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Plant response to salinity: an analysis of ROS formation, signaling, and antioxidant defense

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Abstract: Reactive oxygen species (ROS) are byproducts of normal plant metabolism and their production is elevated under environmental stresses such as drought, extreme temperature, and salinity. Among these, salinity is a worldwide problem that impacts the fertility of arable lands and sustainability of food security, which is getting more attention due to climate change. Halophytes can survive and reproduce in soils containing high concentrations of salt and have developed adaptation mechanisms at physiological, biochemical, and molecular levels including maintenance of ROS metabolism. In this review, we aim to summarize findings related to ROS production, signaling, scavenging, and especially ROS avoidance mechanisms under salt stress. In addition, expressions of antioxidant genes in *Arabidopsis thaliana* and its close relative, the model halophyte *Schrenkiella parvula*, are compared. Moreover, time-course expression levels of genes encoding major antioxidant enzymes in the model plant *A. thaliana* are analyzed with publicly available data to understand rapid responses of antioxidant defense under salt stress. The role of ROS-Ca^{+2} interaction and involvement of NADPH oxidases in this process are also discussed in the context of the perception and signaling of salt stress.

Key words: Antioxidant defense, halophytes, NADPH oxidase, reactive oxygen species, salt stress

1. Introduction

Salt stress is among the most prominent environmental stressors that limit plant growth and development and inevitably yield. More than 20% of the arable land on our planet is challenged by soil salinity, mostly with high Na^+ levels, which are referred to as sodic soils (Qadir et al., 2014). Although not in parallel with the severity of the problem, there has been credible scientific effort to understand plant responses to salt stress and ways to alleviate its effects on plants. Accordingly, mechanisms related to salt stress perception, signal transduction, and regulation of membrane transporters have been elucidated, accompanied with identification of new membrane transporters involved in ion homeostasis and stress-responsive proteins that are essential for salt stress adaptation. Among these salt stress-responsive proteins, proteins that are related to antioxidant defense have been intensively studied in the last 30 years. The antioxidant defense system in plants comprises enzymatic and nonenzymatic components that are responsible for the scavenging of reactive oxygen species (ROS) (Mittler et al., 2004). Besides the primary effects of salt stress, such as osmotic or ionic effects, it has been documented that loss of balance between different metabolic processes can cause generation of excess ROS resulting in oxidative stress (Ozgur et al., 2013). Therefore, the idea that plants with increased capacity to combat oxidative stress might be more tolerant to salt stress paved the way for years of research on this topic (Perez and Brown, 2014), which is still ongoing. In particular, comparative studies conducted with salt stress-tolerant relatives of crop plants demonstrated that there is a correlation between antioxidant capacity and salt stress tolerance (Bor et al., 2003; Demiral and Turkan, 2005; Seckin et al., 2010). Comparison of halophytes (plants that are adapted to live in saline areas) to glycophytes has been a topic of similar research (reviewed by Ozgur et al., 2013 and Ozfidan-Konakci et al., 2016). There is a huge body of literature on elucidation of the role of antioxidant defense and ROS regulation at transcriptomic, proteomic, and/or biochemical levels in various plant species. However, it should be noted that most of these works that utilize top-down approaches, such as transcriptomics or proteomics, use glycophytes as plant material. Therefore, knowledge about halophytes is rather limited, especially at the molecular level.

Moreover, since the 2000s it has been established that ROS not only have damaging roles, but at low concentrations they can act as vital signal molecules that have various roles during growth and development and stress responses (Baxter et al., 2013). This role was further supported with the identification of plant NADPH oxidase encoding genes (respiratory burst oxidase homolog, RBOHS) (Suzuki et al., 2011).

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The importance and pioneering role of ROS in salinity tolerance research has not diminished. On the contrary, it was reinforced by new findings related to the involvement of ROS in stress signaling (Suzuki et al., 2012), regulation of cellular energy budget (Shabala et al., 2015), growth and development (Swanson and Gilroy, 2010), and control of membrane transporter activity (Pottosin et al., 2014), all of which have direct implications on plant performance under salt stress.

In this review, we mostly aim to compile the knowledge related to the secondary effects of salt stress, i.e. ROS production and scavenging. We also discuss how ROS production can be avoided by plants, which is an underexplored topic. In addition, we aim to explore the short-term response of ROS metabolism of plants under salinity with special emphasis on ROS-Ca^{+2}-NADPH oxidase interaction. Throughout the manuscript, we particularly try to relate the research conducted in halophytes with glycophytes to identify gaps in the knowledge.

2. Salinity stress and its effects

The impact of high salinity on plants occurs in two phases, which are osmotic stress and ion toxicity. Osmotic stress develops rapidly, within hours, and it is the first constraint caused by high salt levels. It reduces the capacity of roots to absorb water, which makes it difficult for the plant to replace water lost from the leaves (Munns, 2008). Osmotic stress is followed by the toxic effect of high concentrations of salt within plant cells. It occurs within days and weeks. Na^+ and Cl^- ions accumulate particularly in the leaves. Na^+ accumulation is toxic, especially in old leaves, as their expansion ceases; hence, dilution of high concentrations of salt cannot take place. Na^+ accumulation also affects photosynthetic components such as enzymes, chlorophylls, and carotenoids (Davenport et al., 2005). Furthermore, excess Na^+ uptake causes inhibition of K^+, Ca^{2+}, causing ion imbalance (Hu and Schmidhalter, 2005). As secondary stress, salt stress also induces ROS production, which leads to oxidative damage in various cellular components by oxidizing proteins, lipids, and DNA, leading to interruption of vital cellular functions in plants (Mahajan and Tuteja, 2005). Conclusively, high salinity causes disruption of membrane integrity, nutrient imbalance, decreased ability to detoxify ROS, and inhibition of photosynthetic activity, ultimately reducing plant growth, development, and survival (Munns and Tester, 2008; Gupta and Huang, 2014).

