Transgenic Approaches for the Improvement of Stress Tolerance using Antioxidant Genes

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ABSTRACT

Although relatively low and tightly regulated under normal conditions, reactive oxygen species (ROS) can increase at very high levels, which eventually overwhelm the plant’s defence mechanisms under stress. High level of ROS can rapidly damage many biomolecules including lipids, proteins and DNA resulting in cellular damage and cell death. With the advent of plant transformation technologies efforts have been made to reinforce the antioxidant metabolism of the cell, manipulating the levels of key antioxidant enzymes in order to provide enhanced ROS scavenging capacity and protection against oxidative stress. Accordingly, transgenic plants overexpressing key antioxidant enzymatic defence mechanisms including superoxide dismutase (SOD), catalases (CAT), ascorbate peroxidase (APX), ascorbate oxidase (AO), glutathione reductase (GR), glutathione-S-transferase (GST), alternative oxidase (AOX) have been generated and evaluated for their response to biotic and abiotic stress. In parallel, the role of these enzymes has been studied in transgenic genotypes with reduced levels of expression generated after silencing the respective genes. More recently, pyramiding antioxidant defence for increased stress tolerance has been attempted overexpressing genes that act together in an antioxidant pathway or in separate antioxidant pathways. Results of these studies are reviewed in this chapter and the effects of these manipulations are discussed.
INTRODUCTION

Environmental conditions are seldom favorable for optimum plant growth and development. Seasonal and diurnal fluctuations of abiotic factors (temperature, humidity, wind, rainfall, sunlight, soil conditions, pollution), as well as biotic factors (pest attacks and diseases) may vary between such extremes that are unfavorable for survival in the natural environment or high yield and increased productivity in the cultivated field. Actually several adverse biotic and abiotic factors combined, are always present in the plant’s surroundings and it is reasonable to assume that every plant grows under a certain burden of stress. Different stresses exert their harmful effects by specific ways that may be not entirely clear, but a common result of many stresses of either biotic or abiotic origin is the increased production of activated oxygen intermediates in the cell (Bartels, 2001). These are common byproducts of aerobic metabolism and are present in normal, but are elevated in stress conditions. During respiration, molecular oxygen (O₂) accepts four electrons to produce two molecules of water. Non-activated oxygen is a biradical with its two outermost valence electrons occupying separate orbitals with parallel spins. To oxidize a nonradical atom or molecule, triplet oxygen requires a partner to provide a pair of electrons with parallel spins that fit into its free electron orbitals. However, pairs of electrons typically have opposite spins, and thus fortunately impose a restriction on the reaction of triplet molecular oxygen with most organic molecules (Apel and Hirt, 2004). From this triplet state it can be activated by energy transfer to reverse the spin on one of the unpaired electrons and form the singlet oxygen (¹O₂), or by electron transfer in a stepwise monovalent reduction mode. The first reduction reaction is endothermic forming superoxide (O₂•⁻). Subsequent reductions form hydrogen peroxide (H₂O₂), hydroxyl radical (HO⁺) and water. Very often biologists refer to the reduction products of oxygen as oxygen free radicals, which is a misnomer because free radicals are atoms or molecules with an unpaired electron that is true for O₂•⁻ and HO⁺ but not for H₂O₂. Thus, the oxygen reduction intermediates are more correctly referred by with the equivalent terms reactive
oxygen intermediates (ROIs), active oxygen species (AOS), or reactive oxygen species (ROS) that will be adopted in this review.

Each of the ROS can react with a variety of biomolecules, altering or blocking their biological activity. Although relatively low and tightly regulated under normal conditions, ROS can increase at very high levels that eventually overwhelm the plant’s defence mechanisms under stress. The combined biological effect of such an increase of ROS on organisms is termed "oxidative stress" (Scandalios et al., 1997).

The major sources of ROS in plants are located within organelles with high oxidizing metabolic activity like chloroplasts, mitochondria and peroxisomes. To minimize the damaging effects of activated oxygen, organisms have evolved various enzymatic and nonenzymatic mechanisms that can reduce oxidative stress by detoxifying harmful oxygen species. The major ROS scavenging enzymatic mechanisms in plants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and alternative oxidase (AOX), while the antioxidant system includes also essential antioxidant molecules like ascorbic acid, glutathione and α-tocopherol. The antioxidative plant machinery is highly coordinated and regulated for efficient removal of ROS. This tight regulation of ROS level is even more important under the light of recent findings uncovering a significant role of ROS as signaling molecules in processes such as programmed cell death, abiotic stress responses, pathogen defense and systemic signaling (Mittler et al., 2004a). Thus, detoxification mechanisms must allow fulfilling this significant role of ROS but simultaneously protecting sensitive cell components from ROS-induced damage.

Plants have mechanisms to sense oxidative stress and react synthesizing antioxidant metabolites or enhancing antioxidant enzyme activities. Tolerant or acclimated plants usually contain higher amount of antioxidant factors to alleviate the harmful effects of ROS (Mylona and Polidoros, 2010). In an effort to improve oxidative stress tolerance several researchers attempted to enhance the antioxidant defense capacity in plants using genetic engineering, and aiming either to increase the amount of endogenous antioxidants, or to transfer and overexpress more efficient antioxidant systems from other species and cellular compartments, to more sensitive ones that produce copious amounts of ROS under stress. Accordingly, it was expected that plants with increased antioxidant capacity would be more
tolerant to several adverse environmental conditions, preventing yield losses and reduced productivity under environmental stress. This biotechnological approach was proven to be difficult task as abiotic stress tolerance seems to be a complex genetic trait involving action and interaction of many genes. Plant biotechnology has been extremely successful in manipulating and engineering the usually monogenic traits of pest resistance and herbicide tolerance that are incorporated into cultivars successful in the global market for nearly two decades. However, this is not yet the case for abiotic stress conditions such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress where research results did not transform into commercial products. Promising but also ambiguous results were obtained from research efforts aiming to combat abiotic stress increasing the capacity of the enzymatic antioxidant machinery of the cell and in this review we will summarize the available data on transgenic plants overexpressing antioxidant genes with emphasis to the rationale behind the observed in each case result.

ENZYMATIC MECHANISMS FOR DEFENCE AGAINST OXIDATIVE STRESS AND MANIPULATION OF EXPRESSION LEVELS

A. Superoxide Dismutase (SOD)

Superoxide radical ($\text{O}_2^{\bullet-}$) is the primary product of $\text{O}_2$ reduction generated in mitochondria and chloroplast electron transport chains (ETC). $\text{O}_2^{\bullet-}$ is also produced in the apoplastic space through the action of the plasma membrane NADPH oxidase (Agrawal et al., 2003). $\text{O}_2^{\bullet-}$ is a short lived, 2-4 μs, highly reactive ROS unable to transverse the phospholipid bilayer cause is charged, thus its action is restricted to the close proximity of its generation site (Bhattacharjee, 2005). Sequentially, ubiquitous SODs are the front line of defense as they rapidly dismutate $\text{O}_2^{\bullet-}$ to $\text{H}_2\text{O}_2$. SODs present in every subcellular compartment including chloroplasts, mitochondria, peroxisomes, glyoxysomes, cytosol, and apoplast comprise a multigene family of nuclear encoded enzymes. The number of SOD genes varies among plant species (Grene-Alscher et al., 2002). According to their metal cofactor at their active site, plant SODs are classified into three groups: the copper zinc SOD (Cu/ZnSOD), the manganese SOD (MnSOD), and the iron SOD (FeSOD). The
Cu/ZnSODs are found in the cytosol, apoplast, peroxisomes and chloroplasts. FeSODs are localized in the chloroplasts, and are resistant to KCN inactivation. MnSODs are found in mitochondria and are resistant to H$_2$O$_2$ inhibition. Noteworthy, that both Cu/ZnSOD and FeSOD are inhibited by H$_2$O$_2$, thus they co-exist with robust H$_2$O$_2$-scavenging systems such as enzymes of the ascorbate-glutathione cycle (Scandalias, 1997, Foyer et al., 1997). The intracellular balance of SODs and the different H$_2$O$_2$-scavenging enzymes is apparently crucial in determining the steady-state level of O$_2$$^{-}$ and H$_2$O$_2$. This balance, along with the sequestering of metal ions by ferritin and other metal-binding proteins, prevents the formation of the highly toxic HO via the metal -dependent Haber-Weiss reaction or the Fenton reaction (Halliwell and Gutteridge, 1999). Plants do not possess enzymatic systems to scavenge HO$^\cdot$ thus preventing its generation by keeping low the steady-state level of O$_2$$^{-}$ and H$_2$O$_2$ to avoid their interaction is the only way of defense.

