorghum SbHO1, Rice OsHO1, and Pine PtHO1. Representatives of the HO subfamily included Arabidopsis AtHO2, tomato LeHO2, and sorghum SbHO2. For animal HOs, a positionally conserved *His* is required for heme-iron binding and subsequent oxidative cleavage (Ortiz de Montelano and Wilks, 2001). *His* is also retained in all algal and cyanobacterial HOs and members of the plant HO1 subfamily but is replaced by an Arg in the plant HO2 subfamily. Recent evidences show that some N-terminal sequences can simultaneously target proteins to both mitochondria and chloroplasts (Silva-Filho, 2003; Rudhe et al., 2004) and one or more AtHOS can be directed to both compartments (Dixit et al., 2013). Hemeoxygenases are important enzymes also due to their structural similarity and divergence among animal systems and lower as well as higher plant species.
During the last decade after initial establishing the role of heme oxygenase in plant, research has been explored on diversity of its role in plant system. The enzymatic property of algal Heme oxygenase from *Cyanidium caldarium* (Tilden) is different from that of animal enzyme. Algal HO from *Cyanidium caldarium* (Tilden) has been enzymatic characterized as a soluble and ferredoxin dependent enzyme (Rhie and Beale, 1994; Troxler et al., 1979). In contrast, animal heme oxygenase is a microsomal enzyme requiring NADPH-cytochrome P450 reductase for heme catabolism. Using data of amino acid sequence product of AtHOI has been predicted to be a soluble protein, because it does not have a hydrophobic domain for microsomal membrane association at its C-terminus as it has been observed in animal HOI. Instead the AtHOI protein contains a transit peptide that was sufficient for the transport of GFP in to the plastids. Experiments provide evidences that AtHO1 is accumulated in plastids so it is a soluble plastid protein (Beale and Comejo, 1984). Amino acid sequence comparisons revealed that all plant HOs have a nonconserved, N-terminal extension of various lengths that is followed by a approximately 220-amino acid region of high similarity. The ChlororP algorhythm (Emanuelsson et al., 1999) predicted that many of these N-terminal extensions encode transit peptides consistent with the plastid location of HO activity in plants (Terry et al., 1993; Muramoto et al., 1999). Following the predicted transit-peptide is a region of substantial conservation that is similar to the HO catalytic domain from animals, algae, and cyanobacteria (Ortiz de Montelano and Wilks, 2001). Animal HOs have hydrophobic C-terminal extensions that serve to anchor the enzymes to microsomal membranes (Schuller et al., 2001). No such extensions were evident in the plant counterparts, implying that they behave as soluble proteins.

However in photosynthetic organisms, HO was first identified in the red algae *Cyanidium caldarium* (Tilden) add author (Troxler et al., 1979). Later Muramoto et al. (1999) have reported the gene for HO in *Arabidopsis thaliana* using the mutant hyl. In reptiles, fish, insects, and egg shells of birds, BV-IX (a) is used directly for pigmentation and it has been shown to function as a signalling molecule during dorsal development in *Xenopus laevis* embryos (Falchuk et al., 2002). Phycobilin pigments are structurally similar to biliverdin or bilirubin; they are attached to
biliproteins and function as accessory photo-system antenna pigments. Light stable phytochrome may play a major role in photoperiodic induction of flowering in short day plants and inhibition in long day plants.

Recently role of hemeoxygenase has been also explored as a member of antioxidant network. It shows strong activity against oxidative stress with other enzymes (catalase, peroxidase and superoxide dismutase). To counteract the toxicity of ROS, plants have evolved various antioxidative defense systems that scavenge ROS as well as alleviate oxidative stress via the nonenzymatic and enzymatic pathways (Noctor and Foyer 1998). Thus, enhancement of antioxidant defense in plants can increase tolerance to different stress factors. It was a well known fact that various antioxidative defense systems include enzymes such as superoxide dismutase (SOD) and catalase (CAT) as well as nonenzyme molecules such as ascorbate, glutathione, carotenoids, and anthocyanins. Increase the expressing of enzymes of the antioxidative defense system indicates that it might confer the plant tolerance against different stresses. Meanwhile, the transgenic technology has enhanced the possibility of greater stress tolerance in plants by the induction of antioxidative defense system expression. Thus, the study on those antioxidative enzymes could be of much more important.