3. Halophytes

Based on their ability to cope with salinity, plants can be divided into two groups as halophytes and glycophytes. The latter encompasses a vast majority of the terrestrial plant species, which are sensitive to salinity. On the other hand, halophytes, a special plant community, can tolerate up to 1300 mM NaCl (Glenn et al., 1997) and are salt-tolerant plants. There are various definitions of halophytes (Grigore, 2019). Khan and Duke (2001) defined halophytes as salt-tolerant plants that are highly evolved and specialized organisms with well-adapted morphological and physiological characteristics allowing them to survive in soils with high salt concentrations. On the other hand, Flowers et al. (1986) defined halophytes as plants with the ability to complete their life cycle at or above 200 mM NaCl. Most sensitive crops, on the other hand, are severely damaged by even 20–50 mM NaCl (Greenway and Munns, 1980).

Halophytes broadly differ in their degree of salt tolerance. Crop plants such as sugar beet, date palm, and barley can survive on irrigation water approaching 85 mM NaCl (Ozgur et al., 2013) and are sometimes considered halophytes. An example of the plants at the high end of salt tolerance is *Salicornia bigelovii*, which can survive at up to 1300 mM NaCl (twice seawater's salinity) and can set seed at this salt concentration (Glenn et al., 1997). In a more recent review, Ozgur et al. (2013) reported the NaCl concentrations at which the first significant increase in lipid peroxidation was observed in shoots. In *Atriplex portulacoides*, for example, 40 days of >1000 mM NaCl treatment could cause oxidative injury (Benzarti et al., 2012). On the other hand, in another halophyte (*Beta maritima*), 6 days of 150 mM NaCl treatment was sufficient to significantly increase lipid peroxidation (Bor et al., 2003).

Halophytes are able to accumulate large amounts of Na^+ in their vacuoles (Khan, 2000). This is achieved by an efficient Na^+/H^+ antiport system in the tonoplast and also requires specially adapted membrane lipids to prevent leakage of Na^+ from the vacuole to the cytoplasm (Joshi et al., 2015). While Na^+ is actively pumped into the vacuole, Cl^- enters passively via anion channels (Pantoja et al., 1992). Another important characteristic of halophytes is that they can excrete salt from their leaves and roots (Warwick and Halloran, 1992). Furthermore, both halophytes and nonhalophytes have the ability to export Na^+ from the cytoplasm to the extracellular space using plasma membrane Na^+/H^+ antiporters, which is known as the SOS (salt overly sensitive) pathway (Zhu, 2001). Halophytes can also produce several compatible osmolytes to reduce their osmotic potential to sustain water absorption from saline soil solutions. Some of these compatible osmolytes can also help to protect cellular structures by detoxifying ROS (Zhu, 2001).

4. ROS metabolism under salt stress

4.1. ROS production

ROS are byproducts of normal metabolism and their production is accelerated under salinity. ROS includes

O_2-derived radicals such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$), and singlet oxygen ($^1O_2$) (del Rio, 2015). Both osmotic and ionic effects of salt stress contribute to increased ROS production in various tissues and subcellular compartments of plants (Ozgur et al., 2013). In leaves, the limitation of gas exchange impairs the influx of CO_2 into leaf mesophyll cells, causing a decrease in internal CO_2 levels (C_i) (Steduto et al., 2000). Low C_i causes a loss of balance between light reactions of photosynthesis that produce ATP and NADPH and the Calvin–Benson–Bassham (CBB) cycle that consumes this energy and reducing power. Uncoupling of light reactions and the CBB cycle leads to ROS accumulation in chloroplasts due to overexcitation of PSII, which results in the production of $^1O_2$ (Asada, 2006). Moreover, a high NADPH/$NADP^+$ ratio due to gas exchange limitation induces the Mehler reaction in PSI, resulting in transfer of an electron to O_2, producing $O_2^{\cdot-}$. Further dismutation of $O_2^{\cdot-}$ produces H_2O_2 in chloroplasts (Asada, 2006). On the other hand, besides decreased CO_2 levels, it has been demonstrated that increased levels of Na^+ or Cl^- can also disrupt the kinetics of CBB enzymes, further amplifying ROS production. For example, 250 mM NaCl in the reaction medium decreased the activity of *Phaseolus vulgaris* RuBisCo below 50% of controls (Osmond and Greenway, 1972) and 25 mM NaCl reduced the activity of chloroplastic fructose-1,6-bisphosphatase of *Oryza sativa* by 50%, which is an enzyme involved in the regeneration phase of the CBB cycle. Interestingly, fructose-1,6-bisphosphatase of *Poteresia coarctata*, a halophytic relative of *O. sativa*, was more tolerant to inhibition by NaCl, which was observed as 10% inhibition up to 400 mM NaCl (Ghosh et al., 2001).

In C_3 plants, which do not utilize a biochemical CO_2 pump to concentrate CO_2 around RuBisCo, photorespiratory H_2O_2 production is one of the major sources of ROS (Kangasjärvi et al., 2012). Under stress conditions, photorespiration can proceed with rates of about 35%–50% of CO_2 fixation, which makes it equal to or second after photosynthesis itself (Carmo-Silva et al., 2008). In relation to different carboxylation pathways (C_3 vs. C_4), it has been demonstrated that salt tolerance is significantly more likely to occur in plant lineages with C_4 photosynthesis when compared to C_3 lineages (Bromham and Bennet, 2014). In this respect, it would be logical to assume that one can explain this with reduced transpiration, increased water-use efficiency, and limited uptake of toxic ions. However, it should be also considered that besides photorespiration, the chloroplastic ROS generation dynamics of C_4 plants, especially those of NADP-malic enzyme (NADP-ME) subtypes, would be innately different due to lack of PSII (which means lack of $^1O_2$ production) in their bundle sheath chloroplasts (reviewed by Turkan et al., 2018). This change of ROS production dynamics in C_4 plants is usually accompanied with changes in antioxidant defense, both in terms of total enzyme activity and isoenzyme pattern (Uzilday et al., 2014b, 2018b). However, there are no studies that investigate how ROS formation occurs in mesophyll and bundle sheath cell chloroplasts of C_4 glycophytes or halophytes under salt stress.

Perturbation of the redox balance in mitochondria causes overload of the electron transport chain, resulting in $O_2^{\cdot-}$ production originating from complex I, II, and III (Saha et al., 2016). Although it is required for oxidation of $FADH_2$ during oxidative phosphorylation, decreased levels of succinate dehydrogenase (complex II) increase plant performance under salt stress, most probably due to lower ROS levels (Jardim-Messeder et al., 2015). However, the consequences of this mutation for plant metabolism and redox regulation, especially that of crop plants, are unknown.

Moreover, the endoplasmic reticulum (ER) can also act as a ROS source (Ozgur et al., 2018). The ER is responsible for oxidative protein folding and hence the formation of disulfide bonds. ER-resident protein disulfide isomerases (PDIs) oxidize target proteins, forming disulfide bonds. In turn, they transfer these electrons to ER oxidoreductase (ERO). ERO transfers these electrons to O_2 and forms H_2O_2 at the ER lumen. Hence, the ERO-PDI system controls the redox status of the ER lumen (Ozgur et al., 2018). Under salt stress, the folding of proteins in the ER can be impaired, causing the formation of incorrect disulfide bonds (Ozgur et al., 2018). These bonds are further broken with GSH and new bonds should be formed (Uzilday et al., 2018a). This line of events increases H_2O_2 formation in the ER and can cause depletion of reduced glutathione in the cell.

ROS also stimulates the overproduction of reactive carbonyl species (RCS), which are derived from lipid peroxides (Yalcinkaya et al., 2019b). Many RCS molecules such as acrolein, 4-hydroxy-(E)-2-nonenal (HNE), and malondialdehyde (MDA) were identified in plants. Membranes in a cell are sources of RCS and Mano et al. (2014) demonstrated that salt stress enhanced the generation of HNE, which originated from membranes. In the same study, protein modification with RCS was linked to salt stress response in *A. thaliana*. RCS are scavenged and detoxified by a complex enzymatic system including alkenal reductase (AER), which uses NAD(P)H as an electron donor to reduce the α,β-unsaturated bonds of an RCS molecule, glutathione S-transferase (GST), which uses GSH to form glutathione conjugates with RCS molecules, aldo-keto reductase (AKR), and aldehyde dehydrogenase (ALDH), which use NADPH to reduce RCS to n-alcohol or NAD^+ to oxidize RCS to carboxylate, respectively. Higher RCS detoxification capacity has been linked to increased salt tolerance. For example, overexpression of AER

stimulates salt tolerance in Arabidopsis (Papdi et al., 2008). Salt stress also enhanced the *AKR4B* gene expressions in tomato (Suekawa et al., 2016) and *AKR1*-overexpressing tobacco plants showed enhanced antioxidant capacity (Vemanna et al., 2017). Yalcinkaya et al. (2019a) compared the response of the redox regulatory system in glycophytic *A. thaliana* and halophytic *S. parvulum* to exogenously applied RCS and found that the H_2O_2 scavenging enzymes of *S. parvulum* were not affected as much as of *A. thaliana* by RCS treatments. Moreover, *S. parvulum* managed to maintain NADPH oxidase-mediated ROS signaling under RCS treatment, while it was reduced in *A. thaliana*.

4.2. ROS scavenging systems

The excess and uncontrolled accumulation of ROS in a cell leads to oxidative stress, which can eventually lead to cell death (Petrov et al., 2015). Oxidative stress is known as a secondary component of other stresses and plants have evolved mechanisms to cope with deleterious effects of oxidative damage. These mechanisms include enzymatic and nonenzymatic antioxidants, which work in coordination to balance ROS levels in the cell (Mittler et al., 2004).

Plants have evolved refined enzymatic ROS detoxification mechanisms that are found in different compartments of the plant cells, which include superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), and glutathione peroxidase (GPX). Besides these enzymes that directly scavenge ROS, there are other enzymes such as monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and glutathione-S-transferase (GST), which are responsible for regeneration of oxidized nonenzymatic antioxidants such as ascorbate (MDHA and DHA to ascorbate) and glutathione (glutathione disulfide (GSSG) to glutathione). Besides ascorbate and glutathione, which act as universal redox buffers in plant cells, there are other low-molecular-weight compounds such as phenolics, carotenoids, and tocopherols that have antioxidant properties (Mittler et al., 2004). The extent of the utilization of these nonenzymatic antioxidants depends on the plant's ability to synthesize a specific molecule by regulating its secondary metabolism and can show great variability between different plant species.

Most of the studies that investigate the role of ROS scavenging capacity under salt stress utilize a sensitive and a tolerant cultivar (Bor et al., 2003; Demiral and Turkan, 2005) or a glycophyte and a close halophytic relative (Seckin et al., 2010; Ellouzi et al., 2014). Metaanalysis of these studies implies that halophytes are able to induce ROS detoxification mechanisms better under salt stress when compared to glycophytes, and the same phenomenon applies to salt-tolerant and salt-sensitive cultivars of the same species.

For example, Srivastava et al. (2015) reported that the SOD, APX, and GR activities of *Sesuvium portulacastrum* (halophyte) were increased with NaCl treatment while those of *Brassica juncea* (glycophyte) decreased under high salt conditions, implying that halophytic enzymes are more robust and stable than glycophytic enzymes. It is also reported that halophytes can have higher constitutive antioxidant defense activity as compared with glycophytes (Ozgur et al., 2013). Halophytic plants also increase their enzymatic antioxidant ability with the severity of the salt stress. For example, halophyte *Atriplex portulacoides* increased its SOD activity in a NaCl dose-dependent manner (Benzarti et al., 2012). Similarly, Uzilday et al. (2014a) determined that in *Schrenkiella parvula* (=*Thellungiella parvula*), the activities of SOD, APX, MDHAR, DHAR, GR, and POX were increased following NaCl treatment. In conclusion, these studies underpin the importance of the enzymatic antioxidant defense in halophytes.

The induction of antioxidant defense is not always observed in some halophytes. For example, since obligatory halophytes have the ability to exclude Na^+ from their cytosol, ROS production related to ion toxicity and hence oxidative stress is reduced. In other words, these plants avoid oxidative stress by different means rather than trying to cope with it. For this reason, they may not need high levels of antioxidants (Bose et al., 2014; Kumari et al., 2015; Surówka et al., 2019).

