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Chapter 1
Salt Stress in Higher Plants: Mechanisms of Toxicity and Defensive Responses

Anabella Fernanda Lodeyro and Néstor Carrillo

Abstract Soil salinity is a major constraint to crop performance. The main contributors to salt toxicity at a global scale are Na^+ and Cl^- ions which affect up to 50 % of irrigated soils. Effects of salt exposure occur at the organismic, cellular, and molecular levels and are pleiotropic, involving (1) osmotic and water deficit syndromes, (2) specific Na^+ and Cl^- inhibitions, (3) nutritional imbalance, and (4) oxidative stress. We review herein the responses elicited by salt-stressed plants to face all these challenges. With the only exception of halobacteria, all other organisms are not halotolerant at the molecular level. Instead, they have developed strategies to keep salts out of the cell. Then, induction of systems for salt extrusion to the rhizosphere and salt compartmentation into the vacuole play key roles in salt tolerance, aided by the synthesis and accumulation of compatible osmolytes and of antioxidant enzymes and metabolites. Expression of these effector genes is modulated by a complex network of salt-responsive transcription factors and signaling molecules. We discuss the progress made towards increasing salt tolerance in crops by engineering genes whose products operate at all these stages, from sensing and regulation to effector proteins, and identify key open questions that remain to be addressed.

Keywords Salt tolerance • Oxidative stress • Nutritional imbalance • Osmotic adjustment • Ion toxicity

1.1 Introduction

Earth is a predominantly salty planet, with most of its water containing ~600 mM NaCl. About 7 % of the firm land, 20 % of the cultivated land, and nearly half of the irrigated land are affected by high salt contents (Zhu 2001, 2002, 2003). The threats of salinity are more obvious in arid and semiarid regions where limited rainfall, high evapotranspiration, and extreme temperatures associated with poor water and

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soil management are the main contributing factors (de Azevedo Neto et al. 2006). While virtually all salts may have deleterious effects on plant welfare, the term salt stress usually refers to the consequences of abnormal accumulation of Na^+ and Cl^- ions, since this is by far the most extended environmental hardship related to salinity.

Salinization might occur by natural causes, primarily by capillary water level elevation and subsequent evaporation of saline groundwater. However, the increase of salinization at a global scale is largely due to human intervention, particularly in arid regions. Irrigation practices lead to groundwater level elevation and a subsequent increase in evaporation. Salts not only cause direct damage on plants but also provoke secondary negative effects, such as increase of the soil pH. Most plants do not grow well at high pH due to iron starvation. Iron is found in nature mostly as ferric oxides that are sparingly soluble, indicating that the main problem of Fe acquisition is not of abundance but of bioavailability (Curie and Briat 2003). The solubility product of ferric oxides decreases three orders of magnitude for each pH unit raise, which represents a major deterrent for agriculture in alkaline, calcareous soils that cover more than one third of the planet's cultivable land (Morel and Price 2003).

Salt stress affects plant physiology at whole plant as well as cellular and molecular levels and at all stages of development from germination to senescence (Hasegawa et al. 2000; Muranaka et al. 2002; Murphy et al. 2003; Ranjbarfordoei et al. 2002). Reported effects include changes in growth rate, ion toxicity, mineral limitation, membrane instability, photosynthesis, and increased respiration (Ashraf and Shahbaz 2003; Hasegawa et al. 2000; Munns 2002). Buildup of high amounts of salts in the leaf apoplast leads to dehydration and turgor loss (Marschner 1995), whereas salt accumulation in the cytosol and organelles results in inhibition of enzymes and metabolic pathways, including photosynthesis (de Lacerda et al. 2003). The reduction of plant growth and biomass accumulation under saline conditions has been reported in several important crops (Tejera et al. 2006).

To facilitate analysis, the unfavorable effects of salt stress are classified in four major groups: (1) osmotically induced water stress, (2) specific salt toxicity inhibiting enzymes and metabolic pathways, (3) nutrient ion imbalance due to high levels of Na^+ and Cl^- competing with the uptake of other essential ions, and (4) increased production of reactive oxygen species (ROS) which damage all types of macromolecules. These different modes of action will be briefly described in the forthcoming sections, followed by a survey of the responses elicited by salt-stressed plants, their regulation, and the use of the knowledge gathered in the last decades to design salt-tolerant plants. Several reviews on salt toxicity, sensing, and tolerance have been published in recent years (Bose et al. 2014; Deinlein et al. 2014; Golldack et al. 2011; Huang et al. 2012; Maathuis 2014). The reader is referred to them for a more extensive treatment of these subjects.

1.2 Osmotic Adjustment and Water Deficit

Salt accumulation in soils reduces the plant's ability to take up water from the rhizosphere, and this leads to water limitation and growth reduction. This process is the first to occur in a salt-stressed plant and is formally analogous to a water-deficit stress. Indeed, some cellular and metabolic processes involved in osmotic responses to salinity are common to drought, including stomatal closure (see below). The rates at which new leaves are produced depend largely on the water potential of the soil solution, in the same way as for a drought-stressed plant. At this stage, salts themselves do not build up in the growing tissues at concentrations that could inhibit growth, as the rapidly elongating cells can accommodate the salt that arrives in the xylem within their expanding vacuoles (Munns 2005). Then, reductions in the rate of leaf and root growth are due to water stress rather than a salt-specific effect (Munns 2002), since Na^+ and Cl^- are usually below toxic concentrations in the growing cells. For example, in wheat exposed to 120 mM NaCl, Na^+ in the growing tissues of leaves was at most 20 mM and only 10 mM in the rapidly expanding zones (Hu et al. 2005). Results of experimental manipulation of shoot water relations suggest that hormonal signals, probably induced by the osmotic effect of the salt on the roots, are controlling the rate of cell elongation (Munns et al. 1999). Inhibition of plant growth due to salinity largely depends on the severity of the stress. Mild osmotic challenges rapidly lead to growth inhibition of leaves and stems, whereas roots may continue to elongate (Hsiao and Xu 2000). The degree of growth inhibition due to this osmotic stress also depends on the time scale of the response for the particular tissue and species and on whether the stress treatments are imposed abruptly or gradually (Munns et al. 1999).

1.3 Specific Ion Toxicity

As salts are taken up by a plant, they tend to concentrate in the old leaves. However, continued transport into transpiring leaves over a long period of time eventually exceeds the ability of the cells to exclude salts from the cell or to compartmentalize them in the vacuole (see below). Salts then would build up in the cytosol and inhibit enzyme activity. Alternatively, they might accumulate in the cell walls and dehydrate the cell (Munns 2005). These specific effects of ions such as Na^+ and Cl^- follow the water and osmotic stresses that initiate the salinity syndrome.

Under normal growth conditions, root cytosolic Na^+ concentrations are probably in the order of 10–30 mM (Tester and Davenport 2003). Leaf Na^+ cytosolic concentrations are considered to be in the same range (Jones and Gorham 2002). Roots must exclude most of the Na^+ and Cl^- dissolved in the soil solution, or the salt concentration in the shoot will gradually increase to toxic levels. Plants transpire about 50 times more water than they retain in their leaves (Munns 2005).

The inhibitory concentrations of Na^+ vary depending on the reaction or metabolism, whereas the concentrations at which Cl^- becomes toxic are less defined. Metabolic routes affected include photosynthesis, nitrogen assimilation via nitrate and nitrite reductases, protein translation, and malate metabolism (Parida and Das 2005). The increase of Na^+ levels inside plant tissues also has toxic effects on seed germination, mainly by affecting the plant water relations and through displacement of Ca^{2+} by Na^+ from critical cell wall binding sites, which could disrupt cell wall synthesis and hence inhibit early plant growth (Xue et al. 2004).

1.4 Nutritional Imbalance

Ionic imbalance occurs in the cells due to excessive accumulation of Na^+ and Cl^- which reduces uptake of other mineral nutrients, such as K^+, Ca^{2+}, and Mn^{2+} (Karimi et al. 2005). Sodium and potassium are imported into the cell using the same suite of transporters, and the cations compete with each other (Greenway and Munns 1980). Excess Na^+ therefore inhibits K^+ uptake, leading to the appearance of symptoms of K^+ deficiency. Many central enzymes and metabolic routes require K^+ to acquire high specific activities compatible with life and development (Booth and Beardall 1991). This cation is also necessary for osmoregulation and protein synthesis, for the preservation of cell turgor and for optimal photosynthetic activity (Ashraf 2004; Freitas et al. 2001). Both K^+ and Ca^{2+} are required to maintain the integrity and functioning of cell membranes (de Lacerda et al. 2003; Munns 2002; Wei et al. 2003). Potassium deficiency initially leads to chlorosis and then necrosis (Gopa and Dube 2003).

The maintenance of calcium acquisition and transport under salt stress is also an important determinant of salinity tolerance (Soussi et al. 2001). Salt stress decreases the Ca^{2+}/Na^+ ratio in the root zone, which affects membrane properties due to displacement of membrane-associated Ca^{2+} by Na^+, leading to dissolution of membrane integrity and loss of selectivity (Kinraide 1998). Externally supplied Ca^{2+} has been shown to ameliorate the adverse effects of NaCl on plants by competition with Na^+, by increasing K^+/Na^+ ratio and by osmotic adjustment, through the enhancement of compatible organic solutes accumulation (Girija et al. 2002; Hasegawa et al. 2000).

Ca^{2+} also plays a critical role in the signaling network of plant cells. Extracellular stress signals can be perceived by the membrane receptors, which activate a large and complex signaling cascade, including the generation of second messengers such as Ca^{2+}. This increase in cytosolic Ca^{2+} concentration primes the signaling pathways for stress tolerance (Mahajan and Tuteja 2005; Tuteja and Mahajan 2007). Moreover, Ca^{2+}-binding proteins (calcineurin B-like proteins, CBLs) provide an additional level of regulation in Ca^{2+} signaling, initiating a phosphorylation/dephosphorylation cascade leading to regulation of gene expression and resulting in the expression of multiple responsive effector genes.

1.5 Oxidative Stress

Exposure of plants to salt stress can upregulate the production of ROS such as $O_2^{\bullet -}$ (superoxide radical), H_2O_2 (hydrogen peroxide), $^1O_2$ (singlet oxygen), and $^{\bullet}OH$ (hydroxyl radical). Excess ROS causes phytotoxic reactions including lipid peroxidation, protein degradation, and DNA mutation (Abogadallah 2010; McCord 2000; Pitzschke et al. 2006; Vinocur and Altman 2005; Wang et al. 2003). In plant cells, ROS are generated in the cytosol, chloroplasts, mitochondria, and the apoplastic space (Bowler and Fluhr 2000; Jacoby et al. 2011; Mittler et al. 2004; Mittler et al. 2011).

The main source of ROS in illuminated plants is the photosynthetic electron transport chain (PETC) of leaf chloroplasts. Salt-induced stomatal closure due to the water deficit and osmotic components of the salinity stress cause a decrease in CO_2 concentration inside chloroplasts leading to knockdown of the Calvin cycle by substrate limitation (Miller et al. 2010). NADPH is continuously produced at the thylakoids, but its oxidation in the regenerative stage of the Calvin cycle is blocked (Apel and Hirt 2004). Under these conditions, the PETC becomes over-reduced, and the propensity of O_2 to subtract electrons from the chain is expected to increase, leading to runaway ROS propagation, mostly $O_2^{\bullet -}$ and H_2O_2 (Foyer and Noctor 2000). In turn, ROS buildup damages the D1 protein of photosystem II causing photoinhibition. Stress-enhanced photorespiration in peroxisomes and misfunction of the respiratory chain of mitochondria also contribute to H_2O_2 accumulation (Miller et al. 2010). Major targets of $O_2^{\bullet -}$ toxicity are the iron-sulfur clusters of dehydratases and electron transfer proteins (Imlay 2006), whereas H_2O_2 may inactivate enzymes by oxidizing their thiol groups (Gill and Tuteja 2010). Toxic effects of H_2O_2 are enhanced by reaction with metal reductants, most conspicuously Fe^{2+}, to form the highly reactive hydroxyl radical, which is able to react with virtually any biological molecule (Halliwell and Gutteridge 1999).

In addition to these sources of salt-driven oxidative stress caused by misrouting of reducing equivalents from key redox pathways, ROS are also produced in the apoplast by a multigenic family of membrane-bound NADPH oxidases (Mittler et al. 2004). Given the many negative effects of ROS, it seemed at first odd that exposure to salinity led to induction of these enzymes, resulting in direct H_2O_2 propagation under conditions in which antioxidant defenses are activated to cope with ROS buildup. However, subsequent studies have shown that ROS can also play a key role in plants as signal transduction molecules involved in mediating responses to pathogen infection, environmental stresses, programmed cell death, and developmental stimuli (Mittler et al. 2004; Torres and Dangl 2005). In a phylogenomic study, Mittler et al. (2011) observed that the basic lot of antioxidants and scavenging enzymes were already present in the algal precursors of higher plants, while apoplastic NADPH oxidase activity is a newcomer to the plant kingdom in evolutionary terms, only found in vascular plants. These results suggest that photosynthetic eukaryotes learned first how to detoxify ROS and only later to use them as signaling molecules.

1.6 Plant Responses to Salt Stress

As indicated, salt stress affects plant physiology at almost all growth stages. Salt tolerance at these various developmental conditions varies widely from species to species. Plants that can survive and reproduce on high concentrations of salt in the rhizosphere are called halophytes. Depending on their salt-tolerant capacity, halophytes are either obligate or facultative, the latter ones displaying broader physiological resources that allow them to thrive in both saline and non-saline environments (Parida and Das 2005). Salt-sensitive species are termed glycophytes. Nearly all crops are glycophytes (Ashraf 2004).

With the exception of a few halophytic bacteria, there are no intrinsic salt-resistant enzymes or metabolisms. Tolerance is achieved by keeping salt out of the cell or into the vacuole and by combatting the damaging consequences of the stress situation. In halophytes, these mechanisms are more efficient than in glycophytes. For example, Jones and Gorham (2002) reported that the higher salt tolerance of *Agropyron junceum* with respect to *Agropyron intermedium* was related to more efficient exclusion of both Na^+ and Cl^-. In another study, Carden et al. (2003) found that a salt-tolerant barley variety maintained a tenfold lower cytosolic Na^+ in the root cortical cells than a more sensitive one. It is well established that most of the damage undergone by salt-exposed plants results from accumulation of Na^+ in shoots, inhibiting key metabolic processes such as protein synthesis and photosynthesis (Munns 2005). Thus, in most halophytes, Na^+ retention in the root is a general trend and hence an important component of salt tolerance (Ashraf 2004).

1.7 Ion Homeostasis

The maintenance of a high cytosolic K^+/Na^+ ratio is critical for salt tolerance (Glenn et al. 1999), and plants have evolved two main types of mechanisms to achieve these goals, those preventing the entry of Na^+ into the plant and those minimizing the concentration of Na^+ in the cytoplasm: extrusion and compartmentation (Fig. 1.1). In *Arabidopsis*, Na^+ influx is controlled by AtHKT1, a low-affinity Na^+ transporter (Rus et al. 2001; Uozumi et al. 2000). The knockout mutant *hkt1* from *Arabidopsis* suppressed Na^+ accumulation and sodium hypersensitivity (Rus et al. 2001), suggesting that AtHKT1 is a salt tolerance determinant. On the other hand, Na^+ efflux is controlled by Salt Overly Sensitive 1 (SOS1), a plasma membrane Na^+/H^+ antiporter (Shi et al. 2003), which is powered by the operation of a H^+-ATPase (Blumwald et al. 2000). In addition to its role as an antiporter, SOS1 may act as a Na^+ sensor (Zhu 2003).

The compartmentation of Na^+ in vacuoles provides an efficient and cost-effective mechanism to prevent its toxic effects in the cytosol (Fig. 1.1). The transport of Na^+ into the vacuoles is mediated by a Na^+/H^+ antiporter (*AtNHX1* in

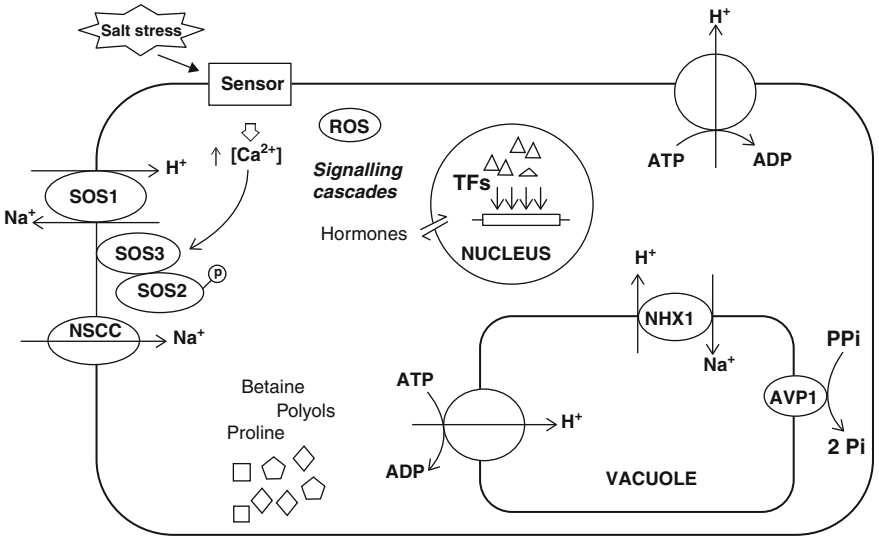

Fig. 1.1 Cellular responses to Na^+ toxicity. Na^+ is passively transported into the cell by nonselective cation channels (NSCCs), where it is sensed by an unidentified sensory system. Next, Ca^{2+}, ROS, and hormones activate signaling cascades which regulate the expression of multiple stress-related genes, resulting in the activation of cellular detoxification pathways. Two mechanisms operate to decrease cytosolic Na^+ concentration in the cell. One is to exclude Na^+ across the plasma membrane by the Na^+/H^+ exchanger SOS1. Another mechanism is to compartmentalize Na^+ into vacuoles by the vacuolar Na^+/H^+ antiporter, NHX1. Both transporter activities require a H^+ gradient across the membranes, which is generated by a plasma membrane H^+-ATPase or by vacuolar H^+-ATPase and H^+-pyrophosphatase (AVP1). Accumulation of osmoprotectants such as betaine, proline, and polyols is also induced by salt stress

Arabidopsis) that is driven by the electrochemical gradient of protons generated by vacuolar H^+-translocating enzymes, the H^+-ATPase and the H^+-pyrophosphatase (Blumwald et al. 2000). Under salinization conditions, Na^+ influx into the cytosol could take place through pathways that normally function in the uptake of K^+, resulting in toxic levels of Na^+ as well as insufficient K^+ concentration for enzymatic reactions and osmotic adjustment. Three classes of low-affinity K^+ channels have been identified (Maathuis and Sanders 1995): these are K^+-inward rectifying channels (KIRCs), K^+-outward rectifying channels (KORCs), and voltage-independent cation channels (VICs). KORCs appear to be particularly important in mediating Na^+ influx into plant cells. These channels, which open during the depolarization of the plasma membrane, could mediate the efflux of K^+ and the influx of Na^+ ions under excess salt. Sodium competes with K^+ uptake through Na^+–K^+ co-transporters and may also block the K^+-specific transporters of root cells (Zhu 2003).

1.8 Synthesis of Compatible Solutes

The cellular response of organisms to both long- and short-term salinity stresses includes the synthesis and accumulation of a class of osmoprotective compounds known as compatible solutes (Fig. 1.1). These organic molecules can build up to high cellular concentrations without inhibiting metabolic processes. They comprise a varied assortment of chemicals including quaternary amines (glycinebetaine), amino acids (proline), soluble sugars such as trehalose, and sugar alcohols such as mannitol, sorbitol, and pinitol (Bose et al. 2014; Chinnusamy et al. 2006; Greenway and Munns 1980; Yeo 1998). These metabolites operate at various levels of the stress response. First, they provide osmotic compensation in the face of soil solutions containing high amounts of NaCl, attenuating the loss of water from the cell. For this reason, compatible solutes are known as osmolytes or osmoprotectants (Bohnert and Jensen 1996; Chen and Murata 2002). They also act as stabilizers of the quaternary structure of proteins and highly ordered states of membranes, preventing protein denaturation and membrane destabilization (Yancey et al. 1982). Finally, some of them (e.g., mannitol) serve as ROS scavengers, especially of those compounds which are too reactive to be detoxified enzymatically, such as the hydroxyl radical (Bose et al. 2014).

Genes involved in biosynthesis of compatible solutes are generally upregulated under salt stress, and concentrations of accumulated osmoprotectants correlate with osmotic stress tolerance (Zhu 2002). As could be expected, this response is largely shared with drought stress. Although enhanced synthesis and accumulation of compatible solutes under osmotic stress has been extensively documented, little is known about the signaling cascades that regulate their biosynthesis in higher plants.

The enhancement of glycinebetaine synthesis has received much attention (Rontein et al. 2002). In spinach and sugar beet, which naturally accumulate glycinebetaine, synthesis of this compound occurs in the chloroplast. The first oxidation to betaine aldehyde is catalyzed by choline monooxygenase and the subsequent oxidation to glycinebetaine by betaine aldehyde dehydrogenase (Rathinasabapathi 2000). In the soil actinobacterium *Arthrobacter globiformis*, the two oxidation steps are catalyzed by a single enzyme, choline oxidase (COD), encoded by the *codA locus* (Sakamoto and Murata 2000). Hayashi et al. (1997) used the *codA* gene of *A. globiformis* to engineer glycinebetaine synthesis in *Arabidopsis*. Tolerance to salinity during germination and seedling establishment was improved markedly in the transgenic lines. Huang et al. (2000) used *codA* from the related species *A. panescens* to transform *Arabidopsis*, *Brassica napus*, and tobacco. In this set of experiments, the COD protein was directed to the cytoplasm and not to the chloroplast. Improvements in tolerance to salinity, drought, and freezing were observed in some transgenic lines from all three species, but the tolerance was variable. The results confirmed that the protection by glycinebetaine is not only osmotic but also as an ROS scavenger. The level of glycinebetaine production in transgenic plants could be limited by the availability of choline, and a

dramatic increase in glycinebetaine contents in *Arabidopsis* was achieved when the growth medium was supplemented with choline (Huang et al. 2000).

1.9 Antioxidant Protection

Stress-induced production of ROS causes oxidative damage to many different cellular components including lipids, proteins, and nucleic acids (Miller et al. 2010), and different reports have shown that amelioration of the deleterious effects of stress-induced ROS could provide enhanced plant resistance to salinity (Ashraf and Harris 2004; Bose et al. 2014; Reguera et al. 2012; Roy et al. 2014). Plants use antioxidants such as reduced glutathione (GSH) and ascorbate (ASC), as well as enzymes specifically involved in ROS detoxification, including superoxide dismutases (SOD), catalases (CAT), glutathione *S*-transferases (GST), glutathione peroxidases (GPX), and ascorbate peroxidase (APX). It has been shown that expression of enzymes responsible for ROS scavenging and synthesis of antioxidants are induced by salt and other sources of environmental stress (Tester and Davenport 2003; Zhu 2001), supporting the notion that containment of cellular ROS buildup represents a major contribution to salinity tolerance and that overexpression of these genes is a promising strategy to develop salt-tolerant lines (see below). Ruiz and Blumwald (2002) investigated the enzymatic pathways leading to GSH synthesis during the response to salt stress of wild-type (WT) and salt-tolerant *B. napus* plants overexpressing a vacuolar Na^+/H^+ antiporter (Zhang et al. 2001). WT plants showed a marked increase in the activity of enzymes associated with cysteine synthesis (the crucial step for assimilation of reduced sulfur into organic compounds such as GSH), resulting in a significant increase in GSH content. On the other hand, these activities did not change with salt stress in the transgenic salt-tolerant plants, and their GSH levels were not modified. These results showed that salt stress induced an increase in the assimilation of sulfur and the biosynthesis of cysteine and GSH in order to mitigate salt-induced oxidative stress.

1.10 Regulation of the Responses

Transcription factors (TFs) are key regulators in salt stress responses, linking sensory pathways to tolerance (Figs. 1.1 and 1.2). Families of TFs are usually classified based on the nature of their DNA binding sites. The most relevant TF families are the basic leucine zipper (bZIP), WRKY, APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF), MYB, basic helix-loop-helix (bHLH), and NAC (Cui et al. 2013; Jiang and Deyholos 2009; Jiang et al. 2009; Kim et al. 2013; Tran et al. 2004; Yang et al. 2009). Genes belonging to different families are differentially expressed during stress, conferring adaptation and/or

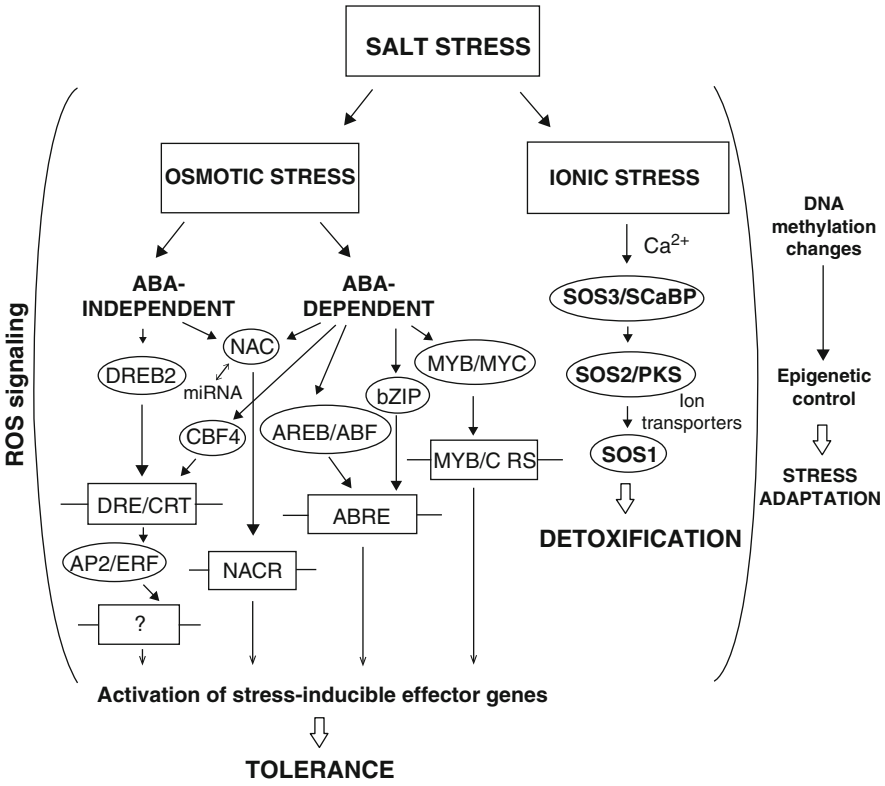

Fig. 1.2 Model of transcriptional signaling network involved in plant adaptation to salinity. Salt stress induces the activation of complex regulatory networks leading to the establishment of a defense response. ABA-dependent and ABA-independent pathways are involved in plant tolerance to salt stress. Osmotic stress signaling generated via salinity is mediated by transcription factors such as DREB2, bZIP, MYB, MYC, CBF4, and NAC, which interact with their corresponding recognition elements in the promoters of stress-dependent genes. Some transcription factors, including NAC and MYB, are themselves targets of drought- and salt-inducible miRNAs. ROS operate as signaling molecules within the regulatory networks. Salt stress detoxification mainly works through the SOS1/2/3 pathway keeping cellular ionic equilibrium. Fine-tuning of the response in plant environmental adaptation also involves epigenetic control of gene expression. Identities of the various components of this regulatory network are given in the text

tolerance to the adverse situation (Golldack et al. 2011). The expression levels of many effector genes, regulated by these TFs, may influence the magnitude of salt tolerance of plants (Deinlein et al. 2014; Golldack et al. 2011). For instance, genes encoding proteins related to ion uptake and osmolyte synthesis are upregulated by salinity, and transcriptional regulation of these stress response genes is mediated by dynamic changes in hormone levels (Geng et al. 2013).

The salt-responsive bZIP TF bZIP24 is induced by salt in *Arabidopsis thaliana* but suppressed in a halophytic *Arabidopsis* relative model species, acting as a key regulator of salt stress adaptation (Yang et al. 2009). RNAi-mediated repression of

this TF conferred increased salt tolerance to *Arabidopsis*, resulting in reduced Na^+ accumulation. Transcript analysis revealed that downstream potential target genes of AtbZIP24 function in osmotic adjustment, ion homeostasis, and plant development. Under normal growth conditions, transgenic *A. thaliana* plants in which bZIP24 levels were decreased by RNAi exhibited activation of stress-inducible genes: the Na^+ transporter HKT1, the Na^+/H^+ antiporter SOS1, the aquaporin PIP2.1, and a glutamine synthetase (Yang et al. 2009).

Endogenous levels of ABA increase in response to osmotic stress, and the phytohormone activates the expression of many genes via ABA-responsive elements (ABRE) in their promoter regions (Fig. 1.2). Transcription factors belonging to the ABRE-binding protein/ABRE-binding factor (AREB/ABF) family regulate the ABRE-mediated transcription of downstream target genes (Fujita et al. 2011). All members of this bZIP subfamily, AREB1, AREB2, and ABF3, are involved in drought stress in *Arabidopsis* via ABA signaling (Yoshida et al. 2010). In rice, a new bZIP TF, OsABF1, was isolated and characterized. It was shown to bind to ABREs and to be induced by anoxia, salinity, drought, oxidative stress, and ABA. In addition, the homozygous T-DNA insertional mutants *Osabf-1* and *Osabf-2* were more sensitive to drought and salt stress than WT plants (Hossain et al. 2010). Constitutive expression of the AREB ortholog TF from tomato (*Solanum lycopersicum*), SlAREB, increased tolerance to water deficit and high salinity in *Arabidopsis* and tomato plants, maintaining photosystem II and membrane integrities, and water content (Hsieh et al. 2010).

Functional specificity of bZIP factors in cellular transcriptional networks might be determined by specific homodimerizations and heterodimerizations which provide the resulting products with distinct DNA- and protein-binding properties, as well as conformational flexibility. Unfolded regions of the TF are responsible for transactivation. These regions contain protein recognition motifs and establish specific interactions with a wide range of protein targets that can be modulated by phosphorylation/dephosphorylation events (Miller 2009). The three TFs AREB1, AREB2, and ABF3 can form homodimers and heterodimers and interact with a SnRK2 protein kinase that regulates AREB1 (Yoshida et al. 2010). AtbZIP24 was targeted to the nucleus forming homodimers in response to salt stress (Yang et al. 2009). However, the salt-responsive TF bZIP1 forms heterodimers with other bZIP TFs (Weltmeier et al. 2009). Moreover, the TBP-associated factor AtTAF10 has a specific function in regulating accumulation of Na^+ and proline (Gao et al. 2006), this function overlapping those of bZIP24 (Yang et al. 2009).

The bZIP factors are located high in the hierarchical network of stress adaptation (Fig. 1.2), but other stress-related proteins play functional roles integrating the main pathways of environmental adaptation and regulating downstream sub-networks (Golldack et al. 2011). Members of the DREB/CBF subfamily of the AP2/ERF TFs play important roles in stress tolerance via ABA-dependent and ABA-independent pathways (Fig. 1.2), regulating a sub-transcriptome with more than 100 target genes (Shinozaki and Yamaguchi-Shinozaki 2000). Constitutive overexpression of DREB/CBF TF resulted in improved tolerance to drought, salt loading, and

freezing. However, the use of the strong constitutive 35S cauliflower mosaic virus (CaMV) promoter also caused severe growth retardation under normal growth conditions. In contrast, expression of DREB1A from a stress-inducible promoter had only marginal effects on plant growth while providing an even greater tolerance to stress conditions (Kasuga et al. 1999). The results illustrate the complexity of the stress adaptive network. Other example of multifunctional regulation is the R2R3-MYB TF AtMYB41 which is transcriptionally induced in response to ABA, drought, salinity, and cold (Lippold et al. 2009).

Transcription factors from the HD-Zip family contain a homeodomain (HD) associated to a leucine zipper (LZ). These TFs have been characterized as active participants in the adaptive response to several abiotic stresses. Their expression is regulated by drought, salt, and ABA (Ariel et al. 2007). Ectopic expression of the sunflower HaHB4 TF in *Arabidopsis* improved tolerance to drought and salinity, among other types of stresses (Cabello et al. 2007). In *Medicago truncatula*, *MtHB1* was identified as a salt stress-regulated gene (Ariel et al. 2007). Cabello and Chan (2012) have shown that the expression of the sunflower HaHB1 or its ortholog from *Arabidopsis* (AtHB13), as well as those of their putative targets, is upregulated by drought and salinity stresses. Transgenic plants overexpressing separately these genes exhibited increased tolerance to severe drought and salinity stresses, displaying cell membrane stabilization and higher chlorophyll contents (Cabello and Chan 2012).

The basic helix-loop-helix TF bHLH92 is induced in response to NaCl, dehydration, mannitol, and cold treatments. Root elongation of *bhlh92* mutants was more sensitive to mannitol and NaCl treatments compared to the WT, whereas overexpression of bHLH92 moderately increased the tolerance to NaCl and osmotic stresses. This TF regulates the expression of at least 19 downstream salt- and drought-responsive genes (Jiang et al. 2009).

WRKY factors modulate diverse plant processes, various biotic and abiotic stresses, and hormone-mediated pathways (Ramamoorthy et al. 2008). When WRKY25 and WRKY33 were overexpressed in *A. thaliana*, the transgenic plants showed increased tolerance to salt stress and ABA (Jiang and Deyholos 2009). Moreover, these TFs are also regulated by oxidative stress (Miller et al. 2010), with their target genes encoding proteins involved in ROS detoxification, such as peroxidases and GST (Jiang and Deyholos 2009). The expression of AREB1/ABF2 TF was affected in *wrky63* knockout mutants, demonstrating the involvement of WRKY factors in drought and salt adaptation via ABA-dependent pathways (Ren et al. 2010). Interestingly, WRKY factors are controlled and regulated by Zat proteins (TFIIIA-type Cys/His2f zinc finger proteins). The regulation of the soybean *GmWRKY54* gene, which confers drought and salt tolerance, by Zat10/SZT has been postulated by Zhou et al. (2008).

The NAC family of TFs is one of the largest ones and is only found in plants (Riechmann et al. 2000). NAC-type proteins play important roles in plant development (Souer et al. 1996; Xie et al. 2000) and have a key function in biotic and abiotic stress tolerance, including drought and salinity (Hegedus et al. 2003). The contribution of rice *NAC* genes to stress adaptation has been characterized;

OsNAC5 and *OsNAC6* are induced by ABA, drought, and salt stress (Rabbani et al. 2003). These factors bind directly to the promoter region of stress-inducible genes as *OsLEA3*, activating its transcription and promoting functional dimerization (Takasaki et al. 2010). Transgenic rice plants constitutively overexpressing the *OsNAC6* gene exhibited growth retardation and low reproductive yields, together with improved tolerance to dehydration and high-salt stresses (Nakashima et al. 2007). Overexpression of the *SNAC1* gene, belonging to this family, in rice plants resulted in stomatal closure, drought resistance, and improved salt tolerance under stressed field conditions (Hu et al. 2006). A recent study has shown that SNAC1-overexpressing cotton plants displayed improved tolerance to both drought and salt stresses under greenhouse conditions, enhancing root development and reducing transpiration rates (Liu et al. 2014). Another rice *NAC* gene, *SNAC2*, was identified as induced by drought, salinity, cold, wounding, and ABA, and its overexpression increased tolerance to salt, cold, and drought during rice seedling development. No common genes were found to be regulated by both SNAC1 and SNAC2 (Hu et al. 2008). Microarray studies demonstrated that two stress-responsive genes that were not affected in either *SNAC1* or *SNAC2* transgenic rice were upregulated in transgenic plants overexpressing OsNAC045, which display enhanced drought and salt tolerance (Zheng et al. 2009). These results indicate that different *NAC* genes have non-redundant functions, even though they are all involved in salt stress responses. Tran et al. (2007) have shown interaction of the drought-, salt- and ABA-inducible zinc finger protein ZFHD1 and an NAC factor.

Transcription factors also participate in adaptive responses to environmental stresses through microRNA (miRNA) pathways. Recently, an NAC-domain TF was identified as a target of miR164 in switchgrass (Matts et al. 2010). Moreover, SCL, MYB, and TCP TFs are targets of drought- and salt-inducible miRNAs as miR159, miR168, miR171, and miR396 (Liu et al. 2008b). In creeping bentgrass, salinity and drought stresses induce augmented expression of miR319, downregulating at least four putative target genes and a homolog of the rice *NAC*-domain gene, AsNAC60. Transgenic creeping bentgrass overexpressing the rice Os-miR319a exhibited enhanced salt tolerance (Zhou and Luo 2014). The regulation of stress-related targets through miRNAs may allow plants to fine-tune their responses to hormone and salt stress.

Epigenetic processes are becoming the new players in plant environmental adaptation. Chromatin structure might be modulated by DNA methylation and posttranslational histone modification (Kim et al. 2010), acetylation, phosphorylation, ubiquitination, biotinylation, and sumoylation (Chinnusamy et al. 2008). Plants growing in hostile habitats may carry memories of stress adaptation and transfer them epigenetically to the next generation (Fig. 1.2). The DNA of mangroves growing under saline conditions was hypomethylated, in contrast to populations from non-saline soils (Lira-Medeiros et al. 2010). In rice, salt stress modifies the expression of cytosine DNA methyltransferases (Sharma et al. 2009). In addition, Sokol et al. (2007) reported salinity-induced phosphorylation of histone H3 and acetylation of histone H4 in tobacco and *A. thaliana*, respectively. Studies

in stress responses related to epigenetics are slowly emerging, but it will be necessary to gain a more detailed knowledge on the specific mechanisms underlying epigenetic regulation under environmental stress to further improve salt tolerance in crop plants.

This brief enumeration, albeit partial, illustrates the bewildering complexity of the regulatory and signaling networks that are called into action when plants are exposed to salinity (Fig. 1.2) and their cross-talk with the responses invoked by other stresses such as drought and cold.

1.11 Strategies for Conferring Salt Tolerance Using Transgenic Plants

Two major approaches have been used to improve stress tolerance: (1) exploitation of natural genetic variations and (2) generation of transgenic plants with novel genes or altered expression levels of the existing ones. Zhang et al. (2004) and Zhu (2001, 2002) have reviewed signaling and transcriptional control in plants under salt stress, while Roy et al. (2014) discussed strategies to improve salinity tolerance of crops in an agronomical context. Since abiotic stress tolerance is multigenic in nature, the main trend has been to generate more tolerant transgenic plants by genetic transformation with multiple genes or with transcription factors. Many crop plants have been engineered using abiotic stress-related genes and have shown increased tolerance under laboratory conditions (Table 1.1, see also Agarwal et al. 2013). Genes whose products combat salt toxicity at various levels (ion homeostasis, synthesis of compatible solutes, and oxidative stress management) have been assayed to improve salt tolerance (Table 1.1). Many of these genes are induced during salinity, and therefore, salt induction has also been used as a criterion to identify tolerance-related traits, assuming that if expression of a gene is induced, it should be involved somehow in defense. In a different context, strategies for augmenting salt tolerance in glycophytes have been based on the expression of genes differentially regulated in salt-resistant and salt-sensitive cultivars. Finally, manipulation of stress-related TFs and signaling components offer the opportunity to modulate a suite of effector genes by a single intervention (Table 1.1).

An example of a protein involved in general processes that confers protection to salt stress is nucleolin. Nucleolin is involved in the assembly of ribosomal proteins with RNA (Didier and Klee 1992). Expression of nucleolin is reported to be regulated by drought, cold, and salinity stresses in an *Arabidopsis* microarray (Seki et al. 2002). Transgenic *Arabidopsis* plants overexpressing a rice nucleolin (OsNUC1) displayed higher relative growth rate, longer root length, and lower H_2O_2 accumulation under salt stress with respect to WT plants (Sripinyowanich et al. 2013). The pea helicase PDH45 was shown to be involved in salt tolerance by expression in transgenic tobacco (Sanan-Mishra et al. 2005) and rice (Sahoo

Table 1.1 Candidate gene families expressed in plants to improve salt tolerance

Transgene	Transgenic plant	Phenotype	References
Transcription factors			
OsDREB1	*Arabidopsis*	Salt tolerance	Zhang et al. (2009b)
	Rice	Salt, drought, and cold tolerance	Wang et al. (2008)
GmDREB2	*Arabidopsis*	Salt and dehydration tolerance	Chen et al. (2007)
GhDREB2	Wheat	Salt, drought, and cold tolerance	Gao et al. (2009)
OsDREB2A	Rice	Salt and dehydration tolerance	Mallikarjuna et al. (2011)
PgDREB2A	Tobacco	Salt and dehydration tolerance	Agarwal et al. (2010)
LcDREB3	*Arabidopsis*	Salt and drought tolerance	Xianjun et al. (2011)
MtCBF4	*Medicago truncatula*	Salt tolerance	Li et al. (2011a)
	Arabidopsis	Salt and drought tolerance	
AtMYB20	*Arabidopsis*	Salt tolerance	Cui et al. (2013)
TaMYB2A	*Arabidopsis*	Salt and drought tolerance	Mao et al. (2011)
CpMYB10	*Arabidopsis*	Salt and desiccation tolerance	Villalobos et al. (2004)
OsMYB3R	*Arabidopsis*	Salt, drought, and freezing tolerance	Dai et al. (2007)
GmMYB76	*Arabidopsis*	Salt and freezing tolerance	Liao et al. (2008)
AtMYB44	*Arabidopsis*	Salt and drought tolerance	Jung et al. (2008)
SlAIM1 (*MYB*)	Tomato	Salt and drought tolerance	Abuqamar et al. (2009)

(continued)

Table 1.1 (continued)

Transgene	Transgenic plant	Phenotype	References
GmERF3	Tobacco	Salt and dehydration tolerance	Zhang et al. (2009a)
OsNAC063	*Arabidopsis*	Salt and osmotic stress tolerance	Yokotani et al. (2009)
GmNAC11	*Arabidopsis*	Salt tolerance	Hao et al. (2011)
AhNAC2	*Arabidopsis*	Salt and drought tolerance	Liu et al. (2011b)
OsNAC5	*Arabidopsis* Rice	Salt and drought tolerance	Song et al. (2011)
OsSNAC2	Rice	Salt and cold stress tolerance	Hu et al. (2008)
OsNAC1	Rice	Salt tolerance	Hu et al. (2006)
DgNAC1	Tobacco	Salt tolerance	Liu et al. (2011a)
TaNAC69	Wheat	Inducible salt and drought tolerance	Xue et al. (2011)
Compatible solutes			
ScTPS1 (trehalose-6-phosphate synthase)	*Arabidopsis* Tomato	Salt tolerance	Miranda et al. (2007), Cortina and Culiáñez-Macià (2005)
ScTPS1-TPS2	Alfalfa	Salt tolerance	Suárez et al. (2009)
OsTPS1	Rice	Salt and drought tolerance	Li et al. (2011b)
otsA/*otsB* (*E. coli* trehalose-6-phosphate synthase/phosphatase)	Rice	Salt, drought, and cold tolerance	Garg et al. (2002)
mt1D (mannitol-1-phosphate dehydrogenase)	*Arabidopsis* Tobacco Wheat	Salt and dehydration tolerance	Thomas et al. (1995), Karakas et al. (1997), Abebe et al. (2003)
VaP5CS (Δ^1-pyrroline-5-carboxylate synthase)	Tobacco Rice Wheat	Salt tolerance	Kishor et al. (1995), Hong et al. (2000), Zhu et al. (1998), Sawahel and Hassan (2002)
AtP5CS	Potato	Salt tolerance	Hmida-Sayari et al. (2005)
codA (*A. globiformis* choline oxidase)	*Arabidopsis* Rice *Brassica juncea*	Salt tolerance	Hayashi et al. (1997), Sakamoto et al. (1998), Sakamoto and Murata (2000), Prasad et al. (2000)
betA (*E. coli* choline dehydrogenase)	Broccoli	Salt tolerance	Bhattacharya et al. (2004)

(continued)

Table 1.1 (continued)

Transgene	Transgenic plant	Phenotype	References
betA/betB (*E. coli* choline dehydrogenase/betaine aldehyde dehydrogenase)	Tobacco	Salt tolerance	Holmstrom et al. (2000)
AtBADH (betaine aldehyde dehydrogenase)	Wheat	Salt tolerance	Guo et al. (1999)
SoBADH	Tobacco	Salt tolerance	Yang et al. (2008), Fan et al. (2012)
	Sweet potato		
ROS detoxification			
AtAPX (ascorbate peroxidase)	Tobacco	Salt and drought tolerance	Badawi et al. (2004)
StAPX	Tobacco	Salt and osmotic stress tolerance	Sun et al. (2010)
PsAPX	Tomato	Salt tolerance	Wang et al. (2005)
OsAPX	*Arabidopsis*	Salt tolerance	Lu et al. (2007)
NtGST/GPX (glutathione *S*-transferase/glutathione peroxidase)	Tobacco	Salt tolerance and chilling	Roxas et al. (1997)
SsGST	*Arabidopsis*	Salt tolerance	Qi et al. (2010)
AmCu/ZnSOD (superoxide dismutase)	Rice	Salt and drought tolerance	Prashanth et al. (2008)
PsCu/ZnSOD/APX	Tobacco	Salt and osmotic stress tolerance	Lee et al. (2010)
AtMnSOD	*Arabidopsis*	Salt and cold tolerance	Wang et al. (2004)
OsDHAR (dehydroascorbate reductase)	*Arabidopsis*	Salt tolerance	Ushimaru et al. (2006)
AtMDAR (monoDHAR)	Tobacco	Salt, osmotic, and ozone stress tolerance	Eltayeb et al. (2007)
AmMDAR	Tobacco	Salt tolerance	Kavitha et al. (2010)
katE (*E. coli* catalase)	Rice	Salt tolerance	Moriwaki et al. (2008)
Ion transporters			
AtNHX1 (vacuolar Na^+/H^+ antiporter)	*Arabidopsis*	Salt tolerance	Apse et al. (1999), He et al. (2005), Xue et al. (2004), Zhang et al. (2001), Zhang and Blumwald (2001), Zhao et al. (2007), Chen et al. (2008), Liu et al. (2008a)
	Cotton		
	Tomato		
	Rapeseed		
	Tall fescue		
	Buckwheat		
	Sugar beet		
	Wheat		

(continued)

Table 1.1 (continued)

Transgene	Transgenic plant	Phenotype	References
SOD2 (yeast Na^+/H^+ antiporter)	*Arabidopsis*	Salt tolerance	Gao et al. (2003), Zhao et al. (2006)
	Rice		
AtSOS1 (plasma membrane Na^+/H^+ antiporter)	*Arabidopsis*	Salt tolerance	Shi et al. (2003), Yue et al. (2012)
	Tobacco		
SbSOS1	Tobacco	Salt tolerance	Yadav et al. (2012)
AtHKT1;1 (Na^+ transporter)	Rice	Salt tolerance	Plett et al. (2010)
HvHKT2;1 (K^+ transporter)	Barley	Salt tolerance	Mian et al. (2011)
AtAVP1 (vacuolar H^+ pyrophosphatase)	*Arabidopsis*	Salt tolerance	Gaxiola et al. (2001), Bao et al. (2009), Pasapula et al. (2011), Li et al. (2010)
	Alfalfa		
	Cotton		
	Creeping bentgrass		

et al. 2012). However, the exact mechanism of PDH45-mediated salinity stress tolerance is not well understood. Gill et al. (2013) have proposed that PDH45 coordinates the action of components of the antioxidant machinery in the transgenic plants. Recently, a gene encoding for a small RNA-binding protein (*S-RBP11*) was isolated as a salt-resistant activation tagging line. *Arabidopsis* plants overexpressing S-RBP11 showed increased tolerance to salt and oxidative stresses compared to WT plants (Lee et al. 2014).

A rational strategy to enhance salt tolerance is by manipulating the expression of effector genes, such as those involved in Na^+ extrusion of the cell or Na^+ import to the vacuole (Table 1.1). Salt-tolerant *B. napus* plants overexpressing the vacuolar Na^+/H^+ antiporter AtNHX1 from *A. thaliana* showed increased Na^+ accumulation in vacuoles and were able to grow in high saline concentration (Zhang et al. 2001). Comparative studies of salt-resistant and salt-sensitive cultivars of wheat have shown differential increase in TaNHX3, a vacuolar Na^+/H^+ antiporter. Expression of TaNHX3 in tobacco significantly enhanced salt tolerance, showing higher fresh and dry weights, contents of chlorophylls, carotenoids and soluble proteins, and increased antioxidant activities (Lu et al. 2014). Overexpression of NHX1 improved salt tolerance in *Arabidopsis* (Apse et al. 1999), tomato (Zhang and Blumwald 2001), maize (Yin et al. 2004), wheat (Xue et al. 2004), rice (Ohta et al. 2002), tobacco (Wu et al. 2004), and tall fescue plants (Tian et al. 2006). All these transformants were able to grow, flower, and set fruit in significantly higher salt concentration compared to their WT siblings.

Overexpression of AtSOS1 in *Arabidopsis* increased salt tolerance by limiting Na^+ accumulation in the xylem and stem (Shi et al. 2003), whereas transgenic

tobacco lines overexpressing AtSOS1 displayed better germination rates, lower chlorophyll loss, and less accumulation of Na^+ under salt treatments compared to WT plants (Yue et al. 2012). It has been recently reported that the expression of a truncated-hyperactive form of durum wheat TdSOS1 conferred significant ionic stress tolerance in *Arabidopsis*. In this context, the authors suggested that selection of hyperactive alleles of SOS1 may pave the way for obtaining salt-tolerant crops (Feki et al. 2014). Another interesting example is the expression of an *Arabidopsis* vacuolar H^+-pyrophosphatase gene (AVP1), which improved drought and salt tolerance in cotton, increasing proline contents and enhancing fiber yield under field conditions (Pasapula et al. 2011).

High salt concentrations lead to secondary stresses by enhancing the production of ROS which ultimately cause oxidative damage (Gill and Tuteja 2010; Munns and Tester 2008). ROS are toxic molecules, but also serve as mobile signals that regulate stress responses. Development of transgenic plants overexpressing one or more antioxidant enzymes is a common strategy used to obtain lines tolerant to salt stress in different species (Table 1.1). Transgenic tobacco plants overexpressing both GST and GPX showed improved seed germination and seedling growth under stress (Roxas et al. 1997). Also, tobacco transformants expressing a cytosolic APX from *Lycium chinense* (LmAPX) exhibited lower H_2O_2 accumulation, higher proline contents, and net photosynthetic rates under salt stress (Wu et al. 2014). Diaz-Vivancos et al. (2013) have reported increased tolerance to salt stress in plum by ectopic expression of cytosolic Cu/Zn SOD and APX. Transgenic plantlets exhibited higher contents of GSH and ASC and lower accumulation of H_2O_2 than the non-transformed control (Diaz-Vivancos et al. 2013). Synergistic effects were observed in cotton plants overexpressing a Cu/Zn SOD and CAT. Plants accumulating the two antioxidant enzymes in their chloroplasts exhibited the highest tolerance to salt stress compared with lines expressing the genes in the cytoplasm or with the single transformants (Luo et al. 2013).

As indicated, compatible solutes play important roles as osmoprotectants. Generally, manipulation of genes whose products are involved in the synthetic and degradative pathways of many osmolytes resulted in enhanced salt and drought tolerance (Table 1.1).