Among the various genes encoding proteins that possess antioxidant characteristics, it has been well established that the inducible hemeoxygenase1 (HO1, EC 1.14.99.3) is a stress response protein and its induction is associated with protection against oxidative stress, in comparison with the behaviour of the constitutive enzymes of HO2/3 (Choi and Alam, 1996). HO1 is an important rate-limiting enzyme in heme degradation pathway with the concomitant release of carbon monoxide (CO), the production of biliverdin IXα (BV) and free iron (Fe²⁺), all of which have been proved playing a significant role in animal oxidative stress responses, respectively (Stocker et al., 1987; Maines, 1988; Ewing et al., 1992; Yamaguchi et al., 1996). HO1 can be induced by multiple stimuli, including heavy metals (Llesuy and Tomaro, 1994; Noriega et al., 2004; Balestrasse et al., 2008; Han et al., 2008), glutathione depletion (Cui et al., 2011), UV radiation (Yannarelli et al., 2006), salinity stresses (Cao et al., 2011; Xie et al., 2008) and hydrogen peroxide (H₂O₂) (Chen et al., 2009; Jin et al., 2011). Thus, the up-regulation of HO1 in plants could
also act as antioxidant barrier against various stresses-triggered oxidative damage (Yannarelli et al., 2006; Noriega et al., 2003; Balestrasse et al., 2008; Chen et al., 2009; Cui et al., 2011) and exhibit hormone-like responses (Xuan et al., 2008). Although HO1 plays an important role in response to a variety of oxidative challenges, previous researches on plant HO1 genes have only focused on a few model plants, including Arabidopsis, Tomato, Pine, Pea, and Rice plants (Shekhawat and Verma, 2010; Fu et al., 2011). Hemeoxygenase (HO) is the first rate-limiting enzyme that yields biliverdin IXa, carbon monoxide (CO) and iron. HO is ubiquitously present in invertebrates, higher plants, algae and even bacteria (McCoubrey and Maines, 1994; Gibbs et al., 1998; Baudouin et al., 2004; Kikuchi et al., 2005). In mammals, three HO isoforms HO1, HO2 and HO3 have been identified (Snyder and Baranano, 2001). HO1 is an inducible enzyme that is expressed in most cell types when exposed to various biotic and abiotic stresses (Dulak and Józkowicz, 2003); HO2 is constitutively expressed and mainly involved in the developmental regulation (e.g. neural system functioning and digestive system) (Liu et al., 2000) and HO3 is also expressed in numerous organs (e.g. liver, spleen, brain and kidney) but its ability to degrade heme is rather limited, and therefore considered as a heme-sensing protein rather than a heme degrading enzyme (McCoubrey et al., 1997).

Another role of hemeoxygenase has been established in developmental biology such as auxin, CO and ABA mediated root development.

Due to different stress, caused by industrial and urban activities plant productivity is getting affected day by day. Perfect reply to all of these problems is to make variety of plants those can survive in these stress conditions. By understanding all mechanism of hemeoxygenase at genetic and biochemical levels, It is possible to make transgenic plants of HO. Those transgenic plants will be able to survive on the lands suffering from pollution due to metals or salts, or in crops affected by exposure to UV-b radiation. The key players of HO activity are biliverdin, CO and Fe++. Still researchers are trying to find out about the targets and interactions of these factors and their specific and separate role in plant physiology. By antisense RNA technology knockdown of the HO gene can be done in different parts of plants and it will help us to know about its regulatory functions in different parts of plants.
By knowing the role of HO in developmental biology important agricultural plants can be modified (e.g. crops) according to environmental changes.

Brassica juncea (L.) Czern. is a species of a worldwide economic importance and is well known for its characteristic resistance to biotic and abiotic environmental stresses, such as heavy metals. This plant has been identified as a high biomass producing plant with the capacity to accumulate Cd at higher concentrations in plant cells (Kumar et al., 1995). Natural hyperaccumulators, such as Brassica juncea (L.) Czern. can accumulate exceptionally high concentrations of heavy metals in the roots (Reeves et al., 1996). In this study, we have used Brassica juncea (L.) Czern. as a test plant because it has been identified as a high biomass producing plant with the capacity to accumulate higher concentrations of metal (Kumar et al., 1995). Activity of HO1 in Brassica juncea has been measured by Li et al., 2012 against Hg induced oxidative stress. They have demonstrated that the newly identified HO1 from Brassica juncea is differentially expressed at various tissues of the plant. Expression of BjHO1 can be induced by Hg exposure. Identification of the promoter sequences of BjHO1 allows us to confirm that BjHO1 is induced for expression under multi environmental stresses. Functional identification of BjHO1 has revealed that expression of BjHO1 leads to less accumulation of Hg in plants. Because of the low level of Hg, the growth status and antioxidant capacity have greatly improved.

This indicates that expression of BjHO1 would be beneficial for the crop growth and production (e.g. rice, wheat and cereal crops), because it is important to limit the uptake or accumulation of heavy metals into eatable part of crops. This work will also provide an example for molecular breeding designed for plants that do not accumulate or accumulate less toxic trace metals growing on heavy metal-contaminated soils.

Soybean is an important crop in the world, offering high-quality protein and increasing the input of combined N into the soil. However, its yield may be adversely affected by heavy metals. Reduction of biomass production and nutritional quality has been observed in crops grown on soil contaminated with heavy metals (Mhatre and Pankhurst, 1997). In roots and nodules of soybean plants high Cd causes oxidative damage and thus affects nitrogen fixation and assimilation (Balestrasse et al., 2001; Balestrasse et al., 2003). Level of proline and polyamine is
also found to be affected by cadmium stress in nodules and roots of *Glycine max* (L.) Merr. Cakmak and Horst (1991) have analyzed affect of aluminium on lipid peroxidation, SOD, Catalase and POD activities in root tips of soybean. Activity of Hemeoxygenase 1 has also been studied in *Glycine max* (L.) Merr. under different abiotic stress. For the first time Balestrasse et al. (2005) have reported involvement of Hemeoxygenase as an antioxidant defense in soybean nodules against Cd induced stress. UV-B induced oxidative stress also caused increase in HO1 enzyme activity in *Glycine max* (L.) Merr. (Yannarelli et al., 2006). Later on they have added HO1 activity is induced by activity of Nitric oxide synthase (NOS) gene (Santa- Cruz et al., 2010). HO1 also shows its alleviation under salinity stress in *Glycine max* (L.) Merr. nodules (Zilli et al., 2008). Correlation has also been found in HO1 and CAT activity in nodules and roots of soybean (Balestrasse et al., 2008).
Chapter 3

Materials and Methods