Given the ubiquitous nature of SOD along with its exceptional role in ROS scavenging, it was the primary antioxidant gene selected to be over-expressed in plants. A list of transgenic plants overexpressing a SOD isoform is shown in Table 1. The first report of transgenic SOD overexpressing plants however was not promising. Transgenic tobacco plants with 30- to 50-fold increased SOD activity levels due to the production of a petunia chloroplastic Cu/ZnSOD were not more resistant to methyl viologen treatment compare to wild type plants (Tepperman and Dunsmuir, 1990). It should be mentioned that tolerance to oxidative stress is easily tested in vitro with methyl viologen (MV, commonly known as paraquat). MV is a light-activated bipyridyl herbicide. In presence of light, MV accepts electrons from photosystem I (PSI), subsequently reduces oxygen to form superoxide radical, O$_2$$^{-}$, and the regenerating oxidized MV engages a successive round of redox cycling. In this way MV mimics fairly well the O$_2$$^{-}$-forming process of illuminated chloroplasts. Noteworthy that MV also accepts electrons from the ETC of mitochondria in light or dark, although, MV-generation of O$_2$$^{-}$ in mitochondria is mainly favored in dark (Halliwell and Gutteridge, 1999). Following, transgenic tomato plants with increased SOD activity due to the same chloroplastic petunia Cu/ZnSOD were not protected against photoinhibitory conditions (i.e. high light intensities, low temperatures and low CO$_2$ concentrations) known to increase oxidative damage (Tepperman and
Dunsmuir, 1990). In addition the same plants were not protected against ozone stress and exogenously applied \( \text{H}_2\text{O}_2 \), although activity levels of \( \text{H}_2\text{O}_2 \)-scavenging enzymes were not assessed. Ozone (\( \text{O}_3 \)) is an atmospheric pollutant that breaks down in the apoplast forming mainly \( \text{O}_2^{•-} \) and \( \text{H}_2\text{O}_2 \). Intriguingly the observed lack of tolerance was attributed to the inability of plants to cope with the elevated levels of produced \( \text{H}_2\text{O}_2 \) due to SOD overexpression (Pitcher et al., 1991). Contrarily, (Gupta et al., 1993b) showed that transgenic tobacco plants overexpressing a pea chloroplastic Cu/ZnSOD to moderate levels were tolerant to high light, chilling stress and low levels of MV. Noteworthy that under high levels of MV, protection of transgenic plants was aborted, attributed to the high levels of produced \( \text{H}_2\text{O}_2 \) acting in feedback inhibition. Notably Cu/ZnSOD is inactivated by the end product \( \text{H}_2\text{O}_2 \), while MnSOD is insensitive. Based on this evidence (Bowler et al., 1991a) generated transgenic tobacco plants overexpressing a chimeric mitochondrial MnSOD with a chloroplast-specific transit peptide leading to efficient import of an active MnSOD into the chloroplast. The generated MnSOD overexpressing tobacco plants were tolerant to low and high concentrations of MV induced oxidative stress. Following tobacco plants overexpressing mitochondria- or chloroplasts-targeted MnSOD exhibited improved ozone tolerance (Van Camp et al., 1994, Van Camp et al., 1996). Accordingly, MnSOD-overproducing plants showed improved tolerance against freezing, water deficit, winter survival (McKersie et al., 1993, McKersie et al., 1999, McKersie et al., 1996), and MV induced oxidative stress (Bowler et al., 1991b, Slooten et al., 1995). Meanwhile it was shown that moderate overexpression of a pea cytosolic Cu/ZnSOD in transgenic tobacco plants increased ozone tolerance (Pitcher and Zilinskas, 1996). It should be noted that transgenic plants were developed using various hosts and assessment of stress tolerance was analyzed by various protocols developed in each research group. Therefore due to the high degree of variation in the methodology of development and analysis of SOD overexpressing plants, detailed comparisons of transgenic performance was difficult. A comparative analysis of transgenic plants overexpressing SOD isoforms was performed by Allen’s group that developed transgenic plants in the same host variety using gene constructs that differed only in the coding sequence of the SOD isoform (Allen et al., 1997). As the levels of SOD overexpression were comparable in
all transgenic lines the observed differences in oxidative stress tolerance was clearly attributed to the biochemical characteristics of the SOD isozymes (isoenzymes) overexpressed. It was speculated that aside of differences in kinetic properties and subcellular localization between MnSOD and Cu/ZnSOD, the poor performance of the later was attributed to its sensitivity to H₂O₂ compare to the former.

On the other hand, leaves of transgenic tobacco plants overexpressing the chloroplastic Cu/ZnSOD were reported (Gupta et al., 1993a, Gupta et al., 1993b) to have substantial protection from photooxidative damage caused by exposure to high light and chilling temperatures, whilst leaves of tobacco plants that express chloroplastic MnSOD had no such protection (Slooten et al., 1995). Specifically, the chloroplastic MnSOD overexpression showed no protection of photosystem II (PSII). This was attributed to the sub-organellar location of overexpressed MnSOD that was unknown compare to the native overexpressed Cu/ZnSOD that is located on the thylakoid membrane in apparent association with PS, in a position to rapidly scavenge O₂•⁻ at its generation site (Ogawa et al., 1995).

Solution to this problem was given by overexpression of a chloroplastic FeSOD in transgenic plants that led to increased MV tolerance, water logging, Mn-deficiency (Yu et al., 1999b, Van Camp et al., 1996, Van Breusegem et al., 1999) and winter survival (McKersie et al., 2000). Remarkably, FeSOD is also inactivated by H₂O₂ as Cu/ZnSOD; however, FeSOD overexpression offers steady tolerance to oxidative stress induced by various factors. Light in that direction was recently shed with the discovery of microRNA (miRNA) molecules that regulate post-transcriptional antioxidant gene expression (Sunkar et al., 2006). Specifically studies in Arabidopsis have identified miR398, a repressor of cytosolic and chloroplastic Cu/ZnSOD namely CSD1 and CSD2, respectively. Transgenic Arabidopsis plants exhibiting down-regulation of miR398 and overexpression of the chloroplastic Cu/ZnSOD showed increased tolerance to MV- and salt-induced oxidative stress, revealing a direct connection between miRNA pathway and CSD1 and CSD2 post-transcriptional regulation. Following, sequence data analysis revealed that miR398 and its target sites on cytosolic and chloroplastic Cu/ZnSOD mRNA are conserved in dicotyledonous and monocotyledonous plants. Therefore the low and moderate levels of Cu/ZnSOD overexpression in transgenic lines in previous studies is
attributed to the presence of the miRNA398 target site sequence in the transgene construct, leading to down-regulation of transgene overexpression in response to oxidative stress. More recent studies showed that posttranscriptional regulation of Cu/ZnSOD genes by miR398 in response to abscisic acid (ABA) or salt stress in plants is twofold: a dynamic regulation within a plant species and a differential regulation between different plant species (Li et al., 2009, Jia et al., 2009). Conclusively, further investigation for complete understanding of the regulation of SOD gene-enzyme system will provide valuable insight for physiologically relevant strategies to enhance antioxidant capacity.

B. Expression of Ascorbate Peroxidase (APX) and Ascorbate-Glutathione Cycle Enzymes

Detoxification of hydrogen peroxide (H₂O₂) in plants is achieved by a complex network of antioxidant enzymes and non-enzymatic antioxidants such as tocopherols, ascorbic acid (AsA) and glutathione (GSH) that work in concert. In the plant cell, removal of H₂O₂ is undertaken by a set of antioxidant enzymes, encoded by nuclear genes, including ascorbate peroxidases (APX), catalase (CAT), glutathione peroxidases (GPX), peroxiredoxins (Prx) and non-specific donor peroxidases (POD) (Smirnoff, 2005). Along with SODs the aforementioned H₂O₂-scavenging enzymes operate collectively in alleviating the detrimental effects of ROS while sustaining an intracellular steady-state level that promotes plant growth, development and cell signaling that reinforces responses to abiotic and biotic environmental stressors (Foyer and Noctor, 2005). APX enzyme, uniquely found in plants and algae, catalyzes the reduction of H₂O₂ to water and oxygen with concomitant consumption of ascorbate as the reductant. Notably APX is found in almost every subcellular compartment including chloroplasts, mitochondria, cytosol, and peroxisomes as well (Del Rio et al., 2006) and references therein. APX exhibits a very high affinity for H₂O₂ thus acting at the micromolar and submicromolar range. Given that, APX is ideally suited for a fine tuning of sensitive redox balances with low intracellular H₂O₂ concentrations that are important for regulatory mechanisms (Neill et al., 2002, Quan et al., 2008).
Elimination of $\text{H}_2\text{O}_2$ by APX depends solely on the availability of reduced ascorbate (AsA), while reduced glutathione (GSH) can be used in some instances. Under normal conditions the cellular pool of ascorbate is kept at the reduced state by a series of redox reactions providing the reducing agent. Ascorbate is then oxidized to monodehydroascorbate (MDHA) that is regenerated in the chloroplast membrane by ferredoxin or in the stroma by MDHA reductase (MDAR) at the expense of NADPH. MDHA can also spontaneously dissociate to form ascorbate and dehydroascorbate (DHA) that is further reduced to ascorbate by dehydroascorbate reductase (DHAR) capable of using reduced glutathione (GSH) as an electron donor. In turn oxidized glutathione (GSSG) is then recycled by NADPH-consuming glutathione reductase (GR) (Mittler et al., 2004b).