Initial strategies aimed at engineering higher concentrations of proline began with the overexpression of genes encoding the enzymes pyrroline-5-carboxylate synthetase (P5CS) and P5C reductase (P5CR), which catalyze the two steps between the substrate (glutamic acid) and the product (proline). P5CS overexpression in transgenic tobacco dramatically elevated the levels of proline (Kishor et al. 1995). However, there is strong evidence that free proline inhibits P5CS (Roosens et al. 1999). Hong et al. (2000) achieved a twofold increase of proline levels in tobacco plants by using a *P5CS* modified by site-directed mutagenesis. The procedure alleviated the feedback inhibition of P5CS activity by proline and resulted in improved germination and growth of seedlings under salt stress. Also, Nanjo et al. (1999) used antisense cDNA transformation to decrease proline dehydrogenase expression in order to increase free proline levels.

On the other hand, the introduction of the enzyme responsible for the synthesis of a proline precursor, Δ^1-pyrroline-carboxylate synthase, provides tolerance to salinity stress in a wide range of transgenic species: tobacco (Hong et al. 2000; Kishor et al. 1995), rice (Su and Wu 2004; Zhu et al. 1998), bread wheat (Sawahel and Hassan 2002), and potato (Hmida-Sayari et al. 2005).

Glycinebetaine is synthesized by a two-step process from choline with the intermediate betaine aldehyde. However, several crop plants could not synthesize betaine aldehyde (Rhodes and Hanson 1993). Genetically engineered tobacco plants synthetizing glycinebetaine in chloroplasts were obtained by introducing the betaine aldehyde dehydrogenase gene. The transgenic plants showed enhanced tolerance to salt stress by protecting photosynthesis (Yang et al. 2008). The gene encoding choline dehydrogenase from *Escherichia coli* has been expressed in *B. oleracea* (Bhattacharya et al. 2004) and maize (Quan et al. 2004), enhancing salt and drought tolerance. Moreover, rice plants expressing an inducible COD gene displayed greater salt tolerance due to increased production of glycinebetaine (Su et al. 2006).

The genes for trehalose synthesis from *E. coli*, *otsA*, and *otsB*, encoding trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively, were introduced in rice (Garg et al. 2002) and potato (Yeo et al. 2000), increasing tolerance to salt, drought, and cold stress in the transgenic plants. In a different approach, the gene encoding the trehalose-6-phosphate synthase from yeast was expressed in rice, tomato (Cortina and Culiáñez-Macià 2005), *Arabidopsis* (Miranda et al. 2007), and alfalfa (Suárez et al. 2009), imparting enhanced salt tolerance to these transgenic plants.

The accumulation of sugar alcohols is a widespread response to environmental stresses including salinity (Table 1.1). Salt tolerance of transgenic tobacco engineered to accumulate mannitol was first demonstrated by Tarczynski et al. (1993). Transgenic tobacco plants were engineered by introduction of the *E. coli* gene encoding mannitol-1-phosphate dehydrogenase. These plants synthesized and accumulated mannitol, increasing their ability to tolerate high salinity (Tarczynski et al. 1992, 1993). Similar results were obtained in *Arabidopsis* (Thomas et al. 1995) and wheat (Abebe et al. 2003). In line with a stress-protective role, overexpression of enzymes involved in the synthesis of inositol (L-myo-inositol-1-phosphate synthase and myo-inositol 1-phosphate phosphatase) from halotolerant plants increased cyclic polyols levels, resulting in salt stress tolerance in tobacco (Majee et al. 2004).

Other examples of genetic engineering directed to the accumulation of compatible solute include the transformation of tobacco cells with genes encoding enzymes for ectoine synthesis from the halophilic bacterium *Halomonas elongata* (Nakayama et al. 2000) and for sorbitol synthesis in *Plantago* (Pommerrenig et al. 2007).

Key transcription factors may regulate the expression of a range of salinity tolerance genes involved in several mechanisms, so it is reasonable to think that manipulation of TFs may result in the greatest effect on crop salinity tolerance with minor genetic modifications. However, the beneficial effects of TFs can be

counterbalanced by the introduction of yield penalties, especially under mild- or non-stress growth conditions. Examples of genetically modified plants using TFs are given in Table 1.1.

The discoveries made in the last few years on the mechanism of salt tolerance should be applied to crops to improve their performance. However, most of the transgenic plants studied are model plants, and stress tolerance has largely been evaluated under laboratory conditions. Salt tolerance must be assayed in the field and during periods of intense or prolonged stress resembling natural conditions. Fine-tuning of the expression of known candidates for stress tolerance genes obeying specific temporal and spatial patterns is essential to rule out negative effects on plant growth. The use of stress-inducible promoters is a good choice for developing stress tolerance while avoiding developmental penalties.

1.12 Conclusion

Environmental stress due to salinity is one of the most serious factors limiting the productivity of agricultural crops, most of which are sensitive to the presence of high concentrations of salts in the soil. About 50 % of irrigated agricultural land is adversely affected by salinity (Flowers and Yeo 1995). The problem of soil salinity is further aggravated through the use of poor quality water for irrigation and inadequate drainage. Soil type and environmental factors, such as vapor pressure deficit, radiation, and temperature, may also alter salt tolerance. The loss of farmable land to salinization is in direct conflict with the needs of the world population, projected to increase by 1.5 billion in the next 20 years (Blumwald and Grover 2006). Engineering crops that are resistant to salinity stress is critical for sustaining food production and achieving future food security. However, progress in breeding for salt-tolerant crops has been hampered by the lack of understanding of the molecular basis of salt tolerance and insufficient availability of genes whose products confer salt tolerance. Also, the evaluation of salt tolerance in transgenic lines has mostly been carried out using a limited number of seedlings or mature plants under laboratory and/or greenhouse conditions different from those which plants would naturally be exposed to in the field (Mittler 2006). The evaluation of field performance under salt stress is difficult because of the variability of salt levels under field conditions and the potential for interactions with other environmental factors, including soil fertility, temperature, light intensity, and water loss through transpiration (Daniells et al. 2001). The lack of success is also due in part to plant geneticists using constitutive promoters such as the CaMV 35S, ubiquitin, and actin promoters (Grover et al. 2003). In general, stress-induced or tissue-specific promoters result in better phenotypes than those obtained by expressing the same genes under control of a constitutive promoter (Kasuga et al. 1999; Zhu et al. 1998). There is a clear and urgent need to introduce these tolerance genes into crop plants, in addition to establishing gene stacking or gene pyramiding.

Although progress in increasing salt tolerance has been relatively slow, there are reasons for optimism. They include, among others, the development of molecular markers and gene tagging methodologies, the complete sequencing of plant genomes, the availability of forward genetics tools such as tilling, and the widespread use of microarray analysis. These powerful resources offer advantages and provide solutions to the complex and intriguing questions of salt resistance.

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Chapter 2
Physiology and Biochemistry of Aluminum Toxicity and Tolerance in Crops

Anjali Aggarwal, Bunichi Ezaki, Ashok Munjal, and Bhumi Nath Tripathi

Abstract Achieving sustainable food production to feed the increasing population of the problematic lands of the world is an enormous challenge. Aluminum (Al) toxicity in the acid soil is a major worldwide problem. Liming and nutrient management technologies are worthless due to high lime requirement, and the effect of liming does not persist for long. Besides this, conventional breeding is useful to manage Al toxicity as some plants have evolved mechanisms to cope with Al toxicity in acid soil. Therefore, understanding of Al tolerance mechanisms is prime necessity for improving Al tolerance in crops. Al resistance mechanisms include mainly Al avoidance (Al exclusion) and/or Al tolerance (detoxification of Al inside the cell) mechanisms. In this chapter, we summarize Al behavior in plant root cell. We include recent findings of Al resistance mechanisms and Al-resistant genes which can be useful to produce cultivars adapted to acid soils.

Keywords Acid soil • Al-resistant genes • Target sites of Al toxicity

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2.1 Introduction

Food security for a growing population is a major challenge in front of every society. The world population is predicted to increase by two billion over the next 40 years. In order to meet the additional demand for food, fuel, fiber, and animal feed, cereal production alone will have to rise about three billion tons per annum, an increase of 50 % of current production levels (FAO 2009). This highlights the need to overcome the major soil constraints currently limiting crop yields. Acid soil is a major soil constrain around the world that limits crop productivity. Acid soils produce multiple stresses to plants including proton toxicity, nutrient deficiency, and metal ion toxicities. Among them, aluminum (Al) ion toxicity severely affects crop production in acid soil due to rapid inhibition of root growth and changes in other metabolic activities of plant cells. Consequently, Al toxicity directly affects the grain quality and plant yield (Kochian et al. 2005; Inostroza-Blancheteau et al. 2012).

2.2 Acid Soil: Formation, Distribution, and Amelioration

Acid soil develops naturally, when basic cations (Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}) are leached down from the soil profile, often with nitrate, and release extra hydrogen ion (H^{+}) which results in low pH of soil solution. Acid rain due to air pollution is the main reason of the acidification of soil. Furthermore, soil acidification can also be directly accelerated by anthropogenic activities such as the use of ammonia and amide containing fertilizers in farming practices. Decomposition of organic matter by microorganism also contributes to soil acidification by releasing CO_2 which forms organic acids and nitrate with soil solution. The United States Department of Agriculture (USDA) classifies acid soils into six levels: ultra-acid ($pH < 3.5$), extremely acid (pH 3.5–4.4), very strongly acid (pH 4.5–5.0), strongly acid (pH 5.1–5.5), moderately acid (pH 5.6–6.0), and slightly acid (pH 6.1–6.5) soils. Soil with $pH \leq 5.5$ adversely affects the production of many crops (Zhou et al. 2011).

Acid soils ($pH < 5.5$) represent between 30 and 40 % of the world's arable soils. Total world acid soils occur in two global belt, northern cold temperate belt and southern tropical belt. In Asia (excluding Australia and New Zealand), acid soil is mainly distributed throughout Southeast Asia and Pacific. The distribution of world acid soil by region is presented in Table 2.1 (von Uexküll and Mutert 1995). Application of lime (primarily calcium carbonate) is the one strategy to ameliorate soil acidity and improve soil suitability for agriculture. However, it is not practical or common in developing countries which rely mainly on small subsistence farming for food production and not an effective strategy for alleviating subsoil acidity. The effects of all kinds of amendment become fade by the huge buffering capacity of soil, as the application of treatments is generally restricted to the soil surface and the

Table 2.1 Distribution of acid soil in world

SN	Region		Total area[a]	Acid soil area[a]	% Acid soil	Contribution to total world acid soil %
1.	Africa		3,010	659	22	16.7
2.	Australia and New Zealand		820	239	29	6.1
3.	Europe		1,018	391	81	9.9
4.	America	North	2,210	698	32	17.7
		South	1,750	916	52	23.2
5.	Asia	East Asia	1,980	217	11	5.5
		Southeast and Pacific	400	314	78	7.9
		North and Central	850	512	60	13
6.	Total world land area		13,150	3,950	30	

[a]Area in million hectares (*Sources*: von Uexküll and Mutert 1995)

properties of the subsoil are hardly modified. Additionally, due to the factors such as acid rain and excess application of ammonium-based inorganic nitrogen fertilizers make this amelioration strategy useless for a long time (Zheng 2010). Besides this, conventional breeding and biotechnology practices are useful to manage acid soil. Many wild and crop plants evolved genetic-based different mechanisms that allow them to survive acid soil better than others, and these mechanisms have allowed plant breeder to develop Al-resistant crops through genetic manipulation (Kochian 1995; Ezaki et al. 2008).

2.3 Aluminum Soil Chemistry: Speciation, Complexation, and Phytotoxicity

The major limitation for crop growth in acid soils is soluble Al ions in soil solution. Al is the third most abundant chemical element in the earth's crust after oxygen and silicon (Kochian 1995). Soluble Al hydrolyzes to form a range of species, and prevalence of these species in the soil is directly dependent on the soil pH. Accordingly, the magnitude of Al toxicity to plant growth depends upon the presence of these species in the soil solution. Therefore, identification of these Al species and estimation of their biological impact are essential.

In acid soil, Al can be found in the following different chemical forms depending upon the soil pH: (1) at pH 4.5 or below, the octahedral hexahydrate $[Al(H_2O)_6]^{3+}$ (commonly referred to as Al^{3+}) predominates in the soil solution, and (2) as soil pH increases, mononuclear hydrolysis species such as $Al(OH)_2^+$ and $Al(OH)^{2+}$ predominate, and (3) at near-neutral pH, the solid phase $Al(OH)_3$ (gibbsite) occurs (4) whereas at slightly alkaline conditions the amphoteric species $Al(OH)_4^-$ (aluminate) predominates. Among them, Al^{3+} is highly toxic to plants, and higher

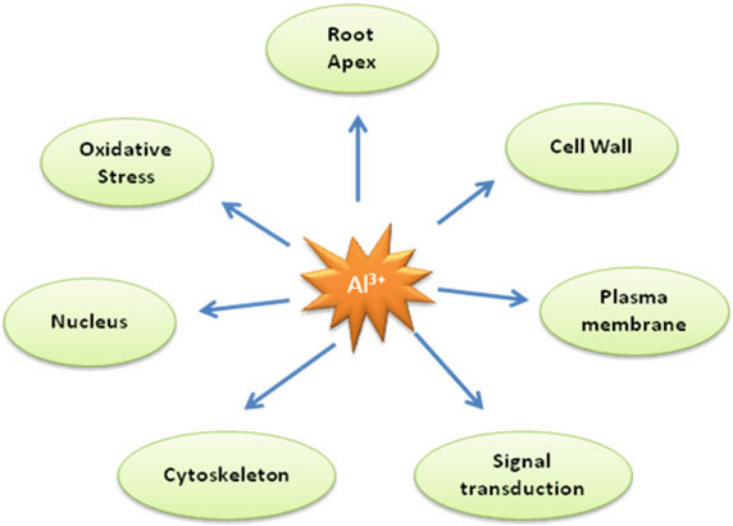

Fig. 2.1 Multiple target sites of Al^{3+} within a root

concentration of Al^{3+} ions in soil is directly related to the low pH of soil solution. Furthermore, Al complexation also plays an important role in the solubility and speciation of Al in soil solution. The major inorganic ligands which make complex with Al^{3+} are F^{-}, H_2O, OH^{-}, PO_4^{3-}, and SO_4^{2-} (Kinraide 1997). Thus, Al may be found in the soil solution as free Al^{3+}, precipitated with other elements, hydroxyl monomers of Al, and complexed with other elements depending upon the soil pH and occurrence of other elements in the soil. The degree to which Al inhibits root growth primarily depends upon the activity of free Al^{3+} ions in solution (Kochian et al. 2004).

However, research on mechanisms of Al toxicity is impeded due to the complexity of Al chemistry in soil solution. Indeed, depending on the concentration and duration of exposure, Al^{3+} ions have multiple target sites within the plant cell as described in the next section (Fig. 2.1).

2.4 Aluminum Toxicity: Target Sites of Al^{3+} Ions

The earliest phytotoxic effect of Al^{3+} ions is root growth inhibition involving many cellular complex processes, which subsequently affect nutrients and water uptake (Foy 1988). Al toxicity alters the morphology of root cell, which results in thick, stunt, and brittle root, poor root hair development, and swollen and damaged root apices (Matsumoto 2000). A large fraction of Al^{3+} ions bind with the apoplast, and a small fraction enter in the symplasm (cytosol) within minutes following the Al exposure (Silva et al. 2000; Taylor et al. 2000) However, it is still a matter of debate

that the primary lesions of Al toxicity are apoplastic or symplastic (Horst et al. 2010).

The promptness of the root growth inhibition upon exposure to Al indicates that Al quickly disrupts root cell expansion and elongation (Kochian et al. 2005). Earlier, it was perpended that the blockage of cell division is the primary mode of Al damage since the cessation of cell elongation and cell expansion was considered as closely correlated (Clarkson 1965). Later on, Llugany et al. (1995) found that the inhibition of root elongation started after less than 30 min exposure to toxic Al^{3+} ions in Al-sensitive maize. Although cell division is a slow process, now it is generally accepted that the inhibition of cell elongation affects first upon Al exposure rather than cell division. Prolonged exposures lead to Al interactions with the root cell nuclei, resulting in disruption of cell division (Silva et al. 2000; Zheng and Yang 2005). However, it is difficult to distinguish the primary targets from the secondary effects so far, understanding of the target sites of Al toxicity is helpful in elucidating the mechanisms by which Al exerts its deleterious effects on root growth.

2.4.1 Root Apex

At the early stage of the research of Al toxicity, it was believed that Al is first absorbed at root cap and initiated the signals that lead to the inhibition of root growth (Fiskesjö 1990; Bennet and Breen 1991). However, Ryan et al. (1993) showed that not the root cap, but the root meristem was the most Al-sensitive site in maize by using divided-chambers method in which agar blocks infused to apply Al to different zones of the root. Further, Sivaguru and Horst (1998) established that the distal transition zone (DTZ, a region 1–3 mm behind the root tip) of the root apex is the primary target of the Al in an Al-sensitive maize cultivar. They demonstrated that roots become increasingly sensitive to Al when treatment zone moved into the DTZ section and recovered increasingly after the removal of Al from this section. Additionally, at short-term exposure to Al, Al toxicity was found to the same extent between the root segments, when Al was individually applied only to the DTZ and on the entire maize root apex while treatments of Al to the other root segments had no effect on root elongation of either Al-resistant or Al-sensitive maize cultivars. In addition, Kollmeier et al. (2000) confirmed that the genotypic differences in Al resistance between maize-sensitive and maize-resistant genotypes are located within DTZ. Moreover, they provide evidence for a signaling pathway in the root apex that mediated the flow of Al information between the DTZ and the elongation zone through alternations in the basipetal auxin transport. Furthermore recently, Sivaguru et al. (2013) found that Al induced the greatest cell damage and generation of reactive oxygen species (ROS) specifically in the root DTZ in *Sorghum bicolor* and also demonstrated that Al-resistant gene (*SbMATE*) and protein expression were higher at DTZ, where root cells

experience the greatest Al stress. These findings indicate that the root DTZ is the primary region of root Al stress.

2.4.2 Cell Wall

Cell wall is the first site in contact with Al^{3+} ions, where plant roots are exposed to Al. It plays a principal role in the absorption and expression of Al toxicity. Several studies conclude that cell wall is the major site of Al accumulation (Horst et al. 1999; Schmohl and Horst 2000; Doncheva et al. 2005). For example, Clarkson (1965) reported that 85–90 % of the total Al accumulated in *Hordeum vulgare* (barley) roots was firmly bound to cell walls. Up to 99.9 % of the total cellular Al was accumulated in the cell wall of giant algal cells of *Chara corallina* (Taylor et al. 2000).

For Al-cell wall interaction, pectin is proposed to be a critical component (Blamey et al. 1993; Horst 1995; Chang et al. 1999). Horst et al. (1999) reported that plant with higher pectin content accumulated more Al in their root apices and were more Al sensitive, whereas other studies have reported that cell wall hemicellulose metabolism is more susceptible to Al stress. For example, Tabuchi and Matsumoto (2001) found that the exposure of Al-sensitive wheat to 10 μM Al for 6 h resulted in the accumulation of hemicellulose. Al treatment resulted in an increase in hemicellulose fraction in the cell wall of the Al-sensitive cultivar of rice sp. *indica* 'Zhefu802' (Yang et al. 2008). Recently, Yang et al. (2011) demonstrated that hemicellulose is the major pool for Al accumulation in *Arabidopsis*.

Further, Al interactions lead to the irreversible displacement of cations (e.g. Ca^{2+}) from cell wall components, which are fundamental to cell wall stability (Matsumoto et al. 1977a; Rincón and Gonzales 1992; Schmohl and Horst 2000; Kochian et al. 2004). Therefore, replaced Al^{3+} ions alter the cell wall structural and mechanical properties by rigidifying the cell wall that prevents its loosening for cell elongation (Kochian et al. 2005; Jones et al. 2006). Horst et al. (2010) summarized the recent progress in understanding the role of the apoplast (cell wall and the outer surface of the plasma membrane) in Al resistance and toxicity and provide physiological, biochemical, and molecular evidence which shows that modification of binding properties of the root apoplast contributes to Al resistance.

2.4.3 Plasma Membrane

Al^{3+} ions have a strong affinity for biomembranes and interact firmly with negatively charged plasma membrane. Al^{3+} ions strongly bind with the choline head of phosphatidylcholine (a lipid constituent of the plasma membrane) by replacing other cations (such as Ca^{2+}) that form bridges between the phospholipid head groups of the membrane bilayer. This process alters the plasma membrane fluidity

and phospholipid packing (Akeson and Munns 1989; Delhaize and Ryan 1995; Kochian 1995). Al^{3+} ions exhibit a 560-fold higher affinity for the phosphatidylcholine surface than Ca^{2+} ions. Callose deposition in the plasma membrane also proposed as one of the early indicators for Al toxicity (Horst et al. 1997; Massot et al. 1999). Since callose synthesis depends on the presence of Ca^{2+} ions, it has been suggested that Al^{3+} ions displacement of Ca^{2+} ions from the membrane surface may increase the apoplastic Ca^{2+} ions pool required to stimulate callose synthesis. Al-induced callose deposition may lead to further cellular damage and block cell-to-cell trafficking of molecules through plasmodesmatal connection (Sivaguru et al. 2000; Kochian et al. 2005).

One of the most noticeable consequences of root Al exposure in the plasma membrane is the Al-mediated induction of membrane potential (Lindberg et al. 1991; Papernik and Kochian 1997). In the cells of fibrous roots of sugar beet, depolarization of the transmembrane potential is reported (Lindberg et al. 1991). Olivetti et al. (1995) exhibit a maximum depolarization of transmembrane potential (55 mV) in Al-tolerant snap bean cultivar at exposure to 150 μM $AlCl_3$. Furthermore, there are several studies which correlate between surface potential and Al tolerance in plants including wheat, maize, and barley (Wagatsuma and Akiba 1989; Kinraide et al. 1992). Ahn et al. (2001) reported that Al^{3+} ions inhibit the H^+-ATPase activity by permanently altering the plasma membrane surface potential in squash roots.

2.4.4 Cytoskeleton

The changes in root morphology induced by Al^{3+} ions in both root and root hair tip indicate that Al-induced alternations in the cytoskeleton might occur (Matsumoto 2000; Sivaguru et al. 2000) and suggest Al could disrupt cytoskeleton dynamics by interacting with cytoskeleton elements (involved in cytoskeleton stabilization; Jones and Kochian 1995). Al^{3+} ions induce morphological alternations in root tip caused by depolymerization of cortical microtubules (Sasaki et al. 1997; Sivaguru et al. 2003) or by the extensive cell-specific reorganization and stabilization of microtubules and actin filaments (Blancaflor et al. 1998). Al^{3+} ions can also affect the mechanisms that control organization of microtubules and polymerization of tubulin which cause a delay in disassembly of microtubules during mitosis (Frantzios et al. 2000). Further, short treatment of Al in tobacco cell lines induces formation of additional cortical microtubulin bundles, while prolonged exposure results in their disorientation (Schwarzerová et al. 2002). Also Al interference with the cortical actin filaments suggested to play a role in fixing the axis of cell division in the correct position (Verma 2001). In addition, Amenós et al. (2009) found that actin cytoskeleton and vesicle trafficking are the primary targets for Al toxicity in the root tips of maize-sensitive genotype.

2.4.5 Signal Transduction Pathways

There are several studies that report Al^{3+} ions interact with signal transduction pathways by disrupting the Ca^{2+} ions homeostasis (Rengel 1992; Zhang and Rengel 1999; Ma et al. 2002). Al toxicity displaces Ca^{2+} ions from the plasma membrane, interrupts the signaling cascades of cytosolic Ca^{2+} ions, and blocks ion-channel pumps (Rengel and Zhang 2003). Al^{3+} ions interact with and inhibit the enzyme phospholipase C of the phosphoinositide pathway associated with Ca^{2+} ions signaling (Jones and Kochian 1995; Jones and Kochian 1997). Zhang et al. (2007) found Al-induced inhibition of genes related to phosphoinositide signaling pathway and hypothesized that gene inhibition could result in disruption of this pathway. Furthermore, NO homeostasis is also a critical site of Al toxicity. NO is an important signaling molecule involved in numerous physiological processes in plants including regulation of root growth and development. Al toxicity reduces endogenous NO levels in root apical cells which effect the hormone signaling pathway. Exogenous NO can ameliorate Al toxicity by improving hormone equilibrium in plants (He et al. 2012a). In addition, it is found that exogenous NO treatments improve Al tolerance by protecting the plant against Al-inducible oxidative damage through activating anti-oxidative capacity to eliminate ROS (He et al. 2012b).

2.4.6 Nucleus

Inhibition of cell division is a potential long-term and lethal consequence of Al toxicity. When roots of pea seedlings, treated with 1 mM $AlCl_3$ and stained with aluminon, it was found that Al^{3+} ions can be bind with the nuclei (Matsumoto et al. 1976). In addition, association of Al^{3+} ions with nuclei was confirmed by the chemical determination in purified nuclei (Matsumoto et al. 1977b). The localization of absorbed Al^{3+} ions in the nuclei was also found in onion root tips (Morimura et al. 1978). The possible target of Al^{3+} ions are phosphate groups of DNA or RNA in nuclei, and it is possible that the binding of Al^{3+} ions to DNA or to chromatin could condense the DNA and inhibit cell division by repressing the DNA template activity for transcription (Matsumoto 1991). Nuclear aberrations, micronuclei, and binuclear cells have been also recorded by Al toxicity (Roy et al. 1989; Zhang 1995). However, at lower Al concentration (1.45 μM) and low exposure time (30 min), Silva et al. (2000) demonstrated that Al^{3+} ions can accumulate in the nuclei of meristematic cells of soybean root tips by staining with lumogallion and a confocal laser scanning microscopy. Moreover, Achary and Panda (2010) underscored the biphasic (hormetic) mode of action of Al that at high doses induced DNA damage and at low nontoxic doses conferred genomic protection, both of which were mediated through ROS but perhaps involving different networks.

2.4.7 *Oxidative Stress*

All aerobic organisms including plants require oxygen for the effective production of energy. Under normal conditions ROS are commonly present in plant cells as a result of normal aerobic metabolism. But in stressed condition, generation of these ROS (superoxide anion radical $O_2^{\bullet -}$, hydroxyl radical $^{\bullet}OH$, hydrogen peroxide H_2O_2, etc.) increase and cause the oxidative stress in plants. ROS are highly reactive and toxic and can lead to cell death by causing damage to protein, lipids, carbohydrate, and DNA (Mittler 2002). Al toxicity also causes excessive generation of ROS and increases in peroxidation and/or breakdown of membrane lipids (Gutteridge et al. 1985; Cakmak and Horst 1991; Ono et al. 1995). Al is not a redox active metal, but it has been proved that it acts as a prooxidant (Exley 2004), and it can facilitate the radical chain reactions mediated by superoxide and iron (Fe) ions and can induce the oxidative damage in plants (Yamamoto et al. 2003). Enhancement of peroxidation of phospholipids in *Glycine max* (soybean) roots was the first report on the involvement of ROS of Al toxicity in plants (Cakmak and Horst 1991). Since then, several lines of evidences from physiological and genetical studies have supported this idea in plant cells (Jones et al. 2006; Achary et al. 2008; Tahara et al. 2008). Recently Yin et al. (2010) reported highly electrophilic α,-β-unsaturated aldehydes (2-alkenals), the lipid peroxide-derived aldehyde, participate in oxidative stress induced by Al in tobacco roots.

2.4.7.1 Superoxide Anion

Superoxide anion is a major ROS and produced at all the locations where an electron transport chain is present including chloroplast, mitochondria, and plasma membranes. Experiment with dihydroethidium (DHE), which is a specific indicator for superoxide anion, indicated ROS production in the pea roots under Al toxicity (Yamamoto et al. 2002). The staining of superoxide with DHE in a pea root tip was increased with an increase in Al concentration. The formation of superoxide anion in the pea roots under Al stress is largely caused by the plasma membrane associated with the NADPH oxidase, and firstly, Matsumoto and Motoda (2012, 2013) reported that the formation of superoxide anion was decreased after the removal of Al stress.

2.4.7.2 Hydrogen Peroxide

As compared to other ROS, H_2O_2 is the weaker oxidizing agent and plays an important role in plants under environmental stresses. H_2O_2 is produced by the cell wall oxidase catalyzing the oxidation of NADH to NAD^+, which in turn reduces O_2 to superoxide anion and is consequently dismutated to produce O_2 and H_2O_2. Al stress induced different types of peroxidase (Ezaki et al. 1996; Tamás et al. 2003).

One of the important functions of peroxidases under Al stress may be the possible participation in lignin formation (Ezaki et al. 2005). H_2O_2 increased only 10 % in the root of wheat (cv. Kalyansona) seedlings exposed to 10 μM Al for 12 h (Hossain et al. 2005), 20–30 % in rice roots exposed to 2 mM Al for 72 h (Ma et al. 2007), and 30 % in pumpkin (*Cucurbita pepo*) root exposed to 50 μM Al for 24 h (Dipierro et al. 2005). H_2O_2 content decreased in Al-tolerant *Melaleuca cajuputi* exposed to 1 mM Al, but increased by 25 % in Al-sensitive *Melaleuca bracteata* compared to the untreated control plants (Tahara et al. 2008).

2.4.7.3 Hydroxyl Radical

Hydroxyl radical is the most reactive ROS and formed by H_2O_2 by the Fenton reaction as a part of the Haber–Weiss reactions by using metal catalysts (Halliwell and Gutteridge 1989). Cell wall-associated peroxidases induce formation of hydroxyl radical which cause various types of oxygen damage, such as the peroxidation of membrane lipids. The Al-enhanced Fe (II)-dependent peroxidation of the lipids was first reported in the root tips of soybean only after a prolonged treatment with Al (Cakmak and Horst 1991). Yamamoto et al. (2001) demonstrated lipid peroxidation in the pea roots is a relatively early symptom induced by Al accumulation but not the primary cause of the root elongation inhibition, because the addition of butylated hydroxyanisole (a lipophilic antioxidant) prevented lipid peroxidation but not the inhibition of root elongation. Similarly, the enhancement of Fe (II or III)-mediated peroxidation of lipids by Al was observed in tobacco-cultured cells (Yamamoto et al. 2002). Recently, Yin et al. (2010) suggested lipid peroxide-derived aldehydes, especially highly electrophilic α,β-unsaturated aldehydes (2-alkenal), participate in Al toxicity.

2.5 Aluminum Resistance: External and Internal Mechanisms

Plants vary considerably in their ability to tolerate the toxic concentrations of Al^{3+} ions in acid soils. This variation is driven by the capacity to which they can exclude Al from their tissues or by their ability to detoxify Al^{3+} ions once it enters the cytosol (Ma et al. 2001; Kochian et al. 2005). In genotypes of some species, most of this variation is controlled by a single major genetic locus, whereas in other species, the trait is more complex including multiple genes and mechanisms which can express constitutively and/or induced upon Al exposure involving various signaling components (Liu et al. 2014).

Al resistance mechanisms have been classified into internal and external mechanisms (Ryan and Delhaize 2010; Fig. 2.2). In external mechanisms, Al resistance in plants involves the exclusion of Al from the root apex by exudation of organic

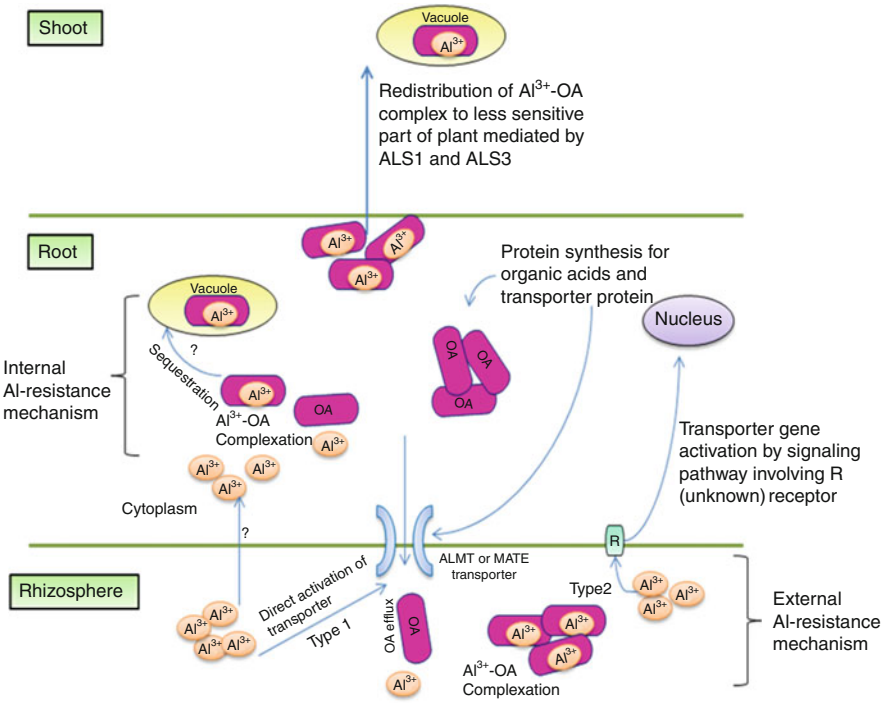

Fig. 2.2 External and internal Al resistance mechanisms in plant. In external mechanism efflux of Al^{3+}-activated organic anion can occur by two types. Type 1 response illustrates the direct activation of organic anion efflux in some species such as wheat where the anion channel is constitutively expressed. In Type 2 response Al^{3+} ions first activates possibly a specific receptor "R" which induces expression of transporters and organic acid protein via signal transduction pathway. Internal resistance mechanism involves chelation of cytosolic Al^{3+} ions by organic anions and the subsequent sequestration into the vacuole. In some hyperaccumulator species, these complexes may be redistributed to the less sensitive part of plants such as leaves

acid anions into the rhizosphere. These organic anions chelate toxic Al^{3+} ions in the rhizosphere, forming stable nonphytotoxic complexes. This mechanism could correspond to an avoidance mechanism. On the other hand, the Al-tolerant genotypes are able to detoxify Al^{3+} ions inside the cell by complexing it in the cytoplasm with organic acid anions or other organic ligands and then compartmentalizing it into vacuole (Kochian 1995). Therefore, there are two main mechanisms that have been proposed to account for resistance, and both can operate parallel in the same plant.

2.5.1 Internal Al Resistance Mechanisms

As mentioned before, many highly Al-tolerant species relies on complexation and detoxification and transport of Al^{3+} ions after it enters the plants. Tea (*Camellia*

sinensis), *Hydrangea* sp., and buckwheat (*Fagopyrum esculentum*) are good example of Al accumulator plant species. Leaves of buckwheat and sepals of *Hydrangea* can accumulate up to 1,500 mg/kg and more than 3,000 mg/kg DW of Al, respectively, when grown on an acid soil (Ma et al. 2001). In *Hydrangea*, after absorption of Al, its sepals change color from pink to blue (Ma et al. 1997a, b). This color change is caused by the formation of Al delphinidin complexes or aluminum caffeoylquinate complexes (Takeda et al. 1985; Ito et al. 2009). Furthermore, Tolrà et al. (2011) suggested that the retention of Al in epidermal leaf apoplast represents the main Al tolerance mechanism in tea plant. High shoot accumulation of Al implies that soluble Al^{3+} ions are transported through the xylem and then stored safely in leaf vacuoles or in the apoplast. In addition, a gene encoding a putative ABC transporter (*ALS3*) was found to be contributing to an Al tolerance mechanism in *Arabidopsis* possibly by facilitating the redistribution of absorbed Al away from the sensitive root tissues (Larsen et al. 2007).

2.5.2 External Al Resistance Mechanisms

The exclusion mechanism is the release of the organic anions from roots (Ma et al. 2001; Ryan et al. 2001). Malate and citrate are the two anions most commonly reported, but oxalate efflux occurs from a few species. As mentioned above, once these anions are released from root cells, they bind with Al^{3+} ions and prevent them from entering in the plant cell (Zhou et al. 2011). Root growth recovered after applying these anions in the solutions containing toxic concentration of Al^{3+} ions, which also confirms the role of these anions in reducing Al toxicity. This exclusion mechanism has been reported in several plant families including Poaceae (e.g., wheat, sorghum, maize, and rye; Ryan et al. 1995; Pellet et al. 1995; Li et al. 2000; Magalhaes 2002; Ma et al. 2014), Araceae (e.g., taro; Ma and Miyasaka 1998), Polygonaceae (e.g., buckwheat; Ma et al. 1997a, b), Brassicaceae (e.g., *Arabidopsis*; Hoekenga et al. 2003), and Fabaceae (e.g., soybean; Yang et al. 2000).

2.5.2.1 Al-Activated Malate Efflux

Malate efflux is the most documented Al resistance mechanism in plants (Delhaize et al. 1993), and the gene controlling this mechanism is *ALMT* which encodes an *Al*-activated *m*alate *t*ransporter. This was the first Al-resistant gene isolated from plants and first member of the *ALMT* gene family. Sasaki et al. (2004) isolated and characterized *ALMT1* gene (*TaALMT1*; *Triticum aestivum*) by cDNA subtractive hybridization using near-isogenic wheat lines ET8 (resistant) and ES8 (sensitive). They also found expression of *TaALMT1* was higher in root tips of ET8 than of ES8 and its expression co-segregated with Al resistance in a segregating population. Heterologous expression of *TaALMT1* in *Xenopus laevis* oocytes, tobacco

suspension cells, barley, wheat, and *Arabidopsis* also increases malate efflux and Al tolerance (Sasaki et al. 2004; Delhaize et al. 2004; Pereira et al. 2010; Ryan et al. 2011). *TaALMT1* is expressed constitutively within root apices and maps to chromosome 4DL in the region of a major quantitative trait locus (QTL) for Al resistance (Sasaki et al. 2004; Raman et al. 2005) that encodes a hydrophobic protein with five to seven predicted membrane-spanning domains that facilitate malate efflux (Ryan et al. 2011).

The coding region of *TaALMT1* has six single-nucleotide polymorphisms (SNPs) that differ by six nucleotides encoding for different amino acids in the predicted proteins between Al-resistant and Al-sensitive wheat near-isogenic lines. Further, the promoter region upstream of *TaALMT1* is highly polymorphic between genotypes. Six different types were identified in the promoter region which differed from one another by the number and pattern of repeated blocks of sequence. A general relationship exists between the number of repeats and the level of the *TaALMT1* expression and Al resistance (Sasaki et al. 2006). Recently, Chen et al. (2013) also found that an increasing number of *cis*-acting elements in the promoter region of the *HIALMT1* gene in *Holcus lanatus* are responsible for enhancing the expression of *HIALMT1* and secretion of malate.

The *TaALMT1* gene encodes a membrane protein, which is constitutively expressed in the root apices of the Al^{3+}-tolerant line at greater levels than in the near-isogenic Al^{3+}-sensitive line (Sasaki et al. 2004), and this protein belongs to ALMT protein family. Ligaba et al. (2009) and Furuichi et al. (2010) suggested that TaALMT1 transport activity is regulated by PKC-mediated phosphorylation, and by the site-directed mutagenesis analysis they concluded that S384 is an essential residue regulating TaALMT1 activity via direct protein phosphorylation. Recently, it has been also identified that the N-domain of TaALMT1 mediates ion transport even in the absence of the C-domain (Ligaba et al. 2013). Other homologues of *TaALMT1* such as *AtALMT1* from *Arabidopsis thaliana* (Hoekenga et al. 2006), *BnALMT1* and *BnALMT2* from rape (*Brassica napus*; Ligaba et al. 2006), and *ScALMT1* from rye (*Seceale cereale*; Li et al. 2000; Stass et al. 2008) have been identified that confer Al-activated malate efflux (Table 2.2).

2.5.2.2 Al-Activated Citrate Efflux

Citrate is another organic anion which is commonly released in response to Al toxicity from plant roots. Similar to malate efflux, citrate efflux is also mediated by anion channel. But different from ALMT1, the transporters encoded belong to the multidrug and toxic compound extrusion (MATE) protein family. From this family, first Al-resistant genes *HvAACT1* and *SbMATE* were isolated through the map-based cloning of the major Al-tolerant loci from barley (Furukawa et al. 2007) and sorghum (Magalhaes et al. 2007), respectively. Later on, MATE orthologs which are citrate transporter have been identified from *Arabidopsis* (*AtMATE1*; Liu et al. 2009), wheat (*TaMATE*; Ryan et al. 2009), maize (*ZmMATE1*; Maron et al. 2010), rye (*ScMATE 2*; Yokosho et al. 2010), and rice (OsFRDL2;

Table 2.2 Al-resistant genes associated with organic anion efflux and their properties

Gene	Species	Location on chromosome	Promoter regulation	Protein function	Expression	References
TaALMT1	Wheat	4DL	Tandemly repeated elements	Malate transporter	Constitutive	Sasaki et al. (2004, 2006)
AtALMT1	*Arabidopsis*	1	*STOP 1*	Malate transporter	Induced by Al^{3+}	Hoekenga et al. (2006), Iuchi et al. (2007)
ScALMT1	Rye	7RS	Gene cluster	Malate transporter	Induced by Al^{3+}	Collins et al. (2008)
HvAACT1	Barley	4H	1-kb insertion in the 5′ UTR	Citrate transporter	Constitutive	Furukawa et al. (2007), Fujii et al. (2012)
SbMATE	Sorghum	3	Tourists-like MITEs	Citrate transporter	Induced by Al^{3+}	Magalhaes et al (2007)
AtMATE1	*Arabidopsis*	–	*STOP 1*	Citrate transporter	Induced by Al^{3+}	Liu et al. (2009)
ZmMATE1	Maize	6	Higher *MATE* copy number	Citrate transporter	Induced by Al^{3+}	Maron et al. (2010), Maron et al. (2013)
TaMATE	Wheat	4BL	Sukkula-like transposable element	Citrate transporter	Constitutive	Ryan et al. (2009), Delhaize et al. (2012), Tovkach et al. (2013)
BnALMT1	Brassica	–	NA	Malate transporter	Induced by Al^{3+}	Ligaba et al. (2006)
ScMATE2	Rye	3R	NA	Citrate transporter	Induced by Al^{3+}	Yokosho et al. (2010)

Yokosho et al. 2011). In a recent study, Zhou et al. (2013) demonstrated that increased expression of the *HvMATE* can also increase citrate efflux in wheat and barley and enhance Al tolerance in both cereal species. The predicted membrane structure for citrate transporters is highly conserved, a large intracellular loop (~100 amino acids) between 2 and 3 transmembrane domains. Amino acid sequence of the first half of the loop is highly conserved, and the rest of the loop may play a role in citrate binding and transmembrane transport.

Transposable elements are capable of altering *MATE* gene expression (Morgante et al. 2007; Delhaize et al. 2012), and there are several examples where they are implicated in enhancing Al resistance. The constitutively greater expression of *HvAACT1* in the root apices of Al-resistant barley is associated with a 1,023-bp insertion in the 50 UTR of *HvAACT1*, 4.6 kb upstream of the coding region (Fujii et al. 2012). This insertion acts as a promoter to enhance the level of *HvAACT1* expression. In addition, to increase overall transcript abundance, the insertion also alters the localization of *HvAACT1* expression in roots. The transposable element causes a mutation that provides an advantage on acid soils by altering the level and localization of gene expression. The 11.1-kb transposable element is inserted 25 bp upstream of the ATG start site and is thought to enhance *TaMATE* expression within root apices resulting in constitutive citrate efflux (Delhaize et al. 2012). In sorghum, tourist-like miniature inverted repeat transposable elements (MITEs) occur upstream of the *SbMATE* gene, and the number of these repeats is broadly correlated with the level of *SbMATE* expression (Magalhaes et al. 2007; see Table 2.2).

2.5.2.3 Other Mechanisms

To combat with oxidative damage induced by Al, Al resistance in plants relies on the antioxidant enzymes such as superoxide dismutase, peroxidase, catalase, ascorbate peroxidase, and glutathione reductase. The activity of SOD enzyme (which degrades superoxide anion into H_2O_2) was induced in maize under Al stress (Boscolo et al. 2003). Transgenically overexpressing Al-induced mitochondrial MnSOD in *Brassica napus* reduced the inhibition of root growth under Al toxicity. In addition, the accumulation of malondialdehyde (MDA), a product of lipid peroxidation, was lower in homozygous transgenic plants compared to wild-type (WT) plants, and the SOD activity was higher in homologous transgenic plants than in WT plants (Basu et al. 2001).

2.5.2.4 Al-Resistant Genes not Associated with Organic Anions

Some genes confer Al resistance by other organic acid effluxes. Several Al-induced genes have been reported from a range of plant species including tobacco (Ezaki et al. 1995, 1996), *Arabidopsis* (Richards et al. 1998), rye (Milla et al. 2002), and sugarcane (Watt 2003) in which some of the induced gene is well known as

antioxidant enzymes (e.g., glutathione S-transference, peroxidase and superoxide dismutase). Furthermore, other defense mechanisms such as in *AtBCB* (*Arabidopsis* blue copper-binding protein) helps to diminish oxidative damage by the lignin formation (Ezaki et al. 2005). Transgenic lines overexpressing the *AtBCB* gene showed constitutive lignin production in whole root and a lower deposition of MDA after Al stress. Al stress-induced lipid peroxides, such as H_2O_2 and various phenoxyl radicals, are consumed during lignin formation by electron transfer for establishing Al resistance. Ezaki et al. (2001) have also characterized genes *NtGD11* (tobacco GDP dissociation inhibitor gene), *parB* (tobacco glutathione S-transferase gene), and *NtPox* (tobacco peroxidase gene) involved in Al resistance mechanisms. Moreover, Sasaki et al. (2002) isolated a cDNA clone exclusively induced by Al^{3+} ions and predicted amino acid sequence exhibited homology to multidrug resistance (MDR) proteins that is known as a member of the ABC protein superfamily, named as *TaMDR1*, and involved in the calcium homeostasis which occurred at early stage of Al toxicity.

STOP1 (Sensitive to proton rhizo-toxicity 1) in *Arabidopsis* and *ART1* (Al resistance transcription factor 1) in rice were identified by mutant analysis and confirmed their role in coordinating the expression of Al-resistant genes and tolerance to low pH (Iuchi et al. 2007; Yamaji et al. 2009). *STOP1* and *ART1* share significant sequence similarity and appear to act as transcription factors to enhance the expression of a range of genes in Al-treated roots. Plant ABC transporters that have been functionally characterized are encoded by the two genes *ALS1* and *ALS3* (Larsen et al. 2007) and inferred that the proteins act to mobilize and sequester Al^{3+} ions within the plant to confer resistance. ALS1 is located at the tonoplast, and the gene is expressed primarily in root apices and the vascular system. By contrast, expression of *ALS3* is induced by Al^{3+} ions and the protein is primarily located at the plasma membrane of leaf hydathode cells, the phloem, and the root cortex. Additionally, *OsSTAR1*/*OsSTAR2* helps in modification of rice root cell wall composition, which could reduce Al binding and accumulation in the cell wall (Huang et al. 2009).

2.6 Future Directions

Plant system is complex and it cannot be understood by focusing on any one aspect of its highly interacting components. From the last decades, our understanding about the Al tolerance mechanisms has increased and some Al-resistant genes have also been isolated. However, most of the proposed mechanisms remain to be thoroughly deciphered. In order to do this, we need to combine a deep knowledge of Al interaction with plants with new high-throughput technologies that enable us to generate Al-resistant crops that better adapted to acid soil. Rather than a single gene or protein, we need to understand how the molecular components of a plant system interact with Al toxicity and their properties arise from those interactions.

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Chapter 3
Adaptive Mechanisms of Photosynthetic Apparatus to UV Radiation

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Abstract Adaptive mechanisms of higher plant photosynthetic apparatus to UV radiation are discussed in the review. Particular attention is paid to the various mechanisms that protect photosynthetic machinery from damage injury and the role of reactive oxygen species, phenolic compounds, and phytochrome system. The effects of the increased content of active form of phytochrome B and its content on photosynthetic activity and adaptation to stress are examined. A relation between the action of UV radiation on photosynthetic activity and the state of phytochromes and pro-/antioxidant balance is considered.

Keywords Adaptation • Photosynthesis • Phenolic compounds • Phytochrome system • Pigments • Reactive oxygen species • Rubisco • Ultraviolet radiation

Abbreviations

ATP	Adenosine triphosphate
Chl	Chlorophyll
D1, D2	Proteins of photosystem II reaction center
LPO	Lipid peroxidation
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
PAR	Photosynthetically active radiation
PhyA (PhyB)	Phytochrome A (B)
PSI	Photosystem I
PSII	Photosystem II
Q_A	Primary quinone acceptor of PSII
Q_B	Secondary quinone acceptor of PSII
RL	Red light
ROS	Reactive oxygen species
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SOD	Superoxide dismutase

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UV-B	Ultraviolet-B radiation
V_{cmax}	Maximum capacity of Rubisco carboxylation

3.1 Introduction

The ultraviolet region of electromagnetic solar radiation is the range of the wavelengths from 100 up to 400 nm. While passing through the atmosphere, UV radiation is absorbed by oxygen molecules and, to a great extent, by the ozone of the stratosphere. This leads to a decrease of intensity and changes in the spectral composition of UV radiation. The beams enter the Earth's surface starting from the wavelengths of 300 nm or in some regions 290 nm. The share of UV radiation makes up about 7 % from the total solar radiation energy, reaching the Earth's surface (Caldwell 1981), and depends on the thickness of the ozone layer (Mc Kenzik et al. 2003; Qing et al. 2004). There is also a clearly defined latitude and longitude gradient of the intensity of UV radiation on the planet (Shulgin 1973; Madronich et al. 1995). Three areas of UV radiation according to their effect on the living objects are specified in biology: UV-C (200–280 nm), UV-B (280–320 nm), UV-A (320–390 nm). UV-C, or rigid UV, is the most destructive for the living organisms, but it is not present in the solar spectrum, reaching the Earth's surface. As the wave length is increased and, consequently, quantum energy is decreased, the negative effect of UV radiation on the living organisms decreases.

Since UV-A and UV-B radiation reach the Earth's surface, a special emphasis is given to studying the biological effect of this region of UV radiation. It is known that soft ultraviolet (UV-A, 320–390 nm) has minimal negative effect as compared to the UV radiation of other wavelengths and can even affect as a stimulator on the plants (Caldwell 1981, 1983; Wellmann 1983; Layakumar et al. 2004).

UV radiation of the wavelengths of 280–320 nm (UV-B) makes up about 1.5 % from the total solar energy, reaching the Earth's surface (Frederick and Lubin 1988). UV radiation of the B area is absorbed by nucleic acids and chromophore protein groups and photosynthetic pigments. An increase of the level of decreasing UV-B radiation causes changes of photosynthetic characteristics in the plants. The presence of UV-A radiation and visible light along with UV-B in solar radiation is important to lower its negative effect on the plants and their photosynthetic apparatus (Caldwell and Flint 1994; Dolzhenko et al. 2010).

3.2 Dependence of Plant Photosynthesis on UV Radiation

Photosynthetic apparatus is one of the most stress-sensitive physiological systems (Berry and Bjorkman 1980). As for UV radiation a major impact site of UV radiation is the chloroplast, its damage leads to the impairment of the photosynthetic apparatus (Iwanzik et al. 1983; Bornman 1989; Vass 1997; Kolli et al. 1998; Lidon et al. 2012). Here, the integrity of the thylakoid membrane and hence chloroplast structure are likely more sensitive as compared with the photosynthetic activities (Lidon et al. 2012).

