Designing Genetic Engineering Approaches for Salinity Induced Oxidative Stress Tolerance in Rice (*Oryza sativa* L)

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Abstract

Abiotic stresses are the main limiting factors for crop loss worldwide. Changing climatic conditions further aggravate these stresses and threaten crop productivity worldwide. Rice, a critical cereal grain crop feeding a big part of world's population, is basically sensitive to salinity stress. High salt concentration in soil imposes both ionic and osmotic stresses on plants and these primary effects lead to secondary stresses by enhancing the production of reactive oxygen species (ROS) which ultimately cause oxidative stress and damage proteins, lipids, carbohydrates and nucleic acids. Many major physiological pathways like photosynthesis, respiration, nitrogen fixation and carbohydrate metabolism have been observed to be affected by high salinity. One of the main goals of modern agriculture is to improve the potential of crop plants to survive under salinity stress for a long time. Transgenic approaches are one of the best approaces for the genetic improvement of crop plants. In the present article, we focus on transgenic rice plants overexpressing various genes for salinity stress tolerance and crop improvement.

Keywords: Abiotic stress, crop productivity, genetic engineering, rice, salinity

Introduction

Rice, a cereal grain, is the most preferred staple food for human population worldwide. Globally, billions of people rely on rice cultivation for their livelyhoods. Furthermore, ~20% of the land area periodically face drought related conditions and ~10% occasionally face low temprature conditions (Lane 2002). Overall, rice is frequently affected by several abiotic stress factors including drought, salinity, and cold. Exposure to environmental conditions outside the acceptable range of tolerance can negatively affect rice growth and production. Due to the rice evolution in semi-aquatic, low-radiation habitats, it exhibits both tolerance and susceptibilities to abiotic stresses (Lafitte et al 2004). Therefore, rice is considered as a model plant for studying monocotyledonous species. Among various abiotic stress factors, salinity stress imposes a major constraint to the sustainability of crop yields, causing significant crop losses. Salinity imparts both ionic and osmotic stresses, thus limiting plant growth and productivity. The production of reactive oxygen species (ROS) is an unaviodable consequence of salinity induced oxidative stress in crop plants which leads to reduction in net photosynthetic rate and ultimately stunted crop yield. (Gill and Tuteja 2010). ROS, including singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$), are generated during aerobic metabolism and abiotic stress conditions. They are capable of unrestricted oxidation of various cellular components and can cause membrane lipid peroxidation, protein oxidation, and enzyme inhibition (Muller 2007; Gill and Tuteja 2010). Plant cells remove excess ROS produced during stress through various enzymatic and non-enzymatic mechanisms.

ROS-scavenging enzymes include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT) (Apel and Hirt 2004). APX and GPX are the most studied scavenging enzymes in rice (Kang et al 2004; Li et al 2000; Teixeira et al 2006). They belong to the plant peroxidase superfamily and catalyse the conversion of H_2O_2 to H_2O. Because ROS also function as secondary messengers, their generation and removal is tightly regulated in different cellular components. In rice, eight

genes encoding for distinct APX enzymes were reported: two cytosolic (*OsAPX1* and *OsAPX2*), two peroxisomal (*OsAPX3* and *OsAPX4*), one mitochondrial (*OsAPX6*), and three chloroplastic isoforms (*OsAPX5, OsAPX7,* and *OsAPX8*) (Teixeira et al 2006). On the other hand, the OsGPX1 is present only in the cytosol (Apel and Hirt 2004; Kang et al 2004). Under salt stress, *OsAPX2*, *OsAPX7* and *OsAPX8* show altered transcript levels (Teixeira et al 2006), but only *OsAPX8* was showed to be induced in rice roots (Hong et al 2007). NaCl, ABA, and H_2O_2 can enhance the expression of *OsAPX8* in rice roots. However, the NaCl induced expression of *OsAPX8* is mediated through the accumulation of ABA but not H_2O_2 (Hong et al 2007). Additionally, even isoforms localized within the same cellular compartment may have distinct functions. Expression analysis studies revealed that OsAPX2 is up-regulated under salt stress conditions (Teixeira, et al 2006). It was demonstrated that *Arabidopsis* plants overexpressing *OsAPX2* exhibit higher ROS-scavenging activity and salt tolerance than those overexpressing *OsAPX1* (Lu et al 2007).