Transgenic plants that express gene constructs of either cytosolic or chloroplast targeted APX have been developed and assessed for oxidative-stress tolerance performance (Table 2). Overexpression of cytosolic APX in chloroplasts or cytosol of transgenic tobacco plants exhibited tolerance to MV-induced oxidative stress as well as to high light and chilling (Pitcher et al., 1994, Allen et al., 1997) and references therein. Contrarily the same transgenic lines were not protected against ozone (Torsethaugen et al., 1997). On the other hand, overproduction of peroxisomal APX in tobacco plants provided complete protection to aminotriazole (Wang et al., 1999). Aminotriazole, an herbicide, inhibits CAT thus provoking accumulation of $\text{H}_2\text{O}_2$. Noteworthy that these transgenic plants were not tolerant to MV compare to control indicating that protection of plant cells during photorespiratory conditions can be achieved by enhancing the $\text{H}_2\text{O}_2$-scavenging system at the specific site of $\text{H}_2\text{O}_2$ production. Following overproduction of cytosolic APX in cotton chloroplasts resulted in increased tolerance to high light and chilling stress (Kornyeyev et al., 2003, Kornyeyev et al., 2001). Similarly overproduction of cytosolic APX in tobacco chloroplasts provided increased tolerance to MV, salt and water deficit induced stress (Badawi et al., 2004a). Accordingly (Murgia et al., 2004) showed protection to MV and NO stress of Arabidopsis plants overexpressing a chloroplast, thylakoid bound APX, but failed to resist high light-, cold-, Fe- and Cu-induced stress, pointing the specificity of the antioxidant mechanism and indicating that overproduction of a particular antioxidant enzyme would not render tolerance
to every type of stress. A number of studies have shown that overexpression of a particular APX in a subcellular compartment rendered transgenic plants tolerant to certain stresses (see Table 2). On the other hand, by combining superoxide radical and H$_2$O$_2$-scavengers better results were envisaged (Kwon et al., 2002).

Given that ascorbate is an antioxidant, a redox buffer as well as an enzyme cofactor, the size of ascorbate cellular pool is critical in attaining redox homeostasis. The ascorbate pool size is influenced not only by its biosynthesis but also by its recycling, thus three distinct approaches have been taken to further increase abiotic stress tolerance: (i) overexpression of biosynthesis enzymes, (ii) overexpression of recycling enzymes and (iii) antisense suppression of ascorbate oxidase (AO). The results of these engineering experiments are summarized in Table 2. Thus overexpression of biosynthesis enzymes solely, MDAR or recycling enzymes DHAR would affect ascorbate accumulation. Specifically, overexpression of cytosolic MDAR in tobacco transgenic plants resulted in increased tolerance to ozone, salt and water deficit (Eltayeb et al., 2007). Accordingly overexpression of recycling DHAR enzyme from cytosol of wheat in transgenic tobacco resulted in a two-fold increase in total ascorbate contents with concomitant increase of ozone tolerance (Chen and Gallie, 2005). Similarly, overexpression of cytosolic DHAR in transgenic plants exhibited tolerance to MV, salt, exogenous applied-H$_2$O$_2$, cold, ozone and water deficit (Eltayeb et al., 2006, Kwon et al., 2003, Ushimarua et al., 2006).

On the other hand, overexpression of ascorbate oxidase (AO), an apoplast located enzyme resulted in reduced redox state and ozone susceptibility of transgenic plants due to decrease in the ascorbate pool (Sanmartin et al., 2003). Interestingly, antisense suppression of AO in the apoplast of transgenic tobacco plants resulted in MV, and salt tolerance due to low levels of H$_2$O$_2$ accumulation with a high redox state of symplastic and apoplastic ascorbate which in turn permitted higher seed yield (Yamamoto et al., 2005). Conclusively, the engineering of ascorbate recycling or oxidation pathway is also potentially useful to the improvement of ascorbate accumulation and its redox state.

Maintenance of the glutathione pool in the reduced state is a critical component of the ROS scavenging system. Reduced glutathione pool size is influenced by synthesis and recycling pathways, thus approaches to enhance either
of these pathways are summarized in Table 2. The rate limiting regulatory step in the synthesis of GSH is the $\gamma$-glutamyl synthetase enzyme, thus over expression of such an enzyme would increase the GSH pool size. Compelling evidence showed that overexpression of cytosolic $\gamma$-glutamyl synthetase in poplar resulted in increase of the cytosolic GSH pool with no concomitant increase in the chloroplastic one (Hartmann et al., 2003). Previous studies reported that increased GSH contributes to antioxidant protection of plants in response to various environmental stresses (Foyer and Noctor, 2005). Recently, it was reported that overexpression of $\gamma$-glutamyl synthetase and glutathione synthetase in Brassica juncea substantially increased GSH level and tolerance to herbicides (Flocco et al., 2004).

The enzyme glutathione reductase (GR) responsible for the regeneration of glutathione (GSH) in the reduced form at the expense of NAD(P)H has been extensively studied. In the primary attempt, for the enhancement of the plant antioxidant capacity, a bacterial, Escherichia coli, GR was used to increase GSH levels in the cytosol of transgenic tobacco plants. Reports on stress response of tobacco expressing a bacterial glutathione reductase do not agree. Leaves of these transgenic plants were reported to have higher ascorbate levels than control plants after exposure to the bipyridylium herbicide MV; however, the glutathione pool of these transgenic plants was unaltered with no significant effects to MV tolerance (Foyer et al., 1991). In contrast to the wild type, the transgenic tobacco suffered lipid peroxidation under moderate light intensities, while it was found to be more resistant towards oxidative stress induced by paraquat or hydrogen peroxide. Transcript levels for violaxanthin deepoxidase and cytosolic Cu-Zn-superoxide dismutase were strongly reduced in transgenic as compared to control plants (Lederer and Böger, 2003). Given that GR is present in the cytosol and the chloroplasts and that MV induces oxidative damage primarily to the chloroplasts, overexpression of GR in chloroplast would render plants tolerant to photoinhibitory conditions. Thus overexpression of the bacterial GR in tobacco chloroplasts resulted in increased tolerance to MV and sulfur dioxide (Aono et al., 1993). Detailed analysis of transgenic poplar plants overexpressing the bacterial GR in the cytosol or in the chloroplasts resulted in higher expression of GR in the chloroplast than in the cytosol. Moreover the chloroplast-targeted GR overexpression led to increased
levels of both ascorbate and glutathione pools thus increasing the antioxidant capacity to withstand oxidative damage (Foyer et al., 1995). Although these plants did not show increased protection to MV-induced inhibition of CO₂ assimilation, they were found to be more resistant to photoinhibition caused by high light intensity and chilling temperatures (Foyer et al., 1995).

Further studies on transgenic tobacco plants overexpressing the bacterial GR in the cytosol exhibited less photoinhibition than wild-type plants, however repair of photoinhibitory damage was not enhanced (Tyystjärvi et al., 1999). Thus far studies have shown that overexpression of GR in the cytosol of transgenic plants led to increased GR activity although; tolerance to oxidative stress is rather ambiguous. Contrarily, chloroplast-targeted overexpression of GR resulted in enhanced oxidative-stress tolerance, with concomitant increases of reduced ascorbate and glutathione pools, thus boosting the antioxidant capacity. Substantial evidence of chloroplastic overexpression of GR in transgenic Indian mustard (Brassica juncea) plants led to increased Cd-tolerance, while cytosolic overexpression of GR had no effect on heavy metal tolerance (Pilon-Smits et al., 2000). Detailed studies of transgenic poplar plants overexpressing GR either in the cytosol or chloroplasts has shown that reduced glutathione levels were increased in both types of transgenic lines, however, overexpression of GR did not provide any tolerance to acute ozone stress. Intriguingly oxidized glutathione (GSSG) contents of leaves increased in cytosol overexpressing GR plants as well as in wild-type plants upon ozone exposure, with the exception of plants overexpressing GR in the chloroplasts (Strohm et al., 2002). Ozone exposure strongly oxidized foliar ascorbate pool except in leaves overexpressing GR in the chloroplast. Apparently, leaves of these plants possess an enhanced capacity for ascorbate regeneration as a consequence of the strongly elevated chloroplast GR activity and the simultaneously increased APX activity and glutathione levels. However, this enhanced antioxidant capacity did not prevent ozone-mediated injury most likely due to different sites of primary ozone reactions (Strohm et al., 2002).

In recent studies development of transgenic plants overexpressing a plant derived GR in the cytosol or the chloroplasts of transgenic plants have shown that chloroplast-targeted GR provides enhanced protection compare to cytosol
overexpression of GR, however as in previous studies this enhancement of the antioxidant capacity rendered tolerance with limitations. Specifically overexpression of GR in chloroplast of transgenic rice plants provides tolerance to low and high concentrations of MV-induced photoinhibition at 25°C, while a moderate increase of temperature (35°C) restricts tolerance to low concentrations of MV (Kouril et al., 2004). Field-studies of transgenic cotton plants overexpressing an Arabidopsis GR in the chloroplasts does not provide any advantage to photosynthetic performance of transgenic plants in a growing season characterized by variable T°C and photon flux density (Kornyeyev et al., 2005).