Irradiation of intact leaves and isolated chloroplasts with UV-B resulted in a decrease of the rates of light responses of photosynthesis (Basiouny et al. 1978; Brandle et al. 1977; Van et al. 1977; Noorudeen and Kulandaivelu 1982; Renger et al. 1991). Thus, after 60 min of irradiation of amaranth chloroplasts with UV-B (30 W m^{-2}), the rate of Hill reaction with potassium ferricyanide or oxidized dichlorophenolindophenol decreased by 85 % as compared to the control value (Noorudeen and Kulandaivelu 1982). By now it has been shown that UV-B damages photosystem 2 (PS II) significantly (Tyystjarvi 2008). Photosystem I turns out to be more resistant to radiation of this region of the wavelengths (Brandle et al. 1977; Van et al. 1977; Noorudeen and Kulandaivelu 1982; Nedunchezhian and Kulandaivelu 1993).

Different hypotheses of the damage mechanism of functioning PSII at UV-B radiation have been suggested. According to Melis et al. (1992), UV-B does not influence on the first charge separation between P_{680} and pheophytin. Inhibition of photoreduction of the primary quinone acceptor Q_A, plastoquinone photoreduction, and a decrease of the content of proteins in PSII RC have been shown. The other researchers (Khalilov and Tikhonov 1992) also supposed that there were several targets in PSII, having different sensitivities to the effect of UV radiation. At the same time manganese complex of the system of water photodestruction is damaged first. According to the data of Nedunchezhian and Kulandaivelu (1993), plastoquinone, the system of water decomposition, and primary quinone acceptor Q_A are not damaged and the place of UV-B radiation is the reaction center of PSII (Noorudeen and Kulandaivelu 1982). First of all the components of the PSII such as Q_A, Q_B, plastoquinone pool, D1 and D2 proteins, and the Mn_4CaO cluster (Rodrigues et al. 2006; Zsiros et al. 2006; Tyystjarvi 2008; Wei et al. 2011; Hou and Hou 2013) are damaged.

The proteins D1 and D2 of PSII RC play a great role in the sensitivity of PSII to UV-B radiation; their degradation occurs as the intensity of UV-B equaled to 1 μE m^{-2} s^{-1} (Jansen et al. 1996). In this case degradation of these proteins can be strongly facilitated in the presence of radiation in the visible spectral range. Facilitated turn of these proteins at UV-B + PAR radiation is connected with the stability of the proteins in the presence of only PAR (Babu et al. 1999). Degradation of D1 and D2 proteins, induced by UV-B, is usually (but not always) followed by the loss of PSII activity, which is characterized by a decrease in maximal quantum efficiency of PSII photochemistry, electron transport rate, and also photosynthesis.

The key effect is damaging in PSII Mn-containing cluster (Melis et al. 1992; Vass et al. 1996; Tyystjarvi 2008; Kreslavski et al. 2009; Wei et al. 2011; Hou and Hou 2013). It is just this damage that promotes destruction of D1 protein. Plastoquinone molecules (Q_A, Q_B, and plastoquinone pool) and the Tyr-Z and Tyr-D redox-activity tyrosine residues are sensitive to UV-B as well (Melis et al. 1992; Vass et al. 2005; Rodrigues et al. 2006).

UV radiation leads also to reductions in CO_2 assimilation rate. This can be due to reduction in the content of light-harvesting complexes, distortion of thylakoid membrane integrity, and inactivation of key enzyme of Calvin cycle, Rubisco, or changes in stomatal conductance (see review of Takeuchi et al. 2002). Several studies have also shown that reduction in CO_2 assimilation is caused by UV-induced changes in stomatal conductance (Jansen and van den Noort 2000).

On the other hand, low doses of UV-A and UV-B can increase the activity of PSII and O_2 evolution as well as intensify the synthesis of chlorophyll and carotenoids (Layakumar et al. 2004).

3.3 Photosynthetic Pigments

UV-B radiation has a great effect on the content and the ratio of pigments of the light-harvesting complexes of the photosystems of chloroplasts—carotenoids and chlorophylls *a* and *b*. Thus, at growing the plants at increased level of UV-B radiation, a decrease of photosynthetic rate and a decrease of the content of chlorophylls in the seedlings of colewort, oats, and soya beans were found. In resistant C_3 and C_4 species (ground nut, maize, sorghum), the concentration of chlorophyll did not change (Basiouny et al. 1978). According to the data of Teramura (1980), irradiation of soya seedlings with UV-B in combination with various PAR modes had a great effect on photosynthesis without changing the total chlorophyll concentration.

High rates of UV-B radiation along with low intensity of PAR led to a significant decrease of chlorophyll concentration in pea (Vu et al. 1981, 1982, 1983), beans, barley, and maize (Tevini et al. 1983). The value of the ratio of chlorophylls *a*/*b* decreased with an increase of UV-B radiation in soya bean, but increased in pea (Vu et al. 1983). The ratio *a*/*b* increased in beans, barley, radish, and maize that enabled us to conclude on the stronger inhibition of chlorophyll *b* biosynthesis as compared to the synthesis of chlorophyll *a* (Tevini et al. 1983). In the work of Basiouny et al. (1978), the absence of changes in the ratio *a*/*b* at a decrease of total content of these pigments in colewort, oats, and soya beans according to the authors testified either on the suppression equally of biosynthesis of both pigments or intensification of chlorophyllase products. Thus, the data on effect of UV-B radiation on the ratio of chlorophylls *a*/*b* are contradictory that is determined by the species differences in plants' responses. The content of carotenoids is less subjected to the effect of UV-B radiation; however, a decrease of them is also marked at irradiation Muzafarov et al. (1995).

A decrease of the pigments' content at UV irradiation along with great damage of the functioning of PSII results in a decrease of ATP synthesis and the rate of NADP reduction, acting indirectly on dark photosynthetic responses.

3.4 Effect of UV Radiation on the Activity of Carboxylase Enzymes

The content and the activity of Rubisco are known to change greatly at the effect of UV-B on many field crops (see review of Lidon et al. 2012). This may affect on V_{cmax} and as a result decrease photosynthesis (Lidon et al. 2012). UV radiation can affect directly by inactivating an enzyme or indirectly by lowering its synthesis. High level of UV-B radiation led to a decrease of Rubisco activity in the leaves of pea and soya beans by 40–60 % as compared to the control plants (Vu et al. 1983). A decrease of enzymatic activity was associated with a decrease of its content and not inactivation. A decrease of the content of soluble protein in the leaves at effect of UV radiation was found that enabled us to suppose this was a reflection of decreased content of Rubisco and PEP carboxylase, making accordingly up to 50 % (at C_3 types) and up to 10–15 % (at C_4 types). At high doses of UV-B radiation, a decrease of the activity of PEP carboxylase and a decrease of protein concentration by 15 % were observed (Vu et al. 1982).

The effect of UV radiation can lead to changes of the activity of an enzyme itself as well as to changes at the cell level that determines the character of response of a biological system to stress (Lyubimov 2003, 2010). Thus, at irradiation of highly purified Rubisco, a decrease of specific activity was observed, the peak of which was at 3 h upon UV effect. The peak of decreasing enzymatic activity with the same time parameter was recorded even in total protein extract. In these parts of both photoreactivation curves, the first type of the mechanism of UV effect appeared, which is destructive in the inhibition of the enzymatic activity. An increase of the effect while increasing the irradiation dose testifies this fact as well. The observed further return of specific activity of the purified enzyme to the control level could be, due to dynamics, by replacement of the damaged molecules.

Significantly different was the influence of UV radiation at the cell level. Activation of an enzyme in total protein extract started later, reaching maximum in 3–9 h upon minimum activity of the purified enzyme. Maximal effect was reached at irradiation of the minimal dose and as the dose was increased, the level of activity decreased. Accounting that absolute value of the effect of Rubisco activation in total protein extract cannot be interpreted only as a result of synthesis de novo, the author supposes that in response to the effect of UV radiation, one of the components of generalized adaptive syndrome (stress reaction) of phototrophic cell is synthesis of a compound (compounds) activating the key enzyme of CO_2 assimilation.

Along with direct effect of UV radiation on photosynthetic plant apparatus and a decrease of photosynthetic rate, due to a decrease of the activity of light and dark processes, significant changes occur at stomatal level.

3.5 Effect of UV Radiation on Stomata

Change of photosynthetic activity at UV irradiation can be associated, to a great extent, with changes of stomatal resistance to the flux of gases (Teramura 1983; Jansen and van den Noort 2000). For example, high level of UV radiation led to a small increase of the stomatal resistance of the leaves of bean, soya bean, and cucumber at a great decrease of total photosynthesis (Bennett 1981). Two-week radiation with relatively low UV-B also resulted in increased stomatal resistance in soya leaves (Teramura et al. 1980). On the other hand, it was shown that at a decrease of photosynthetic rate upon 4 h of UV-B irradiation on pea leaves, the stomatal resistance remained unchanged in this case (Brandle et al. 1977). Dennis et al. tested whether ambient levels of UV-B radiation decrease stomatal density and increase water-use efficiency in field-grown soya bean (Dennis et al. 2013). The authors concluded that photomorphogenic responses to UV-B had an influence on stomatal density and water-use efficiency in soya bean. Solar UV-B decreased stomatal density due to increased season-long WUE and decreased internal CO_2 concentration of the leaf.

At irradiation of cucumber plants with moderate doses of UV light, the stomatal resistance after 24 h increased by three times and remained high within 8–9 days. This level of UV-B radiation led to a small increase of stomatal resistance in radish only after 12 days of exposition (Teramura 1983). It follows from data available in literature that stomatal response on increased level of UV radiation can be a regulator of photosynthetic apparatus activity.

3.6 Role of Reactive Oxygen Species

The role of reactive oxygen species (ROS) in abiotic stress management has become a subject of considerable research interest, particularly since ROS have been reported to be involved in the processes leading to plant stress adaptation. One can call the maintenance of prooxidant/antioxidant balance, i.e., dynamic balance between the products of prooxidants in the organism and their utilization with antioxidant systems, as among the very important processes for plant resistance. As an effect of unfavorable factors, changes occur in this system, which are characterized as oxidative stress (Merzlyak 1989). In phototrophic tissues, oxidative stress is associated first of all with chloroplasts (Merzlyak 1989; Foyer et al. 1994; Schmitt et al. 2014). An increase of ROS products leads to activation of oxidative processes including lipid peroxidation (LPO) (Pradedova et al. 2011). LPO intensification may lead to change of the properties of lipid membrane matrix

and modification of metabolism of the whole cell; however, its level is restrained by antioxidative systems, including enzymes and low-molecular compounds. Thus, for example, in the work of Singh et al. (2012), they studied the influence of low and high fluence rates of UV-B on the growth, oxidative stress, and antioxidant system of two cyanobacteria. The levels of ROS such as superoxide radicals and hydrogen peroxide were significantly increased due to UV-B high light exposure which in turn accelerated LPO evaluated by malondialdehyde level and protein oxidation evaluated by reactive carbonyl groups. Activities of enzymatic antioxidants, such as superoxide dismutase, peroxidase, and glutathione-*S*-transferase, were increased by UV-B. Conversely, high light UV-B significantly reduced catalase activity. In contrast to this, low dose of UV-B did not influence on the growth of cyanobacteria as well as H_2O_2, malondialdehyde, and reactive carbonyl group contents.

In addition, a signal role in the development of general adaptive reaction of the plants is attributed now to fluctuations of prooxidative–antioxidative balance (Kugranova et al. 1999; Sun and Oberley 1996) that modulate the expression of various genes, including those encoding antioxidant enzymes and modulators of H_2O_2 production (Gechev et al. 2002; Suzuki et al. 1997). Many photomorphogenic UV-B responses have been shown to be mediated through UV resistance locus (UVR8) protein (for review, see Nawkar et al. 2013). A lot of works are devoted to elucidation of the role of this protein as a receptor of UV-B radiation. For example, in the work of Brown and Jenkins (2008), the availability of UV-B-specific signaling component that regulated UV-protective responses was shown. Using the uvr8 mutant, genetically distinct UVR8-dependent and UVR8-independent pathways were identified that stimulate different sets of genes in mature *Arabidopsis* leaf tissue. It has been shown that high UV-B fluence rate responses are mediated by DNA damage signaling by producing excess ROS and do not involve specific receptors (Kim et al. 1998; Frohnmeyer and Staiger 2003). Moreover, it has been shown that low UV-B irradiance responses involve specific receptors and appear to be photoregulatory rather than resulting from damage induced by stress. (Frohnmeyer and Staiger 2003; Brown and Jenkins 2008).

Recently, it was demonstrated that the UVR8 protein acts as a receptor for UV-B radiation (Rizzini et al. 2011; Christie et al. 2012; Wu et al. 2012). Low levels of UV-B exposure initiate signaling through UVR8 and induce secondary metabolite genes which are involved in protection against UV radiation, while higher dosages are considered as damaging to plants (Nawkar et al. 2013).

3.7 Protective Role of Phenolic Compounds

UV radiation not only damages different target molecules and the systems of photosynthetic apparatus but simulates many protection systems (Solovchenko and Merzlyak 2008; Katerova et al. 2012). Low-molecular compounds absorb UV, the synthesis of which can be amplified, and antioxidant system relates to them (Bharti and Khurana 1997; Häder et al. 2003; Strid et al. 2008; Kotilainen et al. 2007).

The role of some secondary metabolites such as anthocyanins is still under question (Hada et al. 2003; Katerova et al. 2012); most authors claim that the production of these compounds (mainly flavonoids and some UV-B absorbing metabolites) in plants subjected to low UV-B doses is a major part of the complex plant defense system (Solovchenko and Schmitz-Eiberger 2003; Jansen et al. 2008; Saewan and Jimtaisong 2013). Thus, Suleman et al. (2014) made conclusion that the protective response of *C. lancifolius* to UV-B stress suggested multiple mechanisms which were increased PAL enzyme activity, increased flavonoid production, and reduced LPO.

The study of 70 plant species showed that 90 % of UV radiation entering on the leaf surface is absorbed by the epidermis (Robberecht and Caldwell 1978). In epidermal cells, flavonols' glycosides, absorbing UV beams, are accumulated. It has been determined that absorption of UV light with epidermal layers at low and moderate levels of radiation is proportional to the content of flavonols, the major among them is kaempferol-3-galactosyl-7-rhamnoside (Flint et al. 1985; Shimazaki et al. 1988). The study of the influence of UV radiation on physiological processes in plants and the products of phenolic compounds enabled us to estimate possible protection of flavonoid system from damages of UV radiation, exceeding the natural level. UV radiation has usually an effect on the level of flavonoids by activation of key enzymes of phenylpropanoid pathway of phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) (Solovchenko and Merzlyak 2008).

A protective role of flavonoids shows itself in protection of DNA due to formation of nucleotide dimers (Stapleton and Wabot 1994). Upon short-term irradiation with UV-C and UV-B, a decrease of the damages of DNA in maize plants was observed, having flavonoids, as opposite to the plants where these compounds were absent. A key enzyme of DNA reparation is DNA photolyase, its synthesis is induced by UV. It is supposed that the activity of an enzyme is inversely correlated to the level of flavonoids' accumulation.

In the studies of plant responses to the effect of UV radiation, a concept on protective action of phenolic compounds is supported by the experiments on mutants *Arabidopsis thaliana* Ler. Thus, several mutant forms of *Arabidopsis thaliana* were obtained. They were used to characterize physiological processes making protection of plants from UV stress.

In particular, the mutants tt4 and tt5 are characterized by the absence or deficiency of flavonoids. Tt4 is the mutant on the gene chalcone synthase, tt5 is the mutant on the gene chalcone isomerase. Chalcone synthase and chalcone isomerase are major enzymes of flavonoid metabolism pathway. In its turn, the mutants tt4 are characterized by the higher content of hydroxycinnamic acids and their ethers. The pathway of biosynthesis for these monocyclic phenol ethers acids is the derivative from the main phenylpropanoid pathway. These compounds, both as flavonoids, are accumulated in the vacuoles of epidermal cells. Recently the role of UV absorbents is given to the ethers of hydroxycinnamic acids.

The ethers of sinapic, ferulic, and caffeic acids are assigned by some of the researchers to the main monocyclic phenol absorbents. They are the main

representatives of the group of hydroxycinnamic acids. Their special feature is the ability to *cis–trans*-isometry. In natural conditions *trans*-forms are dominant in the plants. However, at UV radiation the equilibrium shifts to the formation of *cis*-form. This peculiarity of hydroxycinnamic acids has an important biological significance, since *cis*-forms stimulate the plants' growth, and *trans*-forms either do not affect on the growth or suppress it slightly. As at transition of *cis*-form into *trans*-form some energy is supplied, then *cis*-forms of hydroxycinnamic acids with higher response capability play an important role in metabolism of the plants.

All these compounds serve as a protection shield, absorbing UV. They also remove an excess of free radicals and active oxygen forms, protecting lipid bilayer and chlorophylls from damage (Solovchenko and Merzlyak 2008).

3.8 Light as a Factor of Photosynthetic Tolerance to UV Radiation

Elucidation of the mechanisms with the help of molecular targets in plant cells that are damaged by UV radiation and repaired, as well as the mechanisms of damage, inhibition, and restoration of photosynthetic apparatus activity, is important to estimate the ecophysiological role of UV-A and UV-B radiation in the plants' life (Strid et al. 2008; Teramura and Sullivan 1994; Greenberg et al. 1996; Jansen et al. 1996; Vass et al. 1996; Häder et al. 2003).

Along with UV-induced activation of antioxidant systems, increased content of phenolic compounds, and synthesis of UV-protective pigments, the visible light of low intensity can play a protective role, primarily in the blue spectral area and/or in UV-A (Han et al. 2001; Häder et al. 2003), as well as the red light acting via phytochrome (Joshi et al. 1991; Yue et al. 2000; Biswal et al. 2003). Thus, it has been shown, for example, that negative effect of UV on photosynthesis is stopped at radiation of the plants with the light of visible area of PAR (Sikora et al. 2003; Kreslavski et al. 2012a, 2013a, b). In intact cells of *Synechocystis*, simultaneous illumination with visible light and UV-B decreased the PSII activity to a lesser degree that it was possible to expect at independent illumination of every spectral area. A protective effect was marked at low intensity of the visible light (130 μE m^{-2} s^{-1}), but it was absent at the intensity of 1,340 μE m^{-2} s^{-1}.

First of all, weak light was required to restore lower PS2 activity; secondly, the light in the region of 320–450 nm was absorbed by photolyase enzyme, sufficient activity of which was important to prevent dimerization of pyrimidine bases that occurred at effect of UV-B and UV-C, leading to DNA damage (Bergo et al. 2003). An opinion has been also expressed that the red component of the spectrum in the visible area activates photosynthetic apparatus with accelerated formation of protochlorophyllide resulting in the lower loss of chlorophyll and other pigments, favoring in keeping the structure and the activity of the chloroplasts during the aging of leaves (Joshi et al. 1991; Biswal et al. 2003).

It should be stressed, however, that in most of the works, the authors studied the effect of UV radiation on plants for a long time interval or used multiple repeated irradiation of the plants with red light and UV for many days (Joshi et al. 1991; Lingakumar and Kulandaivelu 1993; Biswal et al. 2003). Such an approach is good and necessary to study the responses of plants to long-term change of the growth conditions (increased level of UV radiation) and to strengthen the effect of the red light. Nevertheless, to elucidate the mechanisms and ways of plant adaptation in response to effect of UV, great significance is given to the study of the character of plants' responses within short time, for example, several hours.

3.9 Role of Phytochrome System in Adaptive Processes

A protective role of orange-red region of the visible light from the damaging effect of UV radiation in plants has been studied to a lesser degree relative to the more short-wave radiation (Joshi et al. 1991; Lingakumar and Kulandaivelu 1993; Kobzar' et al. 1998; Kreslavski et al. 2004).

Several factors are important to consider to understand the mechanism of protective and adaptive effect of orange-red light to UV radiation: (1) what photoreceptor participates in transduction of the light signal and (2) the role of the light in primary resistance and reparation of the damages (repair upon photosynthetic apparatus damage) caused by UV radiation. In this connection, the literature considers a protective effect of the light which acts before UV radiation and the reactivating effect which acts after irradiation (Han et al. 2001). For example, photosynthesis can be restored with the light in UV-A/blue region (Han et al. 2001).

One of the key photoreceptors in orange-red spectral region, which can participate in the protection of photosynthetic apparatus from UV radiation, is the different types of phytochromes, in particular PhyB (Joshi et al. 1991; Lingakumar and Kulandaivelu 1993; Biswal et al. 1997; Kreslavski et al. 2004, 2013a, b). An evidence for a role of phytochromes in plant stress tolerance is explored and reviewed. Thus, it is advisable to study their characteristics in detail as well as to consider the participation of this photoreceptor in protective reactions of the light. Five types of Phy are identified, between them PhyA and PhyB are basic types. PhyA is the primary photoreceptor responsible for perceiving and mediating various responses to far-red light (FRL), whereas PhyB is the predominant phytochrome regulating responses to RL (Li et al. 1993; Kreslavski et al. 2012b).

The other spectral regions and, consequently, other photoreceptors can also be efficient in the induction of plant resistance. For example, possible formation of photoinduced resistance of tomato plants to the virus of tobacco mosaic under the influence of long-term supplementary illumination of the plants with blue light has been shown (Kuznetsova 2004). It is supposed to involve endogenic phytochromes in photoinduced resistance: abscisic acid and cytokinins. The other variant of the suggested photoreceptor, in addition to phytochrome, is the molecules of the

precursors of chlorophyll synthesis which have maximal absorption in the region of 620–640 nm. This is in agreement with the work of Cropat and Beck (1998), where a participation of the precursor of Chl *a* synthesis in light induction of the gene HSP70 responsible for the formation of the heat shock protein of 70 kDa in green microalga *Chlamydomonas* was supposed. This was supposed on the fact that maximum in the spectrum of effect of light induction of accumulation mRNA was observed at low light intensity, being at $\lambda_m = 600$ nm. One cannot deny the availability of such mechanism in increasing the resistance via the light activity of the gene responsible for the synthesis of the heat shock proteins and in the case of higher plants. However, evidences on possible participation of such photoreceptor like phytochrome, preferably PhyB, in protection of photosynthetic apparatus from the effect of various stresses are not many (Lingakumar and Kulandaivelu 1993; Thiele et al. 1999; Kreslavski et al. 2004, 2013a, b). Thus, protective effect of short-term radiation with RL ($\lambda_m = 612$ nm), presumably due to induction of formation of active form of PhyB, was found at the study on the effect of UV-B on the activity of photosynthetic apparatus in cotyledonous leaves of the seedlings of *Vigna sinensis* L. (Lingakumar and Kulandaivelu 1993). It is likely that not only the ratio of active form of PhyB to its total pool but the content of Phy is one of the factors which influence on the photosynthetic apparatus resistance and resistance of plants as a whole not only to UV radiation but to other stress factors like light of high intensity (Thiele et al. 1999; Biswal et al. 1997, 2003).

A number of our works studied how an increase of the ratio of active form of PhyB to its total could increase the resistance of photosynthetic apparatus to the damage induced by UV radiation (Kreslavski et al. 2004, 2012a, b, 2013a, b). In this case the content of active form of PhyB can be increased by short-term preirradiation of the plants with red light (RL) of low intensity under conditions when the pool of active form of PhyB was initially low. It has been found that irradiation of the plants or separate leaves of spinach, lettuce, and *Arabidopsis* with RL (620–660 nm) of low intensity in the end of the dark period leads to increased resistance of PS2 photosynthesis and photochemical activity to UV-A and UV-B radiation (Figs. 3.1 and 3.2) and lower degradation of photosynthetic pigments at their following exposition in the dark (Kreslavski et al. 2013a).

In so doing the effect of red light was removed partially or completely with PAR that testified the involvement of PhyB in the formation of the resistance mechanism to UV radiation.

One of the mechanisms of protective and adaptive effect of orange-red region of the visible light can be induction of antioxidative system. The induction of the activity of antioxidant enzymes and accumulation of low molecular weight antioxidants can be a result of the developing weak oxidative stress. Thus, Qi et al. (2000, 2002) showed that preirradiation of seeds with strong red light decreased oxidative stress induced by UV-B that was in line with reductions in malondialdehyde concentration as compared to the control. The activity of the antioxidant enzymes such as superoxide dismutase, peroxidases, and catalase as well as the concentration of low-molecular antioxidants such as ascorbic acid and UV-B absorbing pigments also increased in preirradiated samples. An increase in antioxidant

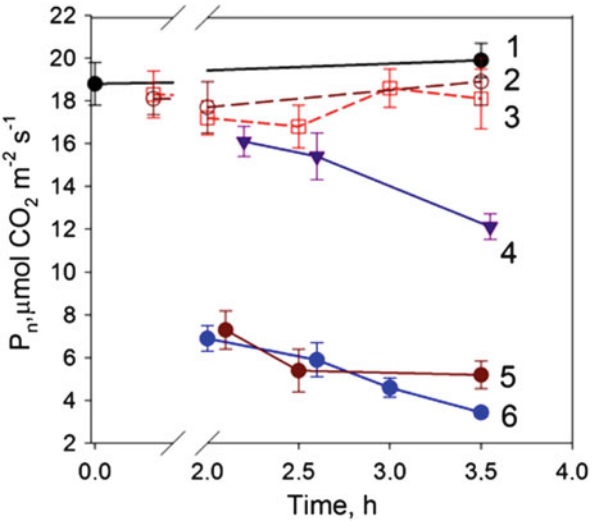

Fig. 3.1 Effect of different light illumination schemes: RL (*3*), FRL (*2*), UV-A radiation (*6*), as well as preillumination with RL (*4*) or RL → FRL (*5*) on the rate of photosynthesis (P_n) in *Arabidopsis* plants, kept in the dark after irradiation for 0, 0.5, and 1.5 h (*1*). The control sample without any illumination is shown as (*1*). The plants were preilluminated with RL (λ_{max}—660 nm, 10 µmol quanta m^{-2} s^{-1}) for 10 min or FRL (λ_{max}—730 nm, 2 W m^{-2}) for 10 min or both RL and FRL. Then a part of plants was illuminated with UV light (365 nm, 8 W m^{-2}). $n = 3$

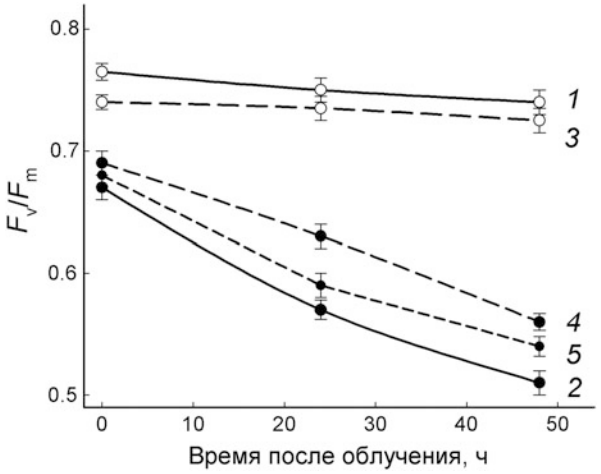

Fig. 3.2 Influence of different variants of radiation of the isolated spinach leaves on the activity of PS2 (the F_v/F_m ratio). The leaves were irradiated with UV-A (UV) for 40 min at light intensity of 15 W m^{-2} and preirradiated with low red light (RL) intensity (1.5 W m^{-2}) for 2 h. In the treatment designated RL–FRL, the leaves were illuminated with RL for 2 h (three 40 min exposures) and with FRL for 30 min (three 10 min exposures). After irradiations all leaves were exposed to the dark for 48 h. The values are average from three biological replications +SE. (*1*) The control, nonirradiated leaves, (*2*) UV, (*3*) RL, (*4*) RL → UV, (*5*) RL → FRL → UV.

activity at irradiation with RL can partly explain the protective and recovery effects of RL in plants.

The same data were obtained in experiments on separate leaves (Kreslavski et al. 2004, 2012a, b, 2013a, b). Preirradiation of leaves with red light resulted in an increase of total peroxidase and ascorbate peroxidase activity (Figs. 3.3 and 3.4) and the content of UV-absorbing pigments (Fig. 3.5) in the leaves, as well as to a

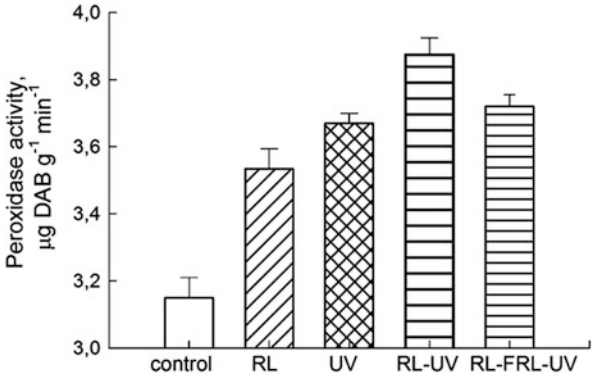

Fig. 3.3 Effect of preillumination with red light (RL) on peroxidase activity in leaves of seedlings irradiated with UV-A (UV) for 2 h and nonirradiated with a light (control). A part of seedlings was preilluminated with RL ($\lambda_{max} = 660$ nm, 2 W m^{-2}) for 10 min and then UV (RL-UV) or red light (RL) only. After exposure of seedlings to UV ($\lambda_{max} = 365$ nm, 10 W m^{-2}) or red light → UV (red light → far-red light → UV), they were exposed to the dark for 0.5 h. After exposure to RL only, the time of dark exposure was 2.5 h up to determination of the activity, which was calculated in relative units at equal protein content in the control and experiment. The values are the means of four biological replicates ±SE

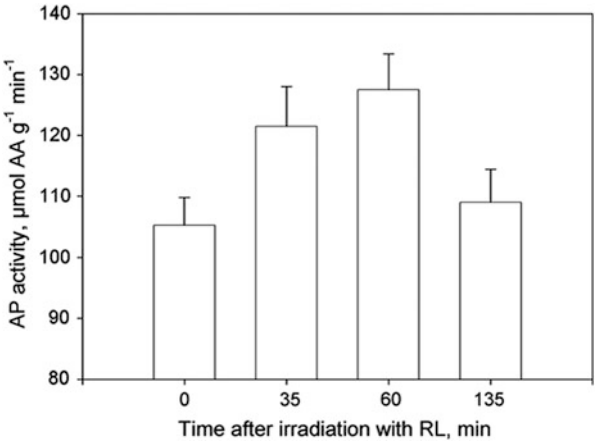

Fig. 3.4 Dependence of the activity of leaf ascorbate peroxidase (AP) on the time of dark exposure of lettuce seedlings after 10 min illumination with red light ($\lambda_{max} = 660$ nm, 2 W m^{-2}). The values are the means of three biological replicates ±SE

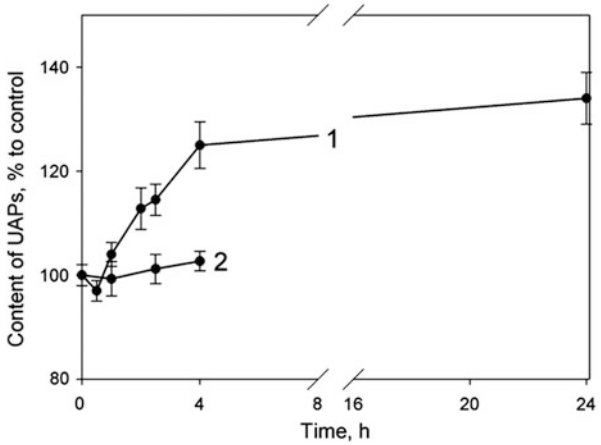

Fig. 3.5 Effect on UV-absorbing pigment contents in lettuce leaves by 10 min illumination of seedlings with RL (*1*) and RL and then FRL (*2*), followed by incubation in the dark. The content of the pigments in the nonirradiated leaves was set to 100 %. RL and FRL parameters: $\lambda_{max} = 660$ nm, 1 W m^{-2} for 10 min and 730 nm, 1.5 W m^{-2} for 10 min, respectively. The values are the means of three biological replicates ±SE

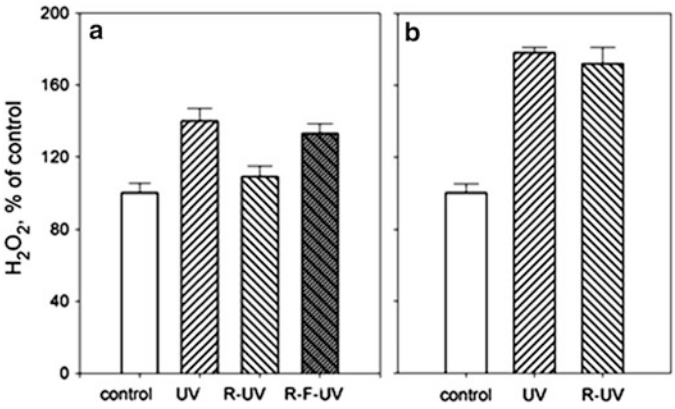

Fig. 3.6 Effect of UV-A (12 W m^{-2}, 2 h) and preillumination with RL (R) and RL → FRL(F) on the content of H_2O_2 defined in WT (**a**) and *hy2* mutant (**b**) of *Arabidopsis* immediately after exposures. The contents of H_2O_2 in WT control and *hy2* mutant were 0.5 ± 0.2 μmol and 0.54 ± 0.15 μmol per 1 g (f.m.), respectively. RL—$\lambda_m = 660$ nm, 2 W m^{-2}, 10 min; FRL—$\lambda_m = 730$ nm, 2 W m^{-2}, 10 min, $n = 3$

decrease of H_2O_2 pool as compared to the control leaves (Fig. 3.6). These effects of RL were partially or completely reversible with the following radiation with far-red light. Activation of antioxidant enzymes at irradiation of the plants with RL is known, but the mechanism in many ways is still unclear (Sharma 1976; Qi et al. 2000, 2002). Based on the data (Figs. 3.1–3.6), it was suggested that red light-induced resistance of photosynthetic apparatus to UV radiation by increasing

total peroxidase activity and the pool of UV-absorbing pigments, and a mediator in forming protective mechanisms at effect of UV radiation was an active form of phytochrome B which was likely to act via signal mediators like Ca^{2+} and transcription factors. Synthesis of different protective compounds including low-molecular antioxidants and UV-absorbing pigments was induced in this case. In other words we have the following sequence of increasing stress resistance of photosynthetic apparatus to UV radiation, the light of high intensity or heat (cold)-induced photoinhibition.

Red light (λ 660 nm) → Phytochrome—active form of phytochrome → (transit accumulation of transcription factors, Ca^{2+} H_2O_2) → increased activity of antioxidant enzymes and the pool of low-molecular antioxidants → increased stress resistance of photosynthetic apparatus (Kreslavski et al. 2012).

One of the possible mechanisms of positive effect of red light radiation can be, in a number of cases, induction in the leaves of weak oxidative stress that increases the activity of antioxidant enzymes and accumulation of low-molecular protective compounds (Kreslavski et al. 2012b). Besides, the formation of stressful proteins is also possible.

An increase in the resistance of photosynthetic apparatus to photoinhibition was also found in transgenic potato plants of superproducers PhyB (Thiele et al. 1999). In contrast, with a decrease in the content of PhyB, a decrease of stress resistance of photosynthetic apparatus should be expected. This work was started in our investigations on *Arabidopsis* plants (Kreslavski et al. 2013b). While studying *hy2* mutant *Arabidopsis* with low synthesis of chromophore of phytochrome B, a decreased resistance of PS2 as well as photosynthesis to UV-A as compared to the wild type of *Arabidopsis* was revealed. UV radiation decreased fluorescence of Chl *a* rather faster in the mutant *hy2* as compared to the wild type. Preirradiation of the *hy2* mutant with red light ($\lambda_m = 664$ nm) did not influence on the activity of PS2 and the level of H_2O_2 in the leaves illuminated with UV. Based on the experiments in which there was found a reversibility of the effects of short-term irradiation of the plants with RL with the following FRL, we supposed that in the formation of the mechanisms of higher resistance of *Arabidopsis* photosynthetic apparatus to UV radiation, an active form of phytochrome B and plants' antioxidative system took part; partially this might occur by induction of transcription activity of the genes of some transcription factors and antioxidant enzymes.

3.10 Conclusion

Investigation of effects of UV-B radiation on photosynthetic apparatus improves our understanding of the molecular background and physiological significance of plant response reactions upon action of ultraviolet radiation. The main interest is associated with estimation of primary targets of UV radiation and signal transduction at the cellular and leaf levels. Our view is mainly focused on the influence of UV-B(A) on plant photosynthesis, contents of photosynthetic pigments and

phenolic compounds, activity of carboxylase enzymes, and the role of light as a factor of photosynthetic tolerance to UV radiation. A special attention was paid to the role of phytochrome system in adaptive processes and link between targets of UV radiation and the processes of adaptation of photosynthetic apparatus occurring during and after exposure of plants to ultraviolet.

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Chapter 4
Signal Perception and Mechanism of Salt Toxicity/Tolerance in Photosynthetic Organisms: Cyanobacteria to Plants

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Abstract High salt concentration represents one of the most significant abiotic constraints, affecting all life forms including plants and cyanobacteria. Soil salinity curtails plant growth by way of osmotic, ionic and oxidative stresses resulting in multiple inhibitory effects on various physiological processes such as growth, photosynthesis, respiration and cellular metabolism. In order to combat high salinity, various adaptive strategies employed include ion homeostasis achieved by ion transport and compartmentalization of injurious ions, osmotic homeostasis by accumulation of compatible solutes/osmolytes and upregulation of antioxidant defence mechanism. The aforesaid processes are executed through SOS and MAPK signalling pathways leading to modulation of gene expression. Salt stress signal transduction pathways initiate through sensing extracellular Na^+ ions causing modification of constitutively expressed transcription factors. This modification is responsible for expression of early transcriptional activators such as CBF/DREB gene family which eventually activate stress tolerance effector genes such as osmolyte biosynthesis genes, detoxification enzymes, and chaperones. Various genes/cDNAs encoding proteins involved in these adaptive mechanisms have been isolated and identified. Bioinformatic predictions through docking revealed interaction of salt across the species at conserved domains and motifs as a possible mechanism for response of a particular protein under salt stress. In this chapter, major aspects of salt stress are reviewed with emphasis on its detrimental consequences and biochemical and molecular mechanisms of signal transduction in plants and cyanobacteria under high salinity.

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4.1 Introduction

Salinity is one of the most widespread abiotic constraints curtailing plant growth and productivity. The source of soil salinization may be primary (natural) or secondary (anthropogenic). Nature-driven salinity may predominantly be due to (i) intrusion of highly salinized water in coastal or continental regions, (ii) deposition of wind- or rain-borne oceanic salts and (iii) weathering of parental rocks. In contrast, agricultural practices, e.g. fertigation (fertilizer application through irrigation) and irrigation with poor drainage, are considered as the major contributor to secondary salinization especially in arid and semiarid regions where higher rates of evapotranspiration cause solutes from the irrigation water to accumulate and eventually reach levels that have an adverse affect on plant growth. Current estimates indicate that 6 % of the world's land and nearly 30 % of all irrigated lands are affected by salinity (UWP 2007).

The effect of salinity is widespread affecting all life forms including plants and cyanobacteria. Study suggests that salinity not only influences survival but also the distribution of cyanobacteria. While low salinity favours the presence of heterocystous cyanobacteria, high salinity mainly supports the survival of non-heterocystous genera (Srivastava et al. 2009). In plants also almost all aspects of developments including seed germination and vegetative and reproductive growth are adversely affected by high salt. On the basis of plant's response to salt, they can be divided into two groups: halophytes and glycophytes. Halophytes are native to the saline habitat equipped with various adaptive mechanisms to thrive under that condition. However, a majority of cultivated plants including horticultural and cereal crops are glycophytes, relatively susceptible to excessive salt concentration.

Under saline environment, organisms employ a variety of mechanisms to maintain their osmotic status and ionic balance. Typically, NaCl is the most abundant salt, and in most cases the negative impact of soil salinity on different physiological processes of an organism is attributed to the increase in Na^+ and Cl^- concentration. In this review, effort has been made to collate the effects of salt, particularly of NaCl on various aspects of growth, mechanisms of salt signalling and variety of strategies employed by plants to cope with salinity stress. In view of close homology between cyanobacteria and plants and their relevance in agriculture, it was decided to include them in the present discussion. In view of the above, we also describe cyanobacterial responses to salt stress and compare with plants.

4.2 Effects of Salinity

Salinity-induced physiological aberrations are mainly because of two reasons: (i) elevated salt causes osmotic effect by reducing soil water potential, influences water and nutrient uptake and leads to cellular dehydration; (ii) a continuous flow of

inorganic ions into the living cells occurs due to its high concentration in the surrounding environment, thus presenting ionic stress (Munns and Termaat 1986; Zhu 2001). 0.1 M of Na^+ concentration is cytotoxic as it directly affects specific biochemical and physiological processes (Serrano 1996). Cl^- accumulation 4–7 mg/g dry weight (DW) is critically toxic for Cl^--sensitive species and 15–50 mg/g DW for Cl^--tolerant species (Xu et al. 2000). Salt stress not only disturbs osmotic and ionic balance within the cell but also exerts some secondary effects (e.g. oxidative stress). Osmotic, ionic and oxidative stresses negatively affecting cell turgor, photosynthesis and transpiration, membrane constitution, and cellular metabolisms are reviewed under separate headings.

4.2.1 Cell Turgor and Water Relation

High salt content of rhizosphere instigates dramatic impact on osmotic relation between the cell interior and the surrounding medium resulting in reduced water uptake but enhanced efflux from the cell (Erdmann and Hagemann 2001). Water loss lowers the leaf water potential (ψ_w) and consequently the leaf turgor potential (ψ_p). Turgor loss is usually the earliest cellular response to water stress since even a decrease of 5–15 % of ψ_w can cause large decrease in plant ψ_p (Hsiao 1973; Turner 1979). Turgor reduction subsequently affects the turgor-dependent activities such as leaf expansion, root elongation and stomatal conductance which may correspond to reduction in transpiration, CO_2 assimilation, plant water uptake and ultimately plant growth. These perturbations are aggravated in environments with high transpirational demands. During the latter phase of water stress, plant always tries to escape from dehydration by reducing osmotic potential (ψ_s) and adjusting with osmolytes so as to maintain positive turgor despite low water potential associated with high rhizosphere salt concentration (Bolaños and Longstreth 1984). Maintaining cell turgor is crucial for proliferation of cyanobacteria which could be achieved by controlled accumulation of sucrose as its high concentration causes damaging effects (Ladas and Papageorgiou 2000). Thus, restoration of cell turgor or cell volume is one of the most important mechanisms to sustain growth and metabolism.

4.2.2 Nutrient Imbalance

Enhanced concentration of NaCl exhibits toxic effect on cell by promoting nutritional deficiencies. Nutritional disturbance may probably be due to (i) displacement of essential ions such as K^+ and Ca^{2+} by Na^+ from the cell surface, (ii) competitive Na^+ uptake through non-specific ion channels and transporters and (iii) altered ion transport or partitioning within the cell (Erdmann and Hagemann 2001; Grattan and Grieve 1999). Mounting evidence points towards the reduced availability and

uptake of K^+, Ca^{2+}, Mg^{2+}, PO_4^{3-} and NO_3^- in salt-stressed organisms exposed to high salinity. The antagonistic effect of excess Na^+ on Ca^{2+} and K^+ uptake resulted in increase in Na^+/Ca^{2+} and Na^+/K^+ ratio in salt-stressed plants and cyanobacterial species that adversely affect cellular metabolism and balanced ion relations.

4.2.3 Membranes

Biomembranes have always been the first to realize environmental fluctuations and respond accordingly by changing their lipid and protein composition. Membrane lipids such as sterols, phospholipids and fatty acids not only have profound role in regulating membrane fluidity and permeability but also responsible for the activity of membrane-associated channels/transporters (e.g. aquaporin) and enzymes (e.g. H^+ ATPase). There are many examples highlighting importance of elevated sterol to phospholipid ratio in lipid bilayer to deal with the hypersaline condition which causes membrane rigidity and reduces NaCl permeability (Wu et al. 2005; Kerkeb et al. 2001; Alvarez-Pizarro et al. 2009). Free sterols, particularly the more planer ones (e.g. cholesterol, campesterol), play pivotal role in controlling membrane permeability (Kerkeb et al. 2001; Douglas and Walker 1984), and their increased level has been reported in many salt-tolerant plant species following exposure to high salinity (Douglas and Walker 1984; Yahya et al. 1995).

A relative compositional change in membrane phospholipids has been witnessed in response to salinity. A salt-tolerant plant generally has an increased ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE) (Wu et al. 2005) while reverse is true for the sensitive plant (Norberg and Liljenberg 1991; Mansour et al. 2002). This is in accordance with the fact that PE in contrast to PC readily attains a non-lamellar-inverted hexagonal structure that may introduce hydrophilic water channels throughout the biological membrane (Caffrey 1985) altering its stability and permeability thereby making plants more vulnerable to salt stress.

Saturation level of membrane fatty acids generally increases with increasing salinity (Wu et al. 2005; Mansour et al. 2002). Though increased fatty acid saturation indicates reduced membrane fluidity and increased leakage, it cannot be considered as an absolute measure of salt sensitivity as changes in fatty acid saturation has been encountered in both halophytes and glycophytes. Alteration in membrane permeability owing to changes in fatty acid saturation was also observed in cyanobacteria. In some species such as *Synechocystis* (Huang et al. 2006) and *Anacystis nidulans* (Molitor et al. 1990), increased proportion of long-chain saturated fatty acids was observed. Whereas in an extreme halophyte alga *Dunaliella salina* when exposed to high salt, a considerable high ratio of unsaturated to saturated fatty acid was reported, thus indicating the protective role of unsaturated fatty acid in salt tolerance (Azachi et al. 2002). The role of unsaturation of fatty acids in membrane lipids in increasing resistance for photosynthetic machinery to salt-induced damage and in repair of Na^+/H^+ antiport system has also been acknowledged (Allakhverdiev et al. 2001).

4.2.4 Photosynthesis

Salinity evokes multiple inhibitory effects on photosynthesis which include alteration in photosynthetic pigments (Chl *a*, Chl *b* and carotenoids), photosystem efficiency, photophosphorylation and CO_2 fixation. In both plants and cyanobacteria, similar effects on chlorophyll content and carotenoids observed were, namely, reduction in chlorophyll contents in salt-sensitive (Singh and Kshatriya 2002; Srivastava et al. 2005) and increase in salt-tolerant species (Saleh 2012; Lu and Vonshak 1999). Thus, chlorophyll content could be used as a parameter for selection of tolerant varieties of crop plants (Eryilmaz 2007). In many plants such as *Zea mays*, *Carthamus tinctorius*, bean and *Paulownia imperialis*, salt-induced reduction in chlorophyll was due to weakening of protein–pigment–lipid complex or increased chlorophyllase enzyme activity (Reddy and Vora 1986; Turan et al. 2007; Rahdari et al. 2012). In contrast, increment in pigment content in some rice cultivar (Doganlar et al. 2010) and purslane (Rahdari et al. 2012) was also reported. These may be due to an increase in the number of chloroplast in the salt-stressed plant leaves (Chaum and Kirdmanee 2009).

Phycobiliproteins (PBPs) specific to cyanobacteria serve as the accessory light-harvesting antenna for PSII and PSI. Salt stress has inhibitory effect on PBPs' content and results in suppressed energy transfer from PBPs to PSII reaction centre (Lu and Vonshak 2002; Zhang et al. 2010).

Apart from the other photosynthetic pigments, carotenoids important for absorption, dissipation and transfer of light energy for photosynthesis show significant increase in salt-stressed plants (Borghesi et al. 2011) and cyanobacteria (Schubert et al. 1993). However, in some cases decreased carotenoids were also observed and correlated with decreased expression of carotenoid biosynthetic genes (Babu et al. 2011) and increased salt sensitivity.

The effect of salinity on photosynthetic electron transport and PSII activity remains a matter of debate in both cyanobacteria and higher plants. Numerous studies have reported severe impairment in PSII activity (Bongi and Loreto 1989; Everard et al. 1994; Rai et al. 2014) while some others indicated that photosynthetic electron transport is relatively insensitive to salt (Jeanjean et al. 1993; Abadia et al. 1999). The reduced PSII activity was supposedly due to the inhibition in electron transport from Q_A to Q_B at the acceptor side (Jafarinia and Shariati 2012) and oxygen-evolving complex at donor side of PSII (Lu and Vonshak 2002). Inhibition in synthesis of D1 protein of PSII (Allakhverdiev et al. 2002) in cyanobacteria and dissociation of 23 kDa polypeptide extrinsically bound to PSII in higher plant (Murata et al. 1992) could also be a cause of salinity-induced damage to PSII. Many studies indicate that salinity blocks PSII but enhances PSI activity (Zhang et al. 2010; Stepien and Johnson 2009) probably promoting cyclic electron flow through PSI.

Furthermore, salt-induced inhibition in photosynthesis partly attributes to stomatal closure that reduces stomatal conductance and partly due to non-stomatal factors involving direct effect of salt ions (Na^+ and Cl^-) on PSII-supported electron

transport and photophosphorylation activity. In *Lycium barbarum*, initial photosynthesis inhibition was attributed to temporary stomatal limitation whereas non-stomatal limitation contributes to reduction in photosynthesis during prolonged salt exposure (Hui et al. 2004).

Dark reaction of photosynthesis (CO_2 fixation) is also equally affected by high salinity. In *Sesbania*, salt stress enhances the oxygenase activity while curtails carboxylase activity of Rubisco (Sivakumar et al. 2000). Similar results were also observed in the cyanobacterium *Anabaena doliolum* (Srivastava et al. 2008). In *Sorghum vulgare* leaves, PEPC catalysing the first step of CO_2 assimilation in C_4 plants significantly increased during salt stress (García-Mauriño et al. 2003).

4.2.5 Cellular Metabolism

Cellular respiration is one of the common phenomena enhanced in both plants (Livne and Levin 1967; Begcy et al. 2011) and cyanobacteria (Molitor et al. 1990; Rai et al. 2014; Jeanjean et al. 1993; Srivastava et al. 2008) when exposed to salt stress. There is a line of evidence supporting the above fact, and this has been linked in some way with higher energy requirement of salt-affected cells in order to maintain turgor, ion homeostasis and production of more osmolytes. However, in some species decreased respiration was also noticed (Flowers 1972). The most dramatic effect of salinity was reported on protein synthesis and nitrogen metabolism. In general, protein synthesis is severely affected by high salt concentration due to inhibition of enzyme activity (Flowers 1972; Greenway and Osmond 1972). Hall and Flowers (1973) observed that microsomal amino acid incorporation fraction from halophilic *Suaeda maritima* is equally sensitive to added salt as salt-sensitive plants. This presumably indicates that salt tolerance in halophytes is related to the spatial separation of salt from cytoplasmic components either by salt exclusion or sequestration in vacuole that eventually lowers cytoplasmic Na^+ concentration.