Under salt stress, plants utilize various regulatory mechanisms at transcriptional, translational, and posttranslational levels (Mazzucotelli et al., 2008). As mentioned above, biochemical studies show that halophytes have a higher antioxidant capacity under salt stress conditions and sometimes even under nonstress conditions (Ozgur et al., 2013). However, this type of data measures the final consequences of all the regulatory pathways. To determine how transcripts of antioxidant enzymes are regulated between glycophytes and halophytes, we utilized RNA-seq data provided by Oh et al. (2014), who used *A. thaliana* and *S. parvula* as plant material. We investigated expression levels of genes that encode well-known antioxidant enzymes in these two species and calculated $\log_2$ ratios (i.e. $\log_2$[*S. parvula*/*A. thaliana*]) (Figure 1). Comparison of *A. thaliana* vs. *S. parvula* has several advantages such as the close relation between the two species, their highly similar genome sequence (~90%), and their similar growth physiology (Dassanayake et al., 2011). As can be seen from Figure 1, at the transcriptional level, in the roots, there is no clear abundance of transcripts in favor of *S. parvula* except for *APX2* (3.56), *MDHAR3* (2.22), and *GPX4* (4.63). However, interestingly, data related to roots clearly indicate that transcript abundances of genes related to antioxidant enzymes are higher in *A.*

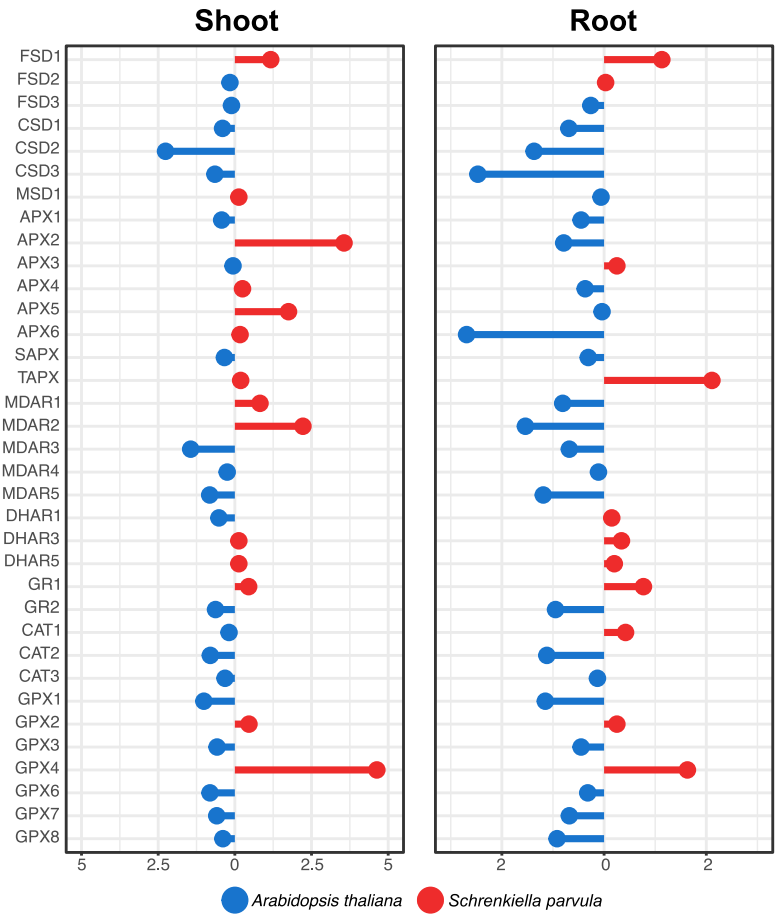

Figure 1. Log_2 ratios of the expression levels of antioxidant enzyme genes in *A. thaliana* and *S. parvula* shoots and roots under nonstress conditions. Data were taken from Oh et al. (2014). Antioxidant enzyme genes were defined according to Mittler et al. (2004).

thaliana when compared to those of *S. parvula*. Among these, *CSD3* (–2.47), *APX6* (–2.69), and *MDHAR2* (–1.54) show the most remarkable differences. Overall, the data imply that under normal conditions, halophytes do not necessarily have higher transcript levels of antioxidant enzymes when compared to glycophytes. This indicates that higher antioxidant capacity might also be related to regulation at the translational and posttranslational levels. A drawback of this comparison is the lack of RNA-seq data under salt stress, which would probably show induced transcript levels in halophyte *S. parvula*. Nevertheless, it illustrates the preconditioning of glycophyte and halophyte plants to salt stress at the transcriptional level.

Nonenzymatic antioxidants are vital for plants because some highly toxic ROS such as $^1O_2$ and $OH^{\cdot}$ cannot be scavenged by antioxidant enzymes and plants rely on the nonenzymatic components of the antioxidant system to scavenge them. These molecules are accumulated at higher concentrations under unstressed conditions in halophytes as compared with glycophytes. The accumulation of proline (Gong et al., 2005; Yaish et al., 2015), α-tocopherol (Ellouzi et al., 2011), carotenoids (Yang et al., 2009), and polyphenols (Ksouri et al., 2012) has been shown to be higher in halophytes than in glycophytes. Taken together, regulation of enzymatic and nonenzymatic components of the antioxidant system enables halophytes to protect themselves against oxidative damage.

Moreover, as mentioned above, excess concentrations of ions can inhibit enzyme activity and this is well documented for some photosynthetic enzymes. However, to the best of our knowledge, there are no data on the effects of different ions on antioxidant enzyme kinetics. In this case, it would be interesting to see if halophyte antioxidant enzymes are more resistant to different ions such as Na^+, K^+, Cl^-, SO_4^{-2}, or NO_3^-. A decrease in the activities of antioxidant enzymes to the same extent as photosynthetic enzymes (up to 50%) with NaCl (Ghosh et al., 2001) may imply that ROS scavenging capacity also

might also decrease with high levels of ions in different compartments of the cell. This topic deserves further scrutiny since it would create opportunities to engineer antioxidant enzymes that are more resistant to inhibition by ionic strength of the medium.

4.3. ROS avoidance mechanisms

As mentioned above, the main reasons for ROS formation in chloroplasts and mitochondria are the overload of electron transport chains. To overcome this, plants developed alternative bypass pathways that oxidize electron transport chain components. In the mitochondria and chloroplasts, these mechanisms involve participation of two proteins, alternative oxidase (AOX) and plastid terminal oxidase (PTOX or IMMUTANS) (Nawrocki et al., 2015; Saha et al., 2016). In essence, both of these proteins function very similarly, with oxidation of the quinone pool of the electron transport chains. In mitochondria, AOX takes electrons from the ubiquinone pool (UQ) and transfers them to O_2 to form H_2O. By doing this, AOX relaxes the electron load on complex I and complex II, preventing the formation of ROS from these complexes. Besides, since AOX bypasses complexes III and IV, the amount of ATP produced per NADH consumed is decreased. With this pathway, $FADH_2$ oxidation does not contribute to the proton motive force (Saha et al., 2016). On the other hand, in chloroplasts, PTOX oxidizes the plastoquinone pool (PQ) to maintain the flow of electrons from PSII, which would otherwise cause production of $^1O_2$ (Nawrocki et al., 2015). This pathway also relaxes PSI due to decreased flux of electrons through the cyt b_6f complex.

Both of these safety valves for electron transport chains have been shown to be involved in salt stress responses of plants. The *A. thaliana* genome encodes 5 *AOX* genes (*AOX1a*, *AOX1b*, *AOX1c*, *AOX1d*, and *AOX2*) and 1 *PTOX* gene (Costa and Svensson, 2015). Among the *AOX* genes, *AOX1a* has been linked to salt stress tolerance. Under salt stress, *AOX1a* expression was induced in *A. thaliana* and plants overexpressing *AOX1a* was more tolerant to salt stress due to lower levels of ROS and lower Na^+ levels, and they had 30%–40% improved growth rates (Smith et al., 2009).

In a comparative study, Stepien and Johnson (2009) demonstrated that *Eutrema salsugineum* (=*Thellungiella salsuginea*) induced PTOX by 4- to 5-fold under salt stress (250 mM NaCl), while PTOX was not induced during this period in *A. thaliana*. Moreover, F_v/F_m and the electron transport rate (ETR) of *A. thaliana* decreased drastically, while these were maintained in *E. salsugineum*. In another study with *Schrenkiella parvula*, it was demonstrated that salt stress (up to 300 mM NaCl) induces *PTOX* gene expression accompanied with increases in ferredoxin thioredoxin reductase (FTR) and NADPH thioredoxin reductase C (NTRC) (Uzilday et al., 2015). Induction of these two thioredoxin reductases involved in transfer of reducing power to thioredoxins from the chloroplastic electron transport chain indicates diversion of the electron flow from photochemistry to defensive responses.

Overall, in chloroplasts, it is evident that plants can divert the electron flow away from photochemistry (i) to plastid terminal oxidases or (ii) to be utilized in defensive responses such as TRX and PRX systems to cope with electron transport chain-related excess ROS production. These adaptive responses both avoid the generation of ROS and provide reducing power to those mechanisms that scavenge ROS. Moreover, plants that are able to utilize these mechanisms more efficiently are more tolerant to salt stress. However, when terminal oxidases are overexpressed in plants, the general outcome is a decrease in plant performance under normal conditions (Krieger-Liszkay and Feilke, 2016). Therefore, there is a need to fine-tune the expression of these proteins. Use of stress-responsive promoters that overexpress terminal oxidases only under stress conditions might increase plant performance under stress without yield penalties for normal conditions. Also, it is not clear how these proteins are regulated in response to salt stress. It is thought that the redox status of the UQ or PQ pool exerts control over activation of alternative electron sinks such as the AOX, PTOX, or FTR-NTRC pathway, but how differential expression between halophytes and glycophytes occurs is not known.

5. ROS-Ca^{+2} hub

In the last decade, new roles of ROS and especially of H_2O_2 and $HO^{\cdot}$ in the regulation of membrane transporter activities have been identified. Acute salt stress can induce production of $HO^{\cdot}$ in plant roots and it has been demonstrated that $HO^{\cdot}$ in the apoplast can activate Ca^{+2} influx and K^+ efflux channels (Demidchik et al., 2010). $HO^{\cdot}$ production in the apoplast is driven by Fenton reaction in the presence of H_2O_2 and metals such as Cu and Fe, the former being more effective in catalyzing the Fenton reaction (Halliwell and Gutteridge, 2015). There are various ROS sources in the apoplast, such as plasma membrane-bound NADPH oxidases, cell wall-bound peroxidases, or amine oxidases (Kärkönen and Kuchitsu, 2015). Among these, NADPH oxidases are thought to be involved in these ion fluxes, because the same ion fluxes were observed when the cell wall was removed (Foreman et al., 2003). Unlike most of their animal counterparts, plant NADPH oxidases contain Ca^{+2} binding EF-hand motifs facing the cytosolic side of the plasma membrane (Oda et al., 2010). This indicates that activities of plant NADPH oxidases are regulated by cytosolic Ca^{+2} levels. Therefore, Ca^{+2} influx upon salt stress can cause activation of NADPH oxidase activity. In turn, increased ROS levels can again activate Ca^{+2} influx channels, as indicated before.

Overall, this chain of events creates a positive feedback mechanism that amplifies itself to induce Ca^{+2} and ROS signaling (reviewed by Demidchik and Shabala, 2017). Indeed, besides posttranslational activation, expressions of plant *RBOH* genes are also induced rapidly under salt stress, as can be seen from Figure 2. Among *RBOH* genes, *RBOHD* and *RBOHF* are especially well known for their response to abiotic stresses (Suzuki et al., 2011), and among others, these two genes respond to salt stress at the transcriptional level within 30 min. Besides *RBOHD* and *RBOHF*, it can be seen that the expression of *RBOHA* is upregulated gradually upon exposure to salt. These data might imply that *RBOHD* and *RBOHF* might be responsible for triggering of ROS-Ca^{+2} signaling. Once triggered, Ca^{+2} signaling via Ca^{+2}-dependent protein kinases (CDPKs), calcineurin B-like (CBL) protein kinases (CIPKs), calmodulin (CaM), and CaM-like proteins (CMLs) might regulate various signaling events (reviewed by Edel et al., 2017 and Huang et al., 2019). However, there has been a very limited number of studies related to the signal role or physiological consequences of K^+ efflux upon salt stress, which is an accelerating area of research (Shabala, 2017).

6. Short- and long-term responses of ROS metabolism

Usually, data collected for salt stress experiments do not have the required temporal resolution to understand very rapid and long-term responses of plants within the same experiment. Accordingly, salt stress studies that deal with antioxidant response can be divided into two as those focusing only on short-term or on long-term adaptive responses. Since salt stress has two different phases, the osmotic and ionic phases, studies in the literature tend to use longer treatment durations to see effects of both phases, in which ionic stress occurs at later stages.

To elucidate the short-term response to salt stress, we have utilized a time-course transcriptomic dataset (Killian et al., 2007). When transcript abundances of major antioxidant enzymes are investigated, it can be seen that the majority of the genes respond (increase or decrease) to salinity within the first 30 min of stress both in shoots and roots (Figure 3). Genes that respond to salinity can be divided into four different clusters according to their time-course trends: (i) genes that are upregulated, (ii) genes that are downregulated, (iii) genes that are first upregulated and then downregulated, and (iv) genes that

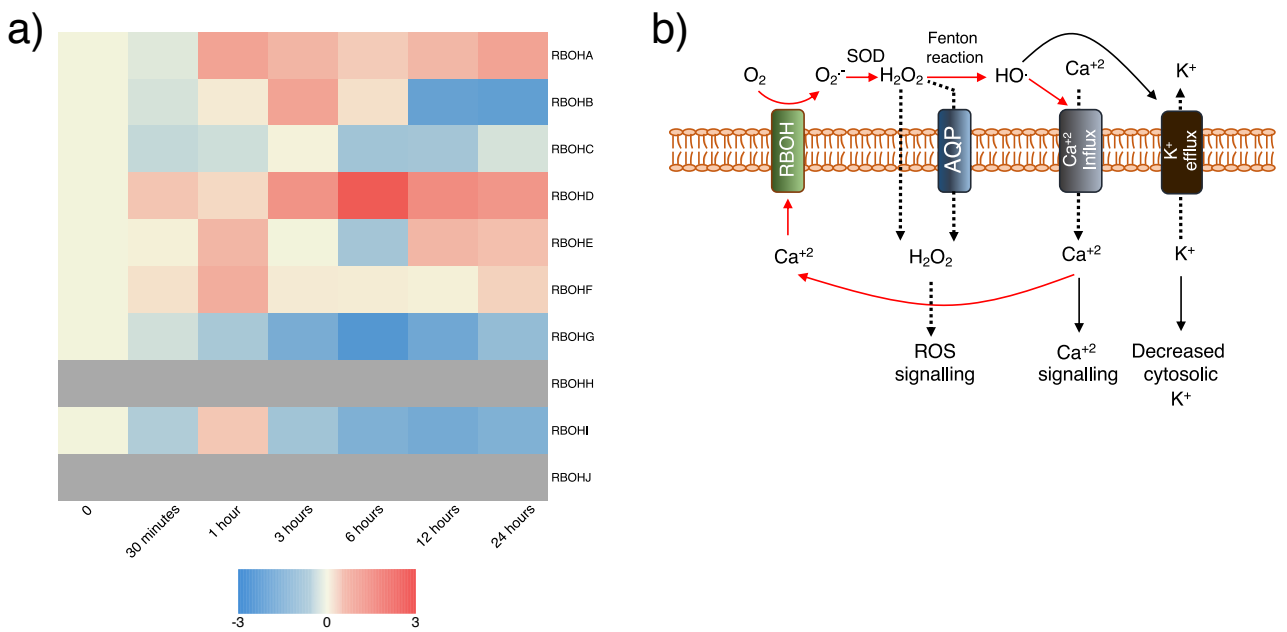

Figure 2. A) Heat map depicting the time-course expression levels of *RBOH* genes in *A. thaliana* roots under salinity stress. Data were taken from Kilian et al. (2007) provided in the eFP Browser (Winter et al., 2007) by using the relative data function, which gives expressions in $\log_2$ ratios. Experimental conditions for the dataset were as follows: plants were grown for 13 days at 24 °C under sterile conditions on polypropylene rafts in growth boxes under long-day conditions (16 h light/8 h dark) at a light intensity of 150 µmol photons m^{-2} s^{-1}. For salt stress 150 mM NaCl was added to MS medium. Plants were harvested at indicated time points and isolated RNA was used to analyze transcriptomic changes with the Affymetrix AHT1 gene chip. *RBOHH* and *RBOHJ* genes were not given as their expression levels were below the threshold defined by the eFP Browser. B) Scheme summarizing ROS-Ca^{+2} self-amplifying loop. $O_2^{\cdot-}$ produced by NADPH oxidase activity (encoded by *RBOH* genes) is converted to H_2O_2, which is simultaneously converted to $HO^{\cdot}$ via Haber–Weiss and Fenton reactions. $HO^{\cdot}$ activates Ca^{+2} inward channels, increasing cytoplasmic Ca^{+2} concentrations. Increased Ca^{+2} in turn induces NADPH oxidase activity. Red arrows indicate ROS-Ca^{+2} self-amplifying loop.