Overall, GR overexpression in transgenic plants provides enhancement of tolerance when is targeted to the chloroplasts, however elucidation of the role of GR in response to oxidative stress was not reported. Light in that direction was recently given with the development of antisense transgenic tobacco plants that have 30-70% decreased chloroplastic GR activity (Ding et al., 2009). These antisense GR transgenic plants exhibited sensitivity to MV-induced oxidative stress accompanied with greater decreases in GSH and reduced ascorbate pools that led to increased H2O2 levels. In conclusion, reduction of glutathione pool by chloroplastic GR plays an important role in maintaining the ascorbate pool and the ascorbate redox state, thus limiting accumulation of H2O2 under adverse environmental conditions.

C. Catalase

While APX with a high affinity for H2O2 is acting at the micromolar and submicromolar range, CAT has lower affinity but is a cellular sink for H2O2 being one of the most active catalysts produced by nature. It decomposes H2O2 at an extremely rapid rate, corresponding to a catalytic center activity of about 10^7 min^-1. At low concentrations (<10^{-6} M) of H2O2, it acts "peroxidatically," where a variety of hydrogen donors (e.g., ethanol, ascorbic acid) can be oxidized in the following manner: RH2 + H2O2 \rightarrow R + 2H2O. At high concentrations of substrate, catalase decomposes toxic H2O2 at an extremely rapid rate using the "catalatic" reaction in which H2O2 acts as both acceptor and donor of hydrogen molecules: 2H2O2 \rightarrow2H2O + O2. Under physiological conditions H2O2 is mainly produced in peroxisomes during
photorespiration. Peroxisomal H$_2$O$_2$ production is by far the biggest producer of H$_2$O$_2$ in photosynthetic cells (Foyer and Noctor, 2005). Catalase is located in peroxisomes/glyoxysomes and is mainly scavenging photorespiratory H$_2$O$_2$ without consuming cellular reducing equivalents. Hence, catalase provides the cell with a very energy-efficient mechanism to remove H$_2$O$_2$ and may be uniquely suited to regulate the homeostasis of H$_2$O$_2$ in the cell (Scandalios, 1997).

The first transgenic approaches manipulating CAT levels were about to unravel the role of catalase in tobacco using sense and antisense technology in order to produce CAT deficient plants. The results of these experiments are summarized in Table 4A. *Nicotiana plumbaginifolia* contains three active genes encoding catalase (*Cat1, Cat2, Cat3*), two of which are expressed in mature leaves (Willekens et al., 1994). CAT1 represents ~80% of leaf catalase activity and is located in palisade parenchyma cells. CAT2 accounts for ~20% and is found in the phloem (Willekens et al., 1997). Transgenic *Nicotiana tabacum* lines deficient for *Cat1* (*Cat1AS*), *Cat2* (*Cat2AS*), or both *Cat*-suppressed *CatGH* lines were generated (Chamnongpol et al., 1996) and were tested under various light conditions. *Cat2*-deficient plants did not show any phenotype, under either low- and high-light conditions. *Cat1*-deficiency had no effect at low light, but caused white necrotic lesions on the leaves when plants were exposed to higher light intensities. It was then proved that photorespiration was required for the development of leaf necrosis in *Cat1*-deficient plants (Willekens et al., 1997). Necrosis was rather occurring as an effect of a change of redox balance in the cell, than an evident increase of H$_2$O$_2$ level. These investigators also demonstrated that catalase, apart from its housekeeping function, is an indispensable component of the antioxidant defence against environmental stress. Sustained exposure of *Cat1AS* plants to excess H$_2$O$_2$ provoked tissue damage, stimulated salicylic acid and ethylene production, and induced the expression of acidic and basic PR proteins (Chamnongpol et al., 1998). This perturbation in H$_2$O$_2$ homeostasis provoked ultrastructural alterations, such as chromatin condensation and disruption of mitochondrial integrity, enhanced transcript levels of mitochondrial defense genes and finally induced cell death in clusters of palisade parenchyma cells (Dat et al., 2003). Unexpectedly, double antisense plants lacking catalase and ascorbate peroxidase were less sensitive to oxidative stress than single
antisense plants lacking either ascorbate peroxidase or catalase (Rizhsky et al., 2002). This peculiar result was due to activation of alternative/redundant defense mechanisms that compensate for the lack of both APX and CAT, but not activated when one of the two major H$_2$O$_2$ scavenging mechanisms is functional. Transgenic tobacco expressing an antisense *Cat1* construct (ASCAT1) developed by another group, had catalase levels less than 10% of the untransformed control and developed necrosis and chlorosis at the lower leaves, elevated SA, accumulated very high levels of PR-1 proteins and showed enhanced resistance to tobacco mosaic virus (Takahashi et al., 1997). Transgenic tomato plants expressing an antisense tomato *Cat1* construct (ASTOMCAT1) displayed susceptibility to H$_2$O$_2$-induced oxidative stress and chilling at 4°C (Kerdnaimongkol and Woodson, 1999).

In Arabidopsis there are also three genes *Cat1*, *Cat2* and *Cat3* encoding the catalase gene family (Frugoli et al., 1996). Here we need to mention that the name of each catalase gene is not inferred by phylogenetic or functional relationship but is rather an arbitrary choice of the researchers who first described each gene. Thus tobacco *Cat1* has as functional equivalent in Arabidopsis the *Cat2* gene. Functional characterization of the catalase gene family in *Arabidopsis* was performed using T-DNA insertion or RNAi knockout lines revealing specificity in the roles of each member under abiotic stress. CAT1 has a prominent role in environmental stress defence, while CAT2 and CAT3 confer ROS homeostasis under light or dark conditions, respectively. *Cat2* is also activated by cold and drought, and *Cat3* by ABA, senescence and oxidative treatments (Du et al., 2008). This network of transcriptional control also detected in the regulation of *Cat* gene expression in every species thus far examined like maize (Mylona et al., 1998, Mylona et al., 2007, Polidoros and Scandalios, 1999, Scandalios et al., 1997), tobacco (Willekens et al., 1995, Willekens et al., 1994), rice (Iwamoto et al., 2000, Iwamoto et al., 2004), pepper (Lee and An, 2005), is indicative of how the complex coordinated regulation of antioxidant defences during development may be modulated and differentially expressed in response to different stresses.

Transgenic Arabidopsis genotypes with reduced CAT2 activity had no phenotypic differences with controls when grown in growth chambers or under low light, but were sensitive to ozone and HL photorespiratory H$_2$O$_2$ induced cell death. Growth of
Arabidopsis knock-out mutants for catalase 2 (cat2) in ambient air caused severely decreased rosette biomass, intracellular redox perturbation and activation of oxidative signalling pathways. These effects were absent when cat2 was grown at high CO2 levels to inhibit photorespiration and data revealed that photoperiod was also a critical determinant of the oxidative stress response (Queval et al., 2007). Transcriptome changes were observed in catalase-deficient plants compared to untransformed controls under HL, affecting antioxidant, pathogenesis related and anthocyanin biosynthetic genes. Induction of these genes under HL was reversed or delayed in CAT-deficient plants indicating that the HL regulation of plant transcriptome response is alleviated by accumulation of photorespiratory H2O2 (Vandenabeele et al., 2004, Vanderauwera et al., 2005). In another report the cat2-1 T-DNA insertion mutant exhibited reduced size, a pale green color and great reduction in secondary roots, increased sensitivity to H2O2, NaCl, norspermidine, high light and cold stress. On the other hand, the germination of the cat2-1 mutant was more tolerant to lithium than the wild type (Bueso et al., 2007).

Several attempts were made to enhance CAT activity in transgenic plants in order to restrict elevation of H2O2 levels under stress. The results of these studies are summarized in Table 4B. Plant catalases have two limitations by nature: they have low affinity for their substrate requiring high amounts of H2O2 in order to act with their rapid catalatic mode and they are located exclusively in microbodies limiting the ability to keep the H2O2 concentrations low enough in other cellular compartments. As plants have no catalase in chloroplasts an attempt to enhance the antioxidant capacity of chloroplasts directing catalase there seemed promising. Some of the first attempts were to protect chloroplasts from salt and HL induced oxidative stress as chloroplastic APX seems to be inactivated under these conditions (Shikanai et al., 1998). It was also reasonable to seek a catalase with higher affinity for H2O2 from other species and express the gene with a transit peptide to direct it in chloroplasts. One such catalase is from Escherichia coli encoded by the katE gene which has a higher affinity for H2O2 than plant catalase. Thus, transgenic plants expressing this gene using a chloroplastic transit peptide were evaluated for their phenotype under various stresses. Photosynthesis of transgenic tobacco plants expressing katE in the chloroplasts was found to be tolerant to high irradiance under
drought conditions (Shikanai et al., 1998). The same was observed against the photo-oxidative stress caused by paraquat treatment under illumination (Miyagawa et al., 2000). And similar results were recorded in tomato (Mohamed et al., 2003). Further studies revealed that the $katE$ transgenic tobacco were also tolerant to salinity. These studies suggested that salt stress enhanced photoinhibition by inhibiting repair of PSII and that the $katE$ transgene increased the resistance of the chloroplast’s translational machinery to salt stress by scavenging hydrogen peroxide (Al-Taweel et al., 2007). Similar results demonstrated that the rice cultivar BR5 was rendered tolerant to salt stress when transformed with the $katE$ gene (Moriwaki et al., 2008). More recently the increased protection of transgenic plants expressing the bacterial $katE$ gene in plastids under increasing salt concentrations was also demonstrated in potato (M’Hamdi et al., 2009). However, when a maize catalase gene was introduced to Chinese cabagge chloroplasts, limited protection against salt stress could be observed, while double transformation with the maize catalase and Cu/ZnSOD conferred increased protection against sulfur dioxide and salt stress (Tseng et al., 2007). Taken together these results demonstrate that strengthening antioxidant defence introducing catalase into the chloroplasts is correlated with an increased tolerance of transgenic genotypes towards photoinhibition and salinity-induced stress in various plant species.