Alteration in nitrate absorption and nitrogen metabolism is also reported in hypersaline condition (Mansour 2000), and the resulting nitrate deficiency may correspond to the reduction in nitrate reductase activity as in the case of *Cucumis sativus* (Sacala et al. 2008). NaCl-induced reduction in ammonia and nitrate has also been reported in a halophyte *Arthrocnemum fruticosum* (Eddin and Doddema 1986). Plant's response to salt not only varies with crop varieties or cultivars but also with the age. For example, in rice older leaves are reported to accumulate higher nitrate content than the younger ones probably because of an upregulated nitrate transporter OsNRT1;2 (Wang et al. 2012). Since many cyanobacterial species are endowed with the N_2-fixing ability, it is relevant to study the effect of high salt on N_2 fixation in general and nitrogenase in particular. Though Na^+ has been recognized to be essential for nitrogenase (Thomas and Apte 1984) and nitrate/nitrite reductase activities (Brownell and Nicholas 1967), its excess is inhibitory for N_2 fixation in many cyanobacteria (Page-Sharp et al. 1998; Fernandes

et al. 1993). However, the inhibition of nitrogenase activity is not universal as many inhabitants of salty estuaries and Baltic Sea such as *Nodularia*, *Anabaenopsis* and *Anabaena* sp. displayed no significant difference in nitrogenase activity when exposed to high salt concentration (Moisnder et al. 2002). Further, the deleterious effect of salinity on N_2 fixation was found to be a consequence of ionic component rather than the osmotic component of salt since nitrogenase activity was found to be completely insensitive to osmotic stress (Fernandes et al. 1993).

The most striking difference was observed in case of ice plant *Mesembryanthemum crystallinum* displaying metabolic shift from C3 to CAM when subjected to water deficit or saline condition. This metabolic shift requires accumulation of numerous enzymes such as phosphoenolpyruvate carboxylase (PVPC), pyruvate orthophosphate dikinase and NADP-malic enzyme (Cushman 2001). CAM metabolism enables plants to improve water-use efficiency, thus having a competitive advantage in day environment.

4.2.6 Growth and Development

Salinity-induced reduction in plant growth and development depends upon many factors including the species, genotype, plant age, ionic strength and composition of the salinizing solution and the organ in question. Major effects of salinity on crop plants can be described by two-phase model proposed by Munns et al. (1995)—phase 1 includes osmotic effect of salt which immediately reduces plant water uptake resulting in significant reduction in shoot growth whereas phase 2 corresponds to the salt-specific or ion excess effect which is due to the penetration of ions in transpiration stream resulting in death of transpiring leaves and reduction in the total photosynthetic leaf area. Thus, in a situation of reduced supply of photosynthate to the plant, the overall carbon balance gets affected thus reducing plant's growth further.

Shoots growth is generally more sensitive to salt stress because elevated salinity leads to reduction in leaf area ratio which in turn decreases water-use efficiency of plants, enabling them to conserve soil moisture and prevent salt build-up in soil (Munns and Tester 2008). Whereas reduced uptake of important mineral nutrients, such as K^+ and Ca^{2+}, are largely responsible for root growth suppression, particularly root tip expansion (Larcher 1980). Reduction in plant growth as a result of salt stress has been reported in several plant species, but greater inhibition was observed in different tolerant genotypes relative to sensitive ones suggesting growth reduction possibly promotes salt tolerance enabling the tolerant plants to save energy for the maintenance of the processes (Mansour et al. 2005).

4.3 Salt Adaptation Mechanisms

Several investigators have demonstrated salt tolerance mechanisms based on factors such as ion accumulation, ion exclusion, accumulation of toxic ions like Na^+ in older leaves, compatible solute production and toxic radical scavenging. Like plants, cyanobacteria possess similar mechanisms for salt tolerance which include Na^+ pumps for active Na^+ extrusion, transport systems for K^+ and osmotically active organic molecules and enzymes creditworthy for their synthesis and qualitative and quantitative modifications of metabolic pathways. The common salt stress coping strategies adopted by these photoautotrophs have been reviewed here.

4.3.1 Intracellular Ion Homeostatic Processes

Compartmentalization of Na^+ and Cl^- into the vacuole, organized Na^+ influx and homeostasis of K^+, Ca^{2+}, NO_3^- and Pi are the phenomenon involved in intracellular homeostasis (Tester and Davenport 2003; Zhu 2003). Plant cell can tolerate sodium ion concentration less than 100 mM, but the basal activity of the cell gets interrupted when the cytosolic Na^+ concentrations increase above 100 mM (Serrano et al. 1999). There are mainly three mechanisms involved in the prevention of excessive Na^+ ion accumulation in cytosol which involves restriction of Na^+ with selective ion uptake, storage of Na^+ in vacuole and exportation of Na^+ back to the apoplastic space (Zhu 2001).

4.3.1.1 Na^+ Influx/Efflux and Limitation of K^+ Loss

Na^+ and K^+ have good similarities between their physicochemical properties and lead to Na^+ competition at transport sites for K^+ entry into the symplast which may result in K^+ deficiency and inhibition of metabolic processes that essentially depend on K^+. Hence, plants tolerance to salt stress strongly depends on the status of their cytosolic K^+–Na^+ ratio which is further promoted by the cooperative action of transport systems located at plasma and vacuolar membranes and probably involves K^+ and Na^+ selective and non-selective pathways (Maathuis and Amtmann 1999). Under normal physiological conditions, cell tries to maintain relatively high K^+ (100–200 mM) and low Na^+ concentrations (1–10 mM) (Binzel et al. 1988).

Plants can deal with external K^+ concentrations ranging from low μM to tens of mM. In general, cellular role of K^+ is to act as (i) counterion for the large excess of negative charge on proteins and nucleic acids hence charge balancing in the cytoplasm; (ii) activator of crucial enzymatic reactions; (iii) endurer of non-lignified plant cells with structural rigidity by contributing to the osmotic pressure of the vacuole and cell turgor. In case of barley roots, it has been reported that the magnitude of the K^+ efflux induced by salt inversely correlates with the

productivity of 62 out of 69 cultivars contrasting in their sensitivity to salt stress (Chen et al. 2007). Unlike K^+, Na^+ as a macronutrient is only required for the translocation of pyruvate across the chloroplast envelope in some C_4 species (Maathuis and Amtmann 1999). Active transport of both the ions occurs via the action of the K^+–Na^+ ATPase that moves K^+ into the cell and extrudes Na^+. Ion transport proteins in the membranes involve three classes of transport proteins, such as 'pumps' with turnover rate of around 10^2 per second fuelled by metabolic energy and able to transport substrates against an electrochemical gradient, 'carriers' with turnover rate of around 10^2–10^3 per second that undergo specific conformational changes during substrate transport are energized via coupling to an electrochemical gradient and 'channels' with turnover rate of around 10^6–10^8 per second catalyse the rapid 'downhill' dissipation of transmembrane ionic gradients and are under control of membrane potential (Maathuis and Amtmann 1999). Role played by K^+ and non-selective cation channels (NSCCs) towards salinity stress is to induce Na^+/K^+ exchange which may be tissue and species specific. For example, in the pea mesophyll membrane, voltage-independent cation channel (VICs) arbitrates both Na^+ influx and K^+ efflux (Shabala et al. 2007). High-affinity K^+ with low-affinity Na^+ channels include inward-rectifying K^+ channels (KIRCs) like AKT1, K^+-outward-rectifying channels (KORCs) and the KUP/HAK gene family of K^+/H^+ symporters (Maathuis and Amtmann 1999; Blumwald et al. 2000; Schachtman 2000). The high-affinity K^+ transporter (HKT1), low-affinity cation transporter (LCT1) and NSCCs are among the transport systems that mediate Na^+-specific cellular influx (Zhu 2003). NORC (non-selective outward-rectifying conductance) does not discriminate between cations and is activated by increased cytosolic Ca^{2+} concentrations (Wegner and De Boer 1997). Plasma membrane H^+-ATPase restricts salt-induced membrane depolarization and its related K^+ efflux. Also, it fuels the Na^+/H^+ antiport for energy-dependent Na^+ efflux further improving the cytosolic Na^+/K^+ ratio. Salt-tolerant genotypes of barley possess intrinsically higher plasma membrane H^+ pump activity, despite having the same level of H^+-ATPase expression (Chen et al. 2007).

Cyanobacteria do not accumulate Na^+ but extrude with the help of Na^+ pumps with energy expenditure and maintain turgor with accumulation of K^+ which lowers later with increase in the level of osmotic solutes. In *Synechocystis* PCC 6803, *Nodularia harveyana and Aphanothece halophytica*, salt tolerance mechanism involved maintenance of a high K^+–Na^+ ratio (Warr et al. 1985).

4.3.1.2 Ion Compartmentalization

Due to the disturbance in ion homeostasis, uptake of ions and their compartmentalization are essential for growth under saline condition which occurs at cellular as well as whole plant level. Plant compartmentalizes Na^+ and Cl^- in the vacuole or in different tissues for facilitating metabolic function, which is essential to minimize cytotoxicity. Na^+/H^+ antiporter in tonoplast plays a key role in Na^+ transport from cytosol to vacuole under salt stress. The activity of such antiporter is controlled by

electrochemical H^+ gradient across the tonoplast generated by V-H^+ATPase and vacuolar type pyrophosphatase (V-H^+PPase). V-ATPase activity is found to be higher than V-H^+PPase as former is required for energizing the tonoplast for ion uptake into the vacuole while the latter plays minor role as supported by the findings of Wang et al. (2001) on halophyte *Suaeda salsa*. Therefore, both V-H^+ATPase and the tonoplast Na^+/H^+ antiporter play significant role in Na^+ compartmentalization. Moreover, in the vacuole, NaCl helps in maintaining osmotic potential hence driving water under salt stress. Salt-sensitive plants involve exclusion of Na^+ across plasma membrane while salt-tolerant plants prefer to accumulate Na^+ in the vacuole (Munns and Tester 2008). Exception to this concept represented by *Thellungiella halophila*, a halophyte, has potential of a good excluder (Gong et al. 2005) whereas some glycophytes can accumulate salt to different degrees on the basis of their capacity to interchange Na^+ for K^+. In a study, concentration of Na^+ and Cl^- was analysed via X-ray microanalysis within mature root cortical cells of *Suaeda maritima* L. Dum. grown in 200 mM NaCl, and it was found to be fourfold higher in vacuoles as compared to cytoplasm or cell wall (Hajibagheri and Flowers 1989). Among the tissues, Na^+ accumulation was found to be highest in the endodermis, followed by those in the exodermis, and stellar tissues in *Salicornia europaea* under salt stress (Lv et al. 2012). In addition, Ferreira et al. (2001) found the highest accumulation of Na^+ and Cl^- in guava leaves followed by the roots, while K^+ and Mg^{2+} level decreased in leaves and the Ca^{2+} showed inverse relationship with Na^+ in the roots. Also Cl^- exclusion is an important mechanism in providing salt tolerance among legumes (Teakle and Tyerman 2010).

4.3.2 Osmotic Homeostasis

Osmotically active compounds known as osmolytes such as proline, glycine betaine, soluble sugars, free amino acids, and polyamines are responsible for osmotic adjustment in plants subjected to salt stress. These compounds show minimal affect on pH or ionic balance of the cytosol or luminal compartments of organelles. They not only raise osmotic pressure in the cytoplasm but act as low molecular weight chaperons by replacing water at the surface of proteins or membranes (Hasegawa and Bressan 2000). An early response to salt stress is the accumulation of proline which balances the water potential of the cytosol with the apoplast and vacuolar lumen hence helps in turgor maintenance of cells. The glutamate and ornithine pathways are the proline biosynthetic pathways in higher plants. Under salt stress, the former is responsible for proline biosynthesis (up to 80 % of amino acid pool under stress as compared to only 5 % under normal condition) as evidenced with the accumulation of pyrroline-5-carboxylate synthetase (P5CS) enzyme and P5C reductase (P5CR) in the chloroplast while the latter is involved in seedling development (Székely et al. 2008; Huang et al. 2013). Proline under salt stress not only acts as osmoprotectant but protects cellular macromolecules, scavenges free radicals and recycles $NADPH^+$ H^+ via its synthesis from the glutamate pathway and in

redox signalling in all plants, including algae (Hare and Cress 1997). By restoring the pool of the terminal electron acceptor of the photosynthetic electron transport chain, proline may provide protection against photo-inhibition under stress (Szabados and Savoure 2009).

Glycine betaine, trigonelline, stachydrine and homostachydrine are aliphatic quaternary ammonium compounds reported to be accumulated under salt stress to serve as intercellular osmoticum. Increased accumulation of glycine betaine in chloroplasts with an increase in the activity of two enzymes, choline monooxygenase and betaine aldehyde dehydrogenase, therein occurs in response to salt stress. Some roles played by glycine betaine under salt stress include protection of membrane and macromolecules, promoting transcription and replication, which might accelerate protein synthesis de novo during recovery from stress. Even at low accumulation level betaine is compartmentalized at certain sites within cells to provide substantive protection against salt stress (Matoh et al. 1987).

The non-protein amino acid γ-aminobutyric acid (GABA) also gets accumulated with increased activity of enzyme involved in GABA metabolism under high salt stress (Renault et al. 2010). Carbon–nitrogen balance and ROS scavenging have been associated with GABA metabolism (Liu et al. 2011).

Polyamines the positively charged small aliphatic molecules in cellular pH mostly include putrescine, spermidine and spermine that help in protecting membrane and alleviating oxidative stress (Hussain et al. 2011). Plants deficient in spermine synthase are hypersensitive to salinity (Yamaguchi et al. 2006).

Salt stress leads to hydrolysis of starch by the β-amolytic pathway thence accumulation of soluble sugars in leaves (Kempa et al. 2008). Soluble sugars like sucrose, trehalose, etc. are involved in several metabolic events and behave as molecular signals regulating different genes involved in photosynthesis, sucrose metabolism and osmolyte synthesis.

Under high salt, these soluble sugars not only act as osmoregulators (Siringam et al. 2012) but prevent protein denaturation by interacting with proteins and membranes through hydrogen bonding. The other prominent roles of soluble sugars are in vitrification, which is the formation of a biological glass in the cytoplasm of dehydrated cells, hence helps in hindering diffusion of reactive compounds in the cell, decreases molecular movements and maintains structural and functional integrity of macromolecules. They also help in chelating Na^+ with starch granules, hence facilitating detoxification (Kanai et al. 2007). In *Synechocystis* sp. PCC 6803, sucrose has been found to transduce a specific signalling pathway in response to salinity at early phase of stress (Desplats et al. 2005). Chemically inert osmolyte trehalose, a nonreducing disaccharide, has been found to accumulate under salt stress and helps in stabilizing membrane and proteins (Paul et al. 2008). Externally applied 5 mM (low concentration) trehalose reduced Na^+ accumulation and inhibited growth while 10 mM (high concentrations) prevented chlorophyll loss in leaf blades and preserved root integrity (Garcia et al. 1997). Cyanobacteria use specific transporters to uptake their compatible solutes diffused in the periplasm, for example, a sucrose-, trehalose- and glucosylglycerol-specific transporter has been discovered for the first time in *Synechocystis* (Mikkat et al. 1996).

Another class of osmoprotectants in plant cell is raffinose family oligosaccharides (RFO) such as raffinose, stachyose and verbascose which accumulate during salt stress in leaves. Galactinol and raffinose also serve as scavengers of ROS and membrane protection. High level of galactinol and raffinose is accumulated by drought- and salinity-tolerant*Arabidopsis* GolS1 and GolS2 (Nishizawa et al. 2008).

Enhanced accumulation of polyols, mannitol and sorbitol has been reported to provide tolerance to salt stress in several plant species. Over-expression of sorbitol-6-phosphate dehydrogenase (S6PDH) from apple prevents photosystem II from salinity impacts in persimmon trees (Gao et al. 2001). The cyclic polyols myo-inositol, and its methylated derivatives D-ononitol and D-pinitol, have also been found with higher level of accumulation under salt stress in several plant species (Sengupta et al. 2008). In halotolerant plants, over-expression of L-myo-inositol-1-phosphate synthase and inositol *O*-methyltransferase results in increased level of cyclic polyols providing salt stress tolerance in tobacco (Patra et al. 2010).

Reduced content of organic acids and TCA cycle intermediates in glycophytes under salt stress are involved in compensating ionic imbalance (Sanchez et al. 2008). Apart from plant, cyanobacterial adaptations to salt stress generally include accumulation of inorganic ions to balance osmotic potential and of osmoprotectants like sucrose, trehalose, glucosylglycerol (2-0-(a-D-glucopyranosy1)-glycerol), glycine betaine, proline and glutamate to prevent denaturation of macromolecules. Among these, sucrose and trehalose are the dominant osmolyte in *Anabaena* strain, glucosylglycerol in *Synechocystis* PCC 6803 and glycine betaine in *Synechococcus* strain. Different osmolytes show difference in their potential to cope with salt stress as the cyanobacteria having major accumulation of glycine betaine show higher salt tolerance (high salt tolerance maximum of 3.0 M NaCl) when compared to those accumulating glucosylglycerol and other polyols (moderate salt tolerance maximum of 1.8 M NaCl) showing better response than those having sucrose and trehalose as their major osmolytes (low salt tolerance, maximum of 0.7 M NaCl) (Reed 1986).

4.3.3 Antioxidant Defence System

Antioxidative defence system includes both enzymatic and non-enzymatic antioxidant systems and their cooperative effort protects the damage generated by salt stress in the plant tissue. Reactive oxygen species (ROS), partially reduced forms of atmospheric oxygen, are generally regarded as the main source of damage to cells under biotic and abiotic stresses. ROS are generated at the plasma membrane level or extracellular in the apoplast; they react with the unsaturated fatty acids of essential membrane lipids in plasma lemma or intracellular organelles. This contributes to the leakage of cellular contents, desiccation and cell death. Intracellular mitochondrial and chloroplast membrane damage causes hindrance to respiratory activity and results in pigment breakdown and loss of the carbon fixing ability,

respectively (Scandalios 1993). ROS of polyunsaturated fatty acid generates aldehydic lipid breakdown product called malondialdehyde (MDA) which is used as a marker of oxidative lipid injury under salt stress (Moller et al. 2007). Lower MDA depicts higher antioxidative ability pondering higher resistance to salt stress.

Carotenoids de novo synthesized by all photosynthetic and many non-photosynthetic organisms are divided into the hydrocarbon carotenes, β-carotene and xanthophylls. β-Carotene bound to the core complexes of PSI and PSII provides protection against damaging effect of ROS further maintaining photochemical processes. Salt-tolerant species show an increase in carotenoids–chlorophyll ratio as well as enhanced anthocyanin, to protect from oxidative damage (Kytridis et al. 2008).

α-Tocopherol, a lipophilic antioxidant located primarily in thylakoid membranes, protects membranes from ROS and lipid radicals and hence helps in membrane stability. Three methyl groups in the molecular structure of α-tocopherol help it in possessing the highest antioxidative activity.

Ascorbate (ASC) has the capacity to directly eradicate ROS including singlet oxygen, superoxide, hydroxyl, peroxyl and alkoxyl radicals. It also helps in maintaining the membrane-bound antioxidant α-tocopherol in the reduced form by reducing α-chromoxyl radical and indirectly eradicates H_2O_2 through the activity of ascorbate peroxidase. Glutathione (GSH, g-glutamyl–cysteinyl–glycine) a thiol metabolite possesses strong reductant capacity that can scavenge toxic reactive oxygen species (ROS) such as $^1O_2$, $O_2^{\cdot -}$ and OH^- directly or in cooperation with other antioxidants like ascorbate and ROS-scavenging enzymes under high salt. H_2O_2 is a stable oxidant; at low concentration, it acts as a signal molecule involved in signal triggering and tolerance to salt stress, and at high concentration, it leads to programmed cell death. It has been reported that 50 % of photosynthesis is inhibited if H_2O_2 is present in chloroplast at a concentration of 10 μM (Kaiser 1979). Excess accumulation of H_2O_2 in the cell can also stimulate Haber–Weiss/Fenton reaction, yielding hydroxyl radicals (•OH) resulting in lipid peroxidation.

Antioxidative enzymes the major part of complex antioxidative defence system developed by living organisms include superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR). Other enzymes such as monodehydro ascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) also help in ROS scavenging under salt stress. Their activity level under salt stress is taken as an indicator of stress tolerance.

SODs found in various cell compartments catalyse the dismutation of superoxide generated into oxygen and hydrogen peroxide. CAT, a tetrameric heme-containing enzyme, and peroxidases such as APX and GPX scavenge hydrogen peroxide to produce H_2O and O_2. CAT having one of the highest turnover rates among all enzymes can convert six million molecules of H_2O_2 to H_2O and O_2 per minute.

APX uses ascorbate as electron donor and possesses higher affinity for H_2O_2 (μM range) as compared to CAT and POD (mM range) and provides tolerance against salinity. Its involvement in ascorbate–glutathione antioxidant pathway results in reduction of hydrogen peroxide.

GR a flavo-protein oxidoreductase localized predominantly in chloroplast converts oxidized glutathione (GSSG) to reduced glutathione (GSH) for maintaining GSH pool (Rao and Reddy 2008), regulates GSH/GSSG ratio and supplies GSH for GPX and DHAR, which further convert hydrogen peroxide to water and reduce oxidized ascorbate, respectively, thus protect against oxidative stress by maintaining the ASH pool.

Although the activity of the above-mentioned enzymes generally increases under salt stress, nevertheless, different plant species show alteration in activity of their enzymes at varying salt concentrations. For example, in *Glycine max*, increased CAT activity level was almost similar both in shoot and root, i.e. about 150 and 352 %, 597 and 188 %, 740 and 547 % under 33, 66 and 99 mM NaCl treatment, respectively, as compared to control (Weisany et al. 2012). While in *B. parviflora*, APX, GPX, GR and SOD activity increased but CAT activity decreased (Parida et al. 2004).

4.3.4 Metabolic Rearrangement and Ionomics

Metabolite fingerprinting and profiling help to understand mechanism underlying stress physiology in plants hence in developing improved breeding strategies towards stress-tolerant crops as the dynamic alteration in metabolite pool or fluxes may decide the phenotype of the organism (Ratcliffe and Shachar-Hill2006). Species adapted to saline environment may be metabolically pre-adapted to salinity hence help in developing salt-tolerant genotypes (Gong et al. 2005; Sanchez et al. 2008).

The ionome represents the composition of inorganic component such as mineral nutrient and trace element of cellular and organismal systems. Quantitative and simultaneous measurement of alteration in elemental composition in response to physiological processes under differing environment helps us to understand the mechanisms of salt tolerance. On the basis of differential rearrangement of shoot nutrient levels, ionomics was applied using inductively coupled plasma-atomic/optical emission spectrometry (ICP-AES) on the extremophile *Lotus creticus* and two glycophytes *Lotus corniculatus* and *Lotus tenuis* upon exposure to salt stress, and it was found that Cl^- exclusion from the shoot tissue was a vital phenomenon in providing tolerance against salinity in *L. creticus* (Sanchez et al. 2011).

4.4 Molecular and Omics Approach Towards Salt Stress

Almost all aspects of life are engineered at the molecular level, and without understanding molecules one can only have a very sketchy understanding of life itself. Molecular analysis to dissect the signal transduction pathways mediating the adaptive strategies employed by plants or cyanobacteria under various

environmental stresses is imperative. Basically, organisms respond to various stresses by modulating gene expression which ultimately causes restoration of cellular homeostasis, detoxification, damage repair and growth recovery. This subsection encompasses molecular aspects of various salt-responsive genes/proteins and associated pathways in plants as well as in cyanobacteria.

4.4.1 Perception and Salt Stress Signal Transduction in Plants

Water deficit due to high salinity initially poses an ionic, osmotic or even a mechanical impact on the cell. It is plausible that all these signals have their own cognate receptors which operate either independently or cooperatively to initiate downstream signalling events. Sensing of salt by plants is an enigma. There is little knowledge about how sodium is sensed in any cellular system. Sodium ion can be sensed either before or after entry into the cell or both. Thus, there could be two different perception mechanisms employed by the cell in order to sense salt stress—direct and indirect. Direct perception includes sensing extracellular sodium ion by a membrane receptor or a putative direct osmosensor, and indirect perception is sensing intracellular sodium ion by sodium ion-sensitive enzymes (induced due to osmotic changes in cell volume, turgor pressure, membrane stability, individual solute concentration, ionic strength and accumulation of macromolecules in cytoplasm). Histidine kinases are among the best candidates for salt and osmotic stress receptors and defined as osmosensors in prokaryotes and yeast; however, these have not been characterized in plants yet. AtHK1 a histidine kinase from *Arabidopsis* structurally related to yeast histidine kinase osmosensor (SLN1) can rescue the salt sensitivity of SLN1 and SHO1 (another transmembrane osmosensor) deleted yeast mutant implying that AtHK1 might have a similar function in plants. In plants SOS1, a plasma membrane Na^+/H^+ antiporter has been proposed to sense Na^+ ions and thus functions as sensor (Chinnusamy et al. 2006). Another potential candidate sensor is a Na^+–K^+co-transporter of *Eucalyptus camaldulensis* (Chinnusamy et al. 2006).

The major routes in salt stress signal transduction consist of pathways for ionic and osmotic homeostasis signalling, ROS (reactive oxygen species) detoxification (i.e. damage control and repair) and growth regulation. SOS (salt overly sensitive) signalling pathway encompasses the entire ionic aspect of salt stress whereas MAPK (mitogen-activated protein kinases) signalling pathway mediates the osmotic homeostasis and/or detoxification responses. All signalling cascades initially modify constitutively expressed transcription factors, causing expression of early response transcriptional activators which ultimately activates downstream delayed responsive stress tolerance effector genes (Fig. 4.1). Early response genes have a transient and quick expression whereas delayed response genes are activated slowly with sustained expression. Several examples of early response genes are

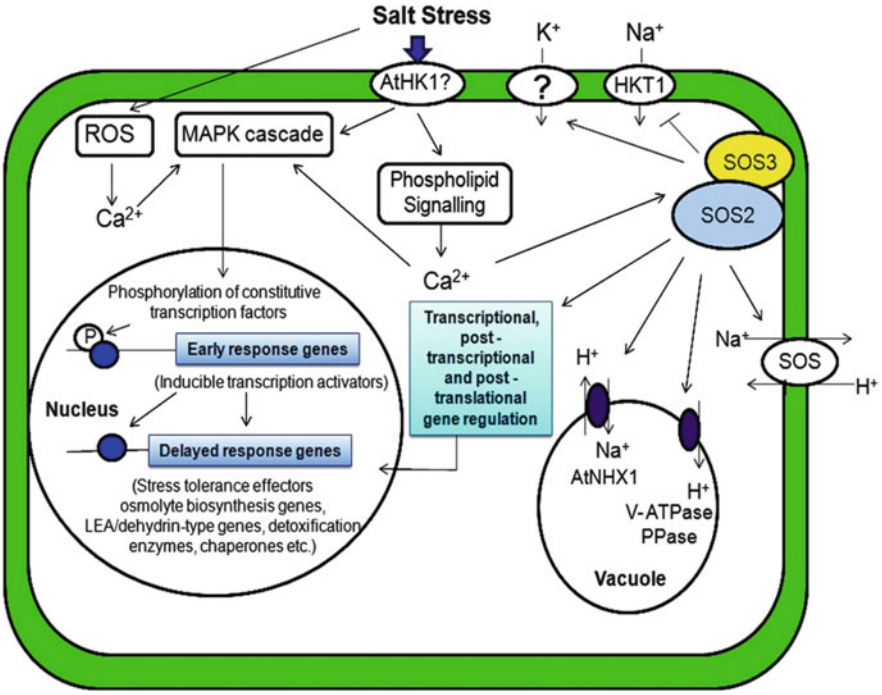

Fig. 4.1 Schematic representation of various signalling pathways responsible for plant responses under high salinity

CBF/DREB (C-repeat binding proteins/dehydration-responsive-element binding proteins) gene family, RD22BP, etc. Delayed response genes include large number of stress-responsive genes such as osmolyte biosynthesis genes, LEA (late embryogenesis abundant)/dehydrin-type genes, detoxification enzymes, and chaperones. (Fig. 4.1).

4.4.1.1 Acute Signalling Pathways

The first response of cells to combat hyperosmolarity is the flux of water and ions across various membranes after which osmolyte accumulation occurs when all the injuries become evident. Therefore, maintenance of ion homeostasis could be an acute response. SOS pathway in *Arabidopsis* represents some aspects of acute adaptive response and is responsible for Na^+ homeostasis and salt tolerance (Zhu 2000). This pathway incorporates three components, SOS1, SOS2 and SOS3. SOS3 is a Ca^+-binding protein which senses the cytosolic calcium ion signal elicited by salt stress (Liu and Zhu 1998). SOS2 is a serine/threonine protein kinase. Approximately, 270 amino acids present at N-terminal of SOS2 comprise the kinase catalytic domain which is inhibited by a 21-amino acid-long FISL motif present

in C-terminal regulatory domain of SOS2. SOS2 is normally inactive, presumably because of an intramolecular interaction between the catalytic domain and the autoinhibitory regulatory domain. Binding of SOS3 to SOS2 at FISL motif removes its inhibitory effect and is determined to be crucial to bind SOS3. Interaction of SOS3 with SOS2 activates the substrate phosphorylation ability of SOS2 in the presence of Ca^{2+}. The activated SOS3–SOS2 kinase complex phosphorylates and activates SOS1, a Na^+/H^+ antiporter on the plasma membrane through which ion homeostasis is maintained (Fig. 4.1). Similarly, for increased expression of other transporter genes activated SOS3–SOS2 kinase complex is perhaps necessary.

4.4.1.2 ROS Signalling Pathways

Salt stress-induced accumulation of ROS is not properly understood in plants. ROS particularly H_2O_2 triggers MAPK cascades, and since osmotic stress signalling also uses some of the MAPK modules it is plausible that there exists a crosstalk between salt stress and oxidative stress signalling at these modules (Xiong and Zhu 2002). It is proposed that Ca^{2+} channels in guard cells are activated by exogenous and ABA-induced H_2O_2 which through activation of MAPK cascade mediates stomatal closure (Fig. 4.1). In *Arabidopsis*, it is reported that MAP kinase kinase ANP1 and downstream genes such as GSTG, HSP 18-2 and GH3 are activated by H_2O_2. Interestingly, tobacco plants overexpressing NPK1, an ANP1 ortholog, exhibit an increased tolerance to salt stress. ROS regulates expression of many genes including ROS scavengers (e.g. superoxide dismutase and catalases) or antioxidant (glutathione, thioredoxins). Transcription factors that bind to the *cis* elements in these gene promoters are well studied in yeast. These include Yap1 group b-Zip transcription factors, having conserved cysteine residues which may act as sensors for the redox status of the cell. Surprisingly, in spite of the presence of all these genes in plants, Yap-1-like transcription factors are missing from *Arabidopsis* genome (Xiong and Zhu 2002).

4.4.1.3 Signalling for Osmolyte Synthesis

Researchers have been attracted to study the underlying mechanism of increased synthesis of osmolytes during hyperosmolarity. In yeast, HOG1 (high osmotic glycerol 1) pathway is the best studied osmolarity signalling cascade, but in case of glycophytes osmolytes do not accumulate to a high level; therefore, the mechanism is not well understood. However, a signalling pathway similar to that of yeast MAPK–HOG pathway may be involved in regulation of osmolyte biosynthesis in plants. At low osmolarity, active form of *Arabidopsis* osmosensor AtHK1 inactivates a response regulator by phosphorylation. Whereas high osmolarity inactivates AtHK1 leading to accumulation of active nonphosphorylated response regulator which in turn activates osmolyte biosynthesis by activating MAPK pathway in

plants. Further studies are required for better understanding of osmolyte signalling pathway.

4.4.2 Perception and Salt Stress Signal Transduction in Cyanobacteria

In living cells, perception of environmental stresses and the subsequent transduction of stress signals are primary events in the acclimation process. Cyanobacteria have several features that make them particularly suitable for the study of stress responses at the molecular level. Homologues of salt-induced genes in cyanobacteria (*Synechocystis*) are also regulated by salt stress in higher plants. Thus, cyanobacteria may serve as a good model system for discerning the molecular mechanism of the stress responses and acclimation of stresses in the plants (Bohnert et al. 2001). In general, sensing salt stress and subsequent signal transduction pathway are not properly understood. In the model cyanobacterium *Synechocystis* 6803, the two component system consists of histidine kinase (Hik) and response regulator (Rre).

The Hik perceives environmental changes by its sensory domain, forms homodimer and gets autophosphorylated at a histidine residue within histidine kinase domain (Stock et al. 2000). The phosphorylated group is transferred from the Hik to a conserved aspartate residue in the transducer, Rre. Phosphorylation leads to the conformational changes and activation of Rre. The activated Rre binds to the promoter regions of many salt-responsive genes such as those involved in ion homeostasis, osmolyte biosynthesis and transport processes (Fig. 4.2).

Upregulation of 38 genes including Hik/Rre pairs Hik33/Rre31, Hik34/Rre1, Hik2/Rre1, Hik16/Hik41/Rre17 and Hik10/Rre3 has been reported after 30 min of addition of 0.5 M NaCl that were particularly involved in perception and transduction of salt stress signal. Hik41, Hik2 and Hik34 are soluble proteins present in cytosol, and Hik33, Hik10 and Hik16 are transmembrane proteins (Hagemann 2011).

4.4.3 Genomic and Proteomic Aspects of Salt Stress

The main response amenable to molecular analysis are metabolic adaptations to salt stress and have led to the identification of a large number of genes induced by salt. Several such novel proteins and polypeptides were identified by both one- and two-dimensional gel electrophoresis. To understand plant osmotic stress at the molecular level, analysis of these genes is required. Based on their predicted physiologic or metabolic functions, these genes can be classified into functional

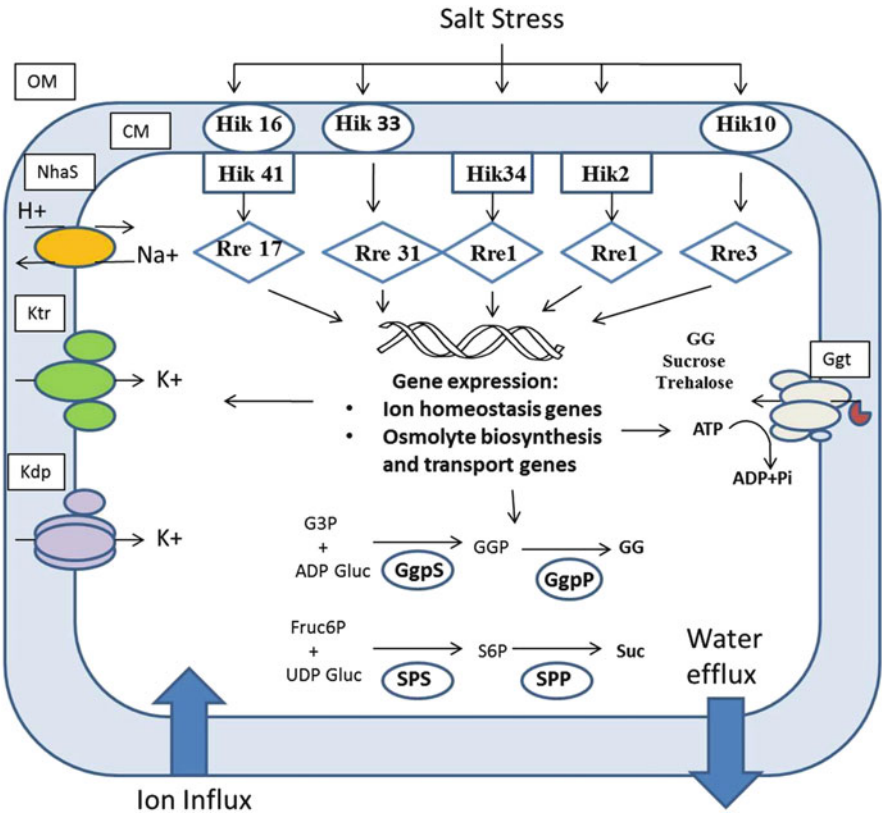

Fig. 4.2 Hypothetical pathways for upregulation of various adaptive processes and transduction of salt stress signal in *Synechocystis*

groups (Sairam and Tyagi 2004). Some genes induced in different plant species under high salinity are listed in Table 4.1.

4.4.3.1 Genes Encoding Proteins Involved in Cellular Protection

Late embryogenesis abundance (LEA) proteins, induced by salinity and water deficit, belong to this functional category. These proteins were originally thought to be associated with desiccation tolerance during seed maturation but are also involved in protection of cellular structure and components from the harmful effects of water deficit during salt stress. Based on sequence and expression kinetics, LEA proteins are categorized in six subgroups.

1. *Group 1 LEA proteins*: The predicted role of this subgroup is water binding, thus providing a protective aqueous environment for cellular components. The

Table 4.1 Selected genes/proteins induced by salt stress

Plant species	Salt-responsive genes/proteins	Characteristic feature(s)	Reference
Arabidopsis thaliana	*Sal* 1	Induced by salt stress, overexpression in *Arabidopsis* or yeast overcomes Na^+ and Li^+ toxicity	Quintero et al. (1996)
Brassica napus	*Bnd* 22	22 kDa protein, level increased by progressive or rapid water stress and salinity	Reviron et al. (1992)
Citrus sinensis	Salt associated 23–25 kDa protein	Induced by salt stress, ABA[a] and water stress	Benhayyim et al. (1993)
Dunaliella salina	P 150	150 kDa protein, induced by salt stress	Sadaka et al. (1991)
Lycopersicon esculentum	TAS-12	Salt- and water stress-induced lipid transfer protein	Torres-Schumann et al. (1992)
Mesembryanthemum crystallinum	*ppc*-1 and *ppc*-2	Encodes PEPcase,[b] induced by salt and water stress	Cushman et al. (1989)
	Isogenes *lmt* 1	Encodes myo-inositol*O*-methyl transferase 1; induced by NaCl and osmotic stress	Vernon and Bohnert (1992)
	Inps 1	Encodes myo-inositol-1-phosphate synthase; shows significant homology to corresponding genes in plants and yeast	Ishitani et al. (1996)
Nicotiana tabacum	Vitronectin- and fibronectin-like protein	Found in membranes and cell wall of NaCl-adapted cells	Zhu et al. (1993a)
	Osmotin	26-kDa protein, protein level enhanced in both NaCl- and PEG[c]-induced water stress-adapted cells but not in unadapted cells	Singh et al. (1987)
Oryza sativa	RAB21	Induced under water, NaCl and/or ABA	Mundy et al. (1990)
	Em	Induced by ABA and salt stress	Bostock and Quatrano (1992)

[a]Abscissic acid
[b]Phosphoenolpyruvate carboxylase
[c]Polyethylene glycol

expression of members of this group of genes in vegetative tissues is induced by salt (Bostock and Quatrano 1992).

2. *Group 2 LEA proteins*: The predicted function of these proteins is preventing denaturation and maintenance of the solvation of structural surfaces.

3. *Group 3 and 5 LEA proteins*: The possible role of these proteins in salt-stressed cells is ion sequestration as these proteins exist as dimers and the polar face of dimerized helices might expose and bind ions via formation of salt bridges. There are reports of induction of group-3 LEA proteins in soybean and barley and also a gene encoding a group-5 LEA homologue of the salt-tolerant line Shamuti orange in response to salt and water deficit (Naot et al. 1995).
4. *Group 4 LEA proteins*: These proteins may serve as reverse chaperones and possess water-binding properties; due to this, they can stabilize the surface of membranes and proteins by binding water and functioning as solvation film. In vegetative tissue, expression of these proteins occurs in response to salinity, drought, low temperature and ABA.
5. *LEA D95*: Being hydrophobic in nature, this protein is unusual. It shows homology to cDNA pcC 27-45 from *Craterostigma plantagineum* and expresses in callus tissue in response to salt stress (Piatkowski et al. 1990).

4.4.3.2 Transporter Genes

Exclusion and ion compartmentation of Na^+ ions via membrane transport is necessary for survival in saline environments. Several ATPase genes of plasma membrane as well as tonoplast are induced by high salt stress. Many of the salinity-induced proteins share sequence similarities with water channels. Salt-induced alteration of the tonoplast H^+-pumping V-ATPase and H^+-pyrophosphatase has been evaluated in hypocotyls of *Vigna unguiculata* seedlings (Otoch et al. 2001). The *Arabidopsis thaliana* AtNHX1 gene encodes a vacuolar Na^+/H^+ antiporter that is important in salt tolerance, and its expression is regulated by salt stress (Shi and Zhu 2002). In *Synechocystis* 6803, six different genes were annotated as Na^+/H^+ antiporters (Kaneko et al. 1996). In other completely sequenced cyanobacterial genomes, also multiple genes for Na^+/H^+ antiporters are present. Over-expression of cyanobacterial genes in defined *E. coli* mutants revealed that at least three antiporters from *Synechocystis* 6803—NhaS1, NhaS3 and NhaS4—are true antiporters among them NhaS3 shows the highest transport activity. NhaS1 shows similarities to the SOS1 Na^+/H^+ antiporter from *Arabidopsis thaliana* and is expressed in *E. coli* mutant defective in Na^+/H^+ antiporter activity which leads to the restoration of Na^+ tolerance (Hamada et al. 2001). Thus, NhaS1 seems to be the most active Na^+/H^+ antiporter in *Synechocystis* 6803, and the gene encoding it (*sll0689*) was the most highly expressed of the Na^+/H^+ antiporter genes. Similar studies were performed in *Aphanothece halophytica* which revealed multiple Na^+/H^+ antiporter genes (Waditee et al. 2001; Wutipraditkul et al. 2005). Out of multiple genes, ApNhaP is related to NhaS1 from *Synechocystis* 6803. K^+ ion is also present in high amount in control as well as salt-loaded cells, in contrast to Na^+, and is also crucial for salt acclimation. Three types of transporters exist in *E. coli*: high-affinity ATP-dependent Kdp system and the low-affinity Trk and Kup systems. The potential candidates for K^+ uptake in cyanobacteria are Kdp subunits, proteins related to Trk but named Ktr and different putative K^+ channels. All cyanobacteria possess

the structural genes for a functional ATP-dependent K^+ transport system consisting of the Kdp ABC subunits. The KdpA subunit is the K^+ permease, the KdpB subunit is a typical P-type ATPase that provides the energy and KdpC is involved in the assembly of the transport system.

4.4.3.3 Osmolyte Biosynthetic Genes

To prevent water loss and to re-establish turgor in order to expand, cells accumulate solutes during osmotic stress. Major organic solutes proline, betaine and ions such as K^+, Na^+ and Cl^- maintain osmotic adjustment. Delta pyroline-5-carboxylate synthetase, a bifunctional enzyme involved in proline biosynthesis (Hu et al. 1992), was induced by high salt stress and dehydration (Delauney and Verma 1993). Similarly, genes involved in pinitol synthesis encoding myo-inositol*O*-methyltransferase were isolated from the ice plant (Vernon and Bohnert 1992) and found to be exclusive in salinity stress. In sugar beet, spinach and barley induced expression of genes and cDNAs encoding choline monooxygenases and betaine aldehyde dehydrogenase involved in conversion of choline to glycine betaine has been reported in response to salinity (Rathinasabapathi et al. 1997; Ishitani et al. 1995). Myo-inositol phosphate synthase, which encodes a precursor for pinitol synthesis, showed sixfold upregulation under salt stress. One more gene mannitol dehydrogenase downregulated by salt stress maintains the high concentration of mannitol in stressed cells in order to function as an osmoprotectant in rice (Williamson et al. 1995; Sairam and Tyagi 2004).

Similar to plants, in cyanobacteria also a large number of genes responsible for compatible solute accumulation are induced. One such compatible solute is sucrose. Salt-induced sucrose biosynthesis is achieved by sucrose phosphate synthase (SPS). Expression of the *Synechocystis sps*A transiently increased after salt shock which corresponds well to the transient sucrose accumulation in *Synechocystis* 6803 (Marin et al. 2004). Glucosyl glycerol (GG) represents another typical compatible solute of cyanobacteria. GgpS (GG-phosphate synthase) as well as GgpP (GG-phosphate phosphatase) involved in GG biosynthesis pathway became activated in crude protein extracts at 100 mM NaCl (Hagemann et al. 1996). The salt stimulation of GgpS and GgpP clearly explains the initial activation of GG synthesis in salt-shocked cyanobacterial cells characterized by transiently high ion contents.

4.4.3.4 Genes Encoding Proteins Involved in General Defence

Screening of salt-induced cDNA libraries resulted in a number of cDNAs associated with plant defence against pathogen or wounding damage which include PRP (pathogenesis-related protein) and β-glucanase from rice and endochitinase from tomato (Umeda et al. 1994; Chen et al. 1994). Osmotin a polypeptide related to a family of PRP is reported to have association with salinity adaptation. These are the

most abundant proteins in salt-adapted tobacco cells (Singh et al. 1985). APX and GPX are the enzymes involved in controlling oxidative stress; genes encoding these enzymes are reported to be induced in salt stress. Methylglyoxal detoxifying enzyme glyoxalase is also induced by salinity stress (Holland et al. 1993; Espartero et al. 1995).

Similar to plants, several cyanobacterial genes involved in defence were found to be induced in high salinity. Nine proteins of defence pathway upregulated under salt stress in *Anabaena* include peroxidase, Alr3090 (similar to catalase), superoxide dismutase (SOD A and SOD B), glutathione reductase and AhpC (alkyl hydroperoxide reductase)/TSA family proteins (Rai et al. 2014).

4.4.3.5 Genes Encoding Proteins Involved in Metabolism

Induced expression of glyceraldehyde-3-phosphate dehydrogenase and phosphoglyceromutase, PEPcase, NADP-malate dehydrogenase and NADP-malic enzyme in *Mesembryanthemum crystallinum* has been reported in response to high salt stress (Cushman et al. 1989; Umeda et al. 1994; Foresthofel et al. 1995). Enhanced mRNA accumulation of both nuclear- and chloroplast-encoded transcripts of photosynthesis-related genes is known in salt-adapted cell cultures (Winicov and Button 1991; Locy et al. 1996).

In response to salt stress, proteins of purine and pyrimidine metabolism were upregulated in *Anabaena*. Some proteins of energy metabolism such as phosphoglycerate kinase, transketolase and FBPase (fructose 1,6-bisphosphatase) registered two–threefold upregulation (Rai et al. 2014). A clear accumulation of transaldolase, ribulose-phosphate 3-epimerase, phosphoglucomutase, transketolase, glycogen phosphorylase, phosphoglycerate kinase and fructose-1,6-bisphosphatase was found in *Synechocystis* sp. strain PCC 6803 in response to high salinity (Fulda et al. 2006).

4.4.3.6 Genes Encoding Proteins Involved in Protein Synthesis, Processing and Degradation

Salt-adapted tobacco cells dramatically accumulate elongation factor 1-alpha, one of the essential components of protein synthesis (Zhu et al. 1997). Protease inhibitors, normally induced upon insect attack, are also induced by salt stress (Downing et al. 1992; Lopez et al. 1994). In *Arabidopsis*, two different cysteine proteinases accumulate in response to salt stress. Several heat-shock proteins which act as chaperons to prevent denaturation and help denatured proteins to regain their native conformation are also expressed under salt stress. In *Atriplex nummularia*, ANJ1, member of the DnaJ family of HSPs, was induced in cell by high salt stress that had been adapted to high salinity but not in normal unadopted cells (Zhu et al. 1993b).

Salt stress in N_2-fixing cyanobacteria *Anabaena* is known to increase protein synthesis and refolding of denatured proteins. Proteins showing remarkable

changes include subunits of ribosome assembly (30S RPs1 in *A. doliolum* and *Anabaena* 7120; 30S RPs6 in *Anabaena* 7120), elongation factors (EfTu in *Anabaena* L31; EfTs in *A. doliolum* and *Anabaena* 7120), posttranscriptional regulators (RNA-binding proteins D and E in *Anabaena* 7120 and *Anabaena* L31) and molecular chaperons HSP1, DnaK and GroEL (Rai et al. 2014). In *Synechocystis* also, GroEL1, DnaK2 and GrpE were found among the accumulated proteins under salt stress (Fulda et al. 2006).

4.4.3.7 Genes Encoding Proteins Involved in Regulating Gene Expression

Salt-responsive regulatory genes, involved in regulation of other salt-responsive genes, are mostly transacting factors and protein kinases. In *A. thaliana*, a receptor-like protein kinase gene and gene encoding components of signal transduction (MAPK) have been reported to be expressed under salt stress. Expression of some other genes like MAPKK and a ribosomal 36 kinase which functions in the MAPK cascade also increases under salinity stress (Sairam and Tyagi 2004). An *myb* homologue in *Arabidopsis* plant regulated at transcriptional level by salt stress may bind to promoter of osmotic stress-regulated genes and regulate their transcription in response to osmotic stress (Zhu et al. 1997). A receptor protein kinase cDNA is also reported in rice (Naot et al. 1995).

4.5 Bioinformatic Predictions of Molecular Targets Under Salt Stress

Though a lot of work has been done on salt toxicity and salinity-induced effects on enzymes and proteins, studies regarding interaction of salt and proteins are still lacking. For proper understanding of salinity-induced loss of enzyme and protein activity, determination of active (functional) sites on proteins and how they interact with ions and salt is necessary. These favourable binding sites relate to locations where a putative ligand could bind. Recently, computational methods for the detection and characterization of functional sites on proteins have increasingly become an area of interest (Campbell et al. 2003). Molecular docking is one such method which simulates the molecular recognition process. The aim of molecular docking is to achieve an optimized conformation for both the proteins and the ligands and their relative orientation such that the free energy of the overall system is minimized. A binding interaction between a small molecule ligand and an enzyme protein may result in activation or inhibition of the enzyme. If the protein is a receptor, ligand binding may result in agonism or antagonism. In order to understand the protein–salt interaction, docking study was performed with certain

commonly upregulated and downregulated proteins/enzymes in cyanobacteria and higher plants under salt stress.

The RNA-binding proteins (RBPs) are proteins that bind to the double- or single-stranded RNA in cells and participate in forming ribonucleoprotein complexes. RBPs have crucial roles in various cellular processes including posttranscriptional RNA maturation, transport, localization and gene regulation. Unfortunately, however, these proteins seem to be salt sensitive not only in plants (Pang et al. 2010) but also in cyanobacteria (Pandhal et al. 2008). The docking study was performed between the selected, downregulated RBP and the ligand NaCl to predict the possible binding site, and interestingly the amino acids which get affected under salt toxicity were found to be common in both higher plants and in cyanobacteria.

The docking study reveals that NaCl successfully docked with the three conserved amino acid residues Val, Ala and Gly present at RRM2 domain of RNA-binding proteins with the calculated binding energy of −52.31 and −47.56 for *Arabidopsis thaliana* and *Anabaena* RBP, respectively. Since RRMs (RNA recognition motifs) are crucial for RNA binding and recognition of specific RNA sequences, its interaction with NaCl could be the plausible explanation as why these proteins are uniformly sensitive under salt stress in both higher plants and cyanobacteria.

During the study, another protein called nitrate reductase of *Nostoc* sp. PCC7120 (Frias et al. 1997) and *Zea mays* (Baki et al. 2000) was found uniformly sensitive to high salt concentrations. The PDB structure of this protein was taken for docking and functional site analysis. The binding energy of salt with nitrate reductase of *Nostoc* sp. PCC7120 and *Zea mays* was found to be −31.75 and −29.75, respectively. The residues that were present in the conserved motif as well as at the active site of the protein, namely, Cys, Phe, Trp, Gly, Thr and Glu, were identified as the targets of NaCl thereby conferring its uniform toxicity on the nitrate reductases family of proteins.