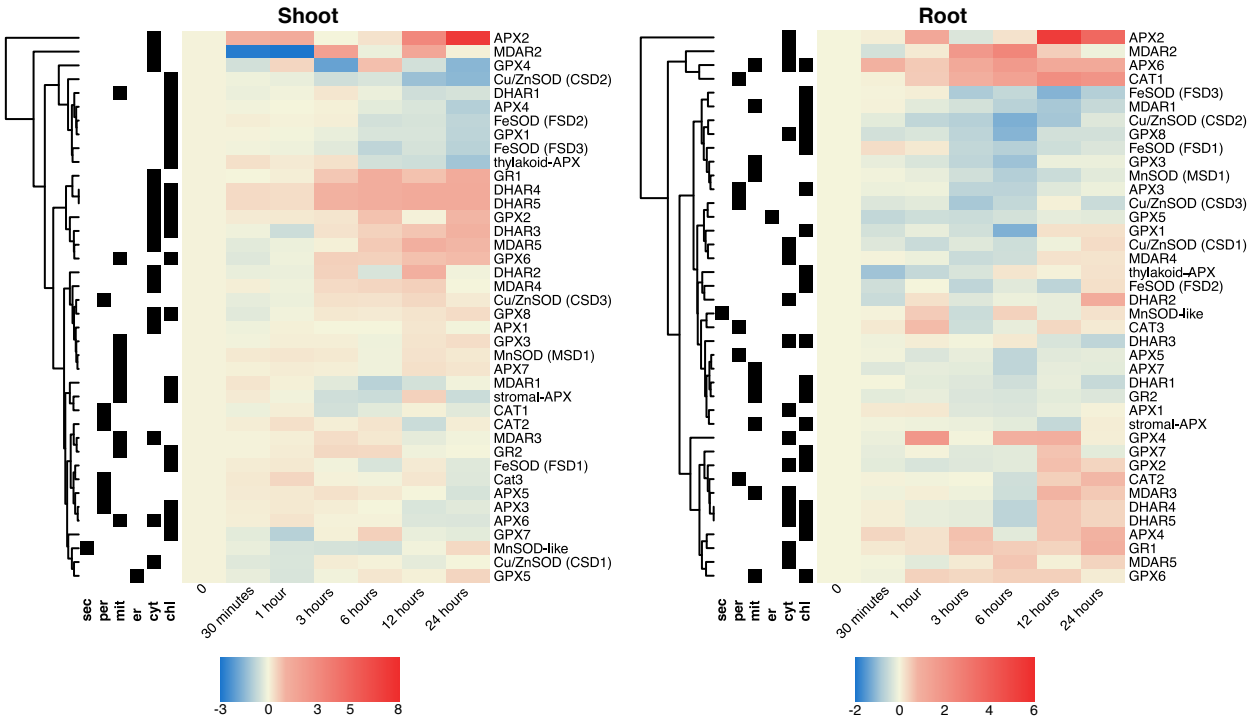

Figure 3. Heat map depicting the time-course expression levels of antioxidant enzyme genes (Mittler et al., 2004) in *A. thaliana* shoot and roots under salinity stress. Data were taken from Kilian et al. (2007) provided in the eFP Browser (Winter et al., 2007) by using the relative data function, which gives expressions in $\log_2$ ratios. For experimental conditions see Figure 2. Plants were harvested at indicated time points and isolated RNA was used to analyze transcriptomic changes with Affymetrix AHT1 gene chip (chl = chloroplast, cyt = cytosol, er = endoplasmic reticulum, mit = mitochondria, per = peroxisome, sec = secretory pathway). Hierarchical clustering was performed on Euclidean distances by using the *hclust* function with complete linkage method in R.

are first downregulated and then upregulated. Among these, interestingly, the first cluster (upregulated genes) includes *GR1*, *MDHAR5*, *DHAR3-5*, and *GPX2* and *-6* in *A. thaliana* shoots, which are all related to glutathione. Moreover, expressions of *CSD2*, *FSD2* and *-3*, *GPX1* and *-4*, *APX4*, and *thylakoid-APX* were downregulated. In roots the highly upregulated ROS scavenging enzymes were *APX6* and *CAT1*, while many other ROS scavengers such as *FeSOD3* and *Cu/ZnSOD3* were downregulated within 24 h. This analysis indicates that, contrary to general opinion, not all components of the antioxidant defense mechanism are upregulated as a rapid response to salt stress in Arabidopsis, but there is a coordinated response to adapt to the new redox environment of the cell. There are no similar transcriptomic data with enough temporal resolution that reflect rapid changes in response to salt in halophytic plants, which is a gap in the knowledge that should be addressed.

The number of studies that investigate rapid biochemical changes (e.g., within 24 h) in terms of antioxidant defense are limited in the literature. Findings of these studies are summarized in Figure 4 to present the changes in antioxidant defense and oxidative stress markers comparatively with two halophytes and with a glycophyte. For example, in the leaves and roots of halophyte *Cakile maritima*, there was a rapid accumulation of H_2O_2 at the onset of salt stress (Ellouzi et al., 2014). However, 16 h after onset of stress H_2O_2 levels started to decrease, indicating an adaptive response. Moreover, MDA followed the same pattern in the leaves under stress. On the other hand, in Arabidopsis H_2O_2 and MDA increased slowly in 3 days both in roots and shoots (Ellouzi et al., 2011, 2014). On the other hand, in Arabidopsis, salt treatment decreased ascorbic acid levels, especially within the first 4 h of treatment. In another study, Huang et al. (2005) demonstrated that ascorbic acid levels increase until 12 h of salinity stress and then start to decrease. Moreover, plants were unable to restore this decrease in the pool of ascorbic acid in the long term (Huang et al., 2005; Ellouzi et al., 2014). Since it is known that ascorbic acid is responsive to the oxidative state of the cell (Foyer and Noctor, 2011), this sudden decrease in the early period in ascorbic acid content may be related to the oxidative burst that occurs in the same period in both leaves and roots (Ellouzi et al., 2011, 2014). Huang et al. (2005) found that GSH levels were significantly enhanced in leaves of Arabidopsis following 48 h of salt

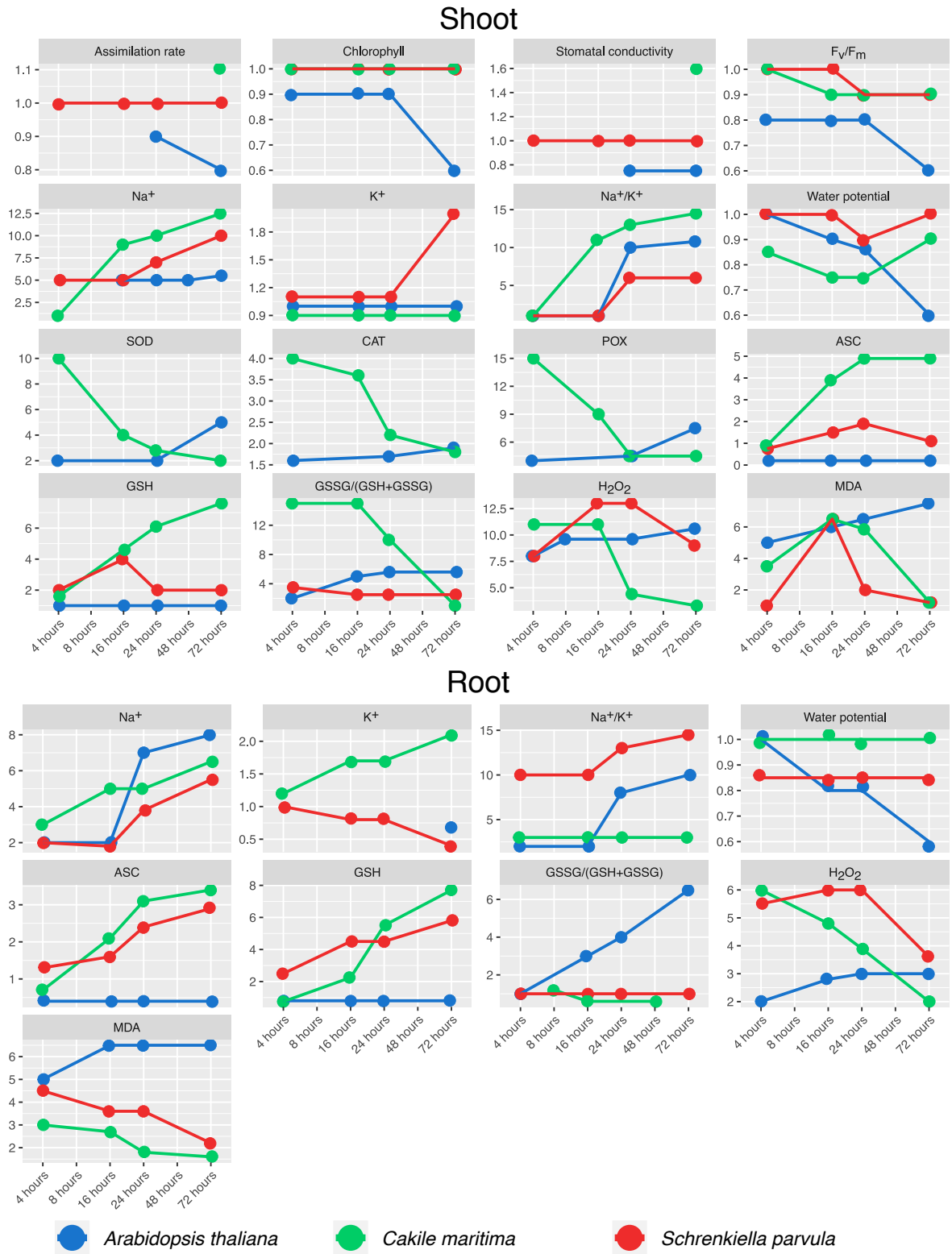

Figure 4. Ratios of the physiological and redox state-related parameters in *A. thaliana*, *C. maritima*, and *S. parvula* shoots and roots. Data for *A. thaliana*, *C. maritima*, and *S. parvula* were taken from Debez et al. (2008), Stepien and Johnson (2009), and Ellouzi et al. (2011, 2014). For salt stress treatments, all three plant species were treated with 400 mM NaCl. Ratios were calculated by dividing the levels in salt-stressed plants by those observed in control plants for each time point. 0 h was omitted on the plots since all of them had a value of 1

treatment. However, Ellouzi et al. (2014) observed that while there was a noticeable decrease in GSH levels in the roots, no significant changes were observed in the leaves of Arabidopsis. In the leaves and roots of *C. maritima*, GSH levels remained high (Ben Amor et al., 2007; Ellouzi et al., 2014). GSH/GSSG and GSSG/(GSH+GSSG) ratios are good indicators of the redox state of the cells. At the onset of salt stress, glycophyte *A. thaliana* and halophyte *C. maritima* showed increases in the GSSG/(GSH+GSSG) ratio. After 4 h the ratio started to decrease in halophyte *C. maritima*, probably due to better regeneration of the GSH pool. On the other hand, the GSSG/(GSH+GSSG) ratio kept increasing in glycophyte *A. thaliana* (Ellouzi et al., 2014).

In *C. maritima* leaves, SOD activity significantly increased, peaking at 4 h, and its activity was high for up to 25 days of salt treatment (Ben Amor et al., 2007; Ellouzi et al., 2011). However, in Arabidopsis, SOD activity increased slowly (Ellouzi et al., 2011). In another study, in *S. protulacastrum* leaves, SOD activity increased in a dose-dependent manner and peaked at 4 days of 1000 mM salt treatment. However, in *B. juncea* SOD activity decreased below control levels after 8 days (Srivastava et al., 2015). Shalata et al. (2001) determined the differential antioxidant responses in roots and compared the responses of cultivated tomato *Lycopersicon esculentum* and its salt-tolerant relative *Lycopersicon pennellii*. When 100 mM NaCl was applied, SOD activity significantly increased up until 16 days of treatment. In the leaves of *C. maritima*, CAT activity peaked at 4 h and remained high up to 10 days. On the other hand, CAT activity increased in the first 4 h and continued to increase for 3 days (Ben Amor et al., 2007; Ellouzi et al., 2011). Another study demonstrated that the CAT activity of *B. juncea* leaves peaked at 2 days of salt treatment.

Regarding the long-term effects of salinity, *C. maritimum* roots showed no significant changes in MDA under 50 mM NaCl treatment, while this concentration might be attributed as a normal condition for this halophyte. On the other hand, the highest antioxidant capacity was determined in plants treated with 50 mM NaCl, while the antioxidant defense was suppressed under 200 mM (Ben Amor et al., 2005). This indicates that for some plant species high salt concentrations might provide a better environment that decreases oxidative stress and hence the antioxidant defense. Yildiztugay et al. (2014) treated *Salsola crassa* plants with 250 mM to 1250 mM NaCl for 15 and 30 days and found that only the highest salt concentrations induced MDA levels and antioxidative capacity of *S. crassa*. These findings indicate that, if given enough time to adjust and acclimate, halophytes can avoid the formation of ROS that would reach toxic levels.

7. Conclusion

For more than three decades, scientists have been actively trying to understand the contribution of antioxidant defense mechanisms to salt stress tolerance of plants, but still, there seem to be gaps in our knowledge, especially related to the dynamics of ROS production in different compartments of the plant cells. Although there is a huge body of literature that investigates antioxidant activities of plants under salt stress and demonstrates that higher antioxidant capacity is favored for salt stress tolerance, there are no transgenic success stories tested under field conditions that utilize a ROS scavenger enzyme.

Still, there seems to be much to be learned from halophytes to understand salt stress tolerance mechanisms. Especially with the development of next-generation sequencing technologies, now we are able to sequence whole genomes or create transcriptomics data much more cheaply and easily, which would inevitably increase the data on halophytic species. Still, increased temporal and spatial (at tissue level) resolution of the transcriptomics data would contribute to our understanding of signaling mechanisms under salt stress at organ or tissue level.

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