Other studies show that overexpression of catalase may provide a protective shield against various stresses in different plants. Overexpression of a $Brassica$ juncea Cat gene in tobacco could reduce the phytotoxicity caused by Cd such as growth inhibition, $H_2O_2$ accumulation, lipid peroxidation and cell death (Guan et al., 2009). Altered photosynthesis was reported in transgenic tobacco plants expressing sense and antisense constructs of tobacco and cotton catalase demonstrating that photorespiratory losses of CO$_2$ were significantly reduced with increasing catalase activities at higher temperatures because of enhanced peroxidation (Brisson et al., 1998). A wheat catalase overexpressed in rice could provide improved tolerance against low temperature stress (Matsumura et al., 2002). Similar results were observed in a low temperature-sensitive rice cultivar transformed with the GST (glutathione S-transferase) or both GST and CAT1 (catalase 1) of Suaeda salsa. GST+CAT1 co-expression conferred greater level of low temperature stress tolerance
to the transformed rice plants compared to the single GST transformed plants (Zhao et al., 2006). These rice plants displayed also markedly enhanced tolerance to salt stress and paraquat (Zhao and Zhang, 2006c). Increase tolerance to paraquat was also reported in transgenic tobacco expressing the maize Cat2 gene (Polidoros et al., 2001). In conclusion, the above data provide enough evidence for a protective role of catalase under environmental stress conditions and the ability to manipulate both, the level and the location of CAT in order to improve stress tolerance in transgenic plants.

However, a different picture has emerged from studies examining the role of catalase manipulation in biotic stress. H$_2$O$_2$ is a signaling and invader killing component in the defence against pathogen attack and perturbation of its levels might affect the ability of the plant to restrict microbes. Hence, in catalase-deficient tobacco, elevated H$_2$O$_2$ level that resulted under high-light conditions was able to trigger local and systemic defence responses similar to the observed in the hypersensitive response (HR) and the programmed cell death (Chamnongpol et al., 1998, Dat et al., 2003, Takahashi et al., 1997). In accordance, the opposite approach revealed that transgenic tobacco genotypes overexpressing the maize Cat2 gene and probably not allowing accumulation of a high enough H$_2$O$_2$ level to kill invading bacteria were less efficient in restricting bacterial growth during manifestation of HR (Polidoros et al., 2001). Similarly, in tobacco plants overexpressing a yeast catalase (CTA1), after infection with tobacco mosaic virus (TMV), less H$_2$O$_2$ accumulation could be detected around necrotic lesions and the size of necrotic lesions was significantly bigger in the infected leaves of the transgenic plants, indicating much lower capacity of these plants to restrict the virus spread (Talarczyk et al., 2002). In contrast, expression of a tobacco catalase transgene activated the endogenous homologous gene and was associated with disease resistance in transgenic potato plants (Yu et al., 1999a). It was speculated that the tobacco and the induced endogenous potato class II catalases served as biological targets for salicylic acid (SA) enhancing its signaling role in disease resistance. The data discussed in this section point to a multifaceted effect of modulating catalase levels that may result in contrasting outcomes under abiotic in comparison to biotic stress conditions. Given that catalase is the major regulator of H$_2$O$_2$ homeostasis in the cell, and the
significant signaling role of H$_2$O$_2$ for deployment of both abiotic and biotic stress defences, it seems that uncontrollable perturbation of the H$_2$O$_2$ levels affect the redox balance and the defence decisions in the cell with contrasting effects that may confer enhanced protection against certain abiotic but reduced protection against biotic insults.

D. Overexpression of Glutathione-S-Transferase Enzymes (GSTs)

Glutathione S-transferase (GST) comprises a family of nuclear encoded enzymes localized mainly in cytosol, whilst plastid, apoplastic and nuclear GSTs were recently reported (Dixon et al., 2009). Plant GST family is classified into a number of subgroups, depending on the species, with distinct structure and specificity. Our view of plant GSTs was profoundly modified by the recent sequencing programs which revealed a number of genes encoding not only the previously identified but also numerous new ones. The primary function of many GSTs is conjugation of reduced glutathione (GSH) to a variety of molecules such as xenobiotics or intermediates of secondary metabolites as well as products of lipid peroxides rendering them less toxic (Marrs, 1996, Mylona et al., 1998, Mylona et al., 2007). Therefore GSTs are key players in phase I (enzymatic modification) and phase II (enzymatic degradation) of detoxification process operating in the cytosol (Abhilash et al., 2009). Certain GSTs play roles as peroxidases or in regenerating ascorbate from DHA (Foyer and Noctor, 2005) (and references therein). GSTs are induced inter alia by ROS and pathogen challenge (Polidoros et al., 2005, Mylona et al., 2007). Taken together enhancement of GST would provide tolerance to abiotic stress. Plant transgenic lines with GST overexpression were only recently developed (see Table 3). Overexpression of cytosolic GST with concomitant glutathione peroxidases (GPX) activity in transgenic tobacco plants enhanced tolerance to heat, chilling and salt stress (Roxas et al., 2000). Overexpression of GST from various plant species in the cytosol of transgenic plants provided tolerance to herbicide, MV, salt, chilling, heat, high light, water logging and Cu-induced stress (Milligan et al., 2001, Pilon-Smits et al., 2000, Takesawa et al., 2002, Yu et al., 2003, Lim et al., 2005, Zhao and Zhang, 2006a, Skipsey et al., 2005). Nevertheless, evidence that overexpression of a
cytosolic GST in Arabidopsis did not provide tolerance to salt stress of the whole plant was attributed to the osmotic imbalance exerted by the stressor (Katsuhara et al., 2005). Similarly, overexpression of tobacco cytosolic GST in transgenic cotton plants failed to provide tolerance to salt, herbicides and cold stress in young seedlings. Intriguingly, antioxidant enzyme activities including APX, SOD, GR, GPX and MDAR among transgenic and control plants were similar (Light et al., 2005). Noteworthy that GST-transgenic plants exhibited increased levels of malondialdehyde (MDA) content, an indicator of lipid peroxidation, under normal conditions while under salt stress MDA content was not changed. In addition, levels of oxidized glutathione were increased under salt stress in GST-transgenic plants compared to wild type, indicating that overexpression of GST in cotton has disrupted the endogenous antioxidant system.

Glutathione peroxidases (GPXs) comprise a group of enzymes capable of eliminating H₂O₂ in the ascorbate-glutathione cycle (Foyer et al., 1997). The major function of GPXs is the reduction of phospholipid hydroperoxides to form corresponding alcohols using thioredoxins (Trx) as preferred electron donors (Navrot et al., 2007). Thereby, the incorrectly named GPXs are actually thiol-dependent peroxidases that protect cell membranes from peroxidative damage, maintaining cellular integrity. Recent evidence has shown that GPX comprise a subgroup of the peroxiredoxin family (Prx). GPXs are present in most subcellular compartments and are involved in the response to both abiotic and biotic stresses acting as general peroxide scavengers (Navrot et al., 2007). Thus GPXs are considered as redox sensors transmitting information of the cellular levels of ROS to the redox network (Foyer and Noctor, 2005). Based on this characteristic overexpression of GPX in the cytosol or chloroplasts of transgenic tobacco plants rendered tolerance to chilling, high light, salt and MV stress (Yoshimura et al., 2004).

E. Alternative oxidase

Besides cytochrome c oxidase, plant mitochondria have an alternative oxidase (AOX), which accepts electrons directly from ubiquinol, dissipating energy as heat. AOX up-regulation occurs under a variety of stresses and its induction by salicylic
acid (SA) has been observed in different plant species. AOX is encoded by a small multigene family typically with 4-5 members in higher plants (Polidoros et al., 2009). Specific Aox alleles are among the first and most intensively stress-induced genes in several experimental systems involving oxidative stress. It has been suggested that the level of AOX influences the antioxidant network establishing the steady-state level of reactive oxygen species in the cell and is related to growth conditions in such a manner that provide metabolic homeostasis and a degree of signaling homeostasis from the mitochondrion (Vanlerberghe et al., 2009). These authors in their recent comprehensive review are examining the functional role of AOX summarizing the results of reverse genetic experiments conducted to manipulate AOX levels of the cell. The availability of the Vanlerberghe et al. (2009) review make the AOX-transgenics results summary in this chapter redundant and the reader is referred to the mentioned paper for an excellent synopsis. We should though mention that among 20 studies cited (Amirsadeghi et al., 2006, Bartoli et al., 2006, Fiorani et al., 2005, Gilliland et al., 2003, Giraud et al., 2008, Guy and Vanlerberghe, 2005, Hiser et al., 1996, Kitashiba et al., 1999, Maxwell et al., 1999, Ordog et al., 2002, Pasqualini et al., 2007, Robson and Vanlerberghe, 2002, Robson et al., 2008, Sieger et al., 2005, Strodtkotter et al., 2009, Sugie et al., 2006, Umbach et al., 2005, Vanlerberghe et al., 1995, Vanlerberghe et al., 2002, Watanabe et al., 2008) most of them have documented changes in ROS levels, expression or activity of ROS-scavenging enzymes or changes in oxidative damage in plants after the alteration of AOX levels, although the functional relationship between AOX and components of the reactive oxygen network still needs clarification.

F. Overexpression of multiple antioxidant enzymes

Recent trend in plant biotechnology is the so called “pyramiding” of traits in a single variety by simultaneous overexpression of two or more genes enhancing different properties e.g pest resistance and herbicide tolerance. Such efforts of pyramiding antioxidant genes with consecutive or different functions have been attempted in several studies. Accordingly, an approach was to couple overexpression of a SOD isoform to dismutate $\mathrm{O}_2^-$ to form $\mathrm{H}_2\mathrm{O}_2$ with overexpression of APX or CAT, both able to reduce the SOD-overproduced $\mathrm{H}_2\mathrm{O}_2$ to water and $\mathrm{O}_2$. Overexpression of
a cytosolic Cu/ZnSOD along with a cytosolic APX in the chloroplasts of transgenic tall fescue plants exhibited tolerance to MV, exogenously applied H$_2$O$_2$ and heavy metals such as copper, cadmium and arsenate (Kouril et al., 2004). Similarly, overexpression of cytosolic Cu/ZnSOD along with peroxisomal CAT in the chloroplasts of transgenic cabbage exhibited increased tolerance to salt and sulfite (Tseng et al., 2007). In another line of research overexpression of γ-glutamyl synthetase and glutathione synthetase in *Brassica juncea* substantially increased GSH level and tolerance to herbicides (Flocco et al., 2004).

Other researchers tried the enhancement of non-related enzymatic defences. For example a low temperature-sensitive rice cultivar was transformed with the GST (glutathione S-transferase) or both GST and CAT1 (catalase 1) of *Suaeda salsa*. The double transformed genotype was more tolerant to low temperature, salt stress and paraquat (Zhao and Zhang, 2006b, Zhao and Zhang, 2006c, Zhao et al., 2006). Interestingly, co-expression of cytosolic forms of both GR and SOD in transgenic tobacco plants provided enhanced tolerance to MV than overexpression of either enzyme alone (Aono et al., 1995). Leaves of these transgenic plants had higher levels of reduced ascorbate and GSH. Conclusively, these results indicate that overexpression of combination of antioxidant enzymes in transgenic plants may have synergistic effects on stress tolerance.

Such studies along with compelling evidence of transcriptome profile analysis of antioxidant genes documenting coordinated responses of multiple members of this defence network under stress, point to the significance of pyramiding antioxidant gene defences in transgenic plants to enhance abiotic stress tolerance.

**CONCLUSIONS**

Stress tolerance in plants is a complex genetic trait and several mechanisms cooperate to achieve the end result. Increases in antioxidant enzyme activities are often related with stress tolerant plant genotypes in various species and overexpression of a particular antioxidant enzyme is anticipated to confer a degree of cross-tolerance against various stresses, although this might be not enough for protection of the organism against other physiological defects. Accumulated
evidence clearly demonstrates that increased expression of specific antioxidant enzymes can provide plants with increased oxidative stress tolerance and a degree of cross-tolerance against different abiotic stress conditions. Pyramiding overexpression of antioxidant genes that either act together in an antioxidant pathway or act in separate pathways has in several cases multiplicative effect in stress tolerance. Nevertheless, performance of the transgenic plants under field conditions and usefulness of the approach for breeding stress tolerant crops still need to be proved.

On the other hand, increased production of ROS under stress conditions may fulfill two different roles: the one is damaging to important components of the cell’s machinery resulting ultimately in cell death, whereas the other is signaling for launching defence responses that might restrict damage and enhance survival of the cell. Therefore ROS scavenging mechanisms are tightly regulated and are decisive for the direction that ROS will lead a cell facing stress. Our attempts to manipulate the expression of these mechanisms in order to strengthen the antioxidant defence network may have unpredicted outcome. There is compelling evidence that uncontrollable increase of specific enzymes, especially those which are involved in scavenging of the important signaling molecule H$_2$O$_2$ may have contrasting effects in the ability of the plant to withstand abiotic than biotic stress. Data show that increased tolerance to abiotic stress may be accompanied with decreased ability to restrict invading pathogens. Therefore, a future research direction should aim to control expression of antioxidant transgenes providing differential regulation under abiotic than biotic stress conditions. This could be achieved by differentially regulated rather than constitutive promoters which have been used in almost all the antioxidant transgenic genotypes so far.

Another important aspect of the use of antioxidant transgenic plants in recent studies is that they provided tools to better understand the antioxidant gene network and the relationship of its members under stress. It is likely that this knowledge will be valuable in our efforts to produce transgenic plants able to tolerate field conditions with multiple environmental stresses.

TABLES
Table 1. Plants overexpressing SOD isoforms.

<table>
<thead>
<tr>
<th>Gene/plant source</th>
<th>Location in Transgenic</th>
<th>Transgenic Plant</th>
<th>Tolerance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu/ZnSOD chloroplastic, petunia</td>
<td>chloroplast</td>
<td>Tobacco, tomato</td>
<td>No protection</td>
<td>(Tepperman and Dunsmuir, 1990)</td>
</tr>
<tr>
<td>Cu/ZnSOD chloroplastic, pea</td>
<td>chloroplast</td>
<td>Tobacco</td>
<td>High light, chilling, MV</td>
<td>(Gupta et al., 1993a)</td>
</tr>
<tr>
<td>Cu/ZnSOD cytosolic, pea</td>
<td>cytosol</td>
<td>Tobacco</td>
<td>Ozone</td>
<td>(Pitcher and Zilinskas, 1996)</td>
</tr>
<tr>
<td>Cu/ZnSOD cytosolic, rice</td>
<td>chloroplast</td>
<td>Tobacco</td>
<td>Salt, water, PEG, MV, SO₃</td>
<td>(Badawi et al., 2004b)</td>
</tr>
<tr>
<td>Cu/ZnSOD cytosolic, Maize CAT peroxisomal, Maize</td>
<td>Cu/ZnSOD chloroplast or CAT Chloroplast or Cu/ZnSOD+CAT chloroplast</td>
<td>Chinese cabbage</td>
<td>Salt, SO₂</td>
<td>(Tseng et al., 2007)</td>
</tr>
<tr>
<td>Cu/ZnSOD cytosolic, Cassava APX cytosolic, Pea</td>
<td>Chloroplast Cu/ZnSOD+APX</td>
<td>Tall fescue</td>
<td>MV, H₂O₂, Heavy metals (Cu, Cd, As)</td>
<td>(Lee et al., 2007a)</td>
</tr>
<tr>
<td>Cu/ZnSOD cytosolic, mangrove</td>
<td>cytosol</td>
<td>Rice</td>
<td>Salt, drought, MV</td>
<td>(Prashanth et al., 2008)</td>
</tr>
<tr>
<td>MnSOD mitochondrial, tobacco</td>
<td>Mitochondria or chloroplasts</td>
<td>Tobacco</td>
<td>MV</td>
<td>(Bowler et al., 1991a)</td>
</tr>
<tr>
<td>MnSOD mitochondria, Tobacco</td>
<td>Mitochondria or chloroplasts</td>
<td>Tobacco</td>
<td>Ozone</td>
<td>(Van Camp et al., 1994, Van Camp et al., 1996)</td>
</tr>
<tr>
<td>MnSOD mitochondrial, FeSOD chloroplastic, Tobacco</td>
<td>Mitochondria MnSOD or Chloroplast MnSOD or Chloroplast FeSOD</td>
<td>Alfalfa</td>
<td>Freezing, drought, winter-survival &amp; -hardiness</td>
<td>(McKersie et al., 1993, McKersie et al., 1996, McKersie et al., 1999, McKersie et al., 2000, Rubio et al., 2002)</td>
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<tr>
<td>MnSOD mitochondrial, Tobacco</td>
<td>chloroplast</td>
<td>Maize</td>
<td>Cold, oxidative stress</td>
<td>(Van Breusegem et al., 1999)</td>
</tr>
<tr>
<td>MnSOD mitochondrial, Tobacco FeSOD chloroplastic, Arabidopsis</td>
<td>Mitochondria MnSOD or Chloroplast MnSOD or Chloroplast FeSOD</td>
<td>Tobacco</td>
<td>Mn deficiency</td>
<td>(Yu et al., 1999b)</td>
</tr>
<tr>
<td>MnSOD mitochondrial, Wheat</td>
<td>mitochondria</td>
<td>Canola</td>
<td>Al, MV</td>
<td>(Basu et al., 2001)</td>
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<td>MnSOD mitochondrial, pea</td>
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<td>Rice</td>
<td>Drought, PEG, MV</td>
<td>(Wang et al., 2004, Wang et al., 2005a)</td>
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<td>MnSOD mitochondrial, Arabidopsis</td>
<td>mitochondria</td>
<td>Arabidopsis</td>
<td>Salt</td>
<td>(Wang et al., 2004)</td>
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<tr>
<td>MnSOD mitochondria, Tamarix</td>
<td>mitochondria</td>
<td>Poplar</td>
<td>Salt</td>
<td>(Wang et al., 2009)</td>
</tr>
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<td>FeSOD chloroplastic, Arabidopsis</td>
<td>chloroplast</td>
<td>Tobacco</td>
<td>Oxidative stress</td>
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</tr>
<tr>
<td>MnSOD mitochondrial FeSOD chloroplastic, Arabidopsis</td>
<td>MnSOD mitochondria or MnSOD chloroplasts or FeSOD chloroplasts</td>
<td>Tobacco</td>
<td>water logging</td>
<td>(Yu et al., 1999b)</td>
</tr>
</tbody>
</table>
**Table 2.** Plants overexpressing enzymes of the ascorbate-glutathione pathway. Ascorbate peroxidase, APX; ascorbate oxidase, AO; mono-dehydroascorbate reductase, MDAR; dehydroascorbate reductase, DHAR; γ-glutamyl synthetase γ-ECS; glutathione synthetase GSHS and glutathione reductase, GR.

<table>
<thead>
<tr>
<th>Gene/Source</th>
<th>Location in Transgenic Plant</th>
<th>Transgenic Plant</th>
<th>Tolerance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>APX cytosolic, Pea</td>
<td>Cytosol or chloroplast</td>
<td>Tobacco</td>
<td>MV</td>
<td>(Pitcher et al., 1994)</td>
</tr>
<tr>
<td>APX cytosolic Pea</td>
<td>Chloroplast</td>
<td>Tobacco</td>
<td>No protection to ozone</td>
<td>(Torsethaugen et al., 1997)</td>
</tr>
<tr>
<td>APX peroxisomal, Arabidopsis</td>
<td>Peroxisomes</td>
<td>Tobacco</td>
<td>Aminotriazole, no MV protection</td>
<td>(Wang et al., 1999)</td>
</tr>
<tr>
<td>APX peroxisomal, Barley</td>
<td>Peroxisomes</td>
<td>Arabidopsis</td>
<td>Heat stress, Zn, Cd, salt</td>
<td>(Shi et al., 2001) (Xu et al., 2008)</td>
</tr>
<tr>
<td>APX cytosolic, Pea</td>
<td>Chloroplasts</td>
<td>Cotton</td>
<td>Photoprotection chilling stress and high light</td>
<td>(Payton et al., 2001, Kornyeyev et al., 2001)</td>
</tr>
<tr>
<td>APX cytosolic, Pea+Cu/ZnSOD or APX+MnSOD</td>
<td>Chloroplast</td>
<td>Tobacco</td>
<td>MV</td>
<td>(Kwon et al., 2002)</td>
</tr>
<tr>
<td>APX chloroplastic, Arabidopsis</td>
<td>Chloroplasts</td>
<td>Arabidopsis</td>
<td>MV, NO No tolerance to high light, cold, Fe, Cu</td>
<td>(Murgia et al., 2004)</td>
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<tr>
<td>APX cytosolic, Pea</td>
<td>Cytosol</td>
<td>Tomato</td>
<td>Chilling, salt stress</td>
<td>(Wang et al., 2005b)</td>
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<tr>
<td>APX peroxisomal, Arabidopsis</td>
<td>Peroxisomes</td>
<td>Tobacco</td>
<td>Drought</td>
<td>(Yan et al., 2003)</td>
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<td>APX cytosolic, Rice</td>
<td>Cytosol</td>
<td>Arabidopsis</td>
<td>Salt</td>
<td>(Lu et al., 2007)</td>
</tr>
<tr>
<td>APX cytosolic Cu/ZnSOD DHAR</td>
<td>APX+DHAR+Cu/ZnSOD chloroplasts</td>
<td>Tobacco</td>
<td>MV, Salt</td>
<td>(Lee et al., 2007b)</td>
</tr>
<tr>
<td>APX chloroplastic, Red alga</td>
<td>Chloroplasts</td>
<td>Arabidopsis</td>
<td>MV, heat</td>
<td>(Hirooka et al., 2009)</td>
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<tr>
<td>MDAR cytosolic, Arabidopsis</td>
<td>Cytosol</td>
<td>Tobacco</td>
<td>Ozone, salt, PEG</td>
<td>Eltayeb, et al 2007</td>
</tr>
<tr>
<td>DHAR cytosol, human</td>
<td>Cytosol</td>
<td>Tobacco</td>
<td>MV, salt, H2O2, cold</td>
<td>(Kwon et al., 2003)</td>
</tr>
<tr>
<td>DHAR cytosolic, Wheat</td>
<td>Cytosol</td>
<td>Tobacco</td>
<td>Ozone</td>
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<tr>
<td>DHAR</td>
<td>Tobacco</td>
<td>Salt tolerance</td>
<td>(Ushimarua et al., 2006)</td>
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<td>DHAR cytosolic, Arabidopsis</td>
<td>Cytosol</td>
<td>Tobacco</td>
<td>Ozone, drought, salt</td>
<td>(Eltayeb et al., 2006)</td>
</tr>
<tr>
<td>DHAR cytosolic, Human APX Cu/ZnSOD</td>
<td>DHAR+APX+Cu/ZnSOD chloroplasts</td>
<td>Tobacco</td>
<td>MV, Salt</td>
<td>(Lee et al., 2007b)</td>
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<td>AO cytosolic, cucumber</td>
<td>Apoplast</td>
<td>Tobacco</td>
<td>Reduced redox state Ozone-, oxidative stress-susceptibility</td>
<td>(Sanmartin et al., 2003)</td>
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<td>AO tobacco</td>
<td>Apoplast</td>
<td>Tobacco</td>
<td>MV and Salt sensitivity, increased seed yield</td>
<td>(Yamamoto et al., 2005)</td>
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<td>γ-ECS E.coli</td>
<td>Cytosol</td>
<td>Poplar</td>
<td>Cytosolic increase of GSH No chloroplastic GSH increase</td>
<td>(Hartmann et al., 2003)</td>
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<tr>
<td>γ-ECS + GSHS</td>
<td>Cytosol</td>
<td>Brassica juncea</td>
<td>Increase of GSH, tolerance to organic pollutants</td>
<td>(Flocco et al., 2004)</td>
</tr>
<tr>
<td>Source</td>
<td>Location</td>
<td>Plant</td>
<td>Tolerance/Effect</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------------------</td>
<td>----------------</td>
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<tr>
<td><strong>E. coli</strong></td>
<td>Cytosol, chloroplast</td>
<td>Poplar</td>
<td>Tolerance to photoinhibition</td>
<td>(Foyer et al., 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tobacco</td>
<td>Tolerance to photoinhibition with chloroplastic GR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tobacco</td>
<td>overexpression</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tobacco</td>
<td>No tolerance with cytosolic GR overexpression</td>
<td></td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>Cytosol</td>
<td>Tobacco</td>
<td>Tolerance to high light at 20°C</td>
<td>(Tyystjärvi et al., 1999)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>Cytosol, chloroplast</td>
<td><em>Brassica juncea</em></td>
<td>Tolerance to Cd stress</td>
<td>(Pilon-Smits et al., 2000)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>Cytosol, chloroplast</td>
<td>Poplar</td>
<td>No protection to ozone stress</td>
<td>(Strohm et al., 2002)</td>
</tr>
<tr>
<td><strong>Brassica campestris</strong></td>
<td>Cytosol</td>
<td>Rice</td>
<td>Tolerance to MV induced photo-oxidative stress at 25°C</td>
<td>(Kouril et al., 2004)</td>
</tr>
<tr>
<td><strong>Arabidopsis</strong></td>
<td>chloroplast</td>
<td>Cotton</td>
<td>No advantage to photoinhibition</td>
<td>(Kornyeyeva et al., 2005)</td>
</tr>
<tr>
<td><strong>Tobacco</strong></td>
<td>Silencing chloroplast GR</td>
<td>Tobacco-antisense</td>
<td>Sensitivity to oxidative stress</td>
<td>(Ding et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tobacco</td>
<td>Low levels of GSH and ascorbate</td>
<td></td>
</tr>
<tr>
<td><strong>bacterial +Cu/ZnSOD</strong></td>
<td>Cytosol</td>
<td>Tobacco</td>
<td>MV tolerance, increased GSH and ascorbate, no ozone tolerance</td>
<td>(Aono et al., 1995)</td>
</tr>
<tr>
<td>Gene</td>
<td>Location</td>
<td>Transgenic Plant</td>
<td>Tolerance</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------------</td>
<td>------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>GST maize</td>
<td>cytosol</td>
<td>wheat</td>
<td>Chloroacetanilide &amp; thiocarbamate herbicides</td>
<td>(Milligan et al., 2001)</td>
</tr>
<tr>
<td>GST rice</td>
<td>cytosol</td>
<td>Rice</td>
<td>Chilling, growth at low temperatures &amp; submergence, herbicide</td>
<td>(Takesawa et al., 2002, Pilon-Smits et al., 2000)</td>
</tr>
<tr>
<td>GST cotton</td>
<td>cytosol</td>
<td>Tobacco</td>
<td>MV</td>
<td>(Yu et al., 2003)</td>
</tr>
<tr>
<td>GST tobacco</td>
<td>cytosol</td>
<td>Arabidopsis</td>
<td>Salt, no whole-plant salt resistance</td>
<td>(Katsuhara et al., 2005)</td>
</tr>
<tr>
<td>GST tobacco</td>
<td>cytosol</td>
<td>Dianthus</td>
<td>Drought, high light, Cu</td>
<td>(Lim et al., 2005)</td>
</tr>
<tr>
<td>GST tobacco</td>
<td>cytosol</td>
<td>Cotton</td>
<td>No protection</td>
<td>(Light et al., 2005)</td>
</tr>
<tr>
<td>GST soybean</td>
<td>cytosol</td>
<td>Tobacco</td>
<td>Herbicides</td>
<td>(Skipsey et al., 2005)</td>
</tr>
<tr>
<td>GST lepidoptera</td>
<td>cytosol</td>
<td>Arabidopsis</td>
<td>cold</td>
<td>(Huang et al., 2009)</td>
</tr>
<tr>
<td>GST+GPX tobacco</td>
<td>cytosol</td>
<td>Tobacco</td>
<td>Heat, chilling, salt</td>
<td>(Roxas et al., 1997, Roxas et al., 2000)</td>
</tr>
<tr>
<td>GST+CAT Suaeda salsa</td>
<td>cytosol</td>
<td>Rice</td>
<td>Salt, MV, chilling</td>
<td>(Zhao and Zhang, 2006a)</td>
</tr>
<tr>
<td>GPX chlamydomonas</td>
<td>Cytosol, chloroplast</td>
<td>Tobacco</td>
<td>MV, salt, chilling, high light</td>
<td>(Yoshimura et al., 2004)</td>
</tr>
</tbody>
</table>
### A. Plants silencing catalase

<table>
<thead>
<tr>
<th>Gene/Genotype</th>
<th>Location</th>
<th>Transgenic Plant</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat1AS</td>
<td>peroxisomes</td>
<td>tobacco</td>
<td>Necrotic lesions under HL, tissue damage, stimulated SA and ethylene production, and induced PR gene expression, chromatin condensation and disruption of mitochondrial integrity, enhanced transcript levels of mitochondrial defense genes, cell death</td>
<td>(Chamnongpol et al., 1996, Chamnongpol et al., 1998, Dat et al., 2003, Willekens et al., 1997)</td>
</tr>
<tr>
<td>Cat1AS and APX double antisense</td>
<td>peroxisomes</td>
<td>tobacco</td>
<td>Resistance to oxidative stress</td>
<td>(Rizhsky et al., 2002)</td>
</tr>
<tr>
<td>ASCAT1</td>
<td>peroxisomes</td>
<td>tobacco</td>
<td>Necrosis, chlorosis in lower leaves, elevated SA and activation of PR1, resistance to TMV</td>
<td>(Takahashi et al., 1997)</td>
</tr>
<tr>
<td>Cat2AS</td>
<td>peroxisomes</td>
<td>tobacco</td>
<td>No phenotype</td>
<td></td>
</tr>
<tr>
<td>Cat2 antisense</td>
<td>peroxisomes</td>
<td>arabidopsis</td>
<td>Sensitivity to Ozone and HL; delayed and reduced HL-dependent induction of antioxidant, HR-related and anthocyanin biosynthetic genes</td>
<td>(Chamnongpol et al., 1996)</td>
</tr>
<tr>
<td>cat2-1 T-DNA mutants</td>
<td>peroxisomes</td>
<td>arabidopsis</td>
<td>Reduced size, pale green color, reduction in secondary roots; increased sensitivity to H₂O₂, NaCl, norspermidine, HL, cold stress; germination tolerant to lithium</td>
<td>(Bueso et al., 2007)</td>
</tr>
<tr>
<td>cat2-1 T-DNA mutants</td>
<td>peroxisomes</td>
<td>arabidopsis</td>
<td>Increased CAT1 and CAT3 expression, higher increase of H₂O₂ levels under cold stress</td>
<td>(Du et al., 2008)</td>
</tr>
<tr>
<td>cat1-01 and cat1-04 (RNAi lines)</td>
<td>peroxisomes</td>
<td>arabidopsis</td>
<td>No phenotype / ND</td>
<td>(Du et al., 2008)</td>
</tr>
<tr>
<td>cat3-03 and cat3-09 (RNAi lines)</td>
<td>peroxisomes</td>
<td>arabidopsis</td>
<td>No phenotype / ND</td>
<td>(Du et al., 2008)</td>
</tr>
<tr>
<td>ASTOMCAT1</td>
<td>peroxisomes</td>
<td>tomato</td>
<td>Susceptibility to oxidative stress and chilling</td>
<td>(Kerdnaimongkol and Woodson, 1999)</td>
</tr>
<tr>
<td>cat2</td>
<td>peroxisomes</td>
<td>arabidopsis</td>
<td>Decreased rosette biomass, intracellular redox perturbation and activation of oxidative signalling pathways when grown in ambient air</td>
<td>(Queval et al., 2007)</td>
</tr>
</tbody>
</table>

### B. Plants overexpressing catalase

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Transgenic Plant</th>
<th>Tolerance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli katE</td>
<td>chloroplast</td>
<td>tobacco</td>
<td>Photosynthetic tolerant to HL under drought</td>
<td>(Shikanai et al., 1998)</td>
</tr>
<tr>
<td>E. coli katE</td>
<td>chloroplast</td>
<td>tobacco</td>
<td>Paraquat under HL</td>
<td>(Miyagawa et al., 2000)</td>
</tr>
<tr>
<td>E. coli katE</td>
<td>chloroplast</td>
<td>tomato</td>
<td>Paraquat under HL</td>
<td>(Mohamed et al., 2003)</td>
</tr>
<tr>
<td>E. coli katE</td>
<td>chloroplast</td>
<td>tobacco</td>
<td>HL, salinity</td>
<td>(Al-Taweel et al., 2007)</td>
</tr>
<tr>
<td>E. coli katE</td>
<td>chloroplast</td>
<td>rice</td>
<td>HL, salinity</td>
<td>(Moriwaki et al., 2008)</td>
</tr>
<tr>
<td>E. coli katE</td>
<td>chloroplast</td>
<td>potato</td>
<td>HL, salinity</td>
<td>(M’Hamdi et al., 2009)</td>
</tr>
<tr>
<td>Z. mays Cat</td>
<td>chloroplast</td>
<td>Chinese cabbage</td>
<td>Limited protection to salinity</td>
<td>(Tseng et al., 2007)</td>
</tr>
<tr>
<td>Z. mays Cat and Cu/ZnSOD</td>
<td>chloroplast</td>
<td>Chinese cabbage</td>
<td>Protection to SO₂ and salinity</td>
<td>(Tseng et al., 2007)</td>
</tr>
<tr>
<td>Brassica juncea CAT3</td>
<td>peroxisome?</td>
<td>tobacco</td>
<td>Reduced Cd phytotoxicity</td>
<td>(Guan et al., 2009)</td>
</tr>
<tr>
<td>N. tabacum CAT-1</td>
<td>peroxisome?</td>
<td>tobacco</td>
<td>Reduced photorespiratory losses of CO₂</td>
<td>(Brisson et al., 1998)</td>
</tr>
<tr>
<td>Species</td>
<td>Enzyme (Gene)</td>
<td>Organism</td>
<td>Trait</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------</td>
<td>-----------</td>
<td>--------------------------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>Wheat</td>
<td>CAT</td>
<td>rice</td>
<td>Cold tolerance</td>
<td>(Matsumura et al., 2002)</td>
</tr>
<tr>
<td>Suaeda salsa</td>
<td>GST and CAT1</td>
<td>rice</td>
<td>Tolerance to salt and paraquat</td>
<td>(Zhao and Zhang, 2006c)</td>
</tr>
<tr>
<td>Suaeda salsa</td>
<td>GST and CAT1</td>
<td>rice</td>
<td>Tolerance to paraquat; less efficient in restricting bacterial growth</td>
<td>(Zhao et al., 2006)</td>
</tr>
<tr>
<td>Z. mays</td>
<td>Cat2</td>
<td>tobacco</td>
<td>Tolerance to paraquat; less efficient in restricting bacterial growth</td>
<td>(Polidoros et al., 2001)</td>
</tr>
<tr>
<td>Yeast</td>
<td>CTA1</td>
<td>peroxisome?</td>
<td>less efficient in restricting necrotic lesion after TMV infection</td>
<td>(Talarczyk et al., 2002)</td>
</tr>
<tr>
<td>N. tabacum</td>
<td>CAT2</td>
<td>potato</td>
<td>constitutive expression of the endogenous potato Cat2St gene; enhanced resistance to P. infestans</td>
<td>(Yu et al., 1999a)</td>
</tr>
</tbody>
</table>
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