Similarly, photosystem II light-harvesting proteins, the intrinsic transmembrane proteins CP43 (PsbC) and CP47 (PsbB) occurring in the reaction centre of photosystem II were commonly downregulated in cyanobacteria as well as in higher plants. In order to trace out the reasons behind salt toxicity at the molecular level, docking study was conducted taking the PDB structures of photosystem II proteins from *Zea mays* and *Synechocystis* sp. PCC 6803 as both have been reported to be highly salt sensitive (Jeanjean et al. 1993; Zörb et al. 2009). Here also the results were almost similar, i.e. the binding of the salt at the active site of the protein which is a part of the conserved motif of the protein's functional domain determining its function. The residues that were found to be involved in the interaction were Met, Ala and Leu in *Zea mays* and Glu, Leu, Ser and Phe in *Synechocystis* sp. PCC 6803. These were the sites of interaction for the salt to confer its toxicity.

Furthermore, proteins commonly upregulated under salt stress in both cyanobacteria and higher plants were subjected to docking analysis. The selected proteins include superoxide dismutases [*Oryza sativa* (Fadzilla et al. 1997) and *Nostoc* sp. PCC7120 (Rai et al. 2014)], glutathione *S*-transferase (GST) [*Oryza sativa* (Chitteti and Peng 2007) and *Nostoc* sp. PCC7120 (Rai et al. 2014)] and heat-

shock protein70 [*Oryza sativa* (Chitteti and Peng 2007) and *Nostoc* sp. PCC7120 (Rai et al. 2014)]. To study the effect of NaCl on SOD activity in terms of their binding affinity with each other, proteins from *Oryza sativa* (Fadzilla et al. 1997) and *Nostoc* sp. PCC7120 (Rai et al. 2014) were taken for docking calculations.

The interaction between salt and SOD in plant and cyanobacteria with a binding energy of −56.39 and −51.89 predicts a stable complex although only few residues were found common between them like His, Asp and Trp, but the point that needs to be noted here is that though these common residues are conserved, still there were residues that showed interaction with the salt but they are neither conserve nor present at the active site of these proteins thereby solubilizing the toxic effect of salt stress on superoxide dismutases and the interaction at the *sod* domain enhances the expression of this protein under salt stress.

Another protein GSTs comprise a family of eukaryotic and prokaryotic phase II metabolic isozymes best known for their ability to catalyse the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification. GST contains a C-terminal structural domain and an N-terminal catalytic domain. The glutathione molecule binds in a cleft between N- and C-terminal domains. Under salt stress, this protein was found to show interaction with the N-terminal domain with Val and Ser being common among both the higher plants and cyanobacteria with equal binding energy of −54.53 for both. In this case too, while these residues are conserved at the N-terminal they are not catalytically important as they do not constitute the functional active site of this protein and therefore, their interaction with the salt does not hamper the protein functioning under salt stress.

The 70 kDa heat-shock proteins (Hsp70s) are a family of conserved ubiquitously expressed proteins. The Hsp70s are an important part of the cell's machinery for protein folding and help to protect cells from stress. Members of the Hsp70 family are strongly upregulated by heat stress and toxic chemicals, particularly heavy metals such as arsenic, cadmium, copper, and mercury. The docking study conducted between the HSP70 and salt depicted good interaction with a binding energy of −46.12 for *Nostoc* and −53.78 for *Oryza sativa*. But this interaction was neither at the conserved motif nor at the functional or catalytic site of this protein. The interacting residues were also found to be entirely different for both of them. Thus, the expression of this protein enhances on interaction with the salt stress making it a salt-tolerant one.

Docking study revealed that salt-interacting residues of some of the salt-responsive enzymes (RBPs, nitrate reductase) are conserved among cyanobacteria as well as higher plants; thus, similar mechanism might be involved in salt-induced toxicity to these enzymes. Since salt-interacting residues of photosystem II light-harvesting proteins, CP43 (PsbC) and CP47 (PsbB), were specific for cyanobacteria and higher plants, these organisms might possess specific mechanisms for salt toxicity. On the other hand, the salt-tolerant proteins showed good interaction with salt with almost equal binding energies as salt-sensitive proteins, but the interacting residues were quite distinct for higher plant and cyanobacteria. Only few conserved interacting residues existed but not located at the active site of the

proteins. Moreover, these proteins had domains that help in their survival under stress. Interaction of salt with these domains triggers their function and thus expression of the protein under stress. One important point emerging from this study was the lack of significant homology between the selected proteins from cyanobacteria and higher plant at the sequence level, but some domains were common across the species unique for each group of proteins. Thus, the interaction of salt at these conserved domains and motifs decides the response of that particular protein under salt stress.

4.6 Conclusion/Future Perspectives

Despite substantial research, we are still far from a proper understanding of salt stress tolerance in plants and/or cyanobacteria. There are some apparent gaps which need attention—first, one cannot draw a clear cut line to identify the real players governing salt sensitivity; second, the molecular pathways for salt stress signal perception and transduction are still to be resolved. Furthermore, since many salt-responsive pathways and associated genes such as LEA, SOD, Prx, and Hsp are found to be common in other stresses, it would be rather interesting if such genes are exploited for development of transgenics tolerant to multiple abiotic stresses and properly tested at field level. In the recent years, bioinformatics has emerged as indispensable for the analysis of genes and proteins. There is a need to create interaction network of genes, proteins and metabolites participating in stress-regulated biological processes.

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Chapter 5
Plant Responses to Soil Flooding

Tamara I. Balakhnina

Abstract The influence of various extent of soil moisture and its aeration status in the root zone of the plants on the physiological status and defensive system against oxidative destruction of the tolerant-to-soil-flooding maize *Zea mays* L. and sensitive pea *Pisum sativum* L. was investigated. The efficiency of the defensive system was evaluated by the activity of SOD as an enzyme neutralizing superoxide anion radicals, by MDA content indicating the rate of free radical lipid oxidation, and by the root and shoot biomass production and pigment concentration in the leaves. Plant resistance to the effects of soil flooding depends not only on the ability to survive at the action of soil hypoxia but also on the subsequent reoxygenation. The effects of prolonged soil hypoxia and subsequent re-aeration on the development of the stress-realizing system bean *Vicia faba major* L. cv. Bartom plants were investigated. In connection with the specificity of the effect of hypoxia on the plants, the specific and nonspecific plant responses to the effect of this stress factor were investigated. In this case special attention was paid to the changes connected with transformations of respiration pathways, with functioning of the root alcohol dehydrogenase in the spring rape *Brassica napus* L. Formation of the reactive oxygen species which appears to be a unspecific plant response to different stress factors, including hypoxia, was estimated by the intensity of oxidative destruction processes and activity of antioxidant enzymes in plant tissues.

Keywords Alcohol dehydrogenase • Ascorbate peroxidase • Flooding • Glutathione reductase • Growth • Guaiacol peroxidase • Photosynthesis • Lipid peroxidation • Proline • Soil hypoxia • Superoxide dismutase

Abbreviations

ADG	Alcohol dehydrogenase
AsP	Ascorbate peroxidase
Chl	Chlorophyll

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DW	Dry weight
E_g	Air-filled porosity
E_h	Redox potential
GR	Glutathione reductase
GPX	Guaiacol peroxidase
LPO	Lipid peroxidation
MDA	Malondialdehyde
ODR	Oxygen diffusion rate
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBARs	Thiobarbituric acid reactive substances

5.1 Introduction

Soil flooding causes displacement of gases when soil pores are filled with water. The low diffusion rate of oxygen in water-filled pore space of the soil results in limitation of oxygen availability for plant roots and soil microorganisms (Drew 1983, 1997; Gliński and Stepniewski 1985) and leads to switch of aerobic metabolism of plant roots into less efficient anaerobic fermentation (Pezeshki 1991), causing a fast depletion of carbohydrate reserves (Bailey-Serres and Voesenek 2008). Hypoxic conditions in soil cause also a decrease of Eh (Pezeshki 1991; Bennicelli et al. 1998; Balakhnina et al. 2009, 2010). This, in turn, stimulates evolution of carbon dioxide, molecular hydrogen, hydrogen sulfide, ethylene, and methane (Smith and Russel 1969; Smith and Restall 2006) and accumulation in the soil of reduced phytotoxins (Fe^{2+}, Mn^{2+}, sulfide, and, at high concentrations, ammonium) which can have a negative impact on plants, causing, among others, growth retardation, reduction in leaf size, wilting of shoots, and necrosis (Snowden and Wheeler 1993; Lucassen et al. 2000, 2002). The ability of plants to survive soil flooding and oxygen starvation is determined by the evolutionary developed resistance to the action of this stress factor (Banach et al 2009). Plant adaptation to soil hypoxia includes series of interconnected reactions directed to survive the periods of hypoxic and anoxic conditions and to maintain homeostasis (Chirkova 1978, 1988; Kennedy et al 1992; Kalashnikov et al 1994; Crawford and Braendle 1996; Vartapetian et al. 2003). Among those adaptations, anatomical and morphological changes (Kennedy et al. 1992; Armstrong et al. 1994; Colmer 2003; Vartapetian et al. 2003; Mommer and Visser 2005; Pederson et al. 2009) help to provide oxygen to the plant tissues.

5.2 Soil Water and Aeration Status

The low diffusion rate of oxygen in water-filled pore space of the soil (hypoxia) appears during short-term flooding when the roots are submerged under water, but the shoots remain in the atmosphere. The total lack of oxygen (anoxia) occurs in soils after long-term flooding, in plants completely submerged by water. The extent of soil moisture and its aeration status can be characterized with the use of several indicators such as total water capacity (TWC), air-filled porosity (E_g), oxygen diffusion rate (ODR), and redox potential (E_h) (Gliński and Stepniewski 1985; Kozlova 2009). Optimal conditions for plant growth are usually created if the soil moisture under watering is brought to 60–70 % of the total water capacity (Kozlova 2009). Under drought conditions, soil moisture decreased up to 30 % and lower; under the full soil flooding, it increased up to 120 % of TWC by the volume (Kalashnikov et al. 1994). The availability of oxygen to roots of plants changed under flooding during a few hours as following: E_g decreased from 25–30 to 0 % (Balakhnina et al. 2009); ODR in the root zone decreased from 120 to 6 μg m^{-2} s^{-1} (Zakrzhevsky et al. 1995) or from 2.28–3.44 to 0.09–0.28 μmol O_2 m^{-2} s^{-1}; Eh decreased from 543 to 70 mV (Balakhnina et al. 2009, 2012). The soil measurements performed during the period of drainage showed that the soil parameters in the flooded treatment variants returned to the control values already after 2 days of free drainage (Balakhnina et al. 2010).

5.3 Plant Responses to Different Levels of the Soil Aeration

Soil hypoxia induces detrimental changes in metabolic processes (Grineva 1975; Chirkova 1978, 1988). Soil flooding results in a wide variation of the root aeration. The oxygen diffusion rate, being an adequate indicator of O_2 available for the root system, may vary in waterlogged soils. In connection with data on seed germination of several plant species (Gliński et al. 1984), ODR can be distinguished by three ranges: (1) the optimal range, ODR_a; variations within this range have practically no effect on seed germination; (2) the limiting range, ODR_b; at medial ODR values in this range, the germination percentage decreases by about 50 %; and (3) the critical range, ODR_c; within this range the germination percentage diminishes to zero. For maize seedlings, the ODR_a extends the value of 40 μg m^{-2} s^{-1}, the ODR_b ranges between 40 and 16 μg m^{-2} s^{-1}, and the ODR_c is below 16 μg m^{-2} s^{-1}.

The effects of various levels of available oxygen in the soil on remotely located protective systems preventing oxidative destruction, i.e., on heterotrophic root cells, the primary target of soil O_2 deficiency, and on phototrophic leaf cells, have been compared (Zakrzhevsky et al. 1995). The latter can be considered as a secondary target because they remain under natural aeration and experience indirect O_2 deficiency due to disturbed interrelation between roots and aboveground organs. The efficiency of the protective system was evaluated from the activity of

SOD as an enzyme neutralizing superoxide anion radicals, from MDA level indicating the rate of free radical lipid oxidation, and from the root and shoot biomass production and pigment concentration in the leaves (Zakrzhevsky et al. 1995).

Selye (1974) classified three phases in an organism response to stress factors: (1) the alarm phase, (2) the phase of elevated resistance, and (3) the exhausting phase (depletion of adaptability). Numerous experiments on animals have revealed the activation of lipid peroxidation during the alarm phase. The products of lipid peroxidation may serve as primary mediators in stress development (Baraboi 1991). The activation of lipid peroxidation during the alarm phase is transient event and, unfortunately, is usually not recorded (Gulyaeva et al. 1988; Baraboi 1991). Nevertheless, during this period, the animal organism undergoes essential biochemical and physiological changes including a massive release of adrenaline and corticosteroids (Gulyaeva et al. 1988) (these substances act as traps for free radicals, superoxide radicals in particular) and perturbation of microcirculation. It was revealed two stages in the alarm phase of stress development. The activation stage of free radical oxidation is preceded by an even shorter primary stage of inhibiting lipid peroxidation. This stage is termed as the stage of urgent adaptation to the stress factor; it ensures the short-term activation of preexisting adaptive mechanisms (Gulyaeva et al. 1988). A large reserve of antioxidants together with an excess of radical-trapping hormones prevents the activation of free radical oxidation in tissues.

During further stress development, the composition of membrane lipids undergoes modification, which makes the membranes more susceptible to lipid peroxidation. This high sensitivity to peroxidation partially results from increasing the fraction of readily oxidizable phospholipids, lowering the level of biological antioxidants and excessive production of oxygen radicals in the site with damaged microcirculation.

During prolonged exposure to unfavorable environmental factors, including hypoxia, the surviving of plant cells depends on the induction of a long-term adaptive mechanism, i.e., activation or biosynthesis of protective enzymes (Monk et al. 1987; Gulyaeva et al. 1988). During this period, cells maintain their viability by entering the phase of elevated resistance. In contrast to the stimulation of lipid peroxidation in the alarm phase (Baraboi 1991), an antioxidant response prevails during the resistance phase due to activation or synthesis of protective enzyme systems (SOD, catalase, peroxidase, and glutathione reductase). These enzymes constitute the “first line of defense,” which protects cells from toxic-free radicals by converting them into harmless compounds (Merzlyak 1989; Braun and Mozhenok 1987).

5.3.1 *SOD Activity and MDA Content in the Roots and Leaves of* Zea mays *L. Plants*

Changes in SOD activity and MDA content depend on the stage of stress development. Table 5.1 shows that after two days of the experiment, the SOD activity in the roots of maize plants, grown at ODR_a, amounted to 1,240 μg^{-1} DW and was 2.5 times lower than that in the leaves. In roots of the plants that were grown at ODR_b (35 and 27 $\mu g\ m^{-2}\ s^{-1}$) for the last 2 days, SOD activity increased by 22 % with respect to control level (ODR_a). Seedlings growing at ODR_c resulted in additional increase in root SOD activity (by up to 56 to 48 %), but there was no significant variation in leaf SOD activity (Table 5.1).

After 8 days of the experiment, the SOD activity in the roots of the plants, grown at ODR_a, decreased to 877–879 μg^{-1} DW (by 30 %), and leaf SOD activity increased to 3,519–3,905 μg^{-1} DW (by 11–19 %). SOD activity in the roots and leaves of the plants grown at ODR_b increased by 23 % and 54 %, respectively, as compared to SOD activities in the plants, grown at ODR_a. The transition to ODR_c resulted in an additional increase in the enzyme activity both in root and leaf tissues (by 91–59 % and 140–129 %, respectively).

Table 5.1 The SOD activity and MDA content in the roots and leaves of maize plants grown for 2, 8, and 12 days at various oxygen diffusion rate (ODR) in soil (error of the mean values does not exceed 8 %)

ODR, $\mu g\ m^{-2}\ s^{-1}$	SOD activity, units g^{-1} DW (%)		MDA, nmol g^{-1} DW (%)	
	Roots	Leaves	Roots	Leaves
2 days				
97	1,240 (100)	3,179 (100)	255 (100)	1,821 (100)
35	1,517 (122)	3,481 (109)	152 (60)	1,816 (100)
27	1,517 (122)	3,223 (101)	149 (58)	1,670 (92)
9	1,933 (156)	3,338 (105)	182 (71)	1,729 (95)
6	1,776 (143)	2,909 (92)	202 (79)	1,547 (85)
8 days				
115	877 (100)	3,519 (100)	263 (100)	1,614 (100)
90	879 (100)	3,905 (111)	256 (97)	1,524 (94)
39	1,076 (123)	5,418 (154)	127 (48)	1,304 (81)
11	1,673 (191)	8,438 (240)	–	905 (56)
7	1,398 (159)	8,049 (229)	207 (79)	956 (60)
12 days				
103	864 (100)	2,989 (100)	259 (100)	1,160 (100)
85	924 (107)	2,689 (90)	243 (94)	885 (76)
18	569 (66)	3,070 (103)	151 (58)	457 (39)
9	707 (82)	10,100 (339)	236 (91)	502 (43)
8	644 (75)	8,354 (280)	232 (90)	409 (35)

*In Tables 5.1–5.5, plants were 10 days old at the beginning of the experiment (Zakrzhevsky et al. 1995)

After 12 days of the experiment in plants grown at ODR_a, the root SOD activity fell by 25–30 %, as compared to that after 2 days of the experiment, whereas the decrease of SOD activity in the leaves was statistically insignificant. SOD activity in the roots of the plants grown at ODR_b increased considerably (by 18–34 %), which contrasts with the SOD activity increase induced by 2- and 8-day-long stresses. In the plants grown at ODR_c, leaves exhibited a 241–180 % increase in SOD activity, which is even higher than under 2- and 8-day stresses. In roots there was no significant variation in SOD activity.

The MDA contents in the roots of maize plants after 2, 8, and 12 days growing at ODR_a were 255, 263–256, and 256–243 nmol g^{-1} DW, respectively (Table 5.1). While imposing the hypoxic stress, we consistently observed the lowest content of MDA in roots grown at ODR_b, i.e., at a moderate reduction in root aeration. The initial amount of MDA in the leaves was much higher than in the roots. On lowering the ODR in the soil, the MDA content in leaves decreased and attained the lowest level at ODR_c as opposed to at ODR_b, ensuring the minimum MDA level in roots.

5.3.2 *SOD Activity and MDA Content in the Roots and Leaves of* Pisum sativum *L. Plants*

Because of the high sensitivity of pea plants to oxygen deficiency, the ODR ranges affecting seed germination are narrower for pea than for maize (Gliński et al. 1984). For pea plants, ODR_a extends value of 50 μg m^{-2} s^{-1}, ODR_b ranges from 50 to 25 μg m^{-2} s^{-1}, and ODR_c is below 25 μg m^{-2} s^{-1}. Disturbance of growth and metabolic processes in pea plants arose from smaller doses of hypoxic treatment than in maize (Gliński et al. 1984). After 8 days of plant growing at ODR_c was observed significant inhibition of SOD activity not only in the root, as was observed with maize plants, but also in the leaves (Table 5.2).

The reduction in SOD activity on changing from ODR_a to ODR_c was accompanied by an increase in the MDA content; this fact suggests acceleration of lipid peroxidation at low ODR values. After 12 days of the experiment SOD activity in the roots of pea plants grown at ODR_a was lower but in the leaves was higher than those after 8 days. When passing from ODR_a to ODR_c, the leaf SOD activity decreased and the MDA content increased, as in 8-day-treated plants. Unlike leaves, roots exhibited diminishing SOD activity (reduction by 84 % on transition from ODR_a to ODR_c) in parallel with decreasing amounts of MDA (decreased by 39 %).

Table 5.2 The SOD activity and MDA content in the roots and leaves of pea plants grown for 8 and 12 days at various oxygen diffusion rate (ODR) in soil (error of the mean values does not exceed 8 %)

ODR, μg m^{-2} s^{-1}	SOD activity, units g^{-1} DW (%)		MDA, nmol g^{-1} DW (%)	
	Roots	Leaves	Roots	Leaves
8 days				
117	660 (100)	3,962 (100)	452 (100)	665 (100)
115	676 (102)	3,219 (81)	430 (95)	689 (104)
103	621 (94)	3,614 (91)	462 (102)	717 (108)
17	430 (65)	2,956 (75)	599 (133)	978 (147)
7	343 (52)	2,422 (61)	660 (146)	1,055 (159)
12 days				
115	557 (100)	5,555 (100)	833 (100)	682 (100)
90	430 (77)	5,168 (93)	809 (97)	673 (99)
39	593 (106)	5,764 (103)	795 (95)	671 (99)
11	123 (22)	4,663 (84)	597 (72)	903 (133)
7	89 (16)	3,199 (58)	510 (61)	796 (117)

5.3.3 *Growth and Chlorophyll Contents in Maize and Pea Plants at Different ODR in the Soil*

Table 5.3 shows the 41 % reduction in root biomass in maize as compared to control after plant growing in the waterlogged soil at ODR_c for 12 days. At the same time, the weight of shoots of maize plants diminished by no more than 15 %.

In contrast, in hypoxia-sensitive pea plants, biomass production declined significantly after 12-day flooding both in the roots and shoots (reduction by 69 % and 66 %, respectively). The damage to shoots was caused by an impaired root system.

In the maize plants subjected to the 12-day hypoxic stress, chlorophyll and carotenoid contents in the leaves diminished by 44 % and 42 %, respectively, as compared to non-stressed plants (Table 5.4). The degradation of the pigments proceeded faster in pea leaves than in maize, which corresponds to a stronger inhibition of biomass production in pea plants under a hypoxic stress. The faster destruction of pigments in pea leaves is clear from comparing the reduced carotenoid levels in the leaves of stressed maize and pea plants (reduced by 42 % and 58 %, respectively.

5.3.4 *Stomata Resistances (R_D) in Leaves of Maize and Pea Plants at Different ODR in the Soil*

Plant stomata are considered as physiological regulators of photosynthetic rate. Therefore, the data on the relationship between stomata resistance in maize and pea leaves and soil-attributed ODR values (Table 5.5) seems to be important. In plants grown under various root aeration, the stomata resistance rose with a lowering of

Table 5.3 Biomass of roots and shoots in maize and pea plants grown for 12 days at various oxygen diffusion rate (ODR) in soil (error of the mean values does not exceed 8 %)

ODR, μg m^{-2} s^{-1}	Dry weight, g plant (%)	
	Roots	Shoots
Maize		
103	0.33 (100)	0.95 (100)
85	0.31 (94)	0.99 (104)
18	0.29 (88)	0.93 (98)
17	0.21 (64)	0.87 (92)
8	0.19 (58)	0.81 (85)
Pea		
117	0.083 (100)	0.33 (100)
103	0.083 (100)	0.73 (99)
83	0.087 (104)	0.70 (96)
14	0.039 (47)	0.32 (44)
9	0.026 (31)	0.25 (34)

Table 5.4 Pigment contents in the leaves of maize and pea plants grown for 12 days at various oxygen diffusion rate (ODR) in soil (error of the mean values does not exceed 6 %)

ODR, μg m^{-2} s^{-1}	Pigment content, μg g^{-1} DW (%)	
	Chlorophylls	Carotenoids
Maize		
103	8.89 (100)	1.74 (100)
85	7.06 (79)	1.57 (90)
18	5.07 (57)	1.25 (72)
9	4.40 (49)	1.09 (63)
8	4.05 (46)	1.01 (58)
Pea		
117	11.65 (100)	2.27 (100)
103	11.59 (99)	2.09 (92)
83	11.29 (97)	2.07 (91)
14	5.68 (48)	1.25 (55)
9	4.65 (40)	0.97 (42)

ODR. When flooding, the roots export aminocyclopropane carboxylic acid along the xylem upward, and the leaves convert it into ethylene, which promotes abscisic acid formation. Abscisic acid controls stomata closure (Kefeli et al. 1989), with a consequent effect on the photosynthetic rate.

The increase in stomata resistance and inhibition of photosynthesis at high light intensities and hypoxic soil conditions should restrict the electron flow to the final acceptor. Excessive supply of electrons into the electron transport chain creates favorable conditions for oxygen activation, because oxygen competes with CO_2 for accepting electrons. Excessive formation of active oxygen forms related to impaired photosynthesis should, in turn, affect the activity of defensive system against oxidative destruction that was shown in by us (Tables 5.1, 5.2, and 5.5).

Table 5.5 Stomata resistance (R_d) in the leaves of maize and pea plants grown for 2, 8, and 12 days at various oxygen diffusion rate (ODR) in soil (error of the mean values does not exceed 6 %)

2 days		8 days		12 days	
ODR, $\mu g \cdot m^{-2} \cdot s^{-1}$	R_d, $s\ cm^{-1}$	ODR, $\mu g\ m^{-2} \cdot s^{-1}$	R_d, $s\ cm^{-1}$	ODR, $\mu g\ m^{-2} \cdot s^{-1}$	R_d, $s\ cm^{-1}$
Maize					
97	6.4	115	5.9	103	6.5
35	4.7	90	6.1	85	7.8
27	7.6	39	6.3	18	8.1
9	6.9	11	9.3	9	11.9
6	10.9	7	10.9	8	12.4
Pea					
117	2.7	117	2.2	117	3.3
115	2.8	115	2.5	103	3.4
103	2.8	103	2.6	83	2.8
17	2.7	17	4.8	14	12.7
7	6.6	7	80.6	9	72.4

5.3.5 *Discussion*

Thus, our data suggest that the resistance phase comprising SOD activation and lipid peroxidation deceleration is preceded by a phase, at which the amount of MDA diminishes, although SOD activity remains practically unchanged. This situation persisted in leaves of maize plants that were grown for the last 2 days at various soil ODR (Table 5.1). In the roots of these plants, only the second stage of the resistance phase, that is, SOD activation concurrent with the decline in lipid peroxidation, was observed. In leaves, this stage of SOD activation, associated with inhibition of lipid peroxidation, started later and was evident on the 8th day of stress.

From studying the effects of prolonged flooding on iris plant metabolism, Monk et al. (1987) proposed that anaerobic conditions may induce an adaptive synthesis of SOD in addition to SOD activation.

The concept of SOD as an anaerobiosis-specific polypeptide deserves, in our view, special investigation because this problem deals with the evolution of mechanisms, by which aerobic organisms protect themselves from active oxygen forms.

In plants exposed to low ODR for a sufficiently long time, the stress load may become critical and lead to exhaustion of adaptive abilities, a lowering of antioxidant activity, and secondary enhancement of lipid peroxidation. These metabolic changes indicate the beginning of the exhaustion phase in the development of stress response in living organisms (Selye 1974). The beginning of SOD inactivation may not coincide with the rise of lipid peroxidation. After 12 days of maize plants growing under stressful conditions (Table 5.1), the rate of lipid peroxidation remained at the low level while the root SOD activity decreased by 25 %. The

beginning of the exhaustion phase, indicated by decreasing activity of protective enzyme SOD, had no immediate effect on overall antioxidant capacity of cells. Apparently, there is some period of cellular resistance to lipid peroxidation: it may rely on either sufficiently high initial SOD activity or operation of addition mechanisms protecting cells from oxidative destruction at another level (mechanism involving antioxidants and phospholipids of cell membranes). For example, unsaturated fatty acids in the phospholipids offer a target for free radical attack, thereby protecting other systems from destruction (Feofilova et al. 1987).

The stage of SOD inactivation accompanied by a secondary rise in the MDA level manifested itself, as a part of the exhaustion phase, in roots and leaves of pea plants after 8 days of soil flooding stress (Table 5.2).

Therefore, the stepwise pattern of SOD kinetics, clearly evident in heterotrophic root cells affected by the soil hypoxia, was also revealed in phototrophic cells of leaves growing in normal air. The indirect effect of oxygen deprivation may result from a deteriorated root metabolism in roots and consequent detrimental changes in assimilatory tissues; such changes would promote generation of the reduced O_2 free radicals. Change in SOD activity appeared in phototrophic leaf cells later than in heterotrophic root cells; however, in both cases, SOD demonstrated a clear phasic pattern.

The duration and magnitude of each phase depend on the stress load and individual resistance of the crop. In maize plants capable of withstanding low ODR, the phase of SOD activation was longer than in hypoxia-sensitive pea plants. The phase of the adaptability exhaustion in the roots and leaves of pea plants took place at comparatively low stress-load doses. In maize plants, these stress doses inhibited the protective systems in roots only but raised SOD activity in leaves.

In maize plants cultivated in flooded soil, the growth of the primary root diminished or ceased, whereas new adventitious roots start growing. Some of the physiological properties of these newly formed roots differ from the primary root; in particular, they have a higher resistance to anaerobiosis (Grineva 1975).

Degradation of pigments in metabolically suppressed phototrophic cells and the influence of SOD activity on the pigment system are common knowledge (Monk et al. 1987; Merzlyak 1989). In waterlogged plants, the supply of kinetin and amino acid from roots to leaves diminishes, which may cause, among other factors, development of chlorosis (Grineva 1975).

The decline in biomass production starts earlier in roots than in shoots (Table 5.3). After 12 days growing at ODR_c, the rate of dry matter of the pea plants gain lessened by 69 % (Table 5.3), and residual SOD activity constituted only 16–22 % with respect to the initial level (Table 5.2). It means the spreading of destructive alterations throughout the organs. These deleterious changes involve membrane-located lipid peroxidation, cell disorganization, modification of the cell surface and cytoskeleton, swelling of mitochondria, elevating Na^+ concentration, and lowering of the level of K^+ and Mg^{2+}. Subsequent increases in the cytoplasmic Ca^{2+} content induce activation of phosphatases, which leads to destruction of phospholipids, formation of free fatty acids, and the final collapse of the cell system (Braun and Mozhenok 1987).

5.3.6 Conclusion

Under conditions of soil hypoxia, antioxidant protective systems change similarly in heterotrophic root cells directly targeted by oxygen deficiency and in phototrophic leaf cells maintaining normal aeration. The magnitude and kinetics of these changes depend on the rates of main physiological process. When oxygen availability in soil decreased and the stress factor dose is increased, the activity of SOD (an enzyme, eliminating superoxide anion radicals) changes. These changes resemble phasic progress of the nonspecific adaptive syndrome in multicellular systems manifesting itself in regulation formation and detoxification of excessive amounts of active oxygen forms. Each phase has its own specific stages that reflect shifting in the dynamic equilibrium between the antioxidative activity and lipid peroxidation under developing stress conditions. The duration of each stage, as well as the magnitudes of SOD activity and free radical oxidation, characterizes the individual plant resistance to oxygen deprivation.

5.4 *Vicia faba major* L. cv. Bartom Plant Reactions to Soil Flooding and Subsequent Drainage

To investigate the effect of soil hypoxia and subsequent re-aeration on the adaptive potential of *V. faba major* L. (cv. Bartom), the plants were exposed to 13–27 days of soil flooding and to subsequent soil drainage of 14 days. The adaptive potential was estimated by the activity of the antioxidant enzymes SOD and GR.

The intensity of oxidative damage was evaluated by TBARs concentration in plant leaves. Under soil flooding, the availability of oxygen to roots of the *V. faba* plants changed during a few hours as following: Eg decreased from 15–17 to 0 %; ODR in the root zone decreased from 2.28–3.44 to 0.09–0.28 μmol O_2 m^{-2} s^{-1}; Eh decreased from 543 to 70 mV. The soil measurements performed during the period of drainage showed that the soil parameters in the flooded treatment returned to the control values already after two days of free drainage.

5.4.1 Shoot Dry Mass and Pigment Concentration

Shoot dry mass of the control plants increased from 0.62 to 4.7 g per plant during 27 days of the experiment (Fig. 5.1). The biomass growth of the plants subjected to flooding did not differ from the control plants during the 15 days of the flooding and began to lag significantly at the end of the experiment.

Chlorophyll (a + b) concentration in the leaves of control plants did not change considerably during 27 days of growth (Fig. 5.2a). Soil flooding caused a significant decrease in this parameter after 15 days of the experiment; at the end of the

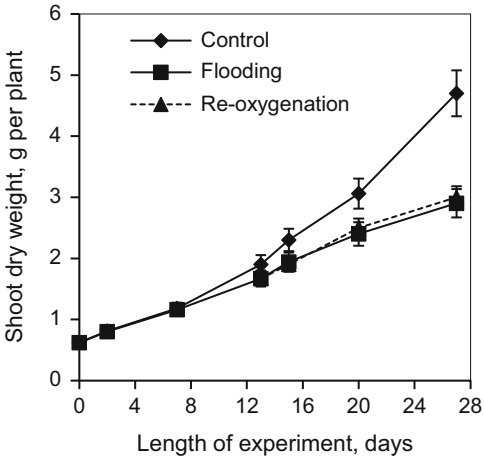

Fig. 5.1 Shoot dry weights of *Vicia faba* plants grown under different soil conditions: control, optimal watering; flooding, 27-day soil flooding; reoxygenation, 14 days of soil reoxygenation following 13-day flooding. The values are means of three replicates (pots) ± standard deviations (SD)

experiment (27 days), chlorophyll concentration in the leaves of flooded plants was 63 % from that in the control ones.

Drainage of the soil did not succeed to increase significantly the shoot biomass and chlorophyll concentration above that of the flood treatment. Like chlorophyll, concentration of carotenoids in the leaves of control plants did not show a tendency to change with time (Fig. 5.2b). Carotenoid concentration in the leaves of flooded plants, as compared with the control ones, decreased significantly after 15 days of soil flooding. Drainage of the soil did not change significantly the concentration of carotenoids during the experiment as compared with the plants which remained flooded.

5.4.2 LPO Processes

The concentration of TBARs in the leaves of control plants showed a typical increasing tendency from 1.7 to 2.6 μmol g^{-1} DW during 27 days of plant growth under experimental conditions (Fig. 5.3). Under soil flooding, the TBARs concentration in plant leaves increased strongly during the first 2 days of flooding, reaching 3.2 μmol g^{-1} DW. The elevated level of TBARs, 3.2–2.7 μmol g^{-1} DW, which was significantly higher than that in control plants, maintained till the 13th day of the flooding experiment. After that the TBARs concentration in flooded plants dropped down and became significantly lower than in the control plants. Drainage of the soil caused a significant reactivation of the oxidative processes in the leaves; the TBARs concentration here was intermediate between the control and flooding treatments (Fig. 5.3).

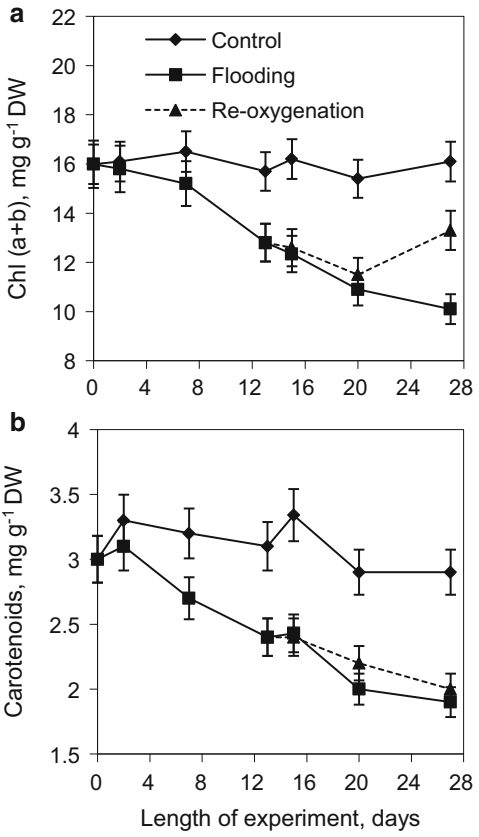

Fig. 5.2 Concentration of chlorophyll (a + b) (**a**) and carotenoids (**b**) in the leaves of *Vicia faba* plants grown under different soil conditions: control, optimal watering; flooding, 27-day soil flooding; reoxygenation, 14 days of soil reoxygenation following 13-day flooding. The values are means of three replicates ± standard deviations (SD). *DW* dry weight

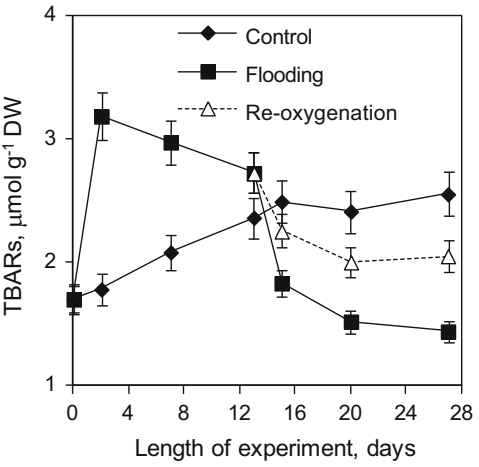

Fig. 5.3 TBARs concentration in the leaves of the *Vicia faba* plants grown under different soil conditions: control, optimal watering; flooding, 27-day soil flooding; reoxygenation, 14 days of soil reoxygenation following 13-day flooding. The values are means of three replicates ± standard deviations (SD). *DW* dry weight

5.4.3 SOD and GR Activities

Activity of SOD in the leaves of control plants increased during the first 15 days of plant growth from 45.3 to 54.5 U mg^{-1} DW and then decreased back to initial value at the end of the experiment (Fig. 5.4). Soil flooding led to a significant increase in the SOD activity by 22–35 % of that in control plants, during the first 13 days of the oxygen stress period. Then the enzyme activity decreased, and there were no significant differences between the flooded and control plants. Under soil drainage the SOD activity increased initially (for 2 days) up to 137 % of that in the control treatment (128 % of the flooding treatment) and then decreased to the level of enzyme activity in the control plants.

Activity of GR in the leaves of control plants was 6.4 μM $NADP^+$ g^{-1} dry mass min^{-1} at the beginning of the experiment and did not change significantly during the 27 days of plant growth (Fig. 5.5). In the flooded treatment, the GR activity was

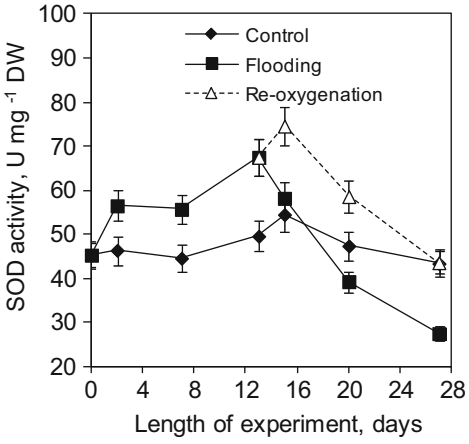

Fig. 5.4 SOD activity in the leaves of the *Vicia faba* plants grown under different soil conditions: control, optimal watering; flooding, 27-day soil flooding; reoxygenation, 14 days of soil reoxygenation following 13-day flooding. The values are means of three replicates ± standard deviations (SD). *DW* dry weight

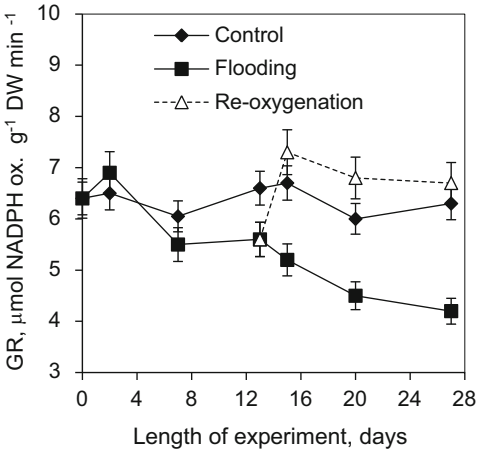

Fig. 5.5 GR activity in leaves of the *Vicia faba* plants grown under different soil conditions: control, optimal watering; flooding, 27day soil flooding; reoxygenation, 14 days of soil reoxygenation following 13-day flooding. The values are means of three replicates ± standard deviations (SD). *DW* dry weight

significantly different from the control one till the 13th day of the flooding but then dropped down and was considerably lower during the 15–27 days period of the flooding. Final value of the GR activity in the flooded plants was about 66 % of the control and of the initial levels. Soil reoxygenation resulted in a significant reactivation of GR up to the level of enzyme activity in the control plants or even higher. Notably, the elevated value of GR activity was significantly higher in the reoxygenation treatment than that in the flooded plants (Table 5.1).

5.4.4 Discussion

Results obtained for *V. faba* exposed to soil flooding allowed us to distinguish two different stages of stress development in leaves of plants with roots in hypoxia. The first stage, 13 days from the stress onset, was characterized by an increase in the TBARs concentration and in the SOD activity. In this stage the shoot dry mass of the stressed plants was close to that of the control ones. It should be noted that in the leaves of another species in the Papilionaceae, flooding-sensitive pea, the intensity of LPO during 12 days of soil hypoxia also increased, but the SOD activity decreased and the shoot dry mass fell below 34 % (Zakrzhevsky et al. 1995). Thus, activation of SOD in the leaves of plants with roots in hypoxia in the first stage of stress development is an indicator of plant adaptive potential. Unlike SOD, the activity of GR in *V. faba* leaves decreased already after 2 days of soil hypoxia. According to Biemelt et al. (1998), the wheat seedlings after 8 days of hypoxic stress development showed a decrease in the GR activity in the roots and an increase in ascorbate peroxidase activity and in the concentration of total ascorbate (from 2.64 to 3.4 μmol g^{-1} fresh weight) and glutathione (from 576 to 657 nmol g^{-1} fresh weight) related to an increase in the reduced forms (AsA and GSH). Authors explained these results as an acclimatization of wheat roots to hypoxia. The analogous decrease in the GR activity was found in the maize leaves (Yan et al. 1996) after 7 days of soil hypoxia, and it was also accompanied by an increase up to 185 % for AsA and 142 % for GSH concentrations as compared to the controls. In our studies (Balakhnina et al. 2009) an increase in ascorbate peroxidase activity up to 200 % was shown in the leaves of flooded barley plants. In the present work we did not measure ascorbate peroxidase activity and concentrations of AsA and GSH and can only speculate that observed decrease in GR activity in *V. faba* leaves under the first stage of stress development is not accompanied with a decrease of low-molecular antioxidants and corresponding inhibition of leaf metabolism. Similar growth of shoot biomass of the control and flooded plants during 15 days of flooding is a non-direct support for such conclusion. The second stage of stress development in *V. faba* was characterized by a stepwise decrease in shoot dry mass and pigment concentration in the leaves. Similar decrease of shoot dry mass was observed just after 2 days of anoxia in young wheat seedlings (Biemelt et al. 1998). In other words, the second stage of hypoxic stress development in *V. faba* is the strong stress. The TBARs concentration as well as SOD and GR

activities also decreased in this stage, indicating the suppression of metabolic activity of the cells. A decrease of the main metabolic processes such as photosynthetic assimilation of CO_2 was shown repeatedly in the leaves of flooded plants (Kalashnikov et al. 1994; Yordanova and Popova 2007). It should be noted that at decreasing metabolic activity, the plant cells can be less sensitive to the effects of unfavorable factors and such response has been considered by Chirkova (1988) as an adaptive feature under strong stress conditions. Many plants are able to survive hypoxia or anoxia but then die during re-aeration, confirming that oxidative damages occur during reoxygenation stage (Hunter et al. 1983; Monk et al. 1987). In our experiment, upon drainage following 13-day flooding, the LPO in the leaves of *V. faba* increased as compared with the plants that remained flooded but did not exceed the corresponding values in the control plants. Increased posthypoxic and postanoxic damages are thought to be the results of the generation of ROS (Albrecht and Wiedenroth 1994). Reactivation of cell metabolism is accompanied with accelerated mitochondrial electron transport to oxygen which leads to excessive formation of ROS, initiating posthypoxic damages (Elstner 1990). Reactivation of cell metabolism in *V. faba* leaves after soil drainage and thus oxygen reentry to roots went with an increase in the SOD and GR activities in leaves. In this case, the levels of enzyme activities reached maximums after 2 days from the beginning of soil drainage and then decreased gradually to the values in the control plants at the end of the experiment. The analogous reactions to re-aeration were shown in flooded rice seedlings (Ushimaro et al. 1992). The authors found that the activities of SOD, GR, and other antioxidant enzymes were lower in seedlings germinated under water for 6 days than in those germinated in air for the same period of time.

When submerged seedlings were exposed to air, the activities of these enzymes increased to or exceeded the levels in aerobically grown controls during 24 h of adaptation to air (Ushimaro et al. 1992). Interestingly, GR activity was found to be increased in cotton leaves exposed to excessive (75 %) O_2 concentration (Foster and Hess 1980). Thus, the increase in GR activity in *V. faba* leaves after oxygen reentry to roots may reflect increased concentration of GSH which neutralizes H_2O_2 produced by superoxide dismutase. In our experiments the concentration of TBARs in the leaves of *V. faba* increased after soil drainage as compared to the flooded plants but was lower than that in the control ones. Concentration of photosynthetic pigments also increased by oxygen reentry to roots, but no significant deviations in the shoot biomass accumulation relative to the flooded treatment were found. These data confirm that *V. faba* is able to keep the LPO intensity nearly at a level that is safe for plants and this feature would contribute to its tolerance in leaves of plants with roots exposed to oxygen deficit.

5.4.5 Conclusion

Thus, *V. faba* can survive the 2-week soil flooding without serious damages to the physiological function of shoots. Under longer term flooding for 27 days, two stages of stress development are described. At the first stage, plant tolerance is related to an increase in the SOD activity. The second stage is characterized by inhibition of shoot growth and activities of antioxidant enzymes, SOD and GR. *V. faba* is also resistant to soil drainage after flooding. In this case, plant tolerance as indicated by leaf functioning might be determined by the activation of SOD and GR.

5.5 Specific and Nonspecific Spring Rape Plants Responses to Soil Flooding

Plant adaptation to soil hypoxia includes series of interconnected reactions directed to survival during the periods of hypoxic and anoxic conditions and to the homeostasis maintenance. Anatomical and morphological changes help to provide oxygen to the plant tissues (Colmer 2003; Pederson et al. 2009; Vartapetian et al. 2003). In connection with the specificity of the effect of hypoxia on plants, of special interest are compensatory changes connected with transformations of respiration pathways. Under oxygen deficiency most plants exhibit intensification of glycolysis accompanied with accumulation of lactate and ethanol (Rocha et al. 2010). Under prolonged and deep hypoxic stress, the formation of ethanol prevails. Production and utilization of this phytotoxic product is connected with functioning of alcohol dehydrogenase (ADH; EC 1.1.1.1). Activity of this enzyme under hypoxia or anoxia increases significantly through induction of the alcohol dehydrogenase gene expression. The ADH gene family consists of 1–4 members, depending on the plant species. The developmental expression and tissue-specific responses of each gene member to hypoxic stress were demonstrated on various species (Garnczanska 2002; Preiszner et al. 2001; Wignarajah et al. 2010).

Formation of reactive oxygen species (ROS) appears to be an unspecific plant response to different stress factors, including hypoxia. In particular ROS may be formed in electron transport chains, because of $NADP^+$ limitation. Due to this oxygen becomes an alternative electron acceptor. Induction of such ROS as superoxide radical (O^{2-}) and hydrogen peroxide (H_2O_2) initiates peroxidation of lipids, proteins, pigments, and other cell compounds (Arbona et al. 2008; Balakhnina et al. 2009, 2010) and leads to serious damage of cells and of the entire organism. Plants possess an evolutionary formed defensive system against oxidative destruction. It consists of low-molecular antioxidants (ascorbic acid, reduced glutathione, tocopherols, and others) and antioxidant enzymes decomposing ROS. Among antioxidant enzymes, superoxide dismutase (SOD; EC 1.15.1.1) has been identified as an essential component in the organism defense mechanism. Besides SOD, superoxide can be scavenged directly by ascorbate or glutathione. Neutralization

of formed H_2O_2 occurs in ascorbate-glutathione cycle with participation of ascorbate peroxidase (AsP; EC 1.1.1.1), glutathione reductase (GR; EC 1.6.4.2), and other enzymes (Asada 2006). The pertinent literature presents data on correlation of the degree of resistance to extreme temperatures, salinity, drought, and other stress factors with the free proline concentration in the plant tissues. According to different authors, the proline accumulation in plant should be considered as one of the links of the common chain of biochemical adaptation mechanisms functioning in stressed plants (Britikov 1975; Radyukina et al. 2008; Shevyakova et al. 2009). Some reports showed an increase in proline concentration in plants under soil flooding (Yordanova and Popova 2007). It was found that under waterlogging conditions, the number of new leaves, root length, plant height, and plant biomass and content of soluble protein in roots of *Malus* species declined, while the content of malondialdehyde (MDA) and proline, the generation rate of superoxide radical, the relative membrane permeability, and the activities of SOD and peroxidase in roots significantly increased (Bai et al. 2008).

To study the specific and unspecific ways of higher plant adaptation to root hypoxia, the spring rape *Brassica napus* L. was exposed to 8 days of soil flooding (Balakhnina et al. 2012). The advancement of soil redox processes was characterized by redox potential. The ADH activity was assessed in the plant roots. The intensity of peroxidation process estimated by concentration of thiobarbituric acid reactive substances (TBARs) and plant antioxidant potential evaluated by the levels of SOD and GR activities were determined in the roots and leaves. AsP activity and proline concentration were measured in the leaves.

5.5.1 The ADH Activity in the Roots of Flooded Plants

Stress responses of spring rape to soil hypoxia were investigated during 8-day flooding. Soil air-filled porosity decreased from 25–30 to 0 %, oxygen diffusion rate from 2.6–3.5 to 0.34 μmol O_2 m^{-2} s^{-1}, and redox potential from 460 to 150 mV within a few hours. Average ODR in soil under control conditions ranged at 2.6–3.5 μmol O_2 m^{-2} $s^{-1}$and for the flood period declined to 0.34 μmol O_2 m^{-2} s^{-1}.

The ADH activity in the roots of control plants did not show significant changes (Fig. 5.1) during the experiment. After the first day of flooding, ADH activity in the roots increased up to sevenfold of the control and remained at the same level till the third day. Then the ADH activity in flooded plants decreased but remained significantly higher (170 %) than that in control plants (Fig. 5.6; Table 5.6).