Kim et al (2008) applied PEG fractionation technique combined with two-dimensional gel electrophoresis in rice root proteomic studies and out of the 295 chosen proteins, 93 were identified by MALDI-TOF mass spectrometry. The proteins were classified as related to metabolism (38.7%), reactive oxygen species (ROS)-related proteins (22.5%), protein processing/degradation (8.6%), stress/defense (7.5%), energy (6.5%) and signal transduction (5.4%). The high percentage of ROS-related proteins found in rice root brings them to assess the role of ROS on rice root growth. Treatment with ROS quenching chemicals such as reduced glutathione (GSH), diphenyleneiodonium (DPI) and ascorbate inhibited root growth in a dose-dependent manner. Forty-nine proteins identified were either up- or down-regulated by GSH treatment, out of which 14 were ROS-related proteins, such as glutathione-S-transferase (GST), superoxide dismutases (SOD) and L-ascorbate peroxidases. The protein levels of four GSTs (NS4, 8, 56 and 57), three APXs (NS46, 49 and 50) and MnSOD (NS45) were strongly reduced by GSH treatment and only slightly reduced by ascorbate and DPI. Ascorbate and DPI strongly inhibited the expression levels of a catalase A (NP23) and an APX (NS65) but did not affect APXs (NS46, 49 and 50) protein levels. Northern blot analysis demonstrated that changes in transcript levels of five genes (GST (NS4), GST (NS43), Mn-SOD (NS45), APX (NS50) and APX (NS46/49)) in response to ROS quenching chemicals were coherent with patterns shown in two-dimensional electrophoresis analyses. The authors suggested that these proteins may play an important role in maintaining cellular redox homeostasis during rice root growth (Kim et al 2008).

Non-enzymatic antioxidants include the major cellular redox buffers, ascorbate and glutathione, as well as carotenoids and tocopherol (Apel and Hirt 2004; Gill and Tuteja 2010). Alleviation of oxidative injury by the use of antioxidants can enhance plant resistance to abiotic stress. Guo et al (2005) found that feeding rice roots with L-ascorbic acid and its immediate precursor protected plants against oxidative damages, suggesting that manipulation of ascorbic acid biosynthesis could be a strategy for improving stress tolerance. During the oxidation process, ascorbate itself is oxidized to dehydroascorbate, while dehydroascorbatereductase (DHAR) re-reduces the oxidized ascorbate. A high ratio of reduced to oxidized ascorbate is important for ROS-scavenging efficiency. Ushimaru et al (2006) reported that overexpression of rice *DHAR1*in *Arabidopsis* increases the ascorbate levels and leads to increased salt tolerance. Plants respond and adapt to these conditions by regulating a wide array of genes. The biotechnology tools, especially genetic engineering approaches, offer a promise to complement existing salinity stress tolerance strategies in rice.

Salinity induced ROS production

Exposure of crop plants to salinity stress can increase the ROS production. ROS are highly reactive and toxic and cause damage to chloroplast, proteins, lipids, carbohydrates and nucleic acids which ultimately results in oxidative stress (Gill and Tuteja 2010; Balestrazzi et al 2011). It has also been noted that plants exposed to various abiotic stresses, including salinity stress, exhibit an increase in lipid peroxidation due to the generation of ROS.

Khan and Panda (2008) studied the response of two *O sativa* cultivars under salt stress and found that it increased the LPO in both; the level was higher in Begunbitchi than that in the cultivar Lunishree. They correlated the higher free radicals scavenging capacity and more efficient protection mechanism of Lunishree against salt stress, with the lower level of lipid peroxidation of Begunbitchi. Similarly, Kukreja et al (2005) noted a marked increase in LPO in *Cicerarietinumroots* under salinity stress. Pan et al (2006) also reported increase in malondialdehyde (MDA) content in liquorice (*Glycyrrhiza uralensis* Fisch) seedlings under salt and drought stress. ROS induced oxidation of a number of protein amino acids, out of which Arg, His, Lys, Pro, Thr and Trp in particular give free carbonyl groups which may inhibit or alter their activities and increase susceptibility towards proteolytic attack (Moller et al 2007). Protein carbonylation may occur due to direct oxidation of amino acid side chains (Shringarpure and Davies 2002). It has been found that various stresses lead to the carbonylation of proteins in plant tissues. Carbonylation

of storage proteins has been noted in dry *Arabidopsis* seeds but carbonylation of a number of other proteins increased strongly during seed germination (Job et al 2005). Plant genome is very stable but its DNA might get damaged due to the exposure to abiotic stress factors and thereby exertsgenotoxic stress (Tuteja et al 2001; Tuteja and Tuteja 2001; Tuteja et al 2009; Balestrazzi et al 2011).

It has been reported that OH is the most reactive and cause damage to all molecular components, damaging both the purine and pyrimidine bases along with the deoxyribose backbone (Halliwell and Gutteridge 1999), $^1O_2$ primarily attacks guanine, while H_2O_2 and $O_2^{\bullet-}$ are less reactive (Wiseman and Halliwell 1996). DNA damage results in various physiological effects, such as reduced protein synthesis, cell membrane destruction and damage to photosynthetic proteins, which affects growth and development of the whole organism (Britt 1999). The literature available on oxidative stress has established that the induction of the cellular antioxidant machinery is important for protection against various stresses (Gill and Tuteja2010).

ROS scavenging machinery and salinity tolerance

ROS basically includes singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) which are highly reactive and toxic and cause damage to chloroplast, proteins, lipids, carbohydrates and nucleic acids which ultimately results in oxidative stress (Gill and Tuteja 2010). Plants possess efficient antioxidant system to protect the photosynthetic machinery and cellular membranes from ROS (Foyer and Harbinson 1994). The non-enzymatic components scavenging ROS include glutathione (GSH), and ascorbic acid, while the enzymatic antioxidants include superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), guaicol peroxidase (GPX) and glutathione reductase (GR; EC 1.6.4.2). ROS induced oxidative stress under salinity stress and their scavenging mechanism through antioxidant machinery is depicted in Fig. 1. SOD constitutes the first line of defense, it is a major scavenger of $O_2^{\bullet-}$ and catalyze the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2. H_2O_2 is scavenged by APX enzyme, which converts H_2O_2 to H_2O using ascorbate as electron donor. Finally, GR catalyzes the NADPH-dependent reduction of oxidized GSSG to the reduced GSH (Noctor et al 2002). It is evidenced from the literature that up regulation of antioxidant systems protect plants from salinity induced oxidative stress (Hoque et al 2008; El-Shabrawi et al 2010; Gill and Tuteja 2010).

Among the non-enzymatic antioxidants, ascorbic acid is an important primary metabolite in plants, representing an enzyme cofactor and a cell signaling modulator in a wide array of crucial physiological processes, including biosynthesis of the cell wall, secondary metabolites and phytohormones, stress tolerance, photoprotection, cell division and growth (Wolucka et al 2005).

Besides, it is also important for the regeneration of membrane-bound antioxidants (Hideg 1999). GSH is a water-soluble tripeptide containing a sulfhydryl group and is a substrate for dehydroascorbatereductase in the ascorbate-glutathione cycle. GSH directly scavenges $OH^{\bullet}$ and $^1O_2$ and may protect enzyme thiol groups (Gill and Tuteja 2010).
Efficient co-ordination of the enzymatic antioxidant machinery components and high concentration of ascorbic acid and GSH help in counteracting the deleterious effects of ROS under salinity stress. Therefore, salinity tolerance may be correlated with induced or constitutively increased high level of the antioxidant activity (Fig. 1).

Genetic engineering approaches for salinity stress tolerance

In order to improve abiotic stress tolerance in plants through genetic engineering, many studies have primarily focused on the transcription factors as gene regulators (Xiong et al 2006; Mazzucotelli et al 2008), then on genes that encode ion transport proteins (Uozumi and Schroeder 2010), compatible organic solutes (Ashraf and Foolad 2007; Chen and Murata 2011), antioxidants (Gill and Tuteja 2010; Kwak et al 2008), heat-shock (Altman et al 2004; Wang et al 2004a), late embryogenesis abundant proteins (Battaglia et al 2008; Tunnacliffe et al 2010) and helicases (Sanan-Mishra et al 2005; Luo et al 2009; Amin et al 2012). A number of transgenic improvements for abiotic stress tolerance have been achieved through detoxification strategy. These include transgenic plants overexpressing enzymes involved in oxidative protection, such as glutathione peroxidase, superoxide dismutase, ascorbate peroxidases and glutathione reductasesetc (Gill and Tuteja 2010).

SOD has been proposed to be important in plant stress tolerance and provide the first line of defense against the toxic effects of elevated levels of ROS. The SODs remove $O_2^{\bullet-}$ by catalyzing its dismutation, the $O_2^{\bullet-}$ being reduced to H_2O_2 and another oxidized to O_2 (Gill and Tuteja, 2010). Significant increase in SOD activity under salt stress has been observed in various plants such as mulberry (Harinasut et al 2003), *C. arietinum* (Kukreja et al 2005) and *Lycopersiconesculentum* (Gapinska et al 2008). Eyidogan and Oz (2005) noted three SOD activity bands (MnSOD, FeSOD and Cu/ZnSOD) in *C. arietinum*under salt stress.

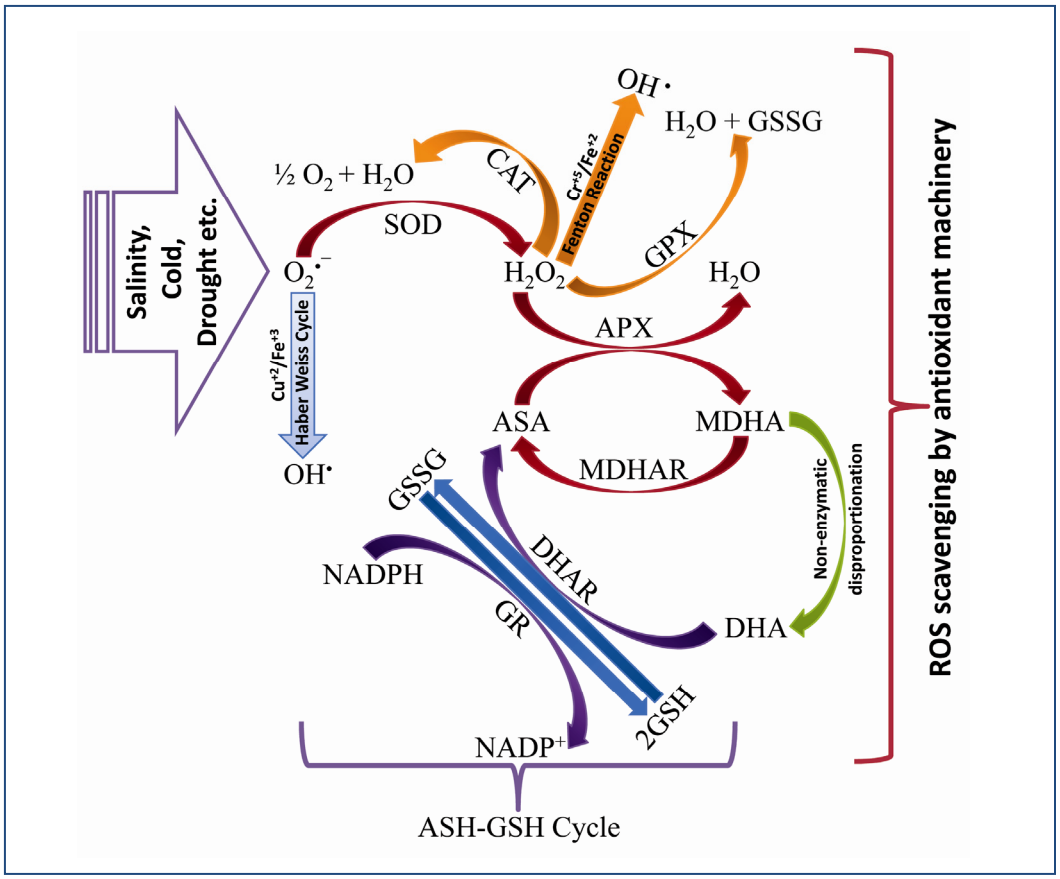

Figure 1. Cartoon represents reactive oxygen species (ROS) induced oxidative stress under salinity stress and their scavenging mechanism through antioxidant machinery. Salinity lead to the overproduction of ROS in plants which results in oxidative stress. ROS comprises both free radical ($O_2^{\bullet -}$, superoxide radicals; OH•, hydroxyl radical; $HO_2^{\bullet}$, perhydroxy radical and $RO^{\bullet}$, alkoxy radicals) and non-radical (molecular) forms (H_2O_2, hydrogen peroxide and $^1O_2$, singlet oxygen). Superoxide dismutase (SOD; EC 1.15.1.1) removes $O_2^{\bullet -}$ by catalyzing its dismutation, one $O_2^{\bullet -}$ being reduced to H_2O_2 and another oxidized to O_2. Catalase (CAT; EC 1.11.1.6) is another potential enzymatic antioxidant which directly dismutates H_2O_2 into H_2O and O_2. Ascorbate peroxidase (APX; EC 1.11.1.11) scavenge H_2O_2 in water-water and ASH-GSH (ascorbic acid-glutathione) cycles and utilizes ASH as the electron donor. APX can be distinguished from plant-isolated guaiacol peroxidase (GPX; EC 1.11.1.7) in terms of differences in sequences and physiological functions. GPX also has a role in defense against various stresses by consuming H_2O_2. Glutathione reductase (GR; EC 1.6.4.2) is a potential enzyme of the ASH-GSH cycle and plays an essential role in defense system against ROS by sustaining the reduced status of GSH. Dehydroascorbatereductase (DHAR; EC 1.8.5.1) regenerates ASH from the oxidized state and regulates the cellular ASH redox state which is crucial for tolerance to various abiotic stresses leads to the production of ROS. Monodehydroascorbatereductase (MDHAR; EC 1.6.5.4) exhibits a high specificity for MDHA as the electron acceptor, preferring NADH rather than NADPH as the electron donor. The reduced enzyme donates electrons successively to MDHA, producing two molecules of ascorbate via a semiquinone form [E-FAD-NADP(P)$^+$]

Furthermore, a significant increase in the activities of Cu/ZnSOD and MnSOD isozymes under salt stress was observed. Many reports of the production of abiotic stress tolerant transgenic plants overexpressing different SODs are available.

Overexpression of a Mn-SOD in transgenic *Arabidopsis* plants also showed increased salt tolerance (Wang et al 2004b). Furthermore, they showed that Mn-SOD activity as well as the activities of Cu/Zn-SOD, Fe-SOD, CAT and POD were significantly higher in transgenic *Arabidopsis* plants than in control (Wang et al 2004b). Cu/Zn-SOD overexpressing transgenic tobacco plants showed multiple stress tolerance (Badawi et al 2004).

Catalase (CAT) are tetrameric heme containing enzymes with the potential to directly dismutate H_2O_2 into H_2O and O_2 and are indispensable for ROS detoxification during stressed conditions. Eyidogan and

Oz (2005) reported a significant increase in CAT activity in *C. arietinum* leaves under salt treatment. Similarly, an increase in CAT activity in *C. arietinum* roots following salinity stress was noted by Kukreja et al (2005). Pan et al (2006) studied the combined effect of salt and drought stress and found that it decreases the CAT activity in *G.uralensis* seedlings. Transgenic rice plants overexpressing *OsMT1a* showed increase in CAT activity and thus enhanced tolerance to drought (Yang et al 2009). Ascorbate peroxidase (APX) is involved in scavenging of H_2O_2 in water-water and ASH-GSH cycles and utilizes ASH as the electron donor. It has also been noted that overexpression of APX in *Nicotiana tabacum* chloroplasts enhanced plant tolerance to salt and water deficit (Badawi et al 2004). In a different study, the expression patterns of APX were analysed in roots of etiolated *O. sativa* seedlings under NaCl stress and the mRNA levels for two cytosolic (*OsAPX1* and *OsAPX2*), two peroxisomal (*OsAPX3* and *OsAPX4*), and four chloroplastic (*OsAPX5, OsAPX6, OsAPX7*, and *OsAPX8*) genes were quantified in rice genome. It was noted that 150 mM and 200 mMNaCl increased the expression of *OsAPX8* and activities of APX, but there was no effect on the expression of *OsAPX1, OsAPX2, OsAPX3, OsAPX4, OsAPX5, OsAPX6*, and *OsAPX7* in rice roots (Hong et al 2007).

Glutathione reductase (GR) is a flavo-protein oxidoreductase, found in both prokaryotes and eukaryotes. Transgenic plants with less GR activity showed enhanced sensitivity to oxidative stress. It was suggested that GR plays an important role in the regeneration of GSH and thus it protects against oxidative stress also by maintaining the ASH pool (Ding et al 2009). MDHAR is a flavinadenin dinucleotide (FAD) enzyme that is present as chloroplastic and cytosolic isozymes. MDHAR exhibits a high specificity for monodehydroasorbate (MDHA) as the electron acceptor, preferring NADH rather than NADPH as the electron donor. Overexpression of MDHAR in transgenic tobacco increased the tolerance against salt and osmotic stresses (Eltayeb et al 2007). Glutathione S-transferases (GST) are a large and diverse group of enzymes which catalyse the conjugation of electrophilic xenobiotic substrates with the GSH. Plant GSTs are known to function in herbicide detoxification, hormone homeostasis, vacuolar sequestration of anthocyanin, tyrosine metabolism, hydroxyperoxide detoxification, regulation of apoptosis and in plant responses to biotic and abiotic stresses. GST *Nt107* expressing transgenic *Gossypium hirsutum* lines were used to investigate the tolerance potential under various stresses like chilling, salinity, and herbicides and it was noted that transgenic seedlings exhibited ten-fold and five-fold higher GST activity under control and salt stress conditions, respectively (Light et al 2005).

Conclusion and future perspectives

It is well known that salinity stress leads to the overproduction of highly reactive and toxic ROS in plants, resulting in oxidative stress. The plant cells are equipped with excellent antioxidant defense mechanisms to counteract the harmful effects of ROS. Therefore, engineering ROS scavenging pathway for salinity tolerance may be an ideal approach for salinity stress tolerance in rice. Several transgenic improvements for abiotic stress tolerance have been achieved through detoxification strategy. These include transgenic plants overexpressing enzymes involved in oxidative protection, such as glutathione peroxidase, superoxide dismutase, ascorbate peroxidases and glutathione reductases etc. Pyramiding of genes of ascorbate-glutathione pathway may also be a preferred target to get salinity tolerance in rice. Recently, we have observed that PDH45, a DEAD–box helicase, functions in salinity stress tolerance by improving antioxidant machinery in rice cultivar PB1. The coordinated and concerted action of antioxidant machinery (SOD, APX, GPX and GR) in PDH45 overexpressing T_1 transgenic rice lines probably helped in avoiding salinity induced oxidative damage (Tuteja's group unpublished data).

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