Biotechnological Perspective of Reactive Oxygen Species (ROS)-Mediated Stress Tolerance in Plants

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Thammineni Chakradhar, Srikrishna Mahanty, Ramesha A. Reddy, Kummari Divya, Palakolanu Sudhakar Reddy, and Malireddy K. Reddy

Abstract

All environmental cues lead to develop secondary stress conditions like osmotic and oxidative stress conditions that reduces average crop yields by more than 50% every year. The univalent reduction of molecular oxygen (O_2) in metabolic reactions consequently produces superoxide anions ($O_2^{\bullet -}$) and other reactive oxygen species (ROS) ubiquitously in all compartments of the cell that disturbs redox potential and causes threat to cellular organelles. The production of ROS further increases under stress conditions and especially in combination with high light intensity. Plants have evolved different strategies to minimize the accumulation of excess ROS like avoidance mechanisms such as physiological adaptation, efficient photosystems such as C4 or CAM metabolism and scavenging mechanisms through production of antioxidants and antioxidative enzymes. Ascorbate-glutathione pathway plays an important role in detoxifying excess ROS in plant cells, which includes superoxide dismutase (SOD) and ascorbate peroxidase (APX) in detoxifying $O_2^{\bullet -}$ radical and hydrogen peroxide (H_2O_2)

T. Chakradhar • S. Mahanty • R.A. Reddy • M.K. Reddy
Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Marg, New Delhi 110 067, India

K. Divya
Cell, Molecular Biology & Genetic Engineering Group, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad 502 324, Telangana, India

P.S. Reddy (✉)
Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Marg, New Delhi 110 067, India

Cell, Molecular Biology & Genetic Engineering Group, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad 502 324, Telangana, India
e-mail: p.sudhakarreddy@cgiar.org; palakolanusreddy@gmail.com

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respectively, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) involved in recycling of reduced substrates such as ascorbate and glutathione. Efficient ROS management is one of the strategies used by tolerant plants to survive and perform cellular activities under stress conditions. The present chapter describes different sites of ROS generation and and their consequences under abiotic stress conditions and also described the approaches to overcome oxidative stress through genomics and genetic engineering.

Keywords
Ascorbate-glutathione cycle • Abiotic stress • Oxidative stress • Reactive oxygen species

Abbreviations

APX	Ascorbate peroxidase
ASA	Ascorbate
CAT	Catalase
DHAR	Dehydroascorbate reductase
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
MDHAR	Monodehydroascorbate reductase
ROS	Reactive oxygen species
SOD	Superoxide dismutase

3.1 Introduction

In daily life, plants encounter different abiotic stresses such as water deficit, extreme temperatures, high salinity, high light intensity, heavy metals and more often combination of these stresses under field conditions. However, plants cannot escape from these harsh environmental stresses due to their sessile life. Although all plants are equipped with adaptive mechanisms, to encounter such environmental cues, difference in their allelic constituency has left few crop plants vulnerable. It is estimated that the average yields are reduced to 50%, due to such abiotic stress factors (Vij and Tyagi 2007). Therefore, to meet the food demand of increasing population and to cope up with ever-changing drastic climatic conditions due to the

global warming effects, there is an urgent need to develop varieties/hybrids that yields better under abiotic stresses. All the primary stresses like drought, salinity and extreme temperatures leads to secondary stresses such as osmotic and oxidative stresses at cellular level. All living organisms utilize oxygen as reducing agent to generate chemical energy, i.e. ATP during active electron transport system. Paradoxically, the univalent reduction of O_2 in metabolic reactions consequently produces superoxide anions ($O_2^{\bullet -}$) ubiquitously in all compartments of the cell. In addition to the respiratory chain, the O_2 becomes an electron acceptor in photosynthetic electron transport chain in plants during light reaction and generates large amount of $O_2^{\bullet -}$, and their production further increases under various environmental stresses and especially in combination with high light intensity (Asada 2006). These $O_2^{\bullet -}$anions dismutate to H_2O_2, and in the presence of metal ions, and further $O_2^{\bullet -}$ anions react again with H_2O_2 to generate highly reactive hydroxyl radicals ($^{\bullet}OH$). These $^{\bullet}OH$ radical can react with DNA, proteins, lipids and other organic constituents of the cell and cause severe damage.

Plants have evolved different mechanisms to minimize the accumulation of excess ROS like avoidance mechanisms such as anatomical adaptation, suppression of photosynthesis and physiological adaptation such as C4 or CAM metabolism. These processes avoid production of excess ROS under environmental stress conditions. Scavenging mechanisms like production of antioxidants and antioxidative enzymes remove excess ROS produced during abiotic stresses. Efficient ROS detoxification is one of the strategies used by stress-tolerant plants for their survival. Therefore, in the present chapter, we focused on generation and maintenance of ROS in plant cell, and discussed approaches to minimize the damage caused by oxidative stress. Efforts made in transgenic technology using antioxidant genes, to overcome oxidative stress, have also been analysed. Finally, opportunities available in modern technologies like genomics and challenges to consider in overcoming oxidative stress tolerance are presented.

3.2 Antioxidants Involved in Scavenging Pathways by Regulating Molecular and Physiological Approaches

The balance between generation and detoxification of ROS is altered when plants are encountered with different abiotic stress conditions. ROS are also involved in signalling of plant growth, development and perception of responses in biotic and abiotic stresses. However, at higher concentration, they react and damage a number of biomolecules and eventually lead to programmed cell death (PCD) (Miller et al. 2008; Petrov and Van Breusegem 2012). Plants have evolved array of antioxidants and antioxidative enzymes along with other small molecules to detoxify ROS in different organelles. However, ROS generation and scavenging is imbalanced under stress conditions leading to oxidative stress (Tripathy and Oelmüller 2012). Enzymatic ROS scavenging of plants include SOD, catalase (CAT), glutathione peroxidise (GPX), glutathione S-transferase (GST), peroxiredoxins (PRX) and ascorbate-glutathione (AsA-GSH) pathway enzymes consisting of APX,

MDHAR, DHAR and GR. Non-enzymatic ROS detoxification system including ascorbate (AsA), glutathione (GSH), tocopherol, carotenoids and flavonoids also plays important role in ROS homeostasis (Khan and Khan 2014; Khan et al. 2014, 2015, 2016; Noctor et al. 2014; Gill and Tuteja 2010; You and Chan 2015).

3.2.1 Enzymatic ROS

The ROS detoxification in chloroplast, peroxisomes and cytosol is carried out by an important antioxidant mechanism in plants called ascorbate-glutathione pathway which comprises enzymes to detoxify the $O_2^{\bullet -}$ using ascorbate and glutathione as reducing substrates. SOD is the first candidate in line of defence in this pathway to reduce H_2O_2 to H_2O and O_2 using ascorbate as reducing agents. In this process, ascorbate is oxidized to monodehydroascorbate, which is spontaneously disproportionated into dehydroascorbate. Ascorbate is regenerated from monodehydroascorbate by MDHAR using reducing power of NADPH and by DHAR using GSH as reducing agent to convert dehydroascorbate into ascorbate. GSH is recycled back from oxidized GSH through GR using NADPH (Pandey et al. 2015).

3.2.1.1 Superoxide Dismutase (SOD)

SODs are metalloenzymes that are designated as initial mode of defence against toxic effects of ROS in all the cellular compartments generating $O_2^{\bullet -}$ in nearly all aerobic organisms (Touati 1997). SODs generate less toxic molecule H_2O_2 by alternately adding or removing electron from $O_2^{\bullet -}$. Two superoxide molecules react with each other and dismutate to O_2 and H_2O_2 and this follows second-order reaction, whereas reaction catalysed by SOD is first order and is necessary to stop damage caused by superoxide radical to different biomolecules. The presence of SOD at the site of radical formation is crucial as phospholipid membrane is impermeable to charged $O_2^{\bullet -}$ radical (Takahashi and Asada 1983). SOD dismutates $O_2^{\bullet -}$ into less toxic H_2O_2, thereby preventing formation of more harmful $^{\bullet}OH$ via Haber-Weiss reaction (Gill and Tuteja 2010). SODs are classified into iron (Fe-SOD), manganese (Mn-SOD), copper-zinc (Cu-Zn SOD) and nickel (Ni-SOD) based on cofactor requirement. Fe-SOD localizes to chloroplast and is sensitive to H_2O_2, while Mn-SOD located both in mitochondria and peroxisomes and resistant to H_2O_2 and Cu-Zn SOD localizes to chloroplast and cytoplasm and is sensitive to H_2O_2 (Gill and Tuteja 2010). Using *in silico* approach, Nath et al. (2014) identified one *SOD* copper chaperone gene and other seven *SOD* isoforms in rice including two cytosolic Cu-Zn SODs, one putative Cu-Zn SOD-like, one plastidic SOD, two Fe-SODs and one Mn-SOD. These isoforms display differential expression during different developmental stages implicating specific role played by each of the gene. Seven *Arabidopsis* SODs were profiled for expression levels in response to oxidative stresses, and that increase in Fe-SOD levels in response to UV irradiation and high light stress was reported, but not to ozone exposure (Kliebenstein et al. 1998). When pea plants were exposed to high salinity,

Fe-SOD levels increased with corresponding decrease in Cu-Zn SOD, but when plants were treated with less severity salt stress, enhanced Cu-Zn SOD and Mn-SOD activities were observed (Gomez et al. 1999; Hernandez et al. 1995).

3.2.1.2 Ascorbate Peroxidise (APX)

APX belongs to class I family of peroxidases, which contains heme as a prosthetic group. APX catalyses transfer of electrons from specific substrate, ascorbate, to H_2O_2, thereby reducing peroxide to water as a part of ascorbate-glutathione pathway. APX activity is highly sensitive to ascorbate concentration, which is reflected in decline of its activity under limited ascorbate concentration. Studies have revealed that APX plays an important role in scavenging excess ROS, therefore maintaining ROS homeostasis and oxidative protection in plants under drought, heat, methyl viologen and high light stress (Caverzan et al. 2012; Zhang et al. 2013). *Arabidopsis thaliana* genome codes for eight isoforms of APXs, including cytosolic (APX1, APX2 and APX6), microsome membrane-bound (APX3, APX4 and APX5) and chloroplastic (stromal APX and thylakoid APX) (Panchuk et al. 2002; Dabrowska et al. 2007). Rice genome also contains eight *APX* genes including two cytosolic (*OsAPX1* and *OsAPX2*), two peroxisomal (*OsAPX3* and *OsAPX4*) and three chloroplastic (*OsAPX5*, *OsAPX7* and *OsAPX8*), and *OsAPX6* localizes to mitochondria (Teixeira et al. 2006). APX isoforms are developmental and stress modulated in response to drought, salinity, extreme temperatures, pathogen attack, UV radiation and heavy metal stress (Shigeoka et al. 2002; Dabrowska et al. 2007). Expression profiles of APX isoforms were studied by exposing rice seedlings to salt stress and found that *OsAPX1, OsAPX3, OsAPX4, OsAPX5* and *OsAPX6* transcripts were not altered, but *OsAPX2, OsAPX7* and *OsAPx8* transcripts were differently modulated under salinity stress (Teixeira et al. 2006). The role of APX in cold stress tolerance was established by Sato et al. (2011) who found that overexpression of cytosolic *OsAPX1* has significantly improved male sterility at continuous cold stress of 12 °C than WT plants that exhibited complete male sterility. This study has lot of impact on improving hybrid rice production. However, *OsAPX2* overexpression enhances salinity tolerance in transgenic *Arabidopsis* and *Medicago sativa* (Guan et al. 2012; Lu et al. 2007). The role of *OsAPX2* in abiotic stress was dissected through knockout mutant and overexpression studies in rice lines. In a study, Zhang et al. (2013) created knockout mutants for cytosolic *APX2* gene in rice, using random T-DNA insertion method, and found that mutants are hypersensitive to all abiotic stresses. Contrasting to this phenotype, rice plants overexpressing *OsAPX2* exhibited increased tolerance to drought, salinity and cold. The transcript levels of pea cytoplasmic APX increases 4-fold in response to drought and significantly increased to 15-fold after the stress withdrawal (Mittler and Zilinskas 1994). The results from above-mentioned experiments suggested that APX transcript and protein are induced in response to various abiotic stress conditions and therefore play important role in oxidative stress tolerance.

3.2.1.3 Monodehydroascorbate Reductase (MDHAR)

Monodehydroascorbate is a primary oxidation product of ascorbate which is reduced back to ascorbate by MDHAR using NADPH as specific electron donor. MDHAR is a FAD enzyme, and the crystal structure of the enzyme from *Oryza sativa* along with its cofactors was elucidated (Park et al. 2016). The overall protein structure is similar to iron-sulphur reductase except the active site forming long loop. Based on point mutation, it was found that Arg320 plays an important role in substrate binding, and Tyr349 is involved reduction reaction in the transfer of electron from NADPH to substrate via FAD (Park et al. 2016). Since ascorbate acts as antioxidant and also as reducing substrate for the APX activity, its levels are to be maintained high during stress conditions. Recycling of monodehydroascorbate to ascorbate can be enhanced by higher expression and activity of MDHAR. Overexpression of MDHAR from mangrove plant confers salinity stress and showed better yield through increased tiller and grain weight in rice (Sultana et al. 2012). In another experiment, transgenic overexpression of LeMDHAR in *Arabidopsis* chloroplast has conferred methyl viologen (MV)-induced oxidative damage through increased levels of reduced ascorbate (Li et al. 2010). Transgenic tobacco plants overexpressing halophytic mangrove chloroplastic MDHAR confer salinity stress tolerance by enhancing redox state of ascorbate and APX activity (Kavitha et al. 2010).

3.2.1.4 Dehydroascorbate Reductase (DHAR)

Oxidation of ascorbate produces short-lived radical monodehydroascorbate, which is converted to ascorbate by MDHAR, or disproportionates non-enzymatically to ascorbate and dehydroascorbate (DHA). DHAR regenerate reduced ascorbate from dehydroascorbate using glutathione as reducing substrate, thus contributing to cellular redox homeostasis (Chen and Gallie 2006). The molecular basis of enzyme DHAR was elucidated from crystal structure of the enzyme isolated from *Oryza sativa* by Do et al. (2016). The structure of the protein in native confirmation along with ascorbate and glutathione-bound form provides information regarding binding sites and interacting residues. The cys20 was involved in electron transfer at the active site to reduce dehydroascorbate to ascorbate by ping-pong kinetic mechanism. DHAR has been isolated, characterized and validated for its role in stress tolerance in different genetic backgrounds and organelles. (Chew et al. 2003; Kataya and Reumann 2010; Shimaoka et al. 2000; Mittova et al. 2000; Teixeira et al. 2005). DHAR plays a vital role in abiotic stress tolerance and this is supported by abundancy of its transcripts under different abiotic stress conditions (Fan et al. 2014; Lu et al. 2008). Mutants with low or no expression of DHAR revealed loss of Rubisco activity, low CO_2 assimilation and increased leaf ageing (Chen and Gallie 2006). In contrast, plants with high DHAR expression exhibited delayed leaf senescence, higher levels of chlorophylls and improved photosynthetic efficiency. Chen and Gallie (2004) demonstrated that high DHAR activity in guard cells interferes ABA-mediated stomatal closure, allowing increased transpiration under drought. This condition might pose negative impact on plant survival of transgenic

plants developed with DHAR overexpression. However, this effect could be minimized through tissue-specific expression of DHAR.

3.2.1.5 Glutathione Reductase (GR)

GR is an important flavoprotein oxidoreductase antioxidative enzyme involved in regeneration of reduced form of glutathione using NADPH as reducing substrate, thereby contributing to redox homeostasis. GR contains FAD and highly conserved GXGXXA motif in the NADPH-binding site, and arginine present therein facilitates binding of NADPH to this domain. Reduction of GSSG to GSH by NADPH at the active site is catalysed by cysteine residues (Trivedi et al. 2013). GR activity has been detected in chloroplast, mitochondria, peroxisomes and cytosol.

3.2.2 Non-enzymatic ROS Scavengers

Non-enzymatic antioxidants essentially include the redox buffers ascorbate and glutathione, as well as tocopherol, flavonoids, alkaloids and carotenoids. Ascorbate and glutathione pools existing at high concentrations (5–20 mM AsA and 1–5 mM GSH) during stress conditions indicate their role in oxidative stress-mediated abiotic defence. This is further evident in transgenic mutants of ascorbate and glutathione, where the plants were found hypersensitive to abiotic stress conditions (Creissen et al. 1999). Maintaining high ratio of reduced/oxidized levels of cellular ascorbate and glutathione is necessary for smooth functioning of ROS-scavenging activity. Little is known about flavonoids and carotenoids in ROS detoxification in plants. However, flavonoids and carotenoids are supposedly acting as auxiliary or secondary or accessory pigments in leaves that absorb the excess light energy not taken by chlorophyll and help in the prevention of photo-oxidation of the same.

3.2.2.1 Ascorbate

Ascorbate is a low molecular weight, water-soluble and highly abundant antioxidant that mitigates the bad effects of enhanced ROS. Ascorbate is generally present in all the tissues and cellular compartments in millimolar concentration with chloroplast stroma reported to contain highest concentration up to 50 mM (Gill and Tuteja 2010; Smirnoff 2000). Ascorbate presents mostly in reduced form under optimal growth conditions. Ascorbate is a multifunctional molecule involved in normal growth and development and in response to stresses by directly detoxifying several ROS and also by donating electrons for detoxification of ROS in enzymatic catalysed reactions (Smirnoff 2000). Ascorbate is also involved in regeneration of vitamin E. The levels of ascorbate are dependent on synthesis, oxidation, recycling and transport. Ascorbate is oxidized to monodehydroascorbate when it reacts with superoxide, singlet oxygen and H_2O_2. Recycling of ascorbate takes place from disproportion of monodehydroascorbate and also through enzymatic reactions catalysed by MDHAR and DHAR. The elucidation of biosynthesis of ascorbate came from experiments of Conklin et al. (1996), where they have isolated and

tested ascorbate-deficient mutants of *Arabidopsis*. Analysis of *vtc*1 mutant which contains only 25% of ascorbate compared to wild type by genetics, molecular and biochemical methods showed that this gene encodes for GDP mannose pyrophosphorylase. The ascorbate levels are closely linked to light and photosynthesis, as *Arabidopsis* plants grown under light contain increased ascorbate than dark-grown plants and also correlate with ascorbate biosynthesis enzyme transcript levels (Yabuta et al. 2007). Foliar application of ascorbic acid in *Zea mays* alleviates ill effects of water stress by reducing antioxidative enzyme activities (Dolatabadian et al. 2009). In response to ozone treatment in winter wheat, apoplastic ascorbate in leaves correlates with sensitive phenotypes of varieties (Feng et al. 2010).

3.2.2.2 Glutathione

Glutathione is a sulphur-contacting tripeptide (GSH, γ-L-glutamyl-L-cysteinylglycine) present in millimolar concentrations in reduced form in different cellular compartments. Glutathione plays a vital role in plant growth and development, evident by its role in phytochrome signalling and cellular redox homeostasis. Its role in stress condition is prominent and discussed largely (Noctor et al. 2011). GSH biosynthesis takes place from cystine, glutamine and glycine in two ATP-dependent reactions. The first reaction is rate-limiting step that is catalysed by glutamate-cysteine ligase to form γ-glutamylcysteine. In the second reaction, glutathione synthetase adds glycine to γ-glutamylcysteine to form glutathione (GSH). The reduced form of GSH is utilized as a substrate for multiple cellular processes to form oxidized GSSG. The ratio of GSH to GSSG is critical for maintaining cellular redox balance. The GSH scavenges $O_2^{\bullet -}$, H_2O_2 and $^{\bullet}OH$ radical and also regenerates powerful antioxidant ascorbate through ascorbate-glutathione cycle. In addition, GSH is also used as a precursor in synthesis of phytochelatins, in response to heavy metal stress and sulphur assimilation (Noctor et al. 2011). Several studies have strongly demonstrated that GSH concentration correlates with abiotic stress-induced oxidative stress tolerance in plants. Two cultivars of *Vigna radiata* L. were analysed for salt stress tolerance and found that variety CO 4 showed greater stress susceptibility and associated with higher GSSG and lower GSH concentration than Pusa Bold (Sumithra et al. 2006). Involvement of GSH in light signalling came from studies of rax1 mutant whose glutathione content is 50% less than wild type that display constitutive expression of high light-induced gene, *APX*2 (Ball et al. 2004).

In addition to antioxidative scavenging mechanism, reduction of ROS generation mechanisms during abiotic stresses also plays an important role in ROS homeostasis. For example, alternative oxidases (AOX) can divert the electrons from electron transport chain in mitochondria, instead of oxygen to produce $O_2^{\bullet -}$, and thereby prevent excess generation of ROS (Maxwell et al. 1999; You and Chan 2015). Other mechanisms include anatomical and morphological changes such as leaf movement and folding and rearrangement of photosynthetic apparatus depending on the amount of light energy to be harvested based on availability CO_2 (Mittler 2002).

3.3 ROS Generation Sites in a Plant Cell

ROS production is an inevitable part of aerobic metabolism of a living organism due to the partial reduction nature of molecular oxygen. ROS are produced continuously at low concentration (below threshold levels) in normal plant cells, at sites that are actively engaged in electron transportation reactions (Choudhury et al. 2013). According to an estimation, about 1% of total O_2 consumed by plants is being utilized to generate ROS in various cellular organelles like chloroplasts, mitochondria and peroxisomes (Bhattacharjee 2005). ROS is also known as reactive oxygen intermediates (ROI) or active oxygen species (AOS). ROS with potent damaging effect includes $O_2^{\bullet -}$, singlet oxygen ($^1O_2$), $^{\bullet}OH$, perhydroxyl radical ($HO_2^{\bullet}$), H_2O_2, alkoxy radical ($RO^{\bullet}$), peroxy radical (ROO) and organic hydroperoxide (ROOH) (Konig et al. 2012; Mignolet-Spruyt et al. 2016).

3.3.1 Chloroplast

The thylakoid membrane system present in chloroplast harbours all components of the light-capturing photosynthetic apparatus. In chloroplast, the presence of triplet chlorophyll and electron transport chain (ETC) in PSI and PSII are the major sites of ROS ($O_2^{\bullet -}$, $^1O_2$ and H_2O_2) production. Under unstressed condition, the electrons flows from the excited photosystem centres to reduce $NADP^+$ to NADPH. Then it enters the Calvin cycle and reduces the final electron acceptor, CO_2. Under water-stressed condition with the combination of limited CO_2 and high light intensity, the deprivation of $NADP^+$ causes overloading in electron transport chain (ETC) system, causing leakage of electrons from ferredoxin to O_2, reducing it to $O_2^{\bullet -}$ (Karupandian et al. 2011; Das and Roychoudhury 2014). This reaction is called Mehler reaction. Transfer of excess electrons or electron leakage to molecular oxygen (O_2) is also observed from 2Fe-2S and 4Fe-4S clusters in PSI and QA-QB complex of PS II leading to the production of $O_2^{\bullet -}$ radical (Sharma et al. 2012). The $O_2^{\bullet -}$continuously dismutated to H_2O_2 enzymatically by SOD found on the external 'stromal' membrane surface or may be protonated to $HO_2^{\bullet -}$ on the internal, 'lumen' membrane surface (Miller et al. 2008). The singlet oxygen ($^1O_2$) is a natural by-product of photosynthesis, mainly formed at PS II even under low-light conditions. At Fe-S centres where Fe2+ is available, H_2O_2 may be transformed to much more toxic $^{\bullet}OH$ ion through the Fenton reaction. So oxygen generated in the chloroplasts during photosynthesis accepts electrons passing through the photosystems, resulting in the formation of ROS (Zolla and Rinalducci 2002; Gill and Tuteja 2010).

3.3.2 Mitochondria

The mitochondrial ETC system and various enzymes present in its matrix produce ROS. Unlike animal cell, plant mitochondria have oxygen-rich environment and

contain photorespiration, a condition that favours ROS generation. Respiratory complex I, II and III are designated major sites for $O_2^{\bullet -}$ and H_2O_2 production in mitochondrial ETC (Huang et al. 2016). Plant mitochondria are also involved in pathogen-mediated defence reaction and PCD (Zhu et al. 2014; Wu et al. 2015). When the concentration of NAD^+-linked substrates is limited at complex I, it leads to reverse electron flow from complex III to complex I. As a result, the production of ROS is enhanced at complex I. The hydrolysis of ATP regulates the reverse electron flow (Turrens 2003; Møller et al. 2007; Noctor et al. 2007; Paterson et al. 2015). In a plant cell under normal aerobic conditions, ETC and ATP synthesis are tightly coupled, but under water stress, the inhibition and modification of ETC components cause the over-reduction of electron carrier like ubiquinone pool which enhances the ROS production (Rhoads et al. 2006; Blokhina and Fagerstedt 2010). During water stress, the lower rate of chloroplast ATP synthesis is compensated by increasing the synthesis rate of ATP in mitochondria; hence, the mitochondrial ROS production is also increased (Atkin and Macherel 2009). The enzymes present in the mitochondria matrix like aconitase directly produce the ROS, and enzyme-like 1-galactono-γ-lactone dehydrogenase (GAL) indirectly produces ROS by supplying electrons to the ETC (Rasmusson et al. 2008). The primary ROS, superoxide $O_2^{\bullet -}$, formed by monovalent reduction in the ETC dismutates quickly into H_2O_2 by the MnSOD (Moller 2001). It has been estimated that 1–5% of total O_2 consumed by mitochondria converts to H_2O_2. This H_2O_2 can be further converted to extremely active hydroxyl radical ($^{\bullet}OH$) by reacting with reduced Fe^{2+} and Cu^+ in the Fenton reaction (Moller 2001; Rhodas et al. 2006). The mitochondria have two major enzymes, alternative oxidase (AOX) and Mn-SOD, to counteract the oxidative stress. The AOX lower down the ROS by catalysing the O_2-dependent oxidation of ubiquinone pool (Jezek and Hlavata 2005). It is found that membrane-bound uncoupling proteins reduce production of ROS by diverting electrons to complex I, III and IV. Transgenic expression of uncoupling protein I in *Arabidopsis* exhibited reduced ROS generation clubbed with tolerance to multiple abiotic stresses (Barreto et al. 2014).

3.3.3 Peroxisome

Peroxisomes are minute subcellular organelle with single lipid bilayer membrane and harbours important oxidative reactions of the cell (fatty acid β-oxidation). H_2O_2 is the major ROS generated in peroxisomes (Luis and López-Huertas 2006). Peroxisome also produces $O^{\bullet -}$, like chloroplasts and mitochondria, as a consequence of the normal metabolic process. The major metabolic chemistries that produce H_2O_2 in peroxisomes include the photorespiratory glycolate oxidase reaction, the fatty acid β-oxidation, the enzymatic reaction of flavin oxidases and the disproportionation of O2•− radical. Under water stress conditions, the stomata remain closed (Sandalio and Rumero puretas 2015). So, there is a considerable reduction in the ratio of CO_2 to O_2, which causes increased photorespiration, leading to glycolate formation. This glycolate is oxidized by the glycolate oxidase

in peroxisome and produces H_2O_2, making it the leading producer of H_2O_2 (Noctor et al. 2002; Mittler et al. 2004; Rodríguez-Serrano et al. 2016). As mentioned earlier, fatty acid β-oxidation is another source of H_2O_2 formation in peroxisomes through action of acyl-CoA oxidase (Palma et al. 2009). It has been established that the $O_2^{\bullet -}$ is generated at two major sites of peroxisome: one is the peroxisomal matrix containing the xanthine oxidase (XOD) that catalyses both xanthine and hypoxanthine into uric acid and generate $O_2^{\bullet -}$ as a by-product and second is the peroxisomal membranes where a small ETC composed of a flavoprotein NADH and Cyt b is involved. The three polypeptides (PMPs) with molecular masses of 18, 29 and 32 kDa integrated in the peroxisomal membrane were found to be involved in $O_2^{\bullet -}$ production. It is found that the 18- and 32-kDa PMPs use NADH as electron donor for $O_2^{\bullet -}$ production. The 29-kDa PMP was clearly dependent on NADPH and was able to reduce cytochrome c with NADPH as electron donor (Lopez-Huertas et al. 2000). Out of the three polypeptides, the 18 kDa is main producer of $O_2^{\bullet -}$, which was believed to be a cytochrome possibly belonging to the b-type group. The PMP32 is very closely related to the MDHAR, and the PMP29 is related to the peroxisomal NADPH, cytochrome P450 reductase. Catalase and SOD are the major ROS-scavenging enzymes found in peroxisomes (Hayashi and Nishimura 2003; Palma et al. 2009). It is noticed that oxidative stress defence mechanism of peroxisomes plays a role in heavy metal stress and xenobiotic stress (2,4-D) (Luis and López-Huertas 2006).

3.3.4 Plasma Membrane

The ubiquitous electron transporting oxidoreductases present in the plasma membrane produces ROS. One such is the NADPH oxidase integrated in the plasma membrane found producing ROS under normal and stressed condition. The plant NADPH oxidase is also known as respiratory burst oxidase homolog (Sagi and Fluhr 2006; Sharma et al. 2012). The core domain of NADPH oxidase is identified by six transmembrane helices supported by two heme groups containing conserved histidines. The NADPH oxidase contains hydrophilic domains for NADPH and FAD at C-terminal and two EF hand motifs at N-terminal for calcium binding. NADPH oxidase catalyses transfer of electrons from cytoplasmic NADPH to exterior O_2 to form $O_2^{\bullet -}$. $O_2^{\bullet -}$ is dismutated to H_2O_2 either spontaneously or by SOD activity (Miller et al. 2009). ROS generated by membrane-bound NADPH oxidase activity acts as signalling molecules under various biotic and abiotic stress stimuli and elicit cellular defence response (Torres et al. 2002; Foreman et al. 2003; Kwak et al. 2003; Jones et al. 2007).

3.3.5 Cell Wall

ROS generates continuously at the outer surface of plasma membrane and cell wall membranes of the epidermis and vascular tissue, where active electron transfer is

involved. H_2O_2 is produced by four possible reactions, importantly being located at the external portion of plasma membrane, and is catalysed by NOX. Enzymes of oxidoreductase peroxidases, laccases, poly(di)amine oxidases (PDAOs), oxalate oxidases (OXOs) and SODs play a role in H_2O_2 generation in different reactions. Unlike PDAOs and OXOs that directly generate H_2O_2, NOX and peroxidases favour the formation of superoxide molecule, which later dismutes to H_2O_2 (Schopfer 2001; Spiteller 2003; Higuchi 2006). The di- and polyamine oxidases present in cell wall membranes of meristematic tissue generate significant quantity of radicle oxygen molecules (H_2O_2), while catalysing polyamines (putrescine, spermidine, cadaverine, etc.) to peroxides. Other cell wall-bound enzyme lipoxygenase (LOX) catalyses hydroperoxidation of polyunsaturated fatty acids (PUFA) making it active source for ROS like $^{\bullet}OH$, $O_2^{\bullet -}$, H_2O_2 and $^1O_2$ (Spiteller 2003; Higuchi 2006; Kim et al. 2010). The lipid hydroperoxides (PUFA-OOH) generated can undergo reductive cleavage catalysed by reduced metals, such as $Fe2^+$. The lipid $RO^{\cdot}$ produced (PUFA-O) initiates sequential reactions that lead to generation of toxic ROS (Liszkay et al. 2004; Karuppanapandian et al. 2006a, b, c, 2009; Karuppanapandian et al. 2008).

3.3.6 Apoplast

The cell wall-located enzymes are responsible for ROS production in apoplast and have been proven (Apel and Hirt 2004; Heyno et al. 2011). CO_2 that enters the apoplastic space will be converted to soluble, diffusible form and enters the cytoplasm to further participate in photosynthesis. The cell wall-associated oxalate oxidase, also known as germin, produces H_2O_2 and CO_2 from oxalic acid (Lane 2002; Cona et al. 2006). Other apoplastic ROS-forming enzymes include cell wall-bound oxidases, peroxidases (POXs), NADPH oxidase and polyamines (Mittler 2002; Kwak et al. 2003). Amine oxidases catalyse the oxidative deamination of polyamines (i.e., putrescine, spermine and spermidine) using FAD as a cofactor. Under extreme environmental conditions, stress signals combined with abscisic acid (ABA) make the apoplast a significant site for H_2O_2 production (Hernandez et al. 2001; Zhu 2001; Hu et al. 2006).

3.3.7 Endoplasmic Reticulum

The endoplasmic reticulum is the active site for ROS generation, as it harbours site for PSII that allows fast electron transportation through CytP450 (Mittler 2002). Organic substrate, RH, interacts with the CytP450 followed by reduction by a flavoprotein to form a free radical intermediate (CytP450 R^-) that can readily react with $3O_2$ to give rise to an oxygenated complex (CytP450-ROO^-); now both the intermediate and $3O_2$ bear one unpaired electron. This oxygenated complex may be reduced by Cyt *b*, or, occasionally, the complex may automatically form the P450-RH by generating $O_2^{\bullet -}$ (Watanabe and Lam 2006; Shi et al. 2010).

3.4 ROS Chemistry and Effect Under Abiotic Stress

The molecular oxygen in its ground state in the cellular environment is stable and it has no deleterious effect. The ground state triplet molecular oxygen is a biradical having two unpaired electrons with parallel spin which makes it paramagnetic (Tripathy and Oelmüller 2012). To participate in oxidation, triplet oxygen molecule would require a partner having pair of electrons with parallel spinning in its outermost orbit. But the ground state O_2 can easily be converted to reactive oxygen species either by absorbing energy or through electron transfer reaction (Apel and Hirt 2004). The former helps in the formation of singlet oxygen, whereas the latter results in the sequential reduction to superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$). However, if the triplet oxygen absorbs sufficient energy, the spin restriction is removed and the spin of one of its unpaired electrons is reversed, favouring the generation of singlet oxygen ($^1O_2$) (Apel and Hirt 2004; Halliwell 2006). In plants, ROS are continuously produced as by-product of the metabolic pathways localized in various cellular compartments and also produced due to the spillage of electron from the electron transport chain system to the cellular environment which reduces the available molecular oxygen (Moller et al. 2007; Miller et al. 2008).

3.4.1 Singlet Oxygen ($^1O_2$)

In plants, light-harvesting complex (LHC) and the photosynthetic reaction centre II are the primary centres for $^1O_2$ production. Inefficient transfer of energy results in the generation of triplet state of chlorophyll that reacts with triplet oxygen to produce the highly reactive $^1O_2$ (Carmody et al. 2016; Zhang et al. 2014):

$$\mathrm{Chl} \rightarrow 3\mathrm{Chl}\ 3\mathrm{Chl} + 3\mathrm{O}_2 \rightarrow \mathrm{Chl} + {}^1\mathrm{O}_2$$

Under conditions of excess light energy, the QA and QB plastoquinone electron acceptors of PSII in the electron transport chain become over-reduced leading to unfinished charge separation between P680 and pheophytin (Dietz et al. 2016). This results in the formation of triplet state of the reaction centre Chl P680 (3P680) thus leading to the formation of $^1O_2$. The release of $^1O_2$ is also detected in isolated PS II particles (Macpherson et al. 1993), thylakoids (Hideg et al. 1998; Fryer et al. 2002) and cytochrome *b6/f* complex (Suh et al. 2000). Alternatively, the nanoradicle $^1O_2$ is formed when superoxide anion (O_2*-) reacts with hydroxyl radicle (*OH) in places where electron transfer is low at pace. Plants under abiotic stress conditions close their stomata partially as adaptive mechanism. This leads to the accumulation of intracellular CO_2, a condition that favours the formation of $^1O_2$ in chloroplast, because of accumulation of electrons at PSII. $^1O_2$ is a highly reactive radical oxygen molecule that is attributed for ROS-induced cell death under high light intensity. Though $1O_2$ has very less half-time period, of about 3 μs (Hatz et al. 2007), its high diffusion capacity (100 nm) can cause the cellular damage under

adverse conditions. Plants have evolved several antioxidant mechanisms to quench off $^1O_2$, and most of them involve non-enzymatic methods. Few of efficient scavengers of $^1O_2$ involve carotenoids, tocopherols and plastoquinones that exist in thylakoid membranes of chloroplast. Apart from the negative effect, $^1O_2$ act as signalling molecule along with chloroplast protein Executar 1 (Ex1) that triggers stress acclimation process under pathogen attack and abiotic stress (Krieger-Liszkay et al. 2008).

3.4.2 Superoxide Radical ($O_2^{\bullet -}$)

Superoxides ($O_2^{\bullet -}$) are produced due to the transfer of electrons to O_2 instead of reducing NADP during excess electron transfer reaction in PSI and PSII. $O_2^{\bullet -}$ first formed ROS in plant cell that further catalysed or dismuted to form more toxic ROS molecules. Superoxide is a moderately reactive ROS with approximately 2–4 μs of half-life and does not cause extensive damage by itself. In plant tissues, about 1–2% of O_2 consumption leads to the generation of $O_2^{\bullet -}$ (Moller 2001; Sharma et al. 2012). Superoxide radical ($O_2^{\bullet -}$) can also undergo further reactions to generate more reactive ROS like $^{\bullet}OH$ and more possibly $^1O_2$ (Bielski et al. 1983; Elstner 1987). $O_2^{\bullet -}$ radical has both oxidizing and reducing property (neutrophilic). The $O_2^{\bullet -}$ undergoes protonation to give up $HO_2^{\bullet}$ a strong oxidizing agent, which directly attacks the PUFA (Bielski et al. 1983). The $O_2^{\bullet -}$ can also donate an electron to iron (Fe_3^+) to yield a reduced form of iron (Fe_2^+) and $^1O_2$. The reduced Fe_2^+ catalysed by SOD produces H_2O_2 that is further reduced by accepting electron to form highly toxic hydroxyl radicle ($^{\bullet}OH$), and this is known as Fenton reaction. Alternatively, $O_2^{\bullet -}$ dismutated to H_2O_2 and led to formation of $^{\bullet}OH$ radicle, a process called Haber-Weiss reaction (Halliwell 2006):

$$O_2^{\bullet -} + Fe^{3+} \rightarrow {}^1O_2 + Fe^{2+}$$

$$2\,O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2Fe_3^+$$

$$Fe_2^+ + H_2O_2Fe_3^+ \rightarrow Fe_3^+ + OH^- + {}^{\bullet}OH(\text{Fenton Reaction})$$

3.4.3 Hydrogen Peroxide (H_2O_2)

H_2O_2 is a potent reactive oxygen species that interacts with organic molecules that contain Fe^{2+}, leading to formation of highly reactive OH radical. The major source of H_2O_2 includes chloroplasts and mitochondria, where imbalance in electron transfer favours its generation. Type III peroxidases, NAD(P)H oxidases, Mehler reaction are few other sites of H_2O_2 generation (Gill and Tuteja 2010). It can occur both non-enzymatically through dismutation of H_2O_2 under low pH conditions and enzymatically, mostly catalysed by SOD. Under water stress conditions, the closure

of stomata limits CO_2 fixation, thus favouring oxygenation of ribulose 1, -5-bisphosphate (RuBisCO) which leads to the formation of H_2O_2. As discussed earlier, H_2O_2 has a dual role in plant growth and development. At low concentrations, it acts as signalling molecule for many physiological processes like senescence (Peng et al. 2005), photorespiration and photosynthesis (Noctor et al. 2004), stomatal movement (Bright et al. 2006) and cell cycle (Tanou et al. 2009). In comparison to other ROS, H_2O_2 has a longer half-life of 1 ms, and hence, it can diffuse longer distances in cell and even cross plant cell membranes through aquaporins (Li et al. 2014) and induce oxidative damage. Generally, H_2O_2 is less toxic than other ROS, but its high intracellular concentration can oxidize cysteine (-SH) and methionine (-SCH3) residues of Calvin cycle enzymes (Dat et al. 2000; Halliwell 2006).

3.5 Approaches for Overcoming the Oxidative Stress Tolerance in Plants

As discussed above, all other stress conditions act through a common mechanism, i.e. oxidative stress. Plants have evolved with different enzymatic, non-enzymatic and antioxidant mechanisms to combat and adapt to the damage caused by oxidative stress. Transferring of genes/genomic regions coding for antioxidants from tolerant genotypes to susceptible species either through transgenic or breeding methods is of prime importance. But, besides the negative consequences, ROS act as sensory molecules in biotic and abiotic stress conditions and also participate in crosstalk with other signalling pathways (Mitler et al. 2004; Suzuki et al. 2012; Baxter et al. 2014; Noetor et al. 2014). Hence, it is important to bear in cognizance, about the threshold levels of ROS molecules, while designing strategies/approaches to develop crops with improved stress tolerance.

3.5.1 Molecular Breeding

Plant breeding techniques are indispensable tools in developing high-yielding varieties/hybrids. The ever-changing global climate, clubbed with continuous evolution of new pests, has thrown challenges to plant breeders in crop improvement. Breeders have exploited genetic variation at all levels, in developing improved varieties. Linkage drag and time involved in transferring of useful alleles and selection of right recombinant are the potential problems associated with traditional breeding methods. Molecular marker technology has greatly facilitated plant breeding in identification of genomic regions (QTLs), linked with trait of interest (Salvi and Tuberosa 2005; Walia et al. 2007; Marino et al. 2009; Pandit et al. 2010). But the recent developments in next-generation sequence-based (NGS) technologies offer SNP-based assays that have revolutionized plant breeding. Genome-wide analysis using NGS methods helps in identification of superior alleles and also helps in identification of evolutionary conserved domains. Genome-wide analysis

of genes of GR in rice has shed light on evolutionary conserved domains, which helps in better understanding of its role under normal and stressed conditions (Trivedi et al. 2013). Genome-wide SNP analysis of antioxidative genes in rice has revealed functional polymorphism between resistant and susceptible genotypes that can be used to identify superior alleles in rice population studies (Prakash et al. 2016). Integration of conventional plant breeding methods, together with advanced genomic tools and phenotyping methods, will rapidly deliver superior breeding material with improved genetic gains.

Parallel to the developments of NGS technologies, research in genetics has discovered novel genetic resources that can utilize the benefits of genomics to gain the maximum genetic gains. Multi-parental crossing designs developed recently combine the alleles across the locations and also increase the diversity and resolution of quantitative trait loci (QTL) mapping studies (Giraud et al. 2014). One such population developed in maize is NAM population by crossing 25 diverse inbred lines with common parent (Elshire et al. 2011) that combines the advantages of both linkage- and association-based mappings. Unfortunately, very limited work has been done in oxidative stress tolerance with reference to molecular breeding. Melonaldehyde is the by-product of lipid peroxidation during extreme oxidative stress damage in crop plants. MDA content is often considered as an index to measure the extent of damage caused by oxidative stress. Jing et al. (2009) identified two QTLs associated with MDA content in rice-inbred population. Markers associated with these QTLs could be used to evaluate the genotypes for oxidative stress tolerance. During drought studies on pearl millet, Kholová et al. (2011) opinioned the possible presence of *APX5* gene in drought-tolerant QTL, as the APX5 activity was high in NIL (near-isogenic line) population introgressed with drought-tolerant QTL. Further studies require confirming this hypothesis, and designing of marker assay will help in selecting the stress-tolerant breeding material in pearl millet. Applying dynamic QTL analysis method, QTLs for antioxidant enzymes SOD, APX and MDA were detected in wheat-inbred population that explains more than 10% phenotypic variation (Jiang et al. 2013). Dynamic QTL analysis, used in this experiment, is a novel and reliable method to detect genomic regions based on both static and dynamic expression of genes, which improves the accuracy and sensitivity of QTL detection. Hence, genomic information generated could be effectively deployed in wheat improvement programmes through marker-assisted selection (MAS). In another study using three different tomato populations, ascorbate-related QTLs were mapped on chromosome numbers 2, 8, 9, 10 and 12. Further characterization of above QTLs revealed the presence of ascorbate regeneration genes *MDHAR* and *GME* in QTL region, indicating the reliability of the experiment (Stevens et al. 2007). The allelic polymorphism in the above QTLs can be exploited for tomato breeding programs on abiotic stress tolerance. In another study on tomato, 28 QTLs were detected governing non-enzymatic antioxidants ascorbate, vitamin C, total phenols and flavonoids, using back cross population (BC_2F_2) (Okmen et al. 2011). Markers associated with these QTLs could be used in marker-assisted breeding (MAB) for improving tomato breeding material.

3.5.2 Genetic Engineering

Limitations in conventional plant breeding in improving crop productivity can be overcome by genetic engineering approach. As discussed above, all abiotic stress like drought, salt and heat conditions leads to induction of secondary stresses like oxidative stress through generation of ROS at the cellular level. Hence, it is most appropriate to target antioxidant genes to overcome abiotic stresses, through gene transfer technology. Though all antioxidant enzymes described participate in scavenging activity, it is proved that CAT, APX and SOD upregulate more abundantly during drought stress, whereas transcripts of GR, POD, MDHAR and DHAR express under cold stress. CAT induces preferentially under salt stress compared to other genes (Zhang et al. 2015). Transgenic plants developed with improved stress tolerance using various enzymatic and non-enzymatic methods have been listed in Table 3.1. As indicated earlier in this chapter, ROS also invokes defence response in plants; hence, expression levels of transgenes are crucial to keep in pace of both metabolic pathways, i.e. signalling activity and scavenging pathway.

3.5.2.1 Non-enzymatic

Plants evolved with an efficient non-enzymatic mechanisms (ascorbate, AsA; glutathione, GSH; a-tocopherol; phenolic compounds; alkaloids; flavonoids; and carotenoids) that could be exploited to control ROS. Many such antioxidants have been isolated, characterized from various sources and tested their efficiency in different backgrounds through transgenic approach. Few of such examples are discussed here. Hemavathi et al. (2010) found that transgenic potato (*Solanum tuberosum* L. cv. Taedong Valley) plants overexpressing the *l-gulono-c-lactone oxidase* (*GLOase*) gene exhibited increased ascorbic acid (AsA) (141%) compared to the control plants, and transgenics exhibited better performance under simulated stress conditions induced by methyl viologen, NaCl and mannitol. Direct correlation between enhanced AsA and abiotic stress tolerance in transgenics was observed in this experiment. Alteration in GSH expression made through transgenic method has also conferred stress tolerance to great extent in plants. In another interesting study, transgenic mustard (*B. juncea*), overexpressing antioxidant enzymes *glutamine synthetase (GS)* and *gamma-glutamylcysteine synthetase (g-ECS)* genes, has displayed tolerance to abiotic stress caused by different heavy metals (Cd, Zn, As and Pb). This tolerance has been attributed to higher accumulation of reduced glutamine (GSH) necessary for phytochelatin synthesis (PC) that actively participates in phytoremediation (Reisinger et al. 2008). Liu et al. (2008) generated tobacco transgenic plants by overexpressing *VTE1* gene and encoding tocopherol cyclase (VTE1), an important enzyme involved in tocopherol biosynthesis. This study revealed that plants overexpressing *VTE1* gene have increased tolerance. Zhang et al. (2012) identified that overexpression of a transcription factor 'AtERF98' in *Arabidopsis* has resulted in higher accumulation of antioxidant ascorbic acid that confers drought and salinity tolerance in transgenics. Further, this study indicated the direct role of ethylene responsive factor (ERF) in AsA biosynthesis. It has also been found that overexpression of proline biosynthetic

Table 3.1 List of transgenic plants developed for ROS-scavenging enzymes in different plants through overexpression show improved tolerance to various abiotic stresses including oxidative stress

Gene	Gene source	Transgenic plant	Function	References
Cu-Zn SOD	*Oryza sativa*	*Nicotiana tabacum*	Salinity and drought	Badawi et al. (2004)
Cu-Zn SOD	*Avicennia marina*	*Oryza sativa*	Salinity, drought and oxidative	Prashanth et al. (2008)
MnSOD	*Nicotiana plumbaginifolia*	*Triticum aestivum*	Oxidative and photo-oxidative	Melchiorre et al. (2009)
MnSOD	*Tamarix androssowii*	*Populus davidiana X Populus bolleana*	Salinity	Wang et al. (2005b)
CAT3	*Brassica juncea*	*Nicotiana tabacum*	Heavy metal	Gichner et al. (2004)
katE	*Escherichia coli*	*Nicotiana tabacum*	Salinity	Al-Taweel et al. (2007)
MDAR1	*Arabidopsis thaliana*	*Nicotiana tabacum*	Salinity, ozone and drought	Etrayeb et al. (2007)
cAPX	*Pisum sativum*	*Lycopersicon esculentum*	Drought, heat, cold and UV light	Wang et al. (2005a)
swpa4	*Ipomoea batatas*	*Nicotiana tabacum*	Salinity, osmotic and oxidative	Kim et al. (2008)
APX1	*Hordeum vulgare*	*Arabidopsis thaliana*	Salinity	Xu et al. (2008)
StAPX	*Solanum lycopersicum*	*Nicotiana tabacum*	Salinity and drought	Sun et al. (2010)
OsAPXa	*Oryza sativa*	*Oryza sativa*	Cold	Sato et al. (2011)
DHAR	*Oryza sativa*	*Arabidopsis thaliana*	Salinity	Chen and Gallie (2005)
DHAR	*Arabidopsis thaliana*	*Nicotiana tabacum*	Drought and ozone	Ushimaru et al. (2006)
DHAR	*Arabidopsis thaliana*	*Nicotiana tabacum*	Salinity and drought	Etrayeb et al. (2007)
GR	*Arabidopsis thaliana*	*Gossypium hirsutum*	Cold and photo-oxidative	Kornyeyev et al. (2003)
GPX	*Chlamydomonas*	*Nicotiana tabacum*	Salinity cold and oxidative	Yoshimura et al. (2004)
GPX-2	*Synechocystis*	*Arabidopsis thaliana*	Salinity, drought, cold, heavy metal, oxidative and MV	Gaber et al. (2006)
VTE1	*Arabidopsis thaliana*	*Nicotiana tabacum*	Drought	Liu et al. (2008)
P5CS	*Arabidopsis thaliana*, *Oryza sativa*	*Petunia hybrida*	Drought	Yamada (2005)

(continued)

Table 3.1 (continued)

Gene	Gene source	Transgenic plant	Function	References
P5CS	*Vigna aconitifolia*	*Triticum aestivum*	Drought	Vendruscolo et al. (2007)
GLOase	Strawberry	*Solanum tuberosum*	Accumulation of vitamin C with enhanced abiotic stress	Hemavathi et al. (2010)
GS	*Escherichia coli*	*Brassica juncea*	Heavy metal tolerance	Reisinger et al. (2008)
AtERF98	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Activation of ascorbic acid synthesis	Zhang et al. (2012)
GLOase	Rat cells	*Solanum tuberosum*	L-ascorbic acid accumulation and tolerance to salinity and MV	Hemavathi et al. (2010)
P5CS	*Arabidopsis thaliana*	*Solanum tuberosum*	Accumulation of proline in response to salinity	Hmida-Sayari et al. (2005)
P5CS	*Vigna aconitifolia*	*Oryza sativa*	Drought and salinity	Su and Wu (2004)
EsSPDS1	*Eutrema salsugineum*	*Nicotiana tabacum*	Drought	Zhou et al. (2015)
MnSOD	Yeast	*Oryza sativa*	Salinity	Tanak et al. (1999)
APX2 and 3	*Arachis hypogaea*	*Arabidopsis thaliana*	Heat tolerance	Chiang et al. (2015)
Cytosolic GR	*Brassica campestris*	*Oryza sativa*	Photo-oxidative stress	Kouril et al. (2003)
GR	*Pennisetum glaucum*	*E. coli*	Heat and MV	Achary et al. (2015)
MDHAR	*Acanthus ebracteatus*	*Oryza sativa*	Salinity	Sultana et al. (2012)
DHAR	*Arabidopsis thaliana*	*Nicotiana tabacum*	Aluminium	Yin et al. (2010)
AtMDHAR1	*Arabidopsis thaliana*	*Nicotiana tabacum*	Salinity, ozone and PEG	Eltayeb et al. (2007)
DHAR	*Eutrema salsugineum*	*Nicotiana tabacum*	Ascorbic acid biosynthesis and drought	Zhou et al. (2015a)
DREB1A/ CBF3	*Arabidopsis thaliana*	*Solanum lycopersicum*	Drought stress	Rai et al. (2013)
APX and Cu-Zn SOD	*Nicotiana tabacum*	*Nicotiana tabacum*	Methyl viologen and oxidative damage	Kwon et al. (2002)
APX and Cu-Zn SOD	*Manihot esculenta*	*Solanum tuberosum*	Heat, oxidative stress and MV	Tang et al. (2006)
APX, Cu-Zn SOD and DHAR	*Manihot esculenta*	*Nicotiana tabacum*	Salinity and paraquat	Xu et al. (2014)

(continued)

Table 3.1 (continued)

Gene	Gene source	Transgenic plant	Function	References
APX and Cu-Zn SOD	*Manihot esculenta*	*Manihot esculenta*	MV and cold	Xu et al. (2014)
APX and Cu-Zn SOD	*Arachis hypogaea*	*Nicotiana tabacum*	Salinity	Negi et al. (2015)
Cu-Zn SOD	*Pisum sativum*	*Nicotiana tabacum*	MV and cold	Gupta et al. (1993)
Cu-Zn SOD	*Kandelia candel*	*Nicotiana tabacum*	Oxidative and salinity	Jing et al. (2015)
Cu-Zn SOD	*Arachis hypogaea*	*Nicotiana tabacum*	Drought and salinity	Negi et al. (2015)
Mn SOD +APX	*Nicotiana tabacum*	*Festuca arundinacea*	Multiple abiotic stresses	Lee et al. (2007)
Cu-Zn SOD + CAT	*Zea mays*	*Brassica campestris*	Salinity and SO_2	Tseng et al. (2007)
SOD +APX	*Spinacia oleracea/Pisum sativum*	*Prunus domestica cv. Claudia Verde*	Salinity	Diaz-Vivancos et al. (2013)
MDHAR + DHAR	*Brassica rapa*	*Arabidopsis thaliana*	Freezing oxidative	Shin et al. (2013)

pathway genes has resulted in enhancing the abiotic stress tolerance in transgenic plants. The potato transgenic plants overexpressing pyrroline-5-carboxylate synthetase (P5CS) cDNA from *A. thaliana* have exhibited increased proline levels under salt stress and showed less altered tuber yield and weight in comparison to control plants (Hmida-Sayari et al. 2005). Su and Wu (2004) reported that both constitutive expression and stress-inducible expression of the P5CS cDNA in transgenic *O. sativa* have led to the accumulation of P5CS mRNA and Pro which resulted in higher tolerance to salt and water deficiency. In a similar study, Vendruscolo et al. (2007) developed wheat transgenics expressing VaP5CS cDNA under the control of stress-inducible promoter AIPC and transgenics, which performed well under water deficit stress. This study also revealed that P5CS induced proline acts in oxidative stress management than osmatic adjustment under water stress. Other than as osmolyte proline also act as scavenging molecule by quenching OH^- and $^1O_2$ radicals (Trovato et al. 2008). Tissue-specific expression of EsSPDS1 encoding a novel cellular polyamine has showed resistance to multiple abiotic stresses through strict regulation of ROS genes (Zhou et al. 2015a).

3.5.2.2 Enzymatic

A number of transgenic plants including *Arabidopsis*, tobacco, rice, tomato, maize, sweet potato and potato have been developed with manipulated expression of

antioxidant enzymes (SOD, APX, DHAR, MDHAR, GR and CAT) that showed increased tolerance to drought, low or high temperatures and salinity stress (Prashanth et al. 2008; Al-Taweel et al. 2007; Kim et al. 2008; Etrayeb et al. 2007; Ushimaru et al. 2006; Table 3.1). Ascorbate (AsA)-glutathione (GSH) pathway is a key metabolic pathway that harbours enzymes like SOD, APX, DHAR, MDHAR and GR, with efficient ROS detoxification capacities. Hence majority of transgenic work is focused on genes involved in this pathway. Majority of the researchers focused on transgenics with overexpression of SOD for improving abiotic stress tolerance (Caverzan et al. 2016). It was noteworthy that overexpression of a single gene could make the transgenic rice tolerant to different stresses. For instance, overexpression of a yeast *MnSOD* gene in transgenic rice resulted in increased salt tolerance (Tanaka et al. 1999), whereas overexpression of the same gene from pea could make the transgenic rice drought tolerant (Wang et al. 2005b). Overexpression of the cytosolic *Cu-Zn SOD* gene from mangrove in transgenic rice could make the plant tolerant to both salinity and drought (Prashanth et al. 2008). This could be due to efficiency of SOD, to detoxify superoxide radical, the first generated ROS in plants that prevents the formation of subsequent ROS molecules, which are more toxic. An *OsAPX* gene overexpressed in transgenic rice could enhance tolerance to chilling at the booting stage (Sato et al. 2011). Overexpression of peanut *APX2* and *3* genes in *Arabidopsis* has improved seed germination rate, and transgenic displayed tremendous heat tolerance compared to WT, through efficient elimination of cellular H_2O_2 (Chiang et al. 2015). Transgenic rice overexpressing a cytosolic GR of *Brassica campestris* showed tolerance towards intensive photo-oxidative stress (Kouril et al. 2003). Overexpression of cDNA clone of GR from *Pennisetum* has conferred heat and methyl viologen (MV) tolerance in *E. coli*. This is due to fat scavenging activity of reduced glutathione pools, generated by overexpression (Achary et al. 2015). Sultana et al. (2012) have developed salt-tolerant rice transgenic by overexpressing a *MDHAR* gene isolated from the mangrove plant (*Acanthus ebracteatus*). Nevertheless, there are exceptions to the general assumptions that transgenic plant would always enhance stress tolerance. For instance, transgenic tobacco and tomato plants overexpressing petunia *Cu-Zn SOD* failed to exhibit any increased tolerance to oxidative or cold stress (Tepperman and Dunsmuir 1990). Likewise, transgenic cotton plant overexpressing an AtGR did not confer any protection from photoinhibition under chilling stress (Logan et al. 2003). Yin et al. (2010) demonstrated that transgenic tobacco plants overexpressing DHAR exhibited better root growth, low levels of H_2O_2 and less lipid peroxidation when compared with their wild counterparts under heavy metal stress (Al). The elevated levels of AsA and APX activity could have performed better scavenging activity in the above experiment. The overexpression of AtMDHAR1 in tobacco increases net photosynthesis rates under salt, ozone and PEG stresses (Eltayeb et al. 2007). Overexpression of dehydroascorbate reductase (DHAR) cDNA in tobacco has improved oxidative damage through fast regeneration of ascorbate (Zhou et al. 2015a). In a significant study, Herbette et al. (2011) made an interesting observation that, along with tolerance to abiotic stresses, transgenic tomato plants

overexpressing GPX become susceptible to pathogen attack. This study has opinioned that the antioxidant role of GPX in both biotic and abiotic pathways and crosstalk mechanism might be responsible for this activity. Controlled expression of GPX may solve this problem.

Apart from various enzymatic and non-enzymatic antioxidants, transgenic expression of signalling molecules and regulatory elements involved in oxidative defence pathways also plays a significant role in developing stress-tolerant crop plants. Regulatory genes or transcription factors (TFs) that control large set of downstream genes involved in ROS-scavenging genes are ideal choice to achieve stress tolerance in crop plants. In *Arabidopsis*, overexpression of mitogen-activated kinase kinase 1 (MKK1) leads to increased tolerance to abiotic stresses through upregulation of genes involved in ascorbate-glutathione pathway (Wrzaczek et al. 2013; Xing et al. 2008). In other example, overexpression of transcription factors *Zat12* or *JERF3*, *Zat10* resulted in upregulation of various transcripts involved in ROS-scavenging pathways leading to higher tolerance to salt, drought or osmotic stresses (Sakamoto et al. 2004; Davletova et al. 2005). Rai et al. (2013) have reported that overexpression of AtDREB1A/CBF3 of *Arabidopsis* under the control of stress-inducible promoter (rd29A) in tomato (cv. Kashi Vishesh) showed enhanced levels of ROS-scavenging enzymes and antioxidants with increased tolerance to drought-induced oxidative stress.

3.5.2.3 Pyramiding of Antioxidant Genes

It is well known that expression of single foreign gene in plants will make them stress tolerant to a certain level in different backgrounds. As science advances, it is realized that simultaneous co-expression of genes involved in metabolic pathway could increase stress tolerance to a great extent (Halpin 2005; Vemanna et al. 2013). Various strategies have been used for multigene transfer including iterative, co-transformation and multigene linking (Halpin et al. 2001). Gene transformation through iterative strategies is more conventional and includes cross-fertilization (Zhao et al. 2003) and retransformation approaches (Singhla-Pareek et al. 2003). In the multigene linking strategy, multiple transgenes are introduced simultaneously into a plant by linking multiple transgene expression cassettes onto a single T-DNA that co-expresses in host plant (Chen et al. 2006, 2010). Among all methods, multigene linking strategy becomes most successful and convenient.

Pyramiding of APX and Cu-Zn SOD has been achieved in tobacco, potato, sweet potato and tall fescue plants that overexpressed in cytosol and chloroplast (Kwon et al. 2002; Tang et al. 2006; Lee et al. 2007; Lim et al. 2007; Xu et al. 2014; Negi et al. 2015). Pyramiding of SOD, APX and DHAR has been done in transgenic tobacco plant (Lee et al. 2007). Transgenic tobacco having overexpressed *Cu-Zn SOD* showing tolerance to oxidative stress (Gupta et al. 1993), upon retransformation with the chloroplastic *APX*, showed enhanced tolerance to paraquat (Kwon et al. 2002). Later into the above transgenic tobacco, a chloroplastic *DHAR* gene was transferred that showed further enhancement of tolerance to oxidative stress (Lee et al. 2007). Likewise, transgenic tobacco through *in vitro* pyramiding of cytosolic Cu-Zn SOD and APX was developed that showed enhanced tolerance to

drought stress as a result of overexpression of both the enzymes (Faize et al. 2011). The pyramiding of Cu-Zn SOD and chloroplastic APX in potato (Tang et al. 2006) and in sweet potato (Lim et al. 2007) enhanced the plant tolerance to chilling, high temperature and paraquat stresses. Transgenic tobacco overexpressing cotton glutathione S-transferase (GST) and *Chlamydomonas* glutathione peroxidase (GPX) showed enhanced resistance to paraquat as well as chilling (Yu et al. 2003; Yoshimura et al. 2004). Also, gene pyramiding with double transgenic plants overexpressing both GST and GPX enhances the seedling growth during chilling and salt stress (Roxas et al. 1997). Co-expression of *Cu* Zn SOD + APX from peanut has greatly improved salt and drought tolerance in tobacco, through minimizing oxidative damage (Negi et al. 2015). It also enhanced germination percentage, indicating the role of oxidative pathway enzymes in seed germination. Similarly co-expression of maize ZmCu-ZmSOD and ZmCAT showed significant increase in photosynthetic performance along with NaCl-induced salt tolerance in transgenic cabbage (*Brassica campestris* L.) better than the independent performance of either ZmCu-Zn SOD or ZmCAT in similar background (Tseng et al. 2007). Xu et al. (2014) have overexpressed native *Cu-Zn SOD* and APX2 in cassava plants that has resulted in higher tolerance to oxidative stress induced by MV and H_2O_2 along with cold tolerance (4 °C for 2 days) (Xu et al. 2014). Lu et al. (2010) have demonstrated that overexpression of SOD and APX in sweet potato have resulted in increased expression of antioxidant enzymes, i.e. SOD, APX and CAT, thus protecting the plant from oxidative stress damage under stress conditions. Zhao and Zhang (2006) explained that co-expression of the *GST* and *CAT1* genes in rice has greatly enhanced their tolerance to salt (200 mM NaCl) and paraquat-induced stresses. While SOD and catalase activity played a crucial role in conferring salt tolerance, GST activity was found only during paraquat treatment. The generation of H_2O_2 and MDA decreased in the transgenics than in non-transgenics under the same conditions. Martret et al. (2011) observed that tobacco chloroplast transformants expressing genes encoding DHAR, GR, and GST exhibit altered antioxidant metabolism that has improved tolerance to salinity and chilling. This improved protection could be explained by synergistic effects of DHAR with GR or GST with GR. The expression of these combinations of transgenes also increased the regeneration of AsA (1.6-fold) and GSH (2.4-fold) and participated in a more rapid scavenging of $O2^{\bullet -}$ and H_2O_2 prior to their interaction with target molecules. Simultaneous expression of Brassica MDHAR and DHAR cDNA clones under the control of oxidative induced promoter SWPA2 has tremendous increased oxidative stress causing freezing tolerance in *Arabidopsis* compared to individual overexpression (Shin et al. 2013). This study combines the benefit of controlled expression and gene pyramiding.

There are many genes other than oxidative pathways that control abiotic stress, through activation of ROS-scavenging pathway. *Expansins* are one such gene belonging to cell wall proteins, inducing cell wall loosening, and participate in plant growth and development processes. *TaEXPB23*, a wheat *expansin* gene, was proved for oxidative stress tolerance, when overexpressed in tobacco plants (Han et al. 2015). The resultant transgenic tobacco plants revealed increased peroxidase

activity particularly in their cell walls conferring oxidative stress tolerance (Chen et al. 2016). This study revealed that *expansin* genes are one of the best probable candidates to produce crop plants tolerant to abiotic stress through transgenic technology.

3.6 Conclusion and Future Prospects

ROS generation is a common mechanism happening in almost all abiotic stresses that creates secondary stress condition, i.e. oxidative stress. A recent study dealing with meta-analysis of publicly available transcriptomes in rice revealed that ROS detoxifiers (scavengers) are the major differentially expressed genes during abiotic stress (de Abreu Neto and Frei 2015). This explains the significance of ROS scavengers in controlling all abiotic stress in crop plants. But progress in science has stressed that maintaining basal level of ROS is essential for plant cell, as it involves various physiological functions including signal transduction and programmed cell death (Mittler 2017). Hence, focus has to be made on controlled expression of transgenes which further depends on the choice of promoters, while designing transgenic approach. T_7 RNA polymerase-based expressing system with induced promoter that has proved successful in our laboratory (unpublished data) could be more judicious for heterologous expression of multigene constructs. Research efforts also have to be made on determining the threshold levels of ROS that makes the cell to function normally. Though overexpression of single genes has proved successful in stress tolerance for long time, improved effect could be achieved by transferring multigenes involved in pathways. Technologies have to be standardized for multigene transfer that express successfully in host plant using a single vector. Site-specific recombination cloning (gateway cloning) holds promising to transfer many genes in single T-DNA. Care must be taken while choosing the genes used for oxidative stress tolerance as antioxidant enzymes perform differentially under different abiotic stress conditions.

Due to the regulatory issues associated with GMOs, transgenic approach may be considered as lost approach, when the gene of interest is not available within the germplasm of host plant. Marker-assisted breeding (molecular breeding) may be considered as the right choice to identify and transfer the QTLs associated with trait of interest. Due to complexity of the trait, and functional sharing with salt, drought and heat stress, oxidative stress was given less focus in QTL identification and subsequent marker development programmes. Not many specific QTLs were identified associated with oxidative stress, but many QTL identified and markers associated were developed for heat tolerance that shares common genes for detoxifying ROS generated through oxidative stress. Bita and Gerats (2013) have described various QTLs associated with heat tolerance in different crops. Characterization of these QTLs revealed ROS-scavenging genes explaining commonality between the pathways. Recent innovations in genomics have resulted in designing of SNP-based assays that are more closely linked to the trait of interest which are more reliable. Along with regular research in transgenic and molecular breeding

efforts to overcome oxidative stress tolerance, focus also has to put on exploring the interacting molecules of *ROS* genes during the combined attack of biotic and abiotic stress conditions.

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