5.5.2 The Intensity Oxidative Processes

The intensity oxidative processes were determined by the MDA or more exactly by the TBARs concentration. The value of this parameter in the roots of the control

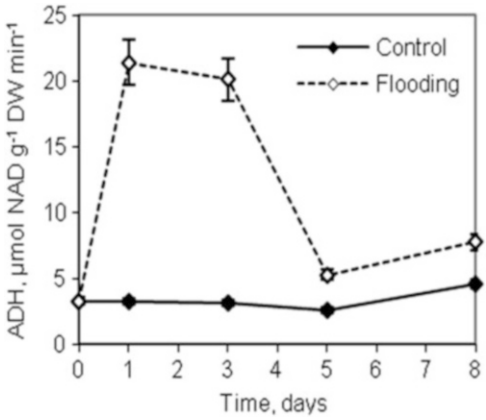

Fig. 5.6 Alcohol dehydrogenase (ADH) activity in roots of *Brassica napus* grown under optimal soil watering (control) and soil flooding (flooding). The values are means of three replicates (pots) ± standard deviations (SD)

Table 5.6 Effects of soil flooding on alcohol dehydrogenase (ADH) activity; thiobarbituric acid reactive substances (TBARs) concentration; superoxide dismutase (SOD), ascorbate peroxidase (AsP), and glutathione reductase (GR) activities; and proline concentration of *Brassica napus* expressed as *P*-values (probability values for rejection of the null hypothesis) from ANOVA test

	Time (days)				
Parameters	0	1	3	5	8
Roots (control/flooding)					
ADH	1.00	0.001***	0.001***	0.001***	0.001***
TBARs	1.00	0.009**	0.002**	0.001***	0.001***
SOD	1.00	0.005**	0.301	0.556	0.001***
GR	1.00	0.121	0.002**	0.002**	0.001***
Leaves (control/flooding)					
TBARs	1.00	0.54	0.001***	0.002**	0.323
SOD	1.00	0.001***	0.001***	0.001***	0.001***
AsP	1.00	0.003**	0.002**	0.351	0.856
GR	1.00	0.088	0.042*	0.553	0,139
Proline	1.00	0.001***	0.001***	0.001***	0.186

Level of significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$
Effects were tested with one-way ANOVAs (GLM1)
Average various traits are given in Figs. 5.6–5.11

plants showed a gradual increase from 0.25 to 0.38 μmol g^{-1} DW during the entire experimental period (Fig. 5.7a). In the leaves of the control plants, the TBARs concentration was by one order of magnitude higher and varied from 2.0 to 2.5 μmol g^{-1} DW (Fig. 5.7b). Under flood conditions, a considerable increase of the TBARs concentration in the roots was observed during experimental period (Fig. 5.2a), the relative values being 138, 175, 152, and 155% of the control after 1, 3, 5, and 8 days, respectively. In the leaves the TBARs concentration increased under flood conditions with a certain delay compared to the roots. It reached 128 and 136 % of the control at the third and fifth days of the experiment,

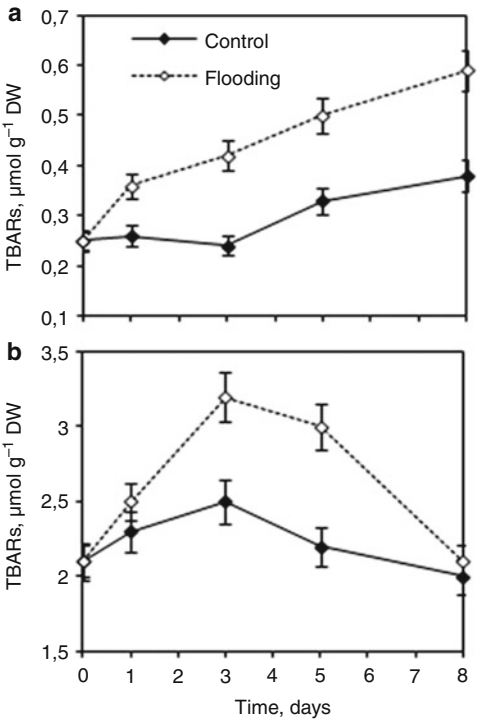

Fig. 5.7 Thiobarbituric acid reactive substances (TBARs) in roots (**a**) and in leaves (**b**) of *Brassica napus* grown under optimal soil watering (control) and soil flooding (flooding). The values are means of three replicates (pots) ± standard deviations (SD)

respectively (Fig. 5.2b). Then it decreased to control level at the eighth experimental day.

5.5.3 *The SOD, AsP, and GR Activities and Proline Concentration in the Leaves of Flooded Plants*

The SOD activity in the roots of control plants was six times lower than that in the leaves (Fig. 5.8). During 8 days of the experiment, the SOD activity in the control plants increased gradually by 43 and 30 % in the roots and in the leaves, respectively. Under flooding conditions, the SOD activity in the roots increased significantly (Fig. 5.8a) during the first 3 days (130 %, 124 % of the control after 1, 3 days, respectively) and later gradually decreased down to 60 % of the control. The SOD activity in the leaves of flooded plants increased up to 150 % of the control after 3 days and then remained 40–44 % higher than the control value until the end of the experiment (Fig. 5.8b).

The leaf AsP activity of the control plants remained at a level of 24.0 μmol AsA g^{-1} DW min^{-1} during the experiment (Fig. 5.9). In the flooded plants the AsP

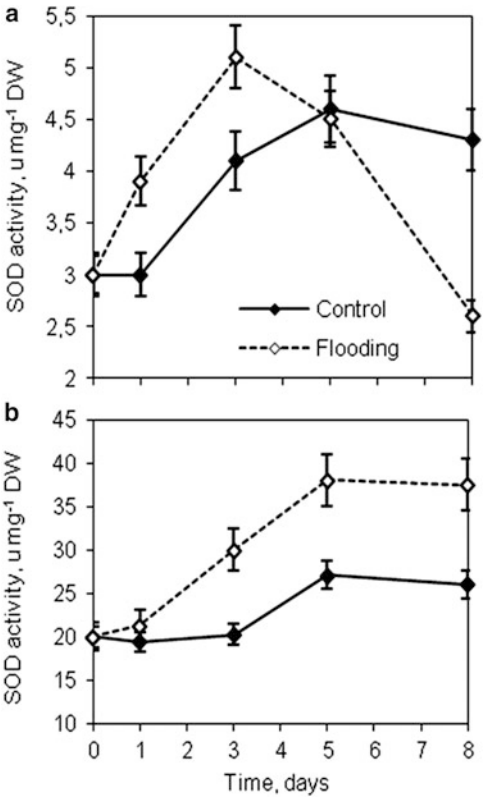

Fig. 5.8 Superoxide dismutase (SOD) activity in roots (**a**) and in leaves (**b**) of *Brassica napus* grown under optimal soil watering (control) and soil flooding (flooding). The values are means of three replicates (pots) ± standard deviations (SD). *DM* dry mass

activity increased significantly (Table 5.6) starting from the first day of the experiment. It reached 137 % at the third day and then dropped to the control level.

The GR activity in the control roots (Fig. 5.10) decreased from 3.4 to 2.3 μmol NADPH g^{-1} DW min^{-1} during 8 days of the experiment. Activity of GR in the roots of the flooded plants oscillated around 3.5 μmol NADPH g^{-1} DW min^{-1} during the entire stress period, and starting from the third day of flooding it was by 45 % higher than that in the control plants. In the leaves of the control plants, the GR activity was about 9.35 μmol NADPH g^{-1} DW min^{-1} during the experimental time and did not differ significantly under flood conditions.

The concentration of free proline in the leaves of flooded plants showed a significant increase after the first day of the experiment (Fig. 5.11; Table 5.6) and rose up to four times of the control value at the third day. Then proline concentration in flooded plants decreased to the control level.

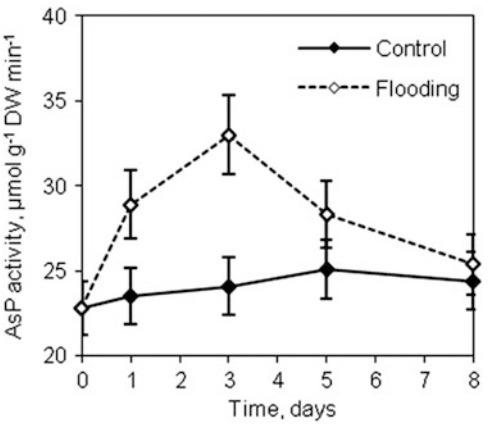

Fig. 5.9 Ascorbate peroxidase (AsP) activity in leaves of Brassica napus grown under optimal soil watering (control) and soil flooding (flooding). The values are means of three replicates (pots) ± standard deviations (SD). DM dry mass

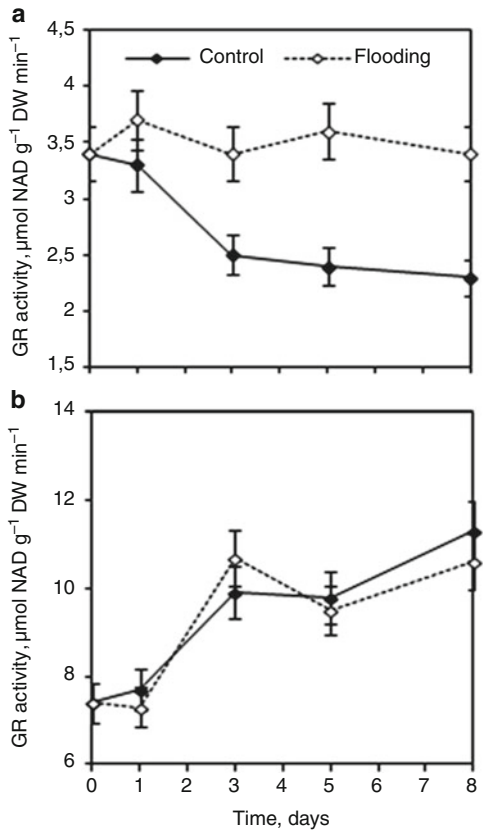

Fig. 5.10 Glutathione reductase (GR) activity in roots (a) and in leaves (b) of Brassica napus grown under optimal soil watering (control) and soil flooding (flooding). The values are means of three replicates (pots) ± standard deviations (SD). DM, dry mass

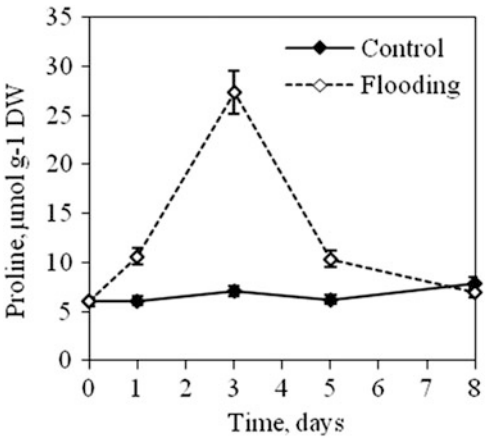

Fig. 5.11 Proline concentration in leaves of *Brassica napus* grown under optimal soil watering (control) and soil flooding (flooding). The values are means of three replicates (pots) ± standard deviations (SD). *DM* dry mass

5.5.4 Discussion

The soil measurements performed in the flood treatment showed that the hypoxic conditions in soil developed as rapidly as in our studies on barley and bean (Balakhnina et al. 2009, 2010). Suppression of plant growth, pigment accumulation, and biomass production under soil flooding was demonstrated. Devkota and Jha (2011) showed that total dry mass of *Centella asiatica* decreased up to one fourth from the control with exposure to extensive water (125 %) as a result of a less by half chlorophyll content. The specificity of the present investigations is connected with integrated studying of the specific and unspecific biochemical responses of spring rape plants to soil flooding. Oxygen deficiency induces anaerobic respiration resulting in ethanol accumulation in plant tissues. Some authors concluded that plant tolerance to hypoxia depends on their ability to multiply activity of ADH, the main enzyme of ethanol oxidation. Really, activity of ADH was shown to be raised in the roots of various plants exposed to soil hypoxia (Chan and Burton 1992; Garnczanska 2002; Wignarajah et al. 2010; and others). Plant tolerance to soil hypoxia was also shown to be dependent on root morphology. In particular, Benz et al. (2007) showed that different genotypes of *Piriqueta caroliniana* can respond to hypoxic soils by producing oxygen-conducting aerenchymous tissue or through induction of ADH. The authors assumed that aerenchyma development is an effective strategy in habitats subject to persistent flooding, while elevating activity of enzymes for ethanolic fermentation is effective only under ephemeral flooding. In our experiments, the ADH activity in the roots of flooded spring rape multiplied up to sevenfold after 1 day of flooding. The following decrease in ADH activity observed after the third day of the experiment is in agreement with papers cited above. But in our case the ADH activity in flooded plants remained higher than in the control ones during the entire experimental period. Intensification of peroxidation process was shown to be a result of increased ROS concentration under stress conditions (Asada 2006; Blokhina et al. 2002). In the present study, the intensity of

oxidative destruction processes as an unspecific response to stress development was examined by the concentration of TBARs as the main products of peroxidation of lipids, carbohydrates, proteins, and other compounds. According to Bach theory (Bach 1912), peroxidation processes occur permanently and unavoidably in the cells of living organisms. The relationship between the concentrations of hydrogen peroxide and MDA in roots and leaves of various plants exposed to soil flooding was repeatedly demonstrated (Asada 2006), and the existence of a direct relationship between stress sensitivity and the early accumulation of MDA was shown (Arbona et al. 2008). Usually soil hypoxia increased the MDA concentration, especially in intolerant species (Chen and Qualls 2003; Yordanova and Popova 2007). In our experiments the TBARs concentration increased and then remained at a high level in roots of flooded spring rape, but in the leaves an initial increase in the TBARs concentration changed to a decrease at the end of the experiment. Early stages of oxidative stress development can be controlled by low-molecular-weight antioxidants and enzymes scavenging ROS (Asada 2006). The positive antioxidant response (activities of SOD, AsP, catalase, and GR) in leaves and roots of citrus genotypes was proposed to be responsible for a higher tolerance to flood stress (Arbona et al. 2008). Wang and Jiang (2007) assumed that SOD and AsP are mainly involved in waterlogging-induced antioxidant responses, and the partial waterlogging could also significantly affect root antioxidant activities, particularly in waterlogging-sensitive cultivars. Blokhina et al. (2002) summarized literature data on different response of SOD and other antioxidant enzymes to oxygen deprivation stress and concluded that increased antioxidant capacity did not always correlate positively with the degree of plant protection. It is suggested that the efficiency of stress protection depends, in the first place, on specificity of a plant species, stage of stress development, and subsequent induction of SOD (scavenging superoxide radicals), peroxidases (scavenging H_2O_2), and GR (Balakhnina et al. 2009, 2010). The SOD activity in spring rape leaves was found to increase after 3 days of flooding and maintained at the high level during the experiment. This fact together with dynamic of TBARs concentration (decrease after initial increase) shows that the leaves are well defended against indirect action of soil flooding. In spring rape roots, induction of SOD was started earlier, after 1 day of flooding, but then, after 5 days, it displayed a gradual decrease below the control which correlated with stress intensification (increased TBARs concentration) (Balakhnina et al. 2012).

Ascorbate-glutathione cycle involving several enzymes, including AsP and GR, is known as an important and efficient defense system for decomposing H_2O_2 (Blokhina et al. 2002). Duration of stress, plant species, and plant organs are the factors influencing AsP activity associated with waterlogging tolerance (Wang and Jiang 2007). In spring rape leaves of flooded plants, AsP activity increased almost on 40 % after 3 days of flooding (Fig. 5.9a) (Balakhnina et al. 2012). GR is required to maintain a high ratio of GSH/GSSH in the presence of NADFH (Smith et al 1989; Asada 2006). Decrease of GR activity in bean leaves after 2 days of soil hypoxia was considered (Balakhnina et al. 2010) to be a reflection of the increased content of reduced forms of antioxidants of ascorbate-glutathione cycle.

In our work the GR activity in spring rape leaves under flood conditions did not differ significantly from control value (Table 5.6), but in the roots it remained about 36–50 % higher than control (Fig. 5.10, Table 5.6). Comparison of these results with the data on SOD activity in spring rape leaves and roots (Fig. 5.8a, b) supports conclusion that stress tolerance correlates well with consequent high levels of CuZn SOD and GR activities. Concentrations of free proline in the leaves of spring rape increased synchronously with SOD and AsP activities in the beginning of soil flooding experiment. Shevyakova et al. (2009) observed on ice plant that changes in SOD activity and proline accumulation in response to paraquat treatment combined with NaCl revealed an opposite dependence to accumulation of proline: the more proline accumulated in leaves, the lower activity of the enzyme. Moreover, exogenous proline decreased not only the rate of lipid peroxidation and content of superoxide radical but also SOD activity (almost fivefold) in leaves of adult plants (Shevyakova et al. 2009). Radyukina et al. (2008) concluded that proline antioxidant effects in common sage are manifested only after 12 h of stress or action, whereas antioxidant enzymes are involved in ROS scavenging during the earlier stage of damaging factor action. Proline accumulation is known to be related to nonenzymatic detoxification of free radicals (superoxide, peroxide, or hydroxyl) that are generated excessively under stress (Alia Prasad and Saradhi 1995; Alia Saradhi and Mohanty 1997; Radyukina et al. 2008; Trovato et al. 2008). The authors explain such an ability of this amino acid by the presence of tertiary carbon which can form stable radical tearing off free radical reactions induced by ROS (Radyukina et al. 2008). Our data showed that the increase of free proline concentration and antioxidant enzyme activities can occur simultaneously in the leaves of spring rape during 3 days of soil flooding. It should be noted that at further stress development, free proline concentration and AsP activity in spring rape leaves decreased with a decrease in TBARs concentration to control levels while SOD activity continued to increase till the fifth day of flooding and then remained high (Balakhnina et al. 2012).

5.5.5 Conclusions

Sevenfold increase in ADH activity (determined by affinity for ethanol) in the roots of *Brassica napus* L. plants under soil hypoxia indicates the activation of the final stage of glycolysis—oxidation of ethanol with the release of CO_2 and a specific reaction resistant to this stress factor cultures.

Resistance of *Brassica napus* L. plants to soil flooding is also provided through the active functioning of superoxide dismutase, glutathione reductase, and ascorbate peroxidase. Antioxidant enzymes neutralize ROS and in the early stages of stress development keep the intensity of lipid peroxidation processes at a safe level for the cell.

Increasing content of proline, natural detoxifier of the ROS, in the leaves at the early stages of the hypoxic stress development should be considered as an important

nonspecific reaction of plant adaptation to oxygen deficiency that occurs also under the action of other adverse factors.

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Chapter 6
Mechanism of Arsenic Toxicity and Tolerance in Plants: Role of Silicon and Signalling Molecules

Meetu Gupta and Ehsanullah Khan

Abstract Arsenic (As) contamination is a major environmental problem. It has become a major limiting factor in the growth and yield of crop plants, affecting the sustainability of agriculture production. Arsenic taken up by the plant tissue causes severe damage to important cellular components, such as lipids, protein, DNA and RNA. Mostly, inorganic forms of As, arsenate and arsenite, are found to be more toxic. To mitigate and reduce the negative effects of As, various prospects have been evaluated. Silicon (Si) has been found to serve as a beneficial element for plant growth and development, and its accumulation is helpful in maintaining sustainable production. Studies have revealed the ability of Si to mitigate various biotic and abiotic stresses in crop plants. It is also known that phosphate transporter recognizes arsenate while arsenite is taken up as a Si transporter. There is a lack of information available on the interactive effects of As and Si, especially in terrestrial or crop plants. On the other hand, signalling molecules are also known to regulate plant metabolism, growth and development under various stresses. The signal pathways either operate independently or may positively or negatively modulate other pathways. This chapter examines the participation and interaction of As and Si in plants. Furthermore, role of signalling molecules is also discussed to mitigate As-induced damages.

Keywords Arsenic • Silicon • Signalling molecules • Reactive oxygen species (ROS) • MAPK cascade

6.1 Introduction

Plants are constantly challenged in an environment by several abiotic and biotic stresses, which adversely affect the normal plant metabolic and developmental programmes. Arsenic (As) is a heavy metal environmental pollutant, toxic to plants

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and animals, considered as carcinogen (Zhao et al. 2010). It occurs naturally in the environment through geological activities. Southeast Asia along with South America is worst affected by arsenic pollution. Arsenic is an element of interest due to the toxic properties of several arsenic compounds. The major inorganic arsenic species found in the environment are arsenite [As(III)] and arsenate [As(V)] and organic arsenic species are monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), trimethylarsine oxide (TMAO), arsenobetaine, arsenosugars and arsenocholine. The inorganic arsenic species arsenite [As(III)] and arsenate [As(V)] are the main phytoavailable forms of arsenic and predominantly found in soil/water. These inorganic forms are interconvertible, depending on the redox condition and may be metabolized by plants from the inorganic to organic form. Methylated species (MMA, DMA) are minor arsenic species in the environment.

In the environment, among the two inorganic forms, arsenate [As(V)] predominates in aerobic environment, while arsenite [As(III)] is predominant in anaerobic condition, such as flooded paddy fields (Zhao et al. 2010). Both the forms of arsenic are accumulated in plants by different ways. Plants accumulate As(V) from soil through phosphate transporter, as it is an analogue of phosphate and interferes with essential cellular processes. It competes with Pi for transportation across root plasma membrane via phosphate co-transport systems in a wide variety of plant species, whereas As(III) binds with sulfhydryl group and interferes in general protein synthesis (Tripathi et al. 2007). After entering into the cell, As(V) reduces into the As(III) by arsenic reductase, and glutathione (GSH) serves as a source of reducing power. Ma et al. (2008) reported As(III) transporter in plants and their role and strategies for reducing As accumulation in rice grains. Plants exposed to arsenic show interruption in several morphological, physiological and biochemical processes, including inhibition of germination, shoot and root growth, biomass production and yield (Abedin et al. 2002; Ahmad and Gupta 2013) as well as the generation of reactive oxygen species (ROS) including superoxide ($O^{2\cdot-}$), hydroxyl radical (·OH), hydrogen peroxide (H_2O_2) and lipid peroxidation (Hartley-Whitaker et al. 2001; Requejo and Tena 2005; Singh et al. 2006; Ahsan et al. 2008; Mallick et al. 2011). The production of ROS can exceed the capacity of the plant's defence mechanisms, an imbalance in intracellular ROS content is established, and this results in oxidative stress (Gill and Tuteja 2010). This metabolic response is highly dependent on the plant species, plant growing state and the type and duration of the stress. ROS can damage proteins, amino acids, nucleotides and nucleic acids and cause peroxidation of membrane lipids which ultimately result in cell death (Møller et al. 2007). Under physiological steady-state conditions, these molecules are scavenged by different antioxidative defence components that are often present in particular compartments.

Plants like other organisms have evolved different mechanisms to maintain physiological concentration of essential metal ions and to minimize non-essential heavy metals. They minimize the damage caused by high concentrations of heavy metals in plants by detoxification, thereby conferring tolerance to heavy metal stress. Another mechanism involved in the elimination of arsenic is sequestration

of this toxic ion within compartments to isolate them from sensitive cellular components. As a first line of defence, plants attempt to prevent or reduce toxic metal uptake into root cells by restricting them, binding to root cell wall or to cellular exudates or by inhibiting long distance transport. If this fails, metals already in the cell are addressed using a range of storage and detoxification strategies, including metal transport, chelation, trafficking and sequestration into the vacuole. When these options also fail to detoxify metal toxicity, plants activate oxidative stress defence mechanisms and the synthesis of stress-related proteins and signalling molecules, such as heat shock proteins, hormones and ROS.

The presence of arsenic is a significant threat to environment and human health. It is a non-essential element, toxic to plants and, due to its accumulation in food crops, may pose a health risk to humans. Thus, there is a need to understand the As toxicity and tolerance mechanisms in plants, as the main environmental exposure to As for humans is through contaminated drinking water and food chain when crops and fodder become contaminated. This chapter compiled the comprehensive knowledge generated in this area related with As toxicity and tolerance mechanisms. Representation of As cycle in the environment and plants is presented in Fig. 6.1

6.2 Uptake, Accumulation and Translocation of Arsenic Species in Plants

Plants have different abilities to accumulate arsenic in their tissues. This variation may be due to translocation factors of arsenic. Their concentrations in the above-ground part of plants growing in uncontaminated soils are less than 1.0 mg kg^{-1} dry weight; those plants, which have low translocation factors (TFs) (<1), are called excluders because of their restricted uptake and restricted translocation of As from roots to shoots. In the case of As hyperaccumulators, they are able to accumulate up to ~2 % As. Between the excluders and hyperaccumulators, there are plant species with intermediate abilities to accumulate As, for example, the Douglas-fir (*Pseudotsuga menziesii*) (Ma et al. 2001; Haug et al. 2004), several *Equisetum* species and *Isatis capadocica* (Meharg 2003; Karimi et al. 2009). Rice (*Oryza sativa*) is also an interesting food crop as it is much more efficient in As accumulation than other cereals such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) with its As TF often one. There are two reasons of high arsenic accumulation in rice: (1) increased As bioavailability under the anaerobic conditions of submerged paddy soils and (2) the inadvertent uptake and transport of arsenite through the Si pathway, which is highly efficient in rice (Su et al. 2010). Determination of As (arsenic) speciation in plants is important for understanding As metabolism in plants and for assessing the toxicity of plant As to the consumers at the higher trophic levels.

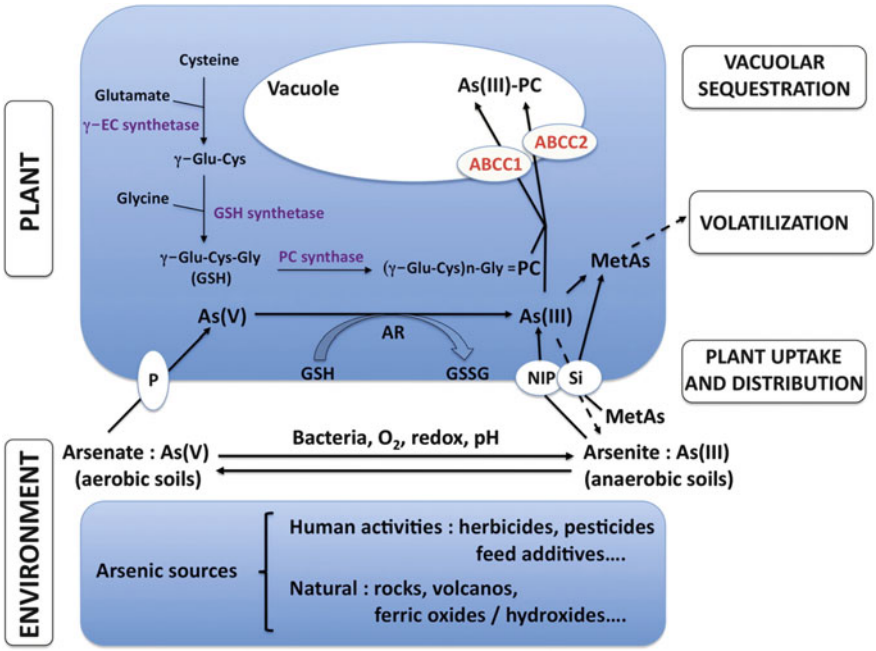

Fig. 6.1 Schematic representation of the As cycle in the environment and plants. Equilibrium between arsenate [As(V)] and arsenite [As(III)] in soil solutions is mainly dependent on the redox conditions. Arsenate is taken up by roots by phosphate transporters (P), and arsenite is taken up by a subclass of aquaporins (NIP), some of them also transporting silicon (Si). Methylated forms of As (MetAs) are also taken up by NIP and Si transporters. Inside plants, these types of transporters are also involved in the distribution of As between organs and tissues. As(V) is enzymatically reduced into As(III) in plant cells by arsenate reductase (AR), leading to the conversion of glutathione (GSH) to its oxidized form (GSSG). Arsenite can be effluxed to the environment by a root Si transporter or methylated. A cascade of methylation can then transform As into a gaseous form that is volatilized. Another pathway of detoxification occurs by the synthesis of phytochelatins (PCs) corresponding to a three-enzymatic-step condensation of three amino acids: cysteine (Cys), glutamate (Glu) and glycine (Gly). PC synthesis and their complexation to As(III) are coordinated to the transport of the PC–As(III) complex to the vacuole subcellular compartment through the activity of two members of a subclass of ATP-binding cassette (ABC) transporters: ABCC1 and -2 (*Source*: Briat 2010)

The most common forms of As in soil solution available for plants are arsenate, arsenite, MMA and DMA. Arsenate [As(V)] is the main As species present in aerobic soils and recognized by phosphate transporter. They are the chemical analogous of corresponding phosphate ions; therefore, both have similar electron configurations and chemical properties and compete for the same uptake carriers in root plasmalemma (Meharg and Hartley-Whitaker 2002). Under low Pi conditions, As(V) may outcompete Pi for entry into the plant, amplifying Pi deprivation symptoms. Conversely, Pi fertilization can protect plants, including the hyperaccumulator *P. vittata*, from As(V) toxicity (Tu and Ma 2003). Once inside the plant cell, As(V) can probably move easily from one cellular compartment to

another, crossing internal membranes through the various Pi transporters. The outcome of this rapid movement would be the rapid equilibrium of As throughout the cell, exposing all parts of cellular metabolism to the toxicant. Although a number of phosphate transporters have been characterized in plants, *A. thaliana* mutants defective in phosphate transport are more tolerant to arsenate and identified for arsenate toxicity screening (Catarecha et al. 2007). Reduced uptake of arsenate is a well-known mechanism of arsenate resistance employed by many plant species, which is achieved through a suppression of the high-affinity phosphate/arsenate uptake system in the resistant plants.

Arsenite [As(III)] is the dominant As species in reducing environments, and it is mainly taken up as the neutral molecule $As(OH)_3$. Like microorganisms and mammalian tissues, plant roots are also capable of rapidly taking up arsenite from the external medium via some aquaglyceroporin channels. In higher plants, the nodulin 26-like intrinsic proteins (NIPs) are the structural and functional equivalents of the microbial and mammalian aquaglyceroporins; NIPs are a subfamily of the plant major intrinsic proteins (MIPs), collectively known as aquaporins or water channels (Wallace et al. 2006). Recent studies have shown that a number of NIPs are permeable to arsenite (Bienert et al. 2008; Isayenkov and Maathuis 2008; Ma et al. 2008) and mediate transport of a range of small neutral molecules including ammonia, urea, boric acid and silicic acid. In rice roots, Lsi1 (OsNIP2;1), which is highly expressed in the distal side of the plasma membranes of the exodermis and endodermis cells where Casparian strips are formed, is a major entry route for silicic acid (Ma et al. 2006) and arsenite. Mutation in this protein resulted in a 60 % loss of the arsenite influx in the short term; however, the effect of Lsi1 mutation on As accumulation in rice shoots is relatively small over a longer growth period (Ma et al. 2008). There are 9–13 NIP genes in the rice and *Arabidopsis* genomes. Some of the rice NIP genes are expressed mainly in the shoot and inflorescence tissues (Sakurai et al. 2005); their roles in As transport toward the grain remain to be investigated. Although some NIP channels allow bidirectional transport of arsenite, efflux of arsenite from the exodermis and endodermis cells in rice roots toward the stele is mediated by the Si efflux carrier Lsi2 (Ma et al. 2006). This process is a crucial step in the accumulation of As in rice shoot and grain; it is also the step in which Si exerts a strong inhibitory effect. In As-hyperaccumulating species, such as *P. vittata*, As is not immobilized in the roots, but is instead rapidly transported as As (III) through the xylem to the fronds. In the fronds, As(III) is sequestered as free As (III) in the vacuole, where it accumulates to extremely high levels (Lombi et al. 2002; Su et al. 2008).

Uptake mechanism of methylated arsenic species is largely unknown at present. The uptake rate is much lower as compared to arsenate and arsenite forms (Raab et al. 2007). Abedin et al. (2002) observed concentration-dependent uptake of MMA in rice roots following Michaelis–Menten kinetics, whereas DMA uptake did not follow Michaelis–Menten kinetics. However, in *Zea mays* DMA uptake followed the Michaelis–Menten kinetics (Abbas and Meharg 2008).

Arsenite is the predominant form of As in the xylem sap, even though plants had been exposed to arsenate. For example, As(III) accounted for 96–100 % of the As in

the roots and shoots of *B. juncea* (Pickering et al. 2000), 97–100 % in the leaves of *A. thaliana* (Dhankher et al. 2002) and 92–99 % in the roots of tomato and rice (Xu et al. 2007). It is a fact that roots have a high capacity for arsenate reduction followed by complexation with thiols and possibly sequestration in the root vacuoles. However, there is no evidence that arsenite in the xylem sap of *B. juncea* or sunflower is complexed with thiol compounds (Pickering et al. 2000). In fact, complexation with thiols decreases arsenite mobility from roots to shoots. Plant species vary widely in the xylem mobility of As, as reflected by the ratio of As concentration in the xylem sap to that in the external nutrient solution. This ratio is well below one in non-hyperaccumulating plants, among which rice stands out as the most efficient in transporting As to the xylem, probably a consequence of the high expression of the Si/arsenite effluxer Lsi2. In contrasts with non-hyperaccumulating plants, As-hyperaccumulator *P. vittata* has extraordinary xylem mobility for As. In a recent study, data shows that As accumulation in the shoots increased markedly when arsenate reductase (*A. thaliana* AtACR2) was silenced using RNAi (Dhankher et al. 2006); this leads to more arsenate in the roots available for xylem transport to the shoots, presumably via the phosphate transport pathway. In *A. thaliana* phosphate mutant pho1, which is defective in xylem loading of phosphate, it showed no effect on As distribution to the shoots. However, pho2 mutant over-accumulates phosphate in the shoots but did not over-accumulate As in the shoots (Quaghebeur and Rengel 2004). These results suggest that As is not loaded into the xylem mainly as the phosphate analogue arsenate. Methylated As is rarely detected in the xylem sap, although DMA is taken up by roots inefficiently compared with other As species, it is transported more efficiently from roots to shoots, and the presence of DMA in the xylem sap of cucumber (*Cucumis sativus*) and tomato plants is of 4 % of the total As (Mihucz et al. 2005). The effect of excess As in plants is given in Fig. 6.2.

There is little knowledge of the extent and mechanisms of As transport in the phloem, such as the form of As transported and the transporters involved in phloem loading and unloading. In rice, As concentrations decrease markedly in the order of roots > stems and leaves > husks > grain (Abedin et al. 2002; Xu et al. 2008), suggesting that remobilization of As from stems and leaves to grain, if any, may be limited. However, the contributions of xylem versus phloem-derived As to the accumulation in grain need to be evaluated experimentally. There is evidence that PCs and other thiol peptides can be transported through phloem from leaves to roots in *A. thaliana* (Chen et al. 2006). As(III)-PC or As(III)-GS complexes that can be transported in phloem has not been investigated. As these complexes are not very stable at pH >7.5, their transport in phloem may not be favoured because of the slightly alkaline pH of phloem sap. It was found that DMA was transported to the immature grain approximately 30 times more efficiently than arsenite (Carey et al. 2010). When the phloem flow was disrupted by stem girdling, transport of arsenite into the grain was decreased by tenfold, but that of DMA by only 50 %. These results suggest that arsenite is delivered to rice grain mainly through the phloem, whereas both phloem and xylem pathways make an equal contribution to the transport of DMA to grain.

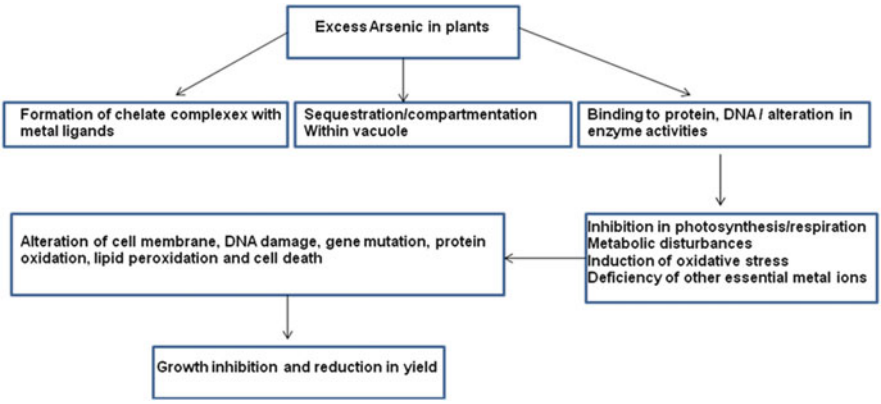

Fig. 6.2 Possible biochemical and molecular mechanisms of arsenic-mediated ROS induction and damage to the development of higher plants

6.3 Arsenic Toxicity and Tolerance in Plants

Toxicity of arsenic depends on the nature of arsenic species present in the environment and plant. Arsenate is rapidly reduced to arsenite; the majority of the toxic effects of arsenate may actually be due to its reduction product, arsenite. The results from a number of hydroponic experiments agree that As phytotoxicity depends on the chemical species supplied to the plant, but disagree on the identity of the most phytotoxic form of As (Carbonell-Barrachina et al. 1998; Abbas and Meharg 2008). These hydroponic experiments provide the clearest insights into the potency of externally supplied As on whole plant growth because they eliminate the complex and confounding phytoavailability issues that arise from differences in the mobility of various As species through the diverse growth substrates of 46 different plant species (Raab et al. 2007) that the uptake of As by plants has the order As(III) > As (V) > MMA(V) > DMA(V), while translocation from the roots to the rest of the plant has the order DMA(V) > MMA(V) > As(V) ≥ As(III). However, no one As form appears to be consistently most phytotoxic. In two *Spartina* species, where the order of uptake was As(III) > As(V) ≈ MMA(V) > DMA(V), the order of phytotoxicity was DMA(V) ≈ MMA(V) > As(III) ≈ As(V)(Carbonell-Barrachina et al.1998). The order for phytotoxicity in maize, a species with the typical order for uptake (Raab et al. 2007), was As(V) > As(III) > DMA(V) (Abbas and Meharg 2008).

The inconsistent order of phytotoxicity of the various As species could be an indication that As has interacted differently with the available nutrients or that the phytotoxic form of As is plant species dependent. Alternatively, the apparent inconsistency of the above results may be due to our incomplete understanding of the relative importance of the various As species to the mechanism of As toxicity. Rice and *Brassica* seedlings grown in arsenic-containing medium showed

retardation in shoot–root growth, decreased photosynthetic yield and other toxic effects (Ahmad et al. 2012; Ahmad and Gupta 2013).

Detoxification of arsenic species is essential to avoid harmful effects on cellular processes. Studies showed that GSH and arsenite form a $(GS)_3$-arsenite complex with cysteinyl sulfhydryl as the arsenite binding site, whereas inorganic arsenate does not form complexes with thiol compounds, but pentavalent As in DMA can bind to GSH when it is activated by sulphide. Raab et al. (2007) identified the dimethyl arsinothioyl glutathione (DMAS-GS) complex from the sulphur-rich plant species *Brassica oleracea*. There is strong evidence that complexation of arsenite by PCs is an important mechanism of As detoxification, and hence tolerance, in As non-hyperaccumulating plants. Exposure to arsenate or arsenite induces a large response in the synthesis and accumulation of PCs in plants (Srivastava et al. 2007; Schulz et al. 2008). The toxicity of arsenite is thought to be caused by the binding of arsenite to the −SH groups of proteins, thus altering protein structure or interfering with the catalytic sites of enzymes. A number of genes or enzymes involved in glutathione synthesis, metabolism and transport are upregulated in rice seedlings exposed to arsenate (Ahsan et al. 2008; Norton et al. 2008), probably reflecting a higher demand for GSH under As stress. Blocking PC synthesis with BSO leads to hypersensitivity to both arsenate and arsenite (Schmöger et al. 2000). An *Arabidopsis* PC-deficient mutant is 10–20 times more sensitive to arsenate than the wild type. Tolerance to arsenate is also enhanced by increased thiol synthesis in transgenic plants overexpressing a bacterial γ-glutamylcysteine synthetase gene (*γ-ECS*) (Dhanker et al. 2002) or the *Arabidopsis* PC synthase gene (*AtPCS1*) (Gasic and Korban 2007). These findings, and the fact that much of the arsenite in plant tissues is complexed with thiol-rich peptides, provide conclusive evidence that thiols, particularly PCs, play a crucial role in As detoxification in As non-hyperaccumulators. The observed effect on arsenate tolerance is through the detoxification of arsenite, the product of arsenate reduction. In contrast, As hyperaccumulators such as *P. vittata* and *P. cretica* do not rely on a PC-based mechanism to detoxify As (Zhang et al. 2004). The PC-arsenite complexes are likely to be stored in vacuoles. The yeast vacuolar transporter Ycf1p, a member of the ATP-binding cassette (ABC) superfamily, confers arsenite resistance by transporting the glutathione-*S*-conjugated arsenite [As(III)-$(GS)_3$] into the vacuole (Ghosh et al. 1999). The PC-arsenite complexes are also likely to be transported into vacuoles by an ABC protein, the identity of which is not yet known. A transporter(s) responsible for arsenite uptake into the vacuoles is not yet known but may be the key determinant of the hypertolerance phenotype in *P. vittata* and other hyperaccumulator plants.

Several enzymes are involved in ROS defence strategies. Highly reactive superoxide can be converted to less active but long-lasting H_2O_2 through the action of superoxide dismutase (SOD). SOD activity in plants varies quite widely with As treatment. In some plants, like *Zea mays*, As-sensitive clones of *H. lanatus* and the As-hyperaccumulator*P. vittata*, the enzyme is induced by low As exposure and either stays at the same level or decreases in activity at higher As levels (Cao et al. 2004). H_2O_2 produced in a plant cell either directly or enzymatically through enzymes such as SOD can be neutralized by catalase (CAT), an enzyme that is

often induced by As exposure (Cao et al. 2004; Duman et al. 2010). In addition to catalase, plants have a two-component system for regulating the balance of H_2O_2, and therefore of ROS, within cells. One component encompasses a group of non-enzymatic antioxidants that includes GSH, PC, ascorbate, carotenoids and anthocyanin. These antioxidants generally accumulate during As exposure. The production of these molecules requires metabolic acclimations, including the diversion of carbon, nitrogen, sulphur and metabolic energy from normal growth and development. The second component of the two-component H_2O_2 neutralizing system is made up of monodehydroascorbate reductase, dehydroascorbate reductase and GSH reductase. These enzymes are responsible to recycle oxidized GSH and ascorbate to allow further cycles of H_2O_2 reduction. The reduction of H_2O_2 through the interdependent ascorbate–GSH cycle requires reducing power in the form of NAD(P)H, diverting this energy from other metabolic processes. The enzymes involved in the recycling of oxidized GSH and ascorbate are also often induced upon exposure of plants to As (Foyer and Noctor 2011). Thus, the interdependent ascorbate–GSH cycle has an important role in maintaining ROS balance in plants, probably, even during As exposure.

6.4 Role of Silicon in Alleviation of Arsenic Stress in Plants

Silicon (Si) is the second most abundant element both on the Earth's crust and in the soil. It is accumulated in plants at a level euivalent to that of macronutrient elements such as calcium, magnesium and phosphorous (Epstein 1999). All plants growing in soils take up Si, but Si content in plant tissues varies greatly among plants, ranging from 0.1 to 10 % (w/w) Si on a dry weight basis (Epstein 1994, 1999). Except for oxygen, silicon (Si) is considered as the most abundant element in the Earth's crust. Evidence showed that Si has favourable effects on plant growth under biotic and abiotic stress (Shi et al. 2005; Guo et al. 2005, 2007; Gottardi et al. 2012). Both Si and As share the similar transporter system through Lsi1 membrane transporter (Ma et al. 2006, 2008). Some evidence suggested that an increase level of Si reduces the arsenic uptake (Alexander et al. 2013; Hu et al. 2013). Si supplementation with heavy metal such as As, Cd, Ni and Cu to plant alleviates the toxicity of these metals. Although the exact mechanism of silicon-mediated signalling in plant is not known, their role in the alleviation/mitigation of heavy metal toxicity indicates that it may affect the signalling process triggered by heavy metals. Rice has been described as natural accumulator of arsenic as compared with other cereal crops and accumulates more As because of a higher bioavailability in the flooded paddy soil (Xu et al. 2008). They use silicon to strengthen their stems and the husks that protect the grain against pest attack. In South and Southeast Asia, where rice is a staple crop, As contamination is widespread and the soils are typically weathered and tend to be depleted in plant-available Si. Thus, adding (or returning) Si to As-contaminated soils may decrease As uptake by plants by providing a more soluble Si substrate. Silicon is essential for high and sustainable production of

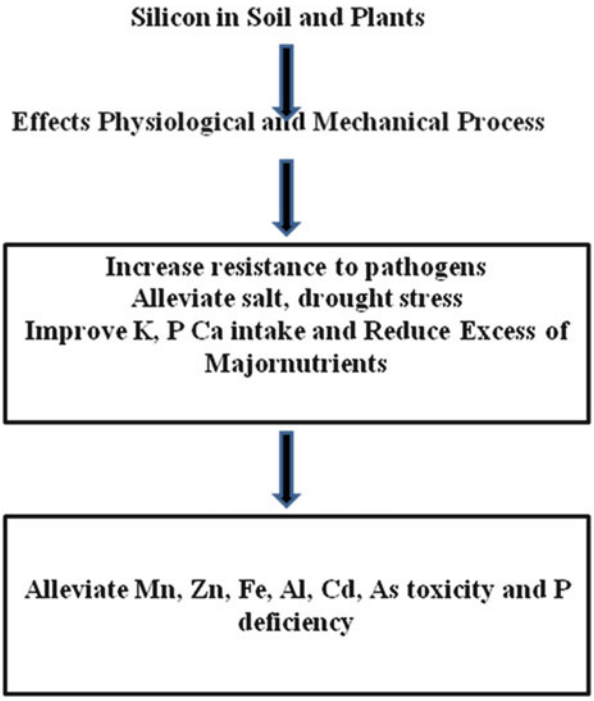

Fig. 6.3 Benefits of silicon for plants under various stresses

rice, and the Si transporter Lsi1 is a major uptake pathway for As(III) in rice (Ma et al. 2008). Lowering the As load by Si supplementation in rice or other crop plants is therefore likely to reduce As-induced stresses and finally would limit food chain contamination. Fleck et al. (2013) reported that silicon application to soil increased the concentrations of Si, Fe, As and P in the soil solution, while the redox potential was unaffected. Arsenic concentrations of straw, flag leaf and husk were reduced by half by Si application. These results confirm that Si reduces As(III) uptake and translocation into the shoot. Silicon nutrition is considered as an important target in an attempt to not only decrease As concentrations but also to ameliorate the photosynthetic performance of rice plants challenged with As (Sanglard et al. 2014). Benefits of silicon in plants and soil under various stresses are presented in Fig. 6.3.

6.5 Signal Transduction in Response to Arsenic

A complicated signal transduction network is activated by sensing the heavy metal, synthesis of stress-related proteins, signalling molecules and finally the transcriptional activation of metal-responsive gene (Maksymiec 2007). The signalling pathways include Ca–calmodulin system, phytohormone, ROS signalling,

calcium-dependent protein kinase (CDPK) and mitogen-activated protein kinase (MAPK), phosphorylation cascades and finally activating the stress-related gene. Different signalling pathways may be used to respond to different heavy metals (DalCorso et al. 2010).

At the cellular level, arsenic induced oxidative damage by the production of ROS which interferes with the antioxidant defence system. The equilibrium between production and scavenging of ROS may be perturbed by a number of adverse abiotic stress factors such as light, drought, heavy metal and low or high temperatures. One of the major consequences of heavy metal accumulation is the production of ROS, which as well as causing widespread damage may also function as signalling molecules. Numerous reports have indicated that arsenic affects transcription factors either by activation or inactivation of various signal transduction cascades. Huang et al. (2012) reported that As(V) induces the activation of NADPH oxidase which produces endogenous ROS, and this activates the MAPK.

Calcium serves as ubiquitous and a central hub in a large number of signalling pathways. Multiple extracellular signals such as light, hormones, biotic and abiotic stimuli elicit changes in calcium levels in the cell (Knight and Knight 2001). In plants, calcium sensor proteins are categorized as calcium sensor responder and sensor relay (Sanders et al. 2002). The calcium relay protein binds calcium and affects their target protein since they themselves do not have enzymatic activity, and a typical example of this is calmodulin (CaM). CaM is best characterized as a calcium sensor, which does not have enzymatic activity, hence activating or deactivating their interacting proteins (Luan et al. 2002). CaM is highly regulated by Ca ion intracellular concentration that acts as secondary messenger, and excess heavy metals disrupt the Ca ion channel, thus increasing the flux into the cell. Excess Ca interacts with CaM and induces signal transduction that ultimately regulates downstream genes involved in heavy metal transport, metabolism and tolerance (Yang and Poovaiah 2003). The Ca–calmodulin system is also involved in the response to heavy metal toxicity, such as Ni, Pb and As. Huang et al. (2012) reported that 7 calmodulin genes are upregulated in response to As which ultimately regulate downstream genes involved in mitigation of arsenic toxicity and transport. CDPKs are the most extensively studied calcium signalling kinases that are triggered by elevated level of Ca ion and activate the downstream signalling in response to heavy metal stress (Martin and Busconi 2001). Two Calmodulin dependent protein kinases (CaMKs) were shown to be activated in response to As (V) in rice root, and this reduces the MAPK activity.

MAPK cascade is one of the major signalling pathways in plants. It is an evolutionary conserved signal transduction module, able to convert extracellular signals to appropriate cellular responses. MAPKs are involved in developmental, hormonal, biotic and abiotic stress signalling. Members of MAPK cascades are activated by more than one type of stress, for example, AtMPK6 is involved in O_3, H_2O_2, ethylene, ABA and JA signalling pathways and also in various important developmental processes such as epidermal patterning, anther and embryo development (Sinha et al. (2011). MAPKs are composed of three protein kinase modules: MAPKK kinases (MAP3K), MAPK kinases (MAP2K) and MAP kinases (MAPK).

MAPK cascades are involved in normal cell metabolism like physiological, developmental, hormonal responses and also play important roles in plant responses to biotic and abiotic stresses, such as pathogen infection, metal stress and ROS. According to Gupta et al. (2009), MAPK activity in response to As(III) treatment in mustard plant indicated the role of this important cascade in transducing As(III)-mediated signals. It has also been shown that OsMPK3, OsMPK4 and OsMKK4 are involved in As(III)-mediated signalling in rice (Yeh et al. 2004). In addition, As(V) treatment increased As accumulation; NADPH oxidases and ROS may be involved in As(V)-induced MAPK activation in roots. These results emphasize a potentially important role for ROS and MAPK signalling components in As(V) stress responses.

Phytohormones are small organic compound synthesized by specific plant cell/tissue and act as signalling molecule in plant growth development (Pasternak et al. 2005a, b). Microarray assay in As-treated rice revealed the changed expression of many phytohormone biosynthesis, inactivation and signalling genes. One important phytohormone, jasmonate, plays a crucial role in the perception and signalling of As stress (Srivastava et al. 2009).

Understanding the molecular mechanism of how the plant cells monitor and respond under arsenic stress is important. Inhibition of uptake, elimination and detoxification are important for arsenic tolerance in cell. Protein kinase, phytohormones and ROS act as signalling molecules and are crucial for generation of signalling to inhibit and to protect the plants from heavy metals like arsenic (As), cadmium (Cd) and other non-essential elements.

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Chapter 7
Response of Wheat Seedlings to Combined Effect of Drought and Salinity

Anatoly A. Ivanov

Abstract In field conditions the plants are most commonly subjected to simultaneous effects of multiple stresses. The mechanisms of plant tolerance to salinity and drought are physiologically connected and overlapping, but some aspects of physiology and metabolism may differ when in the experiment salt and water stress is used separately or both stresses are used simultaneously. Physiological and biochemical reactions of the plants under combined effect of the drought and salinity are unique, which cannot be directly extrapolated from respective responses to each of these stresses individually. Drought and salinity reduce individually the availability of water for plants. However, the presence of salt in the soil inhibits the rate of the development of drought, enabling the plant to survive in unfavorable period of short-term drought without violation of basic physiological functions. At increased NaCl concentration in the soil during the combined stress, basic physiological and biochemical functions of the plants remain constant until a critical threshold, after which the plants' productivity decreases dramatically. This article shows the features of the combined stress and its difference from the drought and salinity individually.

Keywords Drought • Salinity • Growth • Relative water content • Transpiration • Photosynthesis • Peroxide hydrogen • Lipid peroxidation • Proline • Superoxide dismutase • Catalase • Peroxidase

Abbreviations

CAT	Catalase
DAE	Days after emergence
DAT	Days after treatment
DW	Dry weight
MDA	Malondialdehyde

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PEG	Polyethylene glycol
POD	Peroxidase
ROS	Reactive oxygen species
RWC_L	Relative water content of leaves
RWC_S	Relative water content of soil
SOD	Superoxide dismutase

7.1 Introduction

Global climatic change is accompanied by long-term droughts due to reduced rainfalls and increasing temperatures in combination with anthropogenic changes in natural hydrological conditions which may result in reduced water volume required for ecological and anthropogenic use (Nielsen and Brock 2009). Drought and irrigation, induced by salinization, are among the major abiotic factors limiting the growth and yield of the crops (Hamdy et al. 2003; Wang et al. 2003a, b; Araus 2004; Chaves and Oliveira 2004). Moreover, the areas affected by the drought and salinity continue to expand (Wang et al. 2003a, b).

Effective approach to control drought and salinity is an increase of sustainability of conventional agricultural crops; however, the benefit in yields is usually not too high (Tester and Davenport 2003). The main way of adaptation of plants to abiotic stress is to develop varieties more resistant to drought (Araus et al. 2002) or salinization (Munns and Tester 2008). However, this approach is not very efficient in selection of varieties, since most characteristic features of the phenotype vary, depending on the type and the level of stress impact (Araus et al. 2008). Most of the researches are aimed at studying every stress individually, although drought and salinity are usually occurred in the field together (Wild 2003; Ceccarelli et al. 2004). Combined stress as compared to the drought and salinity considered individually reduces to a great extent the availability of water to plants and therefore decreases crop yields more significantly (Katerji et al. 2009). In spite of the fact that accounting of interaction between these two factors is more suitable for analysis of the productivity in agriculture, a relatively small number of researchers investigated total effect of water deficit and salt stress on plants (Jensen 1981; Richards 1992; Shalhevet 1993). There have been only a few attempts to define criteria for the selection of plants that are resistant to combined effect of drought and salinity (Passioura 1996; Shaheen and Hood-Nowotny 2005). However, the issue still needs further research in detail.

7.2 Physiological Responses of Plants to Drought and Salinity: Similarity and Difference

The drought is believed to be temporal or long-term change of water status, affecting its functioning, and this is due to reduced water content in the soil (Wilhite and Glantz 1985; Katerji 1990). Nevertheless, in case of increasing soil salinity the water status of the crops is also changed, but these phenomena are not associated with lower water content in the soil (Katerji et al. 2003). As the amount of salt in the soil increases, its water potential is decreased that, in its turn, reduces the availability of soil water to plants, lowering the growth (Munns 2002, 2005; Mahajan and Tuteja 2005). Osmotic stress of the plant occurs as a result of high ion concentrations in the soil and an excess of salt absorption that leads to high accumulation of salts in the intercellular space. The influence of soil salinity on the plants can be made with several mechanisms, including Na^+ or Cl^- toxicity, competition for uptake of other cations, or osmotic effect on water absorption (Rengasamy et al. 2003; Dang et al. 2008; Katerji et al. 2009). Adaptation of plants to salinization can be classified by three types: osmotic stress tolerance to high ion concentrations in the soil and intercellular space; Na^+ or Cl^- isolation; and tolerance of the tissues to accumulation of Na^+ or Cl^- inside a cell (Munns and Tester 2008). Taking this into consideration, the mechanisms of salt resistance stay focused on different levels of control, including the growth rate and morphology of the plants, resistance to water stress (a decrease of water potential), regulation of CO_2 and H_2O exchange through stomata, and prevention of toxicity and imbalance of ions (Munns 1993, 2002; Koyro 2003; Rengasamy et al. 2003).

The reaction of plants to drought and salt stress has many features in common. Both drought and salinity induce water stress (Munns 2002). Water stress reduces the ability of plants to absorb water, causing a rapid decline in the growth due to reduction of carbon assimilation, tissue expansion, and cell number (Hsiao 1973; Tardieu et al. 2000) and the impact on the metabolism of nitrogen (Hirel et al. 2007). Salinization also leads to several metabolic changes similar to the effects of the drought, such as closing of stomata, inhibition of photosynthesis, oxidative stress, and damage of the cellular structures (Wang et al. 2008). Apparently, the mechanisms of these changes are individual (Tardieu et al. 2011), but this does not exclude existence of the ways interacting according to the type of feedback.

The plants growing in such environmental conditions should have increased salt resistance, as well as the ability to survive in the conditions of limited water resources, i.e., not only be tolerant to the toxicity of Na^+ but also be able to adapt to secondary effects, such as water deficiency/depletion (Munns 2002). Under field conditions, negative effects of salinity on the growth and yield of the plants depend on the level of stress development, time of exposure to stress factor, precipitation degree during the growth season, and physicochemical properties of the soil (Maas and Grattan 1999). All these factors can interact in a dynamic form throughout the growth season (Maggio et al. 2002). The complication of plant growth conditions can lead to significant variations in salinity resistance threshold (Maas and

Hoffmann 1977), physiological basis of which has been discussed critically (Dalton et al. 1997; De Pascale et al. 2003). The alternative indices have been developed for evaluation of salt resistance of the plants, namely, salinity stress index (SSI) (Dalton et al. 2001) and water stress day index (Katerji et al. 2003). The degree of salt resistance depends mainly on the type of a plant; however, even within the same species among different varieties or ecotypes stress resistance can vary significantly. However, some crops due to inherent plasticity can grow on soils with high content of the salt that was shown on certain varieties of wheat (Sairam et al. 2002; Wang et al. 2003a, b), wild species of wheat (Farooq et al. 1989), and other cultures (Francois and Maas 1999).

With an increase of salinity levels, the productivity of crops remains constant as long as the critical threshold value will not be achieved after which the yield is reduced in proportion to the increasing level of salinity. Quantification of plants' responses to salinity can be defined as a piecewise linear regression (threshold-threshold model, model of slope) (Maas and Hoffmann 1977). According to this model, the salt-sensitive cultures have thresholds below 1.5 dS m^{-1} (15 mM Cl^{-}), while salt-tolerant crops have threshold values ranging from 6 to 10 dS m^{-1} (60–100 mM Cl^{-}) (Maas and Hoffmann 1977). The main concept of modeling the yield, based on the level of salinity, is to compute common index of root zone salinity (Feddes et al. 1974), which is defined as an osmotic component of total water potential, reflecting colligative properties of water in the soil, and not the peculiarities of chemical composition.

However, osmotic potential of the root zone is not the main factor determining the threshold (Dalton and Poss 1989) which, most likely, depends on average salinity aboveground organs and their biochemical mechanisms. Alternative indices were described to assess plant salt tolerance such as the salinity stress index (SSI) (Dalton et al. 2001) and the water stress day index (Katerji et al. 2003). On the other hand, dynamical salinity stress index takes into account the whole plant response to salinity and is defined in terms of the dynamic flow of the dominant salinizing anion to the plant shoot relative to the plant growth. An integral part of this approach is biophysical properties of the root system, which controls the loading of salts into aboveground organs at different levels of salinity and atmospheric conditions, affecting transpiration and growth (Dalton et al. 1997, 2001).

7.3 Significance of Study of Combined Water and Salt Stress

Physiological and biochemical reactions of plants to the combined effect of drought and salinity are unique and cannot be directly extrapolated from the respective responses to each of these stresses individually (Mittler 2006). However, there is insufficient information on the effects of combined stress of drought and salinity on the plants. Most studies of abiotic stresses take place in controlled laboratory

conditions and do not reflect real situation faced by the breeders in creating sustainable crop in the field. By this it is possible to explain why plants with increased tolerance to specific abiotic stress, created in a laboratory, do not exhibit stress resistance in field tests (McKersie et al. 1999; Mohamed et al. 2001). For example, salt-resistant varieties that can withstand salinity to 10 dS m^{-1} (Qureshi 1985) are sensitive to reduction of available water in the soil. Hence, one should pay particular attention to the study of molecular, physiological, and metabolic aspects of plant resistance to multiple stress factors that more realistically reflects the situation in field conditions. These generalizations have practical implications for the strategies of crop plant irrigation on saline soils (Richards 1992; Shalhevet 1993), and they can also be related to the strategy for growing the plants adapted to arid conditions (McNaughton 1991).

The mechanisms of plant resistance to salinity and drought are physiologically related and overlapping, but some aspects of physiology and metabolism may differ when salt and water stress is used separately or both stresses are used simultaneously in the experiment (Sucre and Suárez 2011). For example, the influence of the drought on the productivity of grains was regardless of the degree of soil salinization. Conversely, the relationship between salinization of the soil and the reduction of the grain harvest did not depend on the degree of drought increase and did not differ from the relationship existing between the reduction of crop yields and soil salinization separately (Katerji et al. 2009).

Adaptive responses of plants to specific stressful conditions depend on accurate accounting of environmental conditions. Molecular, biochemical, and physiological processes, induced by a particular stress, may differ from those ones activated at other environmental parameters. Transcriptome profiles, studied at the effect of high and low temperature, drought, salinity, high-intensity light, or mechanical stress, were individual, differing from each other. Each different stress condition tested prompted a somewhat unique response, and little overlap in transcript expression could be found between the responses of plants to abiotic stresses (Cheong et al. 2002; Fowler and Thomashow 2002; Kreps et al. 2002; Rossel et al. 2002; Rizhsky et al. 2004). Although the formation of reactive oxygen species takes place at various biotic and abiotic stresses, however, an expression of different sets of ROS genes has been observed under different stressful effects (Mittler et al. 2004). Thus, each individual stress leads to a unique adaptive response, and the combination of two or more different stresses can cause a response, which is also unique.

7.4 Physiological Responses of Plants to Combined Water and Salt Stress: Comparison of the Effect of Drought and Salinity

One of the physiological responses of plants to drought and salinity is the reduction of water content in the cells (a decrease of water potential) and accumulation of organic matter to restore turgescence. A combined effect of water and salt stress is often additive and accompanied by a decrease in gas exchange, chlorophyll, biomass accumulation, and the growth (Brown and Pezeshki 2007). In addition, matrix potential of the soil has also an additive effect on reducing free energy of water in the soil. Thus, it was assumed that water stress on saline soils should only aggravate a toxic effect of the salt, limiting water absorption by the roots and the ability of plants to withstand drought (Shalhevet and Hsiao 1986; Shalhevet 1993; Glenn and Brown 1998; Grewal 2010). Indeed, in the works of some researchers the plants exposed to combined water and salt stress were less viable than at drought and salinization separately (Razzaghi et al. (2011); Ahmed et al. 2013). Yield reduction of leguminous plants was associated with an increase in salinity of the soil. This process was reinforced under combined water and salt stress. However, a comparison of these results with those obtained in similar experiments on wheat and barley has shown that these plants have contrast patterns of behavior in case of double water-salt stress (Katerji et al. 2011). For proper evaluation of physiological effects of various stresses, it is required to consider their intensity and duration (Munns et al. 2000a, b; Munns 2002; Chaves et al. 2009).

The presence of salt in the soil increases significantly its water-holding capacity. Table 7.1 shows changes of relative water content (RWC_S) in the soil at combined water and salt stress depending on the concentration of NaCl. The reduced rate of the development of the drought in the presence of salts in the soil seems to be associated with a reduction of mobility of water molecules due to hydration of ions Na^+ and Cl^- in solution. As the concentration of NaCl increases in the soil, the rate of the development of drought is decreased. For example, in the case of sandy soil, the reduction of RWC_S achieved up to ~5 % for 4 days in the absence of salt in the soil and for 6 and 9 days at 0.05 and 0.1 M NaCl, respectively. Further increases in concentrations of salt up to 0.2 and 0.3 M NaCl in the soil retarded the development of drought still more, but increased the effect of salt toxicity and the risk of exceeding the threshold of salt resistance of the plants dramatically. In our case, at high concentrations of salt the wheat seedlings were dying. Thus, the presence of small quantities of salt in the soil helped to reduce the rate of the development of drought and to expand the period of adaptation of wheat plants under environmental conditions changed.

Hence, contrary to initial expectations that stress factors had additive nature on plants' performance, the productivity of amaranth was higher at double water-salt stress (Omami and Hammes 2006) that, according to the authors, was associated with reduction of water loss through transpiration and withering of old leaves. Richards (1992) also reported on beneficial effects of salinity on the plants grown in

Table 7.1 Relative water content in soil (RWC_S, %) under different NaCl concentrations during combined drought and salinity stress

DAT	NaCl concentrations in soil				
	0	0.05 M	0.1 M	0.2 M	0.3 M
1	60.4	62.8	65.9	73.5	84.1
2	28.8	43.4	48.1	60.4	68.7
3	11.9	23.0	31.6	47.4	57.1
4	5.8	14.2	22.7	37.7	44.9
5		8.9	16.3	29.2	35.0
6		5.3	11.6	22.8	30.2
7			8.6	18.6	
8			5.8		
9			4.3		

dry soil. According to Shalhevet (1993), one of the explanations of the increased performance of plants at salinization was the reduced size and the growth rate of the leaves that reduced the rate of water depletion from soil and, thus, favored an increase of plants' lifetime.

A combined effect of different stress factors can lead to simultaneous activation of various ways of plants adaptation. They may have synergistic or antagonistic character at interaction with each other, or a specific path can be triggered at combination of the stresses (Rizhsky et al. 2004). Interaction between jointly activated pathways may include integration between different systems of transcription factors and mitogen-activated protein kinase (MAPK) pathways (Cardinale et al.2002; Xiong and Yang 2003), perform cross-interaction of various stress hormones, such as ethylene, abscisic and jasmonic acid (Anderson et al. 2004), Ca^{2+} and/or ROS signaling systems (Bowler and Fluhr 2000; Mittler et al. 2004), as well as the title of the interaction of different signaling systems (Casal 2002).

7.4.1 Growth Processes in Leaves at Different Stresses

As water deficit and soil salinization adversely affect the plant growth, causing reduction of the rate of leaf expansion and their final sizes, reduced productivity of the leaves and their accelerated aging (Greenway and Munns 1980; Munns 2002; Munné-Bosch and Alegre 2004; Parida and Das 2005). However, NaCl can stimulate the growth of halophytes with increased concentration of salt in the soil up to a certain limit (Flowers et al. 1977; Erdei and Kuiper 1979; Rubinigg et al. 2004).

Wheat is a plant species with an average sensitivity to soil salinization. In the absence of salinization in well-watered soil, the seedlings of this plant show a linear increase of the leaves, at least within 10 days from the moment of formation (Fig. 7.1a, index W). In our experiments, on the fifth day of formation (or 10 days from germination), the seedlings were exposed to three types of stress: drought, salinization, and combined water and salt stress. In drought conditions the accumulation of dry matter has been stopped almost immediately after the start

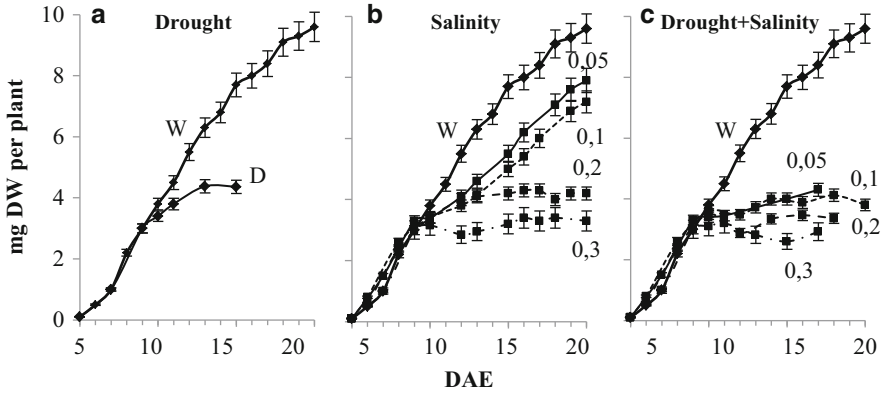

Fig. 7.1 Dry weights (DW) of young leaves of wheat seedlings (*Triticum aestivum* L.) growing under different stress conditions: (**a**) drought stress (D) and control, optimal watering (W); (**b**) salt stress under different NaCl concentrations in the soil; (**c**) combined drought and salt stress under different NaCl concentrations in the soil. Stress effects on the 10th day after emergence. 0.05–0.05 M NaCl in the soil, 0.1–0.1 M NaCl in the soil, 0.2–0.2 M NaCl in the soil, 0.3–0.3 M NaCl in the soil. The values are the average of three replicates (pots) ± standard deviations (SD)

of stress exposure (Fig. 7.1a). The similar situation was observed at combined water and salt stress at all the concentrations of NaCl in the soil (Fig. 7.1c). On the contrary, complete inhibition of the plant growth at single salt stress was observed only at high (0.2 and 0.3 M) concentrations of NaCl. At low concentrations of NaCl (0.05 and 0.1 M), biomass still continued to be accumulated under stress, though slowly (Fig. 7.1b).

A high tolerance to salt stress of the species is often associated with slow growth and the development of transport mechanisms, preventing the absorption of salts (Flowers and Yeo 1995; Koyro 2000; Koyro 2006). Under salinization the loss of water by plants should be reduced to a minimum, since biomass production depends mainly on the ability of plants to maintain net photosynthesis high and the low rate of water loss. From this point of view, the production of plant biomass should always be considered in connection with consumption of energy and gas exchange (Koyro 2006). The main cause of reduced growth of glycophyte at salt stress may be toxic ions, ion imbalance, or properties of cell membranes (Flowers and Yeo 1986; Yeo et al. 1991; Munns 2002; Rodríguez et al. 2005).

Water and salt stress, as well as their combination initiated osmotic stress, which was accompanied by reduced water absorption in the growing area, where xylem water potential suddenly fell (Cramer and Bowman 1991; Passioura and Munns 2000). There has also been a rapid decline of leaf elongation and growth inhibition observed in many species, having a nonspecific nature (Termaat and Munns 1986; Thiel et al. 1988; Cramer and Bowman 1991; Yeo et al. 1991; Passioura and Munns 2000). Moreover, since the developing water stress reduces tissue expansion before stomatal closing (Hsiao 1973), Tardieu et al. (2011) came to the conclusion that deficit of water affected the plant growth through reduction of tissues extension,

carbon absorption, and the cell number; the first process was the most important. However, Turner (1986) showed that osmotic adjustment supported the cell turgor after water stress and, subsequently, helped in growth restoration. As a result of osmotic adjustment, the amaranth plants showed increased turgor of the leaves under stress with reduced extension of the leaf. These results support an idea of Munns (1993) that although turgor is a potential energy, providing cells expansion, it is not the parameter that controls the growth process.

In long-term prospect, inhibition of leaves' growth can be considered as adaptations of the plants to water deficit and salinization, since this enables the leaf surface to be reduced in order to lower water loss via a decrease of transpiration. All these factors increase lifetime of the plants in stressful conditions (Ruiz-Sánchez et al. 2000; Ramoliya and Pandey 2002; Munné-Bosch and Alegre 2004; Rouhi et al. 2007; Chaves et al. 2009).

The factor, determining reduction of the leaves' area at drought and salinity combined stress, can be also an increase of the number of dead leaves (Greenway and Munns 1980; Yeo et al. 1991; Munns 2002; Munné-Bosch and Alegre 2004). Drought-induced senescence of the leaves plays an important role in survival of some species, as aging of the leaves helps to move nutrients from the old leaves and to avoid great water loss, thereby optimizing utilization of the nutrients and water at the level of the whole plant (Ruiz-Sánchez et al. 2000; Munné-Bosch and Alegre 2004).

On the contrary, leaves' necrosis under saline stress is normally associated with accumulation of the salt on the toxic levels, and maintaining the higher levels of leaf production relative to the dying leaves is an important characteristic of plant physiological resistance to stresses (Greenway and Munns 1980; Yeo et al. 1991; Munns 2002).

The influence of water and saline combined stress has frequently less harmful effects on the plants as compared with every stress in particular. At succulents (Slama et al. 2008), mangrove plants (Atreya et al. 2009) and *Ipomoea* (Sucre and Suárez 2011) similar effect of double stress was on the growth rate of the leaves and their total area. At amaranth in salt stress and in combined water-salt stress, biomass accumulation was higher as compared to the plants at only water stress (Omami and Hammes 2006). Pérez-Alfocea et al. (1993) found that several genotypes of tomatoes had similar response to salinity and drought.

7.4.2 Water Exchange in Plants at Different Stresses

Soil salinity and drought have a similar impact on physiological state of the plants (Shalhevet and Hsiao 1986; Munns 2002), since in this case difference in water potential between the substrate and the plant is induced that restricts water flow from soil into the leaves. Drought induces both water and osmotic stress in plants. Low osmotic potential (as a result of dissolved salts) and low capacity of the soil matrix (associated with reduced water content) cause reduction of water potential in

plants. In this case matrix and osmotic potentials of the soil have additive effect in reducing free energy of water in the soil (Shalhevet 1993). As a result, this leads to a decrease in the rate of expansion of leaves, photosynthesis and, ultimately, to a reduce in the growth rate (Rawson and Munns 1984).

Water and osmotic potentials are reduced at all kinds of stresses that ensure maintenance of water flow in the leaves and cell turgor pressure. Osmotic potential of the leaves should always be less than water potential of the soil. This can be achieved by osmotic adjustment at accumulation of soluble substances in the cells (Turner and Jones 1980; Premachandra et al. 1995).

Lower relative water content of the leaves (RWC_L) as a result of dehydration of the cells also contributes to the maintenance of osmotic potential at the low level. However, no significant changes of relative water content in the leaves during initial period of osmotic stress suggest that dehydration cannot be the most important factor in lowering the osmotic potential. Hence, xerophyte saltbush (*Atriplex halimus*) RWC_L at water stress decreased more significantly than in the presence of NaCl (Martínez et al. 2005). In other species of halophytes, RWC_L declined slightly after days or months of exposure to saline stress (Ramani et al. 2006; Redondo-Gómez et al. 2007), while at drought reduced RWC_L was more noticeable (Turner and Jones 1980; Tezara et al. 2003; Pérez-Pérez et al. 2007).

The similar situation was observed in glycophyte plants with short-term stress. During the first days of the development of drought, RWC_L of the leaves of wheat seedlings remained at the initial level (Fig. 7.2a). Then, upon reaching relative water content in the soil (RWC_S) ~12 %, RWC_L began to decline sharply, reaching ~30 % at RWC_S ~6 %. With start of irrigation, RWC_L restored to its initial level. As a control (W) the diagram shows the value of RWC_L plant leaves with full watering of the soil which remained unchanged throughout the experiment.

Under salt stress at low concentrations of NaCl in the soil (0.05 and 0.1 M), RWC_L remained unchanged and coincided with full-watering control throughout the 14 days of the experiment (Fig. 7.2b, c). At NaCl concentration of 0.2 and 0.3 M in the soil, RWC_L was maintained on the initial level for 6 and 8 days, respectively, from the beginning of salinity and then there was a sharp decrease in water content in the leaves (Fig. 7.2d, e).

Osmotic potential of the plants is reduced to a greater extent under saline stress as compared under water stress. This can be explained by the fact that inorganic ions are predominantly accumulated under salt stress in the plants, whereas at drought accumulation of organic matter is more significant (Torrecillas et al. 1995). More serious consequences of water deficit relative to saline stress at maintaining turgor can be due to incomplete osmotic adjustment under drought, because osmotic adjustment via absorption of inorganic ions is more efficient as compared to rearrangements by production of soluble organic substances (Shalhevet and Hsiao 1986; Martínez et al. 2004; Rodríguez et al. 2005; Slama et al. 2008). Raven (1985) suggested that osmotic adjustment by salt accumulation requires less power and carbon than by accumulation of dissolved organic matter. Similar comments were made by Alian et al. (2000) on tomatoes.

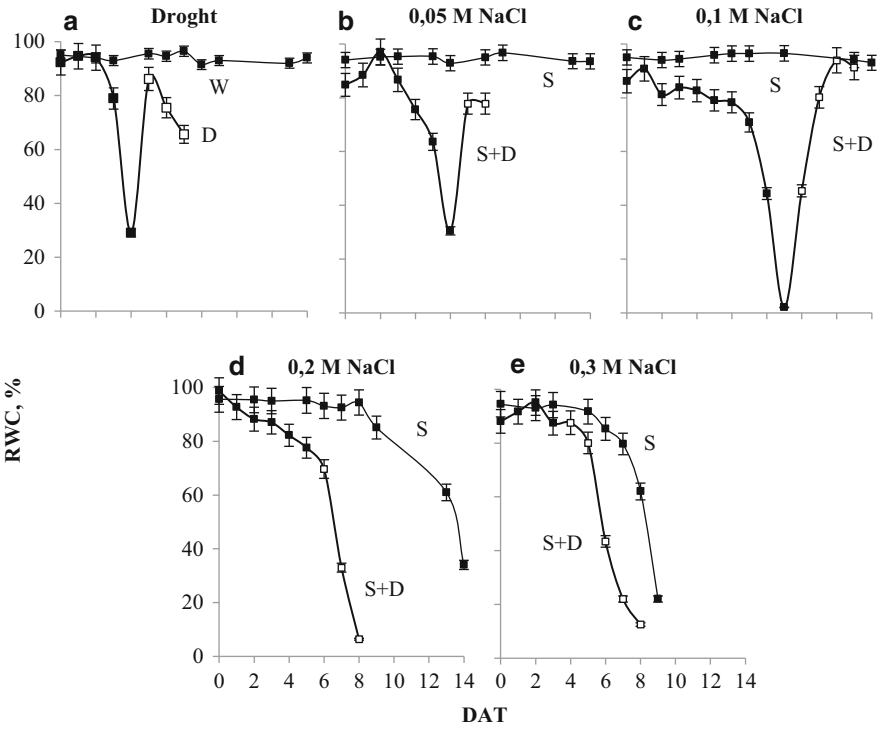

Fig. 7.2 Relative water content in young leaves (RWC_L) of wheat seedling (*Triticum aestivum* L.) after treatment of drought (D), salinity (S), and combined drought and salinity stress (D + S) conditions: (**a**) drought stress (W—control, optimal watering); (**b**) 0.05 M NaCl in the soil; (**c**) 0.1 M NaCl in the soil; (**d**) 0.2 M NaCl in the soil; (**e**) 0.3 M NaCl in the soil. The values are the average of three replicates (pots) ± standard deviations (SD). *White marks* correspond to the data after restart of plant watering

However, it should be considered that in sensitive varieties at long stress with increasing levels of salinity a gradual increase of the cell damage and a reduction of RWC_L are observed, as salinity decreases water potential in the roots. In this case reduction of RWC_L could be considered as "water stress" (Shafqat and Farooqe 2006). For example, at 250 mm NaCl in the soil, RWC_L was reduced up to 30 % after which the wheat plants did not survive even after subsequent watering (Blum 1996).

The cumulative impact of salt and reduced RWC on sensitive varieties cause high level of cellular damage (74 %) that negatively correlates with RWC (Sairam et al. 2002). Salt-resistant varieties absorb less Na^+ as compared to the salt-sensitive plants; however, reduced RWC can be also a cause of cells damages at the same level of salinity.

Under combined water and salt stress at low concentrations of NaCl in the soil (0.05 and 0.1 M), RWC_L of the seedlings was slowly decreased up to ~70 % within the first days of stress development, remaining, nevertheless, at rather high level to

ensure normal work of biochemical processes in the plants (Fig. 7.2b, c). Upon reaching RWC_S ~9 %, a sharp decline of water content in the leaves (5 and 7 days, respectively) occurred. In particular, in the variant with 0.1 M NaCl a very severe dehydration of the tissue was observed. However, the leaves kept their vitality. As watering continued RWC_L of the plants was restored. Another picture was observed at high concentrations of NaCl in the substrate (0.2 and 0.3 M) (Fig. 7.2d, e). RWC_L in these variants was reduced throughout the experiment. This decline continued even after restart of watering. Hence, it is followed that the presence of salts in the soil in small concentrations contributed to the maintenance of RWC_L at high level under developing drought for a longer time than in the absence of salt in the soil. Further increase of salt concentrations resulted in the death of plants that could be associated with achievement of the tolerance threshold of wheat plants, when positive effect of NaCl changed on sharply negative one.

Thus, the plants' response at combined water and salt stress was more complex than simple additive effect from these two stressors (Shalhevet 1993). In case of double stress, maximum osmotic adjustments in plants' tissues occur (Jensen 1981) that allow the plants to continue the growth for a longer period of the drought as compared to the plants at the same water stress. Simultaneous action of salt and water stress accelerates recovery of turgor after osmotic stress as compared with both stresses applied individually (Sucre and Suárez 2011). Martínez et al. (2005) and Slama et al. (2008) ascertained that the presence of salts in growing conditions of the plants helped to prevent dehydration of leaves' tissues at drought and to restore water balance as compared to the nonsalinized plants. However, exact mechanisms, giving for plants an advantage under conditions of simultaneous salt stress and water deficit as compared to the plants exposed to only one of the stresses are not fully understood (Martínez et al. 2005; Pérez-Pérez et al. 2007).

The presence of salt in the soil can actually reduce some negative effects of water scarcity. For example, the plants at water stress usually live longer in saline than in non-saline soils, because the plants at salt stress are less grown and, therefore, exhaust water reserves more slowly in the soil than the non-salt-stressed plants (Richards 1992; Shalhevet 1993). The study of the combined effect of salt and water stresses on the growth of sorghum (Richardson and McCree 1985) showed that although the salinity decreased the rate of leaf expansion in well-watered conditions, it also allowed the plants to continue leaf expansion, even in case of decreasing water potential of the leaves at water stress. This is probably a result of osmotic adjustment upon accumulation of salts that enables us to maintain the plants turgor at low water potential of the soil (Jensen 1981).

7.4.3 Na^+ Accumulation in Leaves at Different Stresses

Absorption and accumulation of Na^+ and Cl^- in different plant organs is a highly controlled process (Munns 1993; Hasegawa et al. 2000). Glenn et al. (1996) indicated that there was a strong positive correlation between the intensity of

absorption of Na^+ and the degree of salt tolerance. On the other hand, PEG-induced water stress in the leaves caused Na^+ accumulation in higher concentrations than expected among the plants growing in nutrient solutions containing low concentration of Na^+ (20 μm). A hypothesis that was made in conditions of water stress Na^+ could carry a positive function, since the plants increased specifically the absorption of Na^+ during drought (Martínez et al. 2004, 2005). There is also a speculation that the salt-tolerant plants are resistant to some extent of water deficit (Farooq and Azam 2002).

Halophytes have built-in mechanisms to control excessive root zone salinity, including effective regulation of salt transport and its distribution throughout the whole plant, organs and subcellular levels (Tattini et al. 2002), as well as transportation systems for selective absorption of sodium and chlorine into the vacuole (Mühling and Läuchli 2002). This system functions as a regulatory metabolic cycle to avoid critical concentration of ions in a cell. This adaptive mechanism has homeostatic functions in supplying basic elements of nutrition, metabolism, and the function of detoxification (Smekens and Tienderen 2001; Koyro 2000, 2003). Compatible soluble substances protect plants at salinization of the root zone, accumulating mainly in the cytosol and maintaining osmotic balance with ions absorbed in the vacuole (Flowers et al. 1977). The concentration of Na^+ in the leaves of halophytes was significantly higher at double water-salt stress as compared to the plants at a single salt stress.

Similar mechanisms may present in the cells and glycophyte plants (Koyro and Stelzer 1988; Flowers et al. 1991; Colmer et al. 1994; Mühling and Läuchli 2002), although the cells of salt-sensitive plants have low ability to retain Na^+ out of the cytoplasm that was demonstrated at wheat varieties (Iqbal et al. 2001).

In our experiments the rates of ion accumulation in young leaves of wheat seedlings both at the salt (S) and double (S + D) stress did not practically differ from each other and were linear throughout the experiment. At low concentrations of NaCl in the soil (0.05 and 0.1 M), the rate of Na^+ accumulation was practically identical, making up 0.08 and 0.09 mmol g^{-1} DW per day (Fig. 7.3a, b). At NaCl concentration of 0.2 M, the rate of ion accumulation reached 0.18 mmol of Na^+ g^{-1} DW per day (Fig. 7.3c) and increased sharply at maximal concentration of the salts (0.3 M) at 0.2 mmol Na^+ g^{-1} DW per day (Fig. 7.3d). With restart of watering in the experiment at double stress, Na^+ accumulation in the leaves decreased slightly in the variants with 0.05 and 0.1 M NaCl. At 0.2 and 0.3 M NaCl in the soil upon watering of the plants, the accumulation of ions was retarded or declined slightly, but with further sharp increase of the salt in the leaves. Thus, it can be seen that at low concentrations of NaCl in the soil Na^+ accumulation in the leaves was slow, but it increased dramatically at high concentrations of the salt. This may be due to exceeding of the threshold of salt tolerance of this plant species (Maas and Hoffmann 1977) or a sharp increase of permeability of the cells' membranes. On the other hand, accumulation of Na^+ can be greatly increased at sharp reduction of RWC_L occurring under the influence of high levels of salinity (Shafqat and Farooqe 2006).

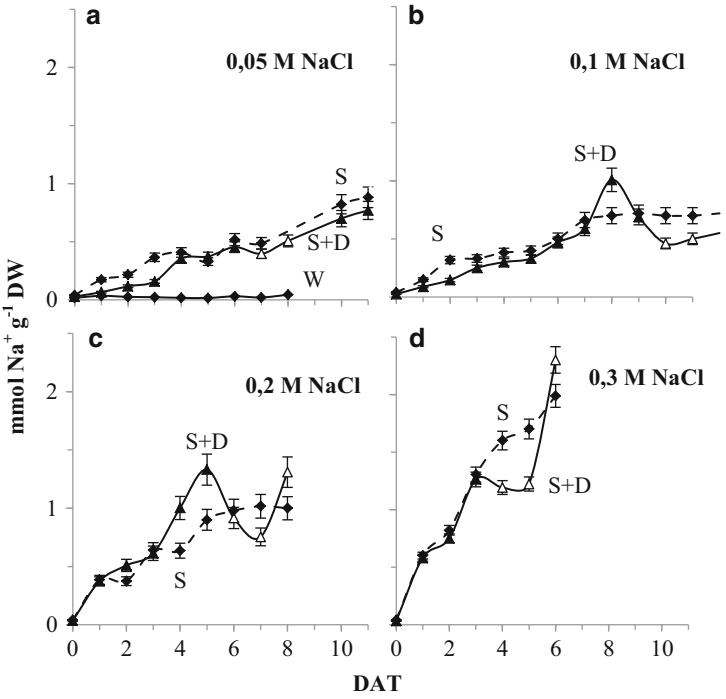

Fig. 7.3 Na^+ accumulation in young leaves of wheat seedling (*Triticum aestivum* L.) after treatment of salinity (S) and combined drought and salinity stress (D + S) conditions: (**a**) 0.05 M NaCl in soil (W—control, optimal watering, no salt in the soil); (**b**) 0.1 M NaCl in the soil; (**c**) 0.2 M NaCl in the soil; (**d**) 0.3 M NaCl in the soil. The values are the average of three replicates (pots) ± standard deviations (SD). *White marks* correspond to the data after restart of plant watering

There is a speculation on involvement of Na^+ in resistance mechanism to water stress and that Na^+ may have a positive effect directly or indirectly on accumulation of other compounds involved in osmotic adjustment (Martínez et al. 2005; Slama et al. 2008). As Shalhevet (1993) pointed out salt stress could allow the plant to be prepared to reduce water potential of the soil by increasing osmotic adjustment, thus improving their ability to survive in drought conditions.

7.4.4 Proline Accumulation in Leaves at Different Stresses

To maintain water status and reduce osmotic potential of the leaves in stressful circumstances, many plants accumulate compatible metabolites in the cytoplasm of the cells (Bohnert et al. 1995; Munns 2002). Accumulation of proline is considered to be the first response of plants exposed to salt stress (Ebenhardt and Wegmann 1989; Madan et al. 1995; Trovato et al. 2008) and water-deficit stress (Boggess and

Stewart 1976; Stewart 1978; Stewart et al. 1977; Handa et al. 1983). There is a linear relationship between the degree of the development of drought and the concentration of proline, as well as between the concentration of proline and NaCl (Somal and Yapa 1998). Kinetics of accumulation of this substance depends on the type of the plant, intensity, and the period of stress (Ashraf and Foolad 2007; Errabii et al. 2007; Cha-um and Kirdmanee 2009). It is considered that accumulation of proline serves to protect plants against stress, acting as a substance of N-storage, osmotically active substance and hydrophobic protector for enzymes and cellular structures (Stewart and Lee 1974; LeRudulier et al. 1984; Chandler and Thrope 1987; Madan et al. 1995).

The higher levels of proline in plants under stress were associated with increased activity of ornithine aminotransferase, pyrroline carboxylate reductase, the enzymes involved in biosynthesis of proline (Charest and Pan 1990; Kohl et al. 1990; Madan et al. 1995), inhibition of proline oxidase, the catabolism enzyme—proline (Stewart et al. 1977; Kandpal et al. 1981; Madan et al. 1995), as well as a decrease of protein synthesis (Stewart et al. 1977).

Accumulation of free proline in glycophyte plants under stress is widely spread. However, several researchers showed that the rate of proline accumulation was relatively low at water or saline stress (Dix and Pearce 1981; Naik and Joshi 1983; Chavan and Karadge 1986; Somal and Yapa 1998). Sucre and Suárez (2011) observed that the content of proline in the leaves increased significantly only at high degree of salinity.

This can be partially explained by the fact that with an increase of salinity the role of other osmotic protectants also increases. In glycophyte plants, such as wheat, osmotic adjustments with accumulation of NaCl in the leaves are associated with accumulation of high levels of K + in the cytoplasm (Sairam et al. 2002) or organic matter such as proline (Hasegawa et al. 2000), glycine betaine (Sakamoto and Murata 2002), and soluble sugars (Maggio et al. 2002). Osmotic function of sugars is well known at wheat (Blum and Ebrecon 1981) and corn (Premachandra et al. 1989), growing in drought or salinization. Shafqat and Farooqe (2006) reported about accumulation of sugars in large quantities. Osmotic regulation of the *Plantago coronopus* was achieved due to sorbitol in most of the organs (Koyro 2006).

On the other hand, as pointed out by Ahmed et al. (2013), at combined water and salt stress accumulation of proline occurred much more rapidly than in the case of a single stress. In this case accumulation of glycine betaine was typical for water stress, whereas at combined stress it was accumulated to a lesser degree (Ahmed et al. 2013). High sustainability of plants at combined stress was closely associated with low Na^+/K^+ ratio, the increasing activity of Ca^{2+} Mg^{2+} ATPase, accumulation of proline and glycine betaine, increased efficiency of water use, and the activity of antioxidant protection against ROS by suppressing the levels of lipid peroxidation (Ahmed et al. 2013).

In our experiments the amount of accumulated proline depended greatly on the type of stress. In the control, in the absence of stress, the content of proline was ~6 μmol g^{-1} DW. In case of water stress, accumulation of proline occurred only under

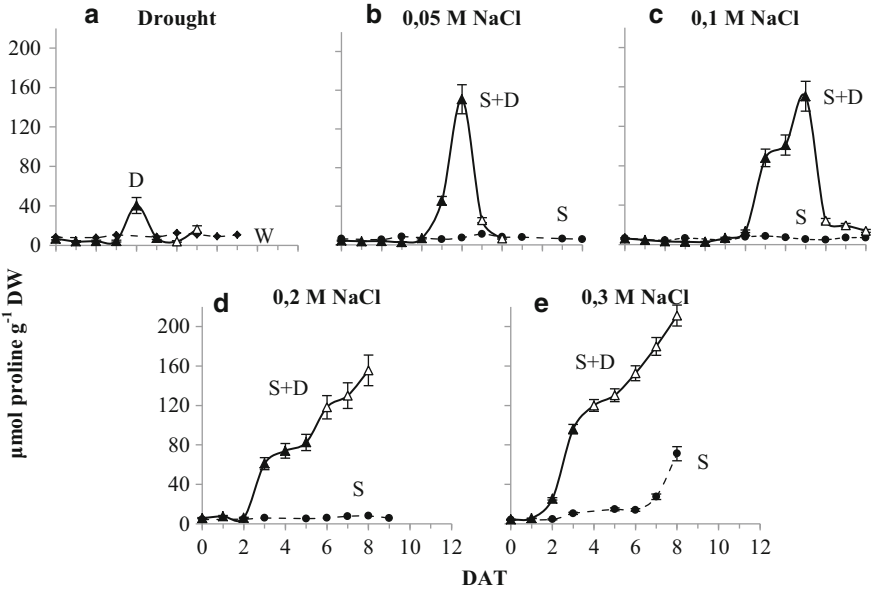

Fig. 7.4 Proline accumulation in young leaves of wheat seedling (*Triticum aestivum* L.) after treatment of drought (D), salinity (S), and combined drought and salinity stress (D + S) conditions: (**a**) drought stress (W—control, optimal watering); (**b**) 0.05 M NaCl in the soil; (**c**) 0.1 M NaCl in the soil; (**d**) 0.2 M NaCl in the soil; (**e**) 0.3 M NaCl in the soil. The values are the average of three replicates (pots) ± standard deviations (SD). *White marks* correspond to the data after restart of plant watering

strong dewatering of the soil at maximal point of the development of drought, reaching ~40 μmol g^{-1} DW (Fig. 7.4a). Upon irrigation proline concentrations decreased to initial values. In case of salt stress, has not been shown accumulation of proline during experiment (8-12 days) in all the variants of salinization except for 0.3 m NaCl in the soil, where a slight accumulation of this amino acid (up to ~70 μmol g^{-1} DW) began for the seventh to eighth day (Fig. 7.4b–e). A rather different picture was observed in case of combined water and salt stresses. Even a slight presence of salt in the soil led to rapid accumulation of proline, the concentration of which reached ~150 μmol g^{-1} DW in the point of maximum stress development in the variants with 0.05 and 0.1 M NaCl (Fig. 7.4b–e). After watering the proline content decreased to initial level, as in the case of water stress. At concentrations of 0.1 and 0.3 M NaCl, a high accumulation of proline has occurred; however, unlike the variants with low concentrations of salt in the soil upon irrigation proline content has not decreased, but increased. Thus, dynamical accumulation of proline is highly dependent on the range of NaCl concentrations used and the stress type. At water stress only a small amount of proline has been accumulated. Salt stress did not cause accumulation of proline. Probably it is necessary to increase the time of stress effect. At combined water and salt stress, accumulation of proline has

dramatically increased. However, at restart of watering the proline level decreased only in those cases when the plants were able to restore the physiological parameters with activation of proline oxidase (Stewart et al. 1977).

Proline accumulation was similar or higher in the plants subjected to PEG impact or mannitol as compared with those plants growing in the presence of NaCl in the same osmotic pressure (Pandey et al. 2004; Errabii et al. 2007; Cha-um and Kirdmanee 2009). It has been suggested that at drought osmotic adjustment is achieved, mainly, due to accumulation of organic substances synthesized in the plant, while at saline stress inorganic ions (Na^+ and Cl^-), easily available in the soil solution, are mainly accumulated (Flowers and Yeo 1986; Shalhevet and Hsiao 1986; Parida and Das 2005).

Both processes, accumulation of Na^+ and osmotically active substances, are associated with energy consumption in addition to the existing metabolic costs. Metabolic regulation of biomass production, storage of metabolites, and respiration is crucial for plants' survival in saline habitats (Martinez-Ballesta et al. 2004). Nevertheless, massive accumulation of soluble carbohydrates or compatible metabolites for osmotic adjustment removes most part of the energy that can be used for the growth (Bohnert et al. 1995; Tattini et al. 2002). Accumulation of these substances and a decrease of sink strength may affect negatively on the rate of net assimilation with the help of feedback mechanisms (Munns and Termaat 1986; Herbers and Sonnewald 1998; Sonnewald 2001).

7.4.5 Gas Exchange and Photosynthesis in Leaves at Different Stresses

Salinization, water stress, and their combined effect induce strong reduction in photosynthesis (Ouerghi et al. 2000; Yousfi et al. 2009, 2010; Omami and Hammes 2006). Usually the first symptom of salt and water stress is a reduction of stomatal conductivity that leads to a reduce of transpiration rate and intercellular CO_2 concentration (Tezara et al. 2003; Meloni et al. 2003; Flexas et al. 2004; Rodríguez et al. 2005; Ramani et al. 2006; Chaves et al. 2009; Cha-um and Kirdmanee 2009). As a result, net photosynthesis is inevitably decreased by reduced availability of CO_2 at the chloroplasts' level (Chaves et al. 2009). This leads to reduced flow of assimilates. Reduced maximal photosynthetic rate may also be related to biochemical restrictions (Tezara et al. 2003; Flexas et al. 2004; Chaves et al. 2009). Excessive concentration of Na^+ has negative effects for photosynthetic electron transport (Muranaka et al. 2002). However, change in stomatal conductivity is considered as a dominant factor limiting CO_2 assimilation, regardless of any metabolic damages (Flexas et al. 2004; Rouhi et al. 2007; Chaves et al. 2009).

A decrease in stomatal conductivity is considered to be the main cause of reduction in leaf transpiration at all kinds of stresses (Omami and Hammes 2006; Sucre and Suárez 2011). A decrease of transpiration in salt and water stress in

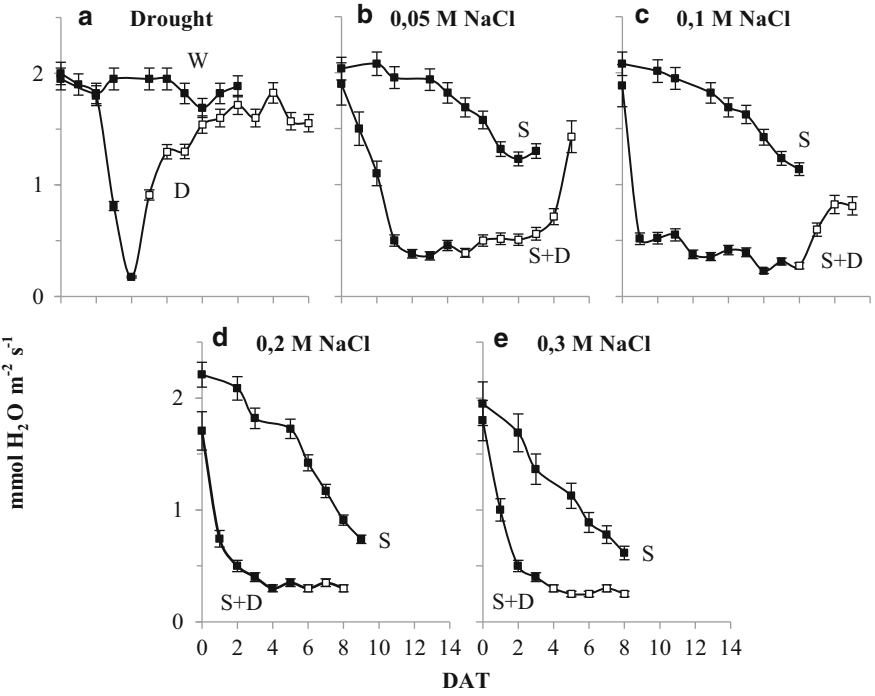

Fig. 7.5 Transpiration rate in young leaves of wheat seedling (*Triticum aestivum* L.) after treatment of drought (D), salinity (S), and combined drought and salinity stress (D + S) conditions: (**a**) drought stress (W—control, optimal watering); (**b**) 0.05 M NaCl in the soil; (**c**) 0.1 M NaCl in the soil; (**d**) 0.2 M NaCl in the soil; (**e**) 0.3 M NaCl in the soil. The values are the average of three replicates (pots) ± standard deviations (SD). *White marks* correspond to the data after restart of plant watering

different plant species was observed (Ashraf 2001; Liu and Stützel 2002). According to our experimental data, dynamical changes in the values of transpiration in the leaves of wheat seedlings under drought resembled the same ones for RWC_L (Fig. 7.2a), i.e., maintaining at high level in the first days of drought and a sharp reduction at maximal development of water stress (Fig. 7.5a). With restart of watering, the rate of leaf transpiration was quickly restored to its initial level. Similar data were obtained at determining photosynthetic activity of O_2. Photosynthetic activity remained unchanged in the early days of stress and then dropped abruptly that also corresponded to the evolution of RWC_L. Upon watering the photosynthetic rate restored, but never reached the initial level. A slight decrease of photosynthetic activity occurred in the control at the absence of stress that might be due to the age-related changes of the plant growth. Thus, at water stress the activity of photosynthetic apparatus depended mainly on the water content in the cells.

Another picture was observed at salt stress of the plants (Fig. 7.5b). The transpiration rate was gradually reduced since the first days of the experiment in

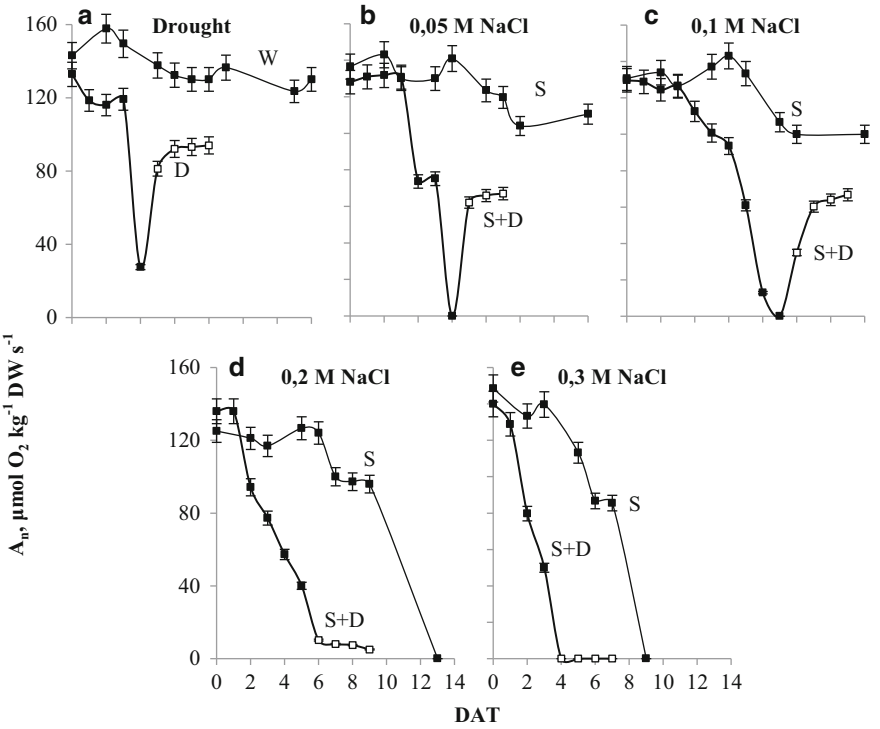

Fig. 7.6 Photosynthetic O_2 evolution (A_n) in young leaves of wheat seedling (*Triticum aestivum* L.) after treatment of drought (D), salinity (S), and combined drought and salinity stress (D + S) conditions: (**a**) drought stress (W—control, optimal watering); (**b**) 0.05 M NaCl in the soil; (**c**) 0.1 M NaCl in the soil; (**d**) 0.2 M NaCl in the soil; (**e**) 0.3 M NaCl in the soil. The values are the average of three replicates (pots) ± standard deviations (SD). *White marks* correspond to the data after restart of plant watering

all the variants and depended on the concentration of NaCl in the soil. In this case the rate of photosynthetic O_2 extraction decreased, but very slowly. At low concentration of the salt in the soil (0.05 and 0.1 M NaCl), the activity of photosynthetic apparatus remained within an experiment (Fig. 7.6b, c). At high concentration of salt (0.2 and 0.3 M), photosynthetic O_2 evolution reduced at a high rate, but did not drop below 50 % of the initial value for 9 and 7 days, respectively (Fig. 7.6d, e). Further there was an abrupt stop of photosynthetic activity.

At salt stress changes of photosynthetic activity depended on the range of concentrations of NaCl and most likely were associated with a threshold of salt tolerance of wheat plants. Should take into account that measurements of O_2 evolution reflects changes of photosynthetic activity, independent on the degree of stomatal opening and shows potential activity of photosynthetic apparatus. Hence, at 0.05 and 0.1 M NaCl potential activity of photosynthetic apparatus changed slightly, despite a decrease of the values in leaf transpiration. Since

RWC_L remained unchanged during the stress period (Fig. 7.2b, c), one could conclude that a decrease of potential activity of photosynthesis in this case was mainly associated with toxic effects of NaCl. Reduction of photosynthetic activity at high salt concentrations (0.2 and 0.3 M) can be due to both the decrease of RWC_L, and ions toxicity.

According to other researchers, among three types of stresses the salinity had really minimal damage to photosynthetic plants' apparatus (Omami and Hammes 2006). This might be due to high water potential of the leaves and turgor maintenance during salinity as compared with the plants at water stresses (Xu et al. 1994). The reduced leaf water potential leads to depression of photosynthesis. Similar to halophytes, salt-induced water stress controls mainly gas exchange of the leaves, while absorption of the salts and ion imbalance in the leaves do not cause irreversible damage of photosynthetic apparatus (Koyro 2006).

There is a significant difference in the ability of the plants to restore at different types of stresses. After water stress and further watering of amaranth, plants restoration of photosynthesis and stomatal conductance occurred to the control level. This testifies that in stress there was no damage to photosynthetic apparatus. Upon removing salts, the plants restored only stomatal conductance with the absence of recovery of photosynthesis that was most likely caused by toxic effect of high salt concentrations on photosynthetic apparatus (De Herralde et al. 1998; Omami and Hammes 2006). In this case, restoration of photosynthesis most likely depended on the concentration of NaCl used, as shown in our experiments.

The mostly marked reduction of photosynthetic intensity was under the action of double water-salt stress as compared to the effect of every stress at one and the same water potential. This was shown in different plant species (Chen et al. 2010; Pérez-López et al. 2012). The simultaneous application of NaCl and PEG in nutrient solution, however, increases restoration of maximal photosynthetic intensity and stomatal conductivity after stress. Similarly, Martínez et al. (2005) have shown that low salinity levels can improve the ability of plants to overcome the PEG-induced water stress. A reduce of photosynthesis can be connected both with the movements of the stomata and the influence of biochemical processes (Farquhar and Sharkey 1982). Thus, a sharp decline of barley photosynthetic intensity, stomatal conductivity, transpiration, and stomatal closure was due to the mechanism of plants resistance to the loss of water at water-salt stress. However, reduced photosynthesis could be also attributed to the inhibition of chlorophyll synthesis at this stress (Ahmed et al. 2013).

According to our data, in the first days upon treatment at combined water and saline stress, there was a sharp reduce in transpiration rate, which remained at the lowest level throughout the experiment in all the variants with different salt concentrations in the soil (Fig. 7.5b). However, as watering restarted, only the leaves with the variants at low concentrations of NaCl in the soil (Fig. 7.5b, c) were able to restore the transpiration. It should be noted that unlike a drought, in this case the increased transpiration after watering occurred very slowly. At concentrations of 0.2 and 0.3 M NaCl in the soil, no recovery of transpiration happened (Fig. 7.5d, e).

At the same time, dynamics of photosynthetic O_2 evolution (Fig. 7.6b) was corresponded to a great extent to that one while measuring of RWC_L that indicated the same situation with water stress (Fig. 7.6a). At low salt concentrations (0.05 and 0.1 M), a rather low decline of photosynthetic activity in first early days changed slowly with a sharp decline to zero at strong dewatering of the leaves and the substrate (Fig. 7.6b, c). Upon watering of the plants, photosynthesis was restored, however reaching only ~50 % of the initial level. At 0.2 and 0.3 M NaCl from the first days of the development of double stress, the photosynthetic rate decreased rapidly up to zero values and did not restore after watering of the plants (Fig. 7.6d, e).

Thus, change of potential photosynthetic activity is mainly determined by the dynamics of water stress, not depending on the degree of stomatal opening. One of the positive peculiarities of the presence of salt in the soil at combined stress is a decrease of the development of drought and, thereby, an enhancement of plants' lifetime at stressful situations while keeping high level of photosynthesis (Fig. 7.6b, c). Toxic effect of NaCl is manifested only at high concentrations of salt in the soil (0.2 and 0.3 M), apparently as a result of exceeding the threshold of salt tolerance of wheat plants. It is interesting to note that Na^+ accumulation in seedling tissues did not depend on the leaf transpiration rate (Figs. 7.3 and 7.5) and was almost exactly the same as that at a salt and double stress. This contradicted Pitman (1988), who considered that Na^+ uptake by plants was proportional to the level of transpiration. On the other hand, Ahmed et al. (2013) showed that accumulation of Na at salt and combined stresses differed insignificantly.

The plants' response to drought and salinity by stomatal closure prevents the development of water deficit in the tissue, preserving the leaf for recovery (Mohammadkhani and Heidari 2008) that is typical both for the glycophytes and halophytes (Shalhevet and Hsiao 1986; Ramani et al. 2006; Redondo-Gómez et al. 2007; Mohammadkhani and Heidari 2008).

Like in other species salinity has a strong impact on leaf gas exchange of the halophytes (Flanagan and Jefferies 1989; Mudrik et al. 2003). In the chloroplasts electron quantum yield and respiration in the dark decreased that could reflect relative increase of alternative processes for consumption of the electrons. An increased ratio of carotenoid/chlorophyll, reducing the flow of electrons via the photosystems (reduced efficiency of photosynthesis), lowers the risk of photoinhibition (Koyro 2006). The growth and net photosynthesis rate changed proportionally with an increase of salinity though it was frequently difficult to ascertain correlation between carbon uptake and the growth of all plants at salt stress (Cheeseman 1998; Clark et al. 1999). However, serious reductions in growth and gas exchange in saline conditions testified their close relationship (Koyro 2006). It is expected that reduced stomatal conductivity and transpiration represent adaptive mechanisms to control excessive salinity and not its negative consequences (Flanagan and Jefferies 1989; Clark et al. 1999; Koyro 2006). The halophytes show a combination of low net photosynthesis, transpiration, minimum stomatal resistance, and minimal internal CO_2 concentration (Koyro 2000, 2003). This strategy aims to reduce the supply of salt into the leaves and helps to increase

lifetime, maintaining concentration of the salt in subtoxic levels longer than it will occur if the transpiration rate is reduced (Everard et al. 1994). The salt-induced water stress controls mainly gas exchange of the leaves, while the absorption of salts and ion imbalance in the leaves does not cause irreversible damage to photosynthetic apparatus (Koyro 2006).

At the same time, the reduced CO_2 assimilation due to the reduced stomatal resistance is useful for optimizing photosynthetic water-use efficiency by the plants (Flexas et al. 2004; Rouhi et al. 2007), which increases significantly at water and salt stress. The higher water-use efficiency is reached due to the lowering of stomatal resistance and maximization of photosynthesis (Xu et al. 1994). At salt stress the plants showed higher values of photosynthetic water-use efficiency as compared to water stress (Omami and Hammes 2006), since a decrease of stomatal conductivity leads to lesser decrease of photosynthetic rate than transpiration. This can be considered as sodium avoidance mechanism (Greenway and Munns 1980; Brugnoli and Bjorkman 1992) which allows the plants to survive in conditions of water deficit.

A number of studies (James et al. 2008; Rahnama et al. 2010) show that screening for high stomatal conductivity can be an efficient way to identify fast-growing genotypes on saline soils. However, according to the other authors, stomatal conductivity or other parameters of gas exchange differed insignificantly between the resistant and susceptible genotypes (Yousfi et al. 2010, 2012).

7.4.6 Oxidative Processes in Leaves at Different Stresses

The levels of reactive oxygen species (ROS) in plant cells increased at different types of environmental stresses, including drought and salinization. That might be associated with the decreased gas exchange at stress and, consequently, the activity of photosynthetic apparatus. The ROS are chemically reactive molecules, obtained as a result of the incomplete recovery of oxygen. These include singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$). The mitochondria and the chloroplasts are important generators of intracellular ROS. In chloroplasts, singlet oxygen can be generated by direct transfer of excitation energy from photon excited chlorophyll to triplet oxygen. Formation of superoxide radicals occurs in photosystem I chloroplast in the Mehler reaction related to the work of the 4Fe-4S clusters, ferredoxin and/or ferredoxin-NADPH reductase, or in the reaction center of photosystem II, probably in QA and QB sites (Asada 1999). When the photoreceptors capture more light energy than what is required for the photosystem, the light quanta can start to react with other substrates that differed from conventional electron carriers. In these reactions ROS are able to be generated in damaging concentrations (Sicher 1999), which increases the risk of photoinhibition or photooxidation. Adaptation to the light harvesting capacity can lead to a reduction (or optimization) of photosynthetic efficiency and, as a result, to oxidative stress (Moorthy and Kathiresan 1999).

NAD(P)H oxidase, localized in plasmalemma (Plas et al. 2002), as well as oxalate oxidases, peroxidases, and amine oxidases in apoplast can be another source of ROS in plants. These enzymes play a role of ROS-delivering system for physiological processes, such as regulation of the cell growth (Foreman et al. 2003) and stomatal closing (Pei et al. 2000; Kwak et al. 2003).

To reduce oxidative damage initiated by ROS, the plants have developed a complex of protective antioxidant system. Antioxidant systems play an important role in maintaining the balance between overproduction and utilization of ROS to keep their quantity at the level required for signaling in metabolic homeostasis (Szalai et al. 2009). To prevent oxidative damages antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase, catalase (CAT), and glutathione reductase, the activity of which is increased under stress, are produced in the plants (Hernandez et al. 2000; Sairam et al. 2002; Ahmed et al. 2013). It is believed that salt tolerance, at least in partially, may depend on the ability of plants to counteract oxidative stress (Badawi et al. 2004). There have been reports describing synergistic effects of the simultaneous expression of ROS-scavenging enzymes on stress tolerance (Kwon et al. 2002).

In addition to activation of antioxidant enzymes, salt tolerance can depend on such antioxidant mechanisms like photorespiration or the path of Halliwell-Asada (Dionisio-Sese and Tobita 1998). The ascorbate-glutathione cycle, also known as the Halliwell-Asada or water-water cycle, utilizes serial enzymes, ascorbate peroxidase, dehydroascorbate reductase, glutathione reductase, and monodehydroascorbate reductase to scavenge superoxide radicals and H_2O_2 in chloroplasts (Asada 1999). Lowering of chlorophyll under stress may also reduce the flow of electrons through the photosystem (a reduce of apparent quantum efficiency) reducing the risk of photoinhibition (Koyro 2006). In addition, it has been shown that salt-induced increase of carotenoids' content in the leaves can be used to dissipate excess energy in photosystems I and II in the form of heat or in the not-damaging chemical reactions (Lu et al. 2003) and can stabilize membranes in the chloroplasts (Havaux 1998).

Some other non-enzymatic reducers, such as reduced glutathione and ascorbate, as substrates for detoxification of H_2O_2 play an important role in recycling of ROS.

Double water-salt stress also induces an oxidative stress that is accompanied by accumulation of H_2O_2 and MDA in the leaves. The varieties of barley, differed by higher stability at double stress, have greater efficiency of antioxidant systems (Ahmed et al. 2013).

ROS and H_2O_2, superoxide, play a significant role in regulation of biological processes, acting as signal molecules in response of plants to various stimuli (Overmyer et al. 2003; Laloi et al. 2004). For example, superoxide, acting as a signal, results in activation of protease (Reeves et al. 2002). H_2O_2 has played a key role in modulation of the growth by reducing the extension of the cellular wall with the help of peroxidase-linked oxidative cross-linking of cell wall polysaccharides and glycoproteins (Schopfer 1996; Liszkay et al. 2004). H_2O_2 has also been implicated in the auxin-dependent gravitropic stimulus (Joo et al. 2001) and abscisic acid-induced stomatal closure (Pei et al. 2000; Kwak et al. 2003).

Furthermore, H_2O_2 behaves as a second messenger in the activation of defense genes (Orozco-Cárdenas et al. 2001) and interacts synergistically with nitric oxide in inducing programmed cell death during the hypersensitive response (Delledonne et al. 1998).

7.4.6.1 MDA Content and SOD Activities

Malondialdehyde (MDA) is formed in the cells when the degradation of polyunsaturated fatty acids by ROS serves as a test of the lipid peroxidation, as well as a marker of oxidative stress. Malondialdehyde (MDA) as the decomposition product of polyunsaturated fatty acids of biomembranes showed greater accumulation under salt stress (Gossett et al. 1994). Membrane lipids in seeds are the primary site for deterioration during the attack by ROS and free radicals (Wilson and McDonald 1986). Cell membrane stability has been widely used to differentiate stress-tolerant and susceptible cultivars of some crops (Blum and Ebrecon 1981), and in some cases higher membrane stability could be correlated with abiotic stress tolerance (Premachandra et al. 1992).

The efficiency of the defense system is often evaluated in terms of free radical lipid oxidation (MDA content) and SOD activity as an enzyme neutralizing superoxide radicals. Superoxide radicals can be converted into H_2O_2, either spontaneously or by SOD. Although both forms of oxygen are moderately reactive, in the presence of transition metal ions, such as Fe^{2+} and Cu^{+} H_2O_2, turns into highly reactive free hydroxyl radical ($OH^{\bullet}$) via reaction of Fenton. SODs (EC 1.15.1.1) catalyze dismutation of two superoxide radicals and water into H_2O_2 and O_2. Three SOD genes are distinguished by their locations and covalently linked catalytic metal ions. Manganese SOD has Mn^{3+} at the active site and is localized in mitochondria (Zelko et al. 2002). Iron SOD has Fe^{3+} at the active site and is found in chloroplasts. CuZnSOD has Cu^{2+} plus Zn (II) at the active site and is found in the cytosol and plastids. Overexpression of CuZnSOD genes in the chloroplasts increased the tolerance to oxidative stresses (Kwon et al. 2002), lowered the amount of ion leakage, and maintained the membrane integrity (Lee et al. 2010).

According to our data, in the absence of stress the content of MDA in the leaves was ~32 μmol g^{-1} DW (Fig. 7.7a). The impact of drought did not lead to the marked increase of the quantity of the substance in the leaves. However, as watering restarted, the concentration of MDA significantly increased with further trend of its decrease. The activity of SOD in the absence of stress was ~1,000 units g^{-1} DW. During the development of drought, the activity of this enzyme did not change, but after watering of the plants it increased drastically (Fig. 7.8a). Further the activity of SOD has decreased to the initial level. Thus, under conditions of water stress a strict correlation of dynamics of MDA accumulation and SOD activity has been observed.

Salinity of the substrate at low concentrations of NaCl (0.05 and 0.1 M) did not lead to changes of MDA content (Fig. 7.7b, c) and the activity of SOD in the leaves

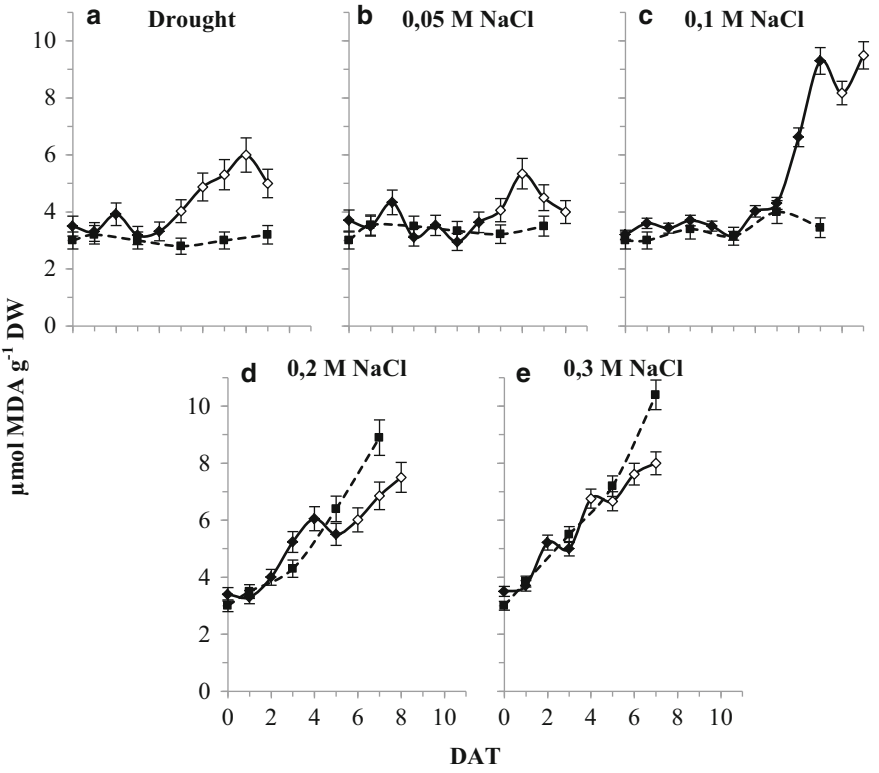

Fig. 7.7 MDA concentration in young leaves of wheat seedling (*Triticum aestivum* L.) after treatment of drought (D), salinity (S), and combined drought and salinity stress (D + S) conditions: (**a**) drought stress (W—control, optimal watering); (**b**) 0.05 M NaCl in the soil; (**c**) 0.1 M NaCl in the soil; (**d**) 0.2 M NaCl in the soil; (**e**) 0.3 M NaCl in the soil. The values are the average of three replicates (pots) ± standard deviations (SD). *White marks* correspond to the data after restart of plant watering

of wheat seedlings (Fig. 7.8b). At high concentrations of NaCl (0.1 and 0.3 M), a linear accumulation of MDA was observed during the experiment (up to 90 and 105 µmol g^{-1} DW, respectively) (Fig. 7.7d, e). However, the activity of SOD did not increase and even had a tendency to decrease that might lead to increase of MDA concentrations (Fig. 7.7d, e). By the end of the experiment, the activity of SOD increased slightly, but this did not affect on the contents of MDA. Perhaps, by this moment destructive processes in the cells have reached very high intensity and are associated with a sharp drop of RWC_L (Fig. 7.2d, e) and photosynthetic activity (Fig. 7.6d, e). That is, correlation between accumulation of MDA and SOD activity can be considered only in case when concentrations of NaCl match the threshold of salt tolerance of wheat plants.

Under combined water and salt stress at 0.05 M NaCl in the substrate, dynamics of MDA accumulation (Fig. 7.7b) and changes of SOD activity (Fig. 7.8b)

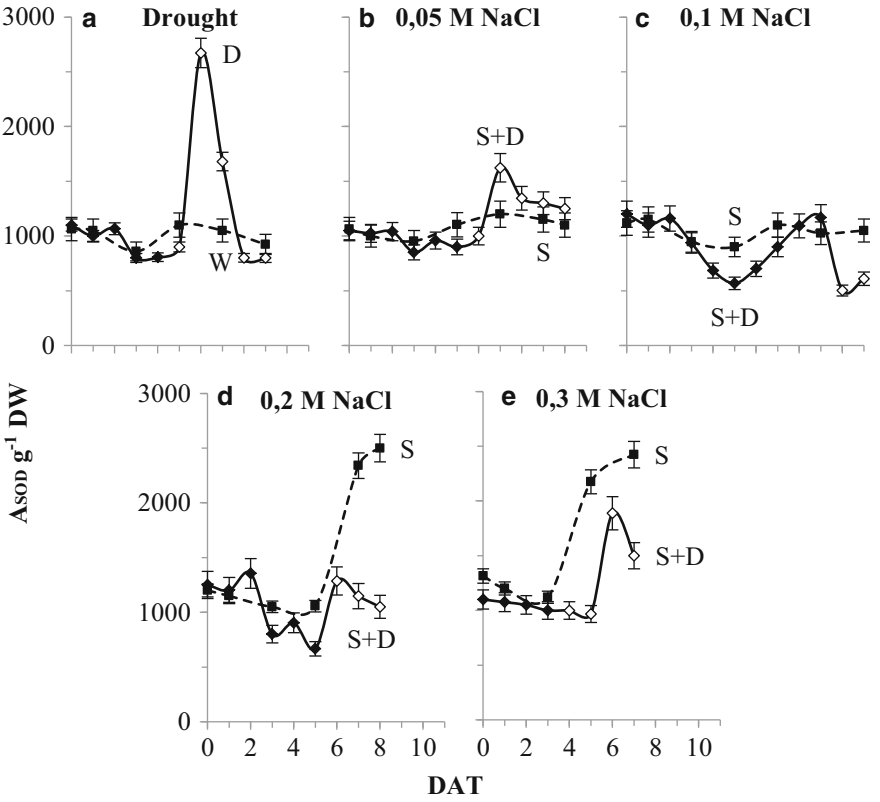

Fig. 7.8 SOD activity in young leaves of wheat seedling (*Triticum aestivum* L.) after treatment of drought (D), salinity (S), and combined drought and salinity stress (D + S) conditions: (**a**) drought stress (W—control, optimal watering); (**b**) 0.05 M NaCl in the soil; (**c**) 0.1 M NaCl in the soil; (**d**) 0.2 M NaCl in the soil; (**e**) 0.3 M NaCl in the soil. The values are the average of three replicates (pots) ± standard deviations (SD). *White marks* correspond to the data after restart of plant watering

resembled that one at water stress (Figs. 7.7a and 7.8a), i.e., the absence of changes at stress and slight increase after watering. At 0.1 M NaCl in the soil, the concentration of MDA in the leaves remained unchanged until a sharp drop of RWC_L (Fig. 7.2c) and further increased significantly up to ~93 μmol g^{-1} DW (Fig. 7.7c). However, there was no significant change in the activity of SOD (Fig. 7.8c), probably due to inhibition of this enzyme at increasing NaCl concentration due to dehydration of the leaves. At high concentrations of NaCl (0.2 and 0.3 M), MDA accumulation corresponded with that one in salt stress, i.e., increased during the experiment and continued to increase even after watering of the plants (Fig. 7.7d, e). However, it was slightly lower than after salt stress. Dynamical changes of SOD activity resembled the dynamics at salt stress, but differed with lower intensity (Fig. 7.8d, e). Thus, when the combined stress the activity of SOD and MDA

accumulation depended mainly on the NaCl presence in the soil and, to a lesser extent, on the degree of drought development.

7.4.6.2 H_2O_2 Content and the Activities of CAT and POD

The H_2O_2 produced is then scavenged by CAT (EC 1.11.1.6) and a variety of POD (EC 1.11.1.7). CAT, which is apparently absent in the chloroplast, dismutates H_2O_2 into water and molecular oxygen, whereas POD decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants.

According to our data, in the absence of stress, H_2O_2 concentration in the second young leaves of wheat seedlings did not exceed ~20 μmol g^{-1} DW (Fig. 7.9a). At water stress accumulation of H_2O_2 and the activity of POD and CAT were in strict correlation with dynamical development of the drought (see Fig. 7.2a). H_2O_2 concentration began to rise after a sharp drop of RWC_L. At the same time, the

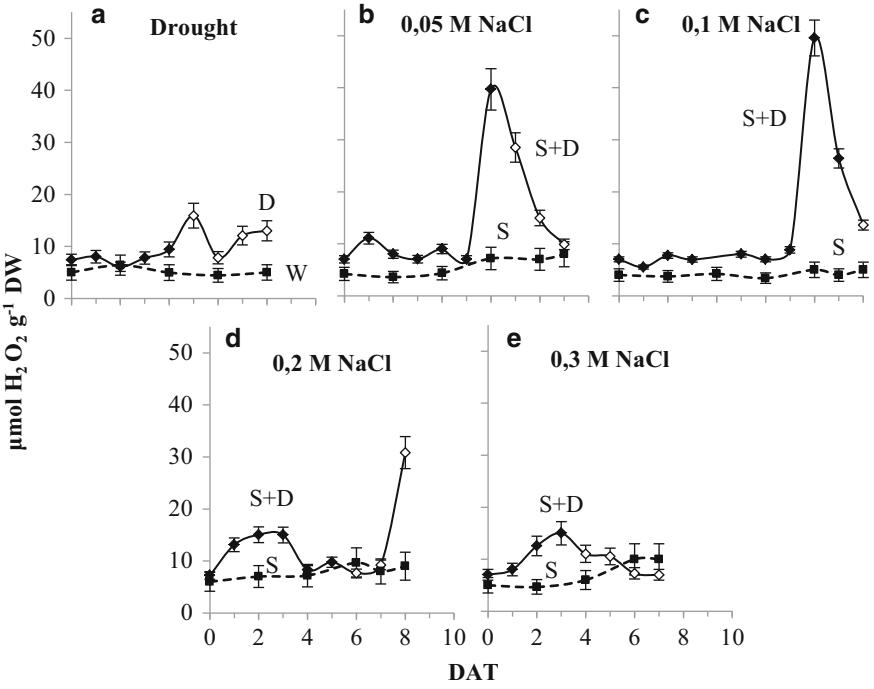

Fig. 7.9 H_2O_2 concentration in young leaves of wheat seedling (*Triticum aestivum* L.) after treatment of drought (D), salinity (S), and combined drought and salinity stress (D + S) conditions: (**a**) drought stress (W—control, optimal watering); (**b**) 0.05 M NaCl in soil; (**c**) 0.1 M NaCl in the soil; (**d**) 0.2 M NaCl in the soil; (**e**) 0.3 M NaCl in the soil. The values are the average of three replicates (pots) ± standard deviations (SD). *White marks* correspond to the data after restart of plant watering

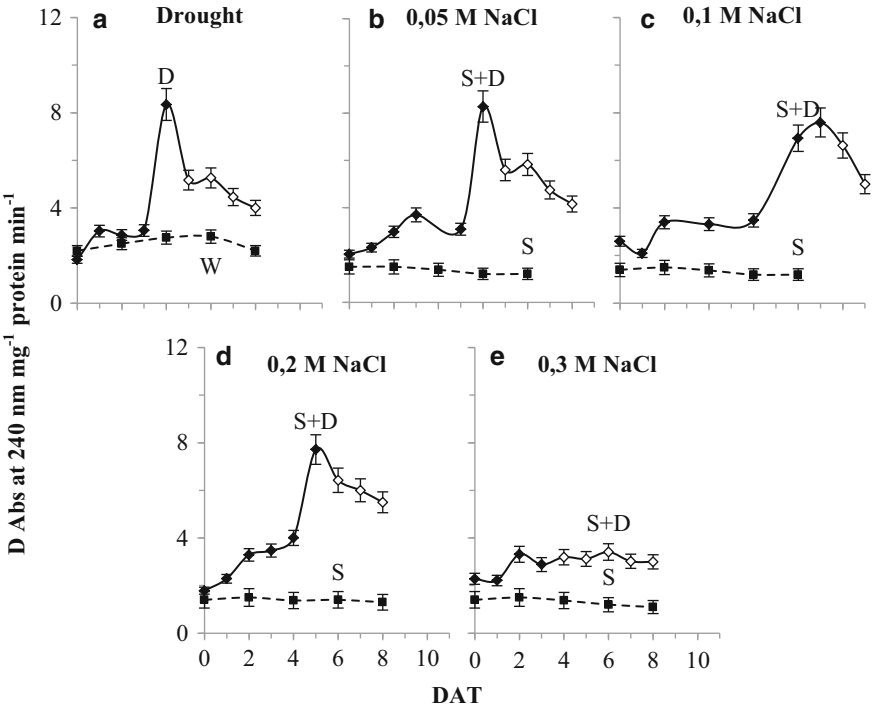

Fig. 7.10 CAT activity in young leaves of wheat seedling (*Triticum aestivum* L.) after treatment of drought (D), salinity (S), and combined drought and salinity stress (D + S) conditions: (**a**) drought stress (W—control, optimal watering); (**b**) 0.05 M NaCl in the soil; (**c**) 0.1 M NaCl in the soil; (**d**) 0.2 M NaCl in the soil; (**e**) 0.3 M NaCl in the soil. The values are the average of three replicates (pots) ± standard deviations (SD). *White marks* correspond to the data after restart of plant watering

activity of CAT and POD increased more than 2.5 times (Figs. 7.10a and 7.11a). After watering of the plants, enzymatic activity decreased to its initial level.

Under salt stress H_2O_2 concentration (Fig. 7.9b), as well as the activity of CAT and POD (Figs. 7.10b–e and 7.11b–e), remained at the control within an experiment at all the concentrations of NaCl used. Only at high content of NaCl in the soil, at the end of the experiment, there was a slight increase of the activity of POD in the leaves. Probably, in salt stress initial enzymatic activity was enough to neutralize H_2O_2 generated.

In case of combined water and salt stress at concentrations of 0.05 and 0.1 M NaCl in the soil, accumulation of H_2O_2 (Fig. 7.9b, c) in the leaves began only upon a sharp drop of RWC_L (Fig. 7.2b, c), reaching 40 and 50 μmol g^{-1} DW, respectively. Upon watering H_2O_2 concentration was reduced to its initial level. It was interesting to note that at high concentrations of salt in the soil (0.2 and 0.3 M), there was only a small accumulation of H_2O_2 (Fig 7.9d, e) within an experiment, at least in young leaves of wheat seedlings. The CAT and POD activities increased

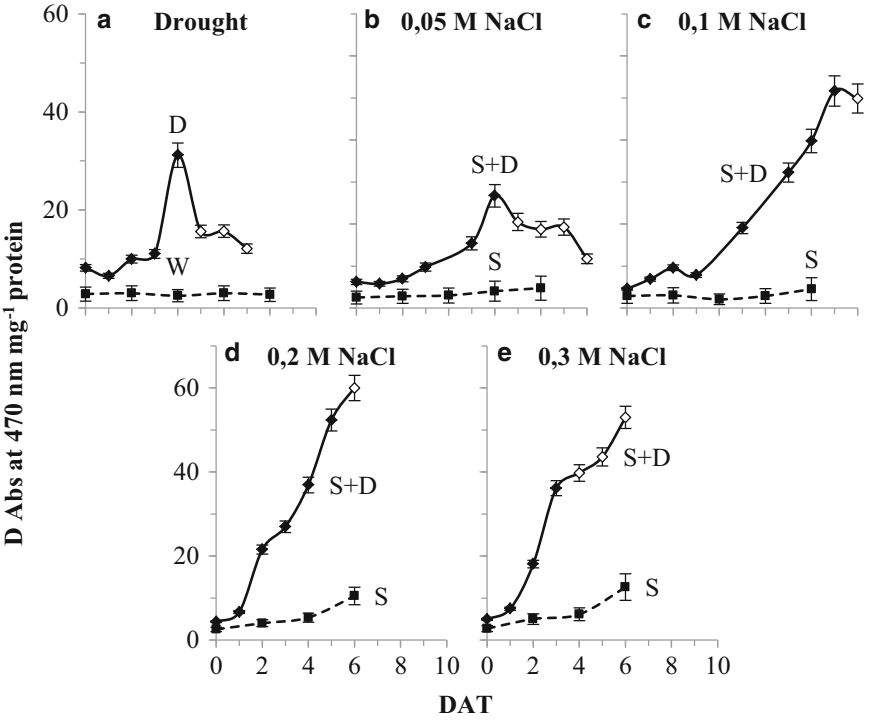

Fig. 7.11 POD activity in young leaves of wheat seedling (*Triticum aestivum* L.) after treatment of drought (D), salinity (S), and combined drought and salinity stress (D + S) conditions: (**a**) drought stress (W—control, optimal watering); (**b**) 0.05 M NaCl in the soil; (**c**) 0.1 M NaCl in the soil; (**d**) 0.2 M NaCl in the soil; (**e**) 0.3 M NaCl in the soil. The values are the average of three replicates (pots) ± standard deviations (SD). *White marks* correspond to the data after restart of plant watering

(Figs. 7.10b, d and 7.11b–e), especially after a drop of RWC_L (Fig. 7.2b). The exception was the variant with 0.2 M NaCl, where no increased activity of CAT was observed, most likely due to inhibition of the enzyme at very high concentration of the salt (Fig. 7.10). Upon watering the CAT and POD activities had a tendency to decrease. Thus, the feature of combined water and salt stress can be considered a significant accumulation of H_2O_2 and a strong increase of the activity of antioxidant enzymes, CAT and POD.

7.5 Conclusion

The plants' responses to environmental conditions vary due to the type and the value of stress effect. Drought and salinity are the most common encountered abiotic factors, having a negative impact on crop yields. In field conditions the

plants are most frequently subjected to the effects of multiple stresses simultaneously. Under drought the concentration of salt in the soil is increased, even on non-saline lands that can cause salt stress. It would be logical to assume that negative effect of every stress should be intensified in combination. However, the response of the plants at combined water and salt stress was more complex than simple additive effect from these two stressors. Drought and salinity reduce individually the availability of water for plants. However, the presence of salt in the soil inhibits the rate of drought development, enabling the plants to survive in unfavorable period of short-term drought without violating the basic physiological functions. These functions remain constant until the level of soil salinity reaches a critical threshold value, above which the main physiological parameters of plants are reduced in proportion to the increase in the concentration of salts in the soil. That model of plant salt tolerance has been developed for conditions of a single salt stress. The concentration range of plants' tolerance in conditions of combined stress has, most likely, to correspond to those calculated for salt stress conditions, but the limiting threshold should manifest itself more clearly. The key issue is in understanding the mechanisms that allow the plants to function successfully within the threshold stress tolerance. Studying of the reactions of plants outside the tolerance is unpredictable, although adaptive mechanisms to control the stress can manifest themselves more clearly. Individual characteristics of double stress are in the fact that with the increased concentration of NaCl in the range of threshold tolerance the plants' vitality increases but drops drastically when exceeding the threshold. The plant's ability to restore after stress can serve a criterion for the threshold value. In it a combination of synergistic and additive nature of double stress is shown due to the concentration of salt in the soil.

The combined water-salt stress has a number of features, differed both from water and salt stress. Na^+ accumulation in the leaves is similar to that at salt stress and independent of drought effects. This process enables the osmotic potential of the plants to be maintained at a rather low level required for leaf turgor and also promotes the absorption of water even in rapid stomatal closure and a reduce of osmotic potential of the salted soil. Rapid closing of stomata also contributes to preservation of water in plants under conditions of combined stress.

Water stress should be considered as the main active component of combined stress, since changes of important parameters like transpiration, photosynthetic activity, accumulation of proline, and antioxidant enzyme activity are in strict accordance with water content in the leaves, i.e., dynamical developments of the drought. The growth of the leaves is also quickly stopped as in drought.

The development of combined stress was characterized by two steps: long absence of major changes of the main physiological parameters of the plants despite continuing soil dehydration and a rapid decline of these indicators in the point of maximal water stress. Reduced turgor leaf should be also considered as a part of adaptive mechanism under heavy drought in case of exhaustion of adaptive possibilities of other mechanisms, since the plants have kept their ability to restore at restart of watering.

One of the most striking features of combined water-salt stress is accumulation of proline in quantities exceeded considerably that once both at water and salt stress. Meanwhile, for synthesis of proline in such quantities it seems to need both components of double stress, the presence of salt in the soil against the developing drought. Along with absorption of Na^+, proline synthesis enables us to maintain osmotic potential of the leaf at low concentrations, contributing to preservation of water in the leaves on the lower level. The possibility of proline synthesis of great quantities can be considered as a criterion of stress tolerance of the plants. However, it should be bear in mind that this may be possible only at salt concentrations which do not exceed the tolerance threshold. At high NaCl concentrations in the soil, accumulation of proline can continue even after irreversible degradation of the cells.

It is considered to be that accumulated proline performs osmoregulatory function. However, as shown in our experiments, at salt stress no marked accumulation of proline is observed, in spite of continuing uptake of Na^+. At the same time at combined stress at the same level of Na^+ accumulation, a powerful synthesis of proline occurs. These data allow us to doubt that synthesis of proline occurs only for alignment of osmotic potentials between the cytoplasm and vacuole while compartmentalization of Na^+ takes place. Moreover, the accumulation of large quantities of proline does not favor to preservation of water in the cells at high concentrations of NaCl in the soil. However, proline accumulation may help to maintain water in the cells at drought during the combined stress, which explains long period of stationary phase of stress development. Keeping photosynthetic activity in this period makes the synthesis of proline energetically justified. The reduced concentration of proline after watering of the plants confirms this concept.

Another feature of combined stress is the absence of oxidative stress in a stationary period at low concentrations of NaCl in the soil. During this period there was no accumulation of ROS, possibly due to initially high activity of antioxidant enzymes and an increase of the activity of CAT and POD at stress development, which was much higher than at saline stress. Oxidative stress has been observed only at strong drop of water content in the leaves that speaks more about the impact of drought than the salt stress.

Thus, combined water and salt stress should be considered as a special event that cannot be extrapolated from the sum of the properties of drought and salinity individually.

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Chapter 8
Plant Response to UV-B: From Tolerance to Toxicity

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Abstract In higher plants, UV-B is known to have two very diverse effects: one in response to the evoked damage and the other in response to the perception of UV-B by postulated receptor, leading to UV-B-induced photomorphogenesis and thus acclimation. The UV-B-specific pathway involves the UVR8-COP1-HY5 pathway. The response depends on wavelength, fluence rate, and duration of the UV-B radiation as well as the extent of adaptation. On the other hand, the damage response pathway includes activation of more general stress responses.

Keywords UV-B • Receptors • Photomorphogenesis • UVR8-COP1-HY5 • Stress

8.1 Introduction

Current stratospheric ozone (O_3) levels are at the lowest point since measurements began in 1970s, and global terrestrial UV-B radiation levels range between 0 and 12 kJ m^{-2}, with near equator and midlatitudes receiving higher doses (McKenzie et al. 2011). The changes in O_3 and UV-B are not uniform over the Earth's surface. The O_3 concentration in the high latitudes is 40–50 % lower than the pre-1980 values; in the midlatitudes, it is 3–6 % lower than the pre-1980 values; and at the equator, minimum variation was observed (Forster et al. 2011). UV-B radiation comprises a very small part of the solar spectrum, although it affects the plants at molecular, cellular, and whole organism level (Jenkins 2009). In higher plants, UV-B is known to have at least two distinct effects: one in response to the evoked damage and the other in response to the perception of UV-B by a postulated receptor, leading to UV-B-induced photomorphogenesis and thus acclimation (Jenkins 2009). The response depends on the wavelength, fluence rate, and duration of the UV-B irradiance as well as the extent of adaptation. In general, UV-B radiation results in the activation of a diverse and distinct set of signaling responses leading to the reprogramming in gene expression (Ulm and Nagy 2005).

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8.2 Balance Between Damage and Acclimation

The damage response pathway includes activation of more general stress response (Ulm 2003), whereas the UV-B-specific pathway leading to acclimation involves the recently described UVR8-COP1-HY5 pathway in *Arabidopsis* (Favory et al. 2009). Plants adaptation to UV-B is intricate and is a manifestation of several mechanisms (Fig. 8.1). Several components are thought to play a role in UV-B responses such as NADPH oxidase-derived reactive oxygen species (ROS) (Kalbina and Strid 2006), jasmonic acid (Mackerness and Jordan 1999), nitric oxide (Izaguirre et al. 2007), and mitogen-activated protein kinases (MAPK) (Holley et al. 2003). These components form part of general stress response network, such as those regulating wound and defense signaling (Stratmann 2003) and are therefore unlikely to be UV-B specific. UVR8 appears as a symmetric seven-bladedβ-propeller homodimer that is stabilized by arginine, primarily Arg286 and Arg338. These arginines facilitate the cation–π interaction with their surrounding tryptophan residues, among which Trp 285 and Trp 233 act as the internal UV-B chromophores. Upon UV-B radiation, dimer of UVR8 is monomerized as a result of disruption of the intramolecular cation–π interactions and intermolecular hydrogen bonds mediated by Arg286 and Arg338 (Wu et al. 2012). This structural conversion is a major determinant for UVR8 to sequester CONSTITUTIVELY PHOTOMORPHOGENIC 1 SUPPRESSOR OF PHYTOCHROME A (COP1-SPA) core complex(es) from the CULLIN 4-DAMAGED DNA BINDING PROTEIN 1 (CUL4-DDB) E3 apparatus. The reorganization of this complex enables COP1 to act as a positive regulator in the UV-B-mediated photomorphogenesis by facilitating the stability and activity of a photomorphogenesis promoting transcription factor ELONGATED HYPOCOTYL 5 (HY5) (Rizzini et al. 2011). UV-B damages macromolecules and inhibits cellular processes. The photoexcited biomolecules can be damaging as well as regulatory. At low fluence rates, UV-B acts as an informational signal that regulates UV-B-protective responses and development processes (Jenkins and Brown 2007).

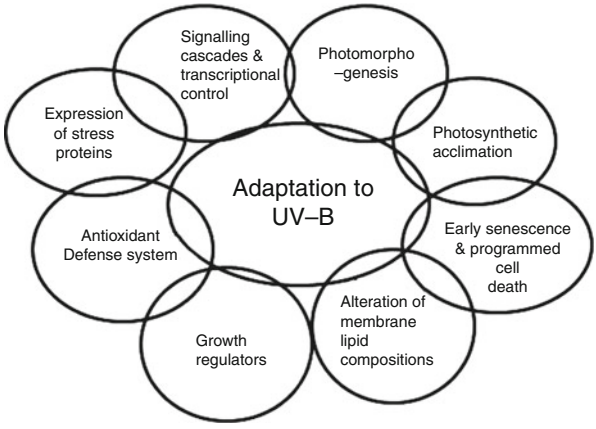

Fig. 8.1 Different adaptation mechanisms of plants to UV-B radiation

At high fluence rates, the damaging effects include damage to biomolecules by generating ROS, which can cause oxidation of lipid and protein and damage to DNA (Kliebenstein et al. 2002). Tolerance to UV-B depends on the balance between several reactions, both repair and acclimation responses, although the analysis of balancing act is difficult to distinct.

Damaging effects of UV-B include damage to DNA, proteins, membranes, impairment of photosynthetic activities and plant growth. Oxidative stress is the key factor in UV-B stress. However ROS, DNA damage, and membrane degradation products play a role in mediating UV-B protection. Imbalances between the production of ROS and antioxidant scavenging capacity led to damaging effect. Several sets of processes at both cellular and molecular levels which are critical to plant growth and development appear to be affected by absorption of UV-B radiation. The imbalance between the rate of energy excitation and the rate of the assimilation capability under UV-B radiation results in an over-excitation of the photosystems. This condition favors the formation of highly ROS that may produce photooxidative damage to the photosynthetic machinery components (pigments, proteins, nucleic acids). Plants have protective mechanisms against photooxidative damage, such as decreased light absorption, removal of excess excitation energy inside the photosystems, scavenging of ROS, and up- and downregulation of photosynthesis-related genes (Demmig-Adams and Adams 1996). ROS are chemical species possessing an unpaired electron in their outermost orbital. Due to presence of one or more unpaired e^{-}, these species are paramagnetic, which makes them highly reactive. Activation and photo-deactivation of important signal molecules, such as hormones and photoreceptors, may also add to the effects of UV-B on plant growth and development. Cell extension in many plants is influenced by indoleacetic acid (IAA) which is absorbed in the UV-B region photooxidized to 3-methyleneoxindole, an inhibitor of hypocotyls growth (Tevini and Teramura 1989). Major UV-B-induced responses are discussed below.

8.2.1 DNA Damage and Repair

UV-B radiation gives rise to a multitude of DNA photoproducts (Sancar and Sancar 1988). There products include cyclobutane pyrimidine dimers (CPDs) and to a lesser extent, pyrimidine pyrimidone photoproducts (6-4 PPs) (Lario et al. 2011). The dimers formed in the most significant quantity are the cis-syn cyclobutane dimer of two thymine bases (Smith and Taylor 1993). The trans-syn thymine dimer (Ling et al. 2003) is formed at a much lower level in single- and double-stranded DNA. By the absorption of around 325 nm of UV-B, the 6-4 photoproduct is converted to Dewar isomer (Taylor et al. 1987). 7,8-Dihydro-8-oxoguanine (GO) is a common oxidative DNA lesion generated by direct modification via ROS. GO lesion is mutagenic and can mispair with adenine during DNA replication (Yang et al. 2001). If the resulting A/GO is not repaired before DNA replication, a C/G–A/T transversion occurs and the opportunity for repair is lost. The A/GO is

repaired via base excision repair (BER) which is initiated by the DNA repair enzyme adenine-DNA glycosylase (Yang et al. 2001). Accumulation of photoproducts can be prevented to maintain the genome integrity and other development and reproductive processes. As plants lack a reserved germ line and mutations occurring in somatic cells will be transmitted to the progeny. Therefore, plants involve mechanisms to filter UV-B and repair systems to repair or tolerate DNA lesions (Kimura and Sakaguchi 2006).

In many organisms, the biological effects of UV radiation can be significantly reduced by subsequent exposure to light in the blue or UV-A region of the spectrum, a phenomenon known as photoreactivation. The photoreactivating effects of visible light are due to the actions of the enzyme photolyase. These proteins bind specifically with the CPDs and upon absorption of photons of appropriate wavelength (350–450 nm), directly reverse the damage. Photolyases carry two prosthetic groups. One chromophore (either methyltetrahydrofolate or 8-hydroxy-5-deazaflavin) absorbs the photoreactivating light and transfers this excitation energy to other chromophore, a fully reduced FAD^{-}. FAD^{-} then transfers an e^{-} to the dimer (Sancar 1994) inducing its reversal.

At the genome level, the accessibility of DNA is determined by the structure of chromatin, which is subjected to epigenetic regulation. Chromatin remodeling has previously been crucial for UV-B damage repair in plants (Campi et al. 2012). Different chromatin landscapes control the accessibility of the DNA repair machinery to damaged DNA. In several organisms, a major factor affecting chromatin accessibility is DNA methylation. The disruption of the interactions of nucleosome-DNA or the remodeling of chromatin can stimulate or repress DNA repair. Transgenic maize plants knockdown for chromatin remodeling genes were known to be acutely sensitive to UV-B at doses that do not cause visible damage to maize lacking flavonoid sunscreens (Casati et al. 2006).

In contrast to photoreactivation, dark repair pathways do not directly reverse DNA damage with new, undamaged nucleotides. These excision repair pathways can be BER, nucleotide excision repair (NER), mismatch repair (MMR), and other DNA repair pathways. The wide class of helix-distorting lesions such as CPDs and (6-4) photoproducts are repaired by NER (Tuteja et al. 2001; Britt 1999). The NER sequentially involves recognition of DNA damage, incision on damaged strand, excision of damage containing oligonucleotides, and DNA synthesis and ligation. There are two sub-pathways of NER designated as global genomic repair (GGR) and transcription-coupled repair (TCR), while GGR repairs the DNA damage over the entire genome, TCR is selective for the transcribed DNA strand in expressed genes. AtRAD1 and AtRAD2, which encode NER endonucleases, have been isolated from *Arabidopsis thaliana* by studying the UV-hypersensitive mutants uvh1 and uvh3, respectively (Liu et al. 2001). *Arabidopsis* mutants of CPD and 6-4 photolyase genes (uvr2 and uvr3, respectively) are hypersensitive to UV radiation (Nakajima et al. 1998). Sato and Kumagai (1993) reported that most *indica* cultivars of rice are more sensitive than *japonica* cultivars. Among *japonica* cultivars, Sasanishiki is resistant to UV-B, whereas Norin is sensitive (Hidema et al. 1996). This cultivar difference in UV resistance is derived from the activity of

photorepair, NER, and accumulation of UV-absorbing compounds (Sato and Kumagai 1993). The UV-sensitive cultivar Norin 1 contains a defective CPD photolyase (Hidema et al. 2000). Oxidized or hydrated bases and single-strand breaks are repaired by BER. DNA glycosylases initiate this process by releasing the damaged DNA, with the cleavage of sugar-phosphate chain, excision of the abasic residue containing oligonucleotide, and DNA synthesis and ligation (Tuteja et al. 2001; Britt 1999). There are two sub-pathways for BER. The short-patch BER is DNA polymerase beta dependent, while the long-patch-dependent BER is DNA polymerase delta/epsilon dependent. Excision repair (BER and NER) is very important for maintaining genome stability and essential for survival of organisms.

8.2.2 Photosynthetic Damage

Photosynthetic organisms are especially sensitive to UV-B due to their requirement for light (Hada et al. 2003), as well as their low genome template stability (Teranishi et al. 2004). UV-B can impair all the three main components of photosynthesis, i.e., the photophosphorylation reaction of the thylakoid membrane, the CO_2 fixation reaction, and the stomatal control of CO_2 supply (Allen et al. 1998). UV-B radiation also affects the photosynthetic pigments, either through inhibition of their synthesis or effects on the enzymes involved in the chlorophyll biosynthetic pathway. However, some studies have shown that UV-B radiation has no effect on chlorophyll and even increased chlorophyll content (Liu et al. 2007). Thylakoid lipids, which have a high proportion of polyunsaturated fatty acids (Gounaris et al. 1986), are potentially very susceptible to oxidative degradation. UV-B-induced destruction of the structural integrity of thylakoid membranes will deleteriously alter photosynthetic function. The main target site of UV-B radiation in the photosynthesis system is PS II (Albert et al. 2005), especially the manganese cluster of the water-splitting apparatus. Based on their absorption in the UV-B range, the quinine e^- acceptors, the catalytic Mn_4Ca cluster of water oxidation, and the tyrosine e^- donors have been suggested as potential target sites of UV-induced damage in PSII (Vass et al. 1996). After absorption of UV-B, the manganese ions are released from the cluster and e^- flow towards PSII reaction center is inhibited (Hakala et al. 2005). Also, aromatic components of PSII like the donor of P680, tyrosine, Yz, or plastoquinones Q_A and Q_B on the acceptor side, may absorb UV-B and be damaged, also resulting in impaired e^- transport (Vass et al. 1996). As a consequence, $P680^+$ may accumulate; an oxidative chain reaction is activated degrading the associated proteins of PSII (Ohnishi et al. 2005). Any inability to supply reductants or ATP from e^- transport will limit the carbon fixation, but UV-B is known to largely inhibit the major enzymes of Calvin cycle directly. UV-B majorly targets the RuBisCO. Levels of mRNA coding for both the large and small subunits of RuBisCO have been reported to decline before any effect at the protein level was evident (Jordan et al. 1992). UV-B induced reductions in the content of sedoheptulose-1,7-bisphosphate but not on chloroplastic fructose-1,6-

bisphosphatase or phosphoribulokinase (Allen et al. 1998). Jordan et al. (1991) observed a complex array of regulatory mechanisms involved in adapting the level of gene expression for chloroplast proteins under UV-B stress conditions.

8.2.3 Programmed Cell Death

The higher doses of UV-B can induce oligonucleosomal DNA fragmentation like a typical apoptotic DNA ladder (Lytvyn et al. 2010). DNA laddering is an integral part of programmed cell death (PCD) in plant systems, confirming the role of UV-B in induction of PCD. In *Arabidopsis*, ATR (ataxia telangiectasia and Rad3-related) and ATM (ataxia telangiectasia mutated) act as sensors of DNA damage. ATR-deficient plants showed hypersensitivity to UV-B radiation and exhibited altered G2-phase cell cycle checkpoints (Cullighan et al. 2004). Numerous genes involved in cell cycle regulation are impaired under UV-B leading to hypersensitivity. The transcriptional responses of maize to UV-B under field condition indicate that UV-B might affect the cell cycle (Jiang et al. 2011). Cell cycle regulators control both cell cycle duration and the number of dividing cells. Cell cycle progression is controlled by checkpoints that mediate the entry into S-phase and mitosis (De Veylder et al. 2003). The progression through these checkpoints is catalyzed by cyclin-dependent kinases (CDKs) and their interaction components, cyclins (De Veylder et al. 2003). In plants, two main classes of CDKs (A- and B-type) have been extensively characterized (Joubès et al. 2000). Multiple cyclins have been identified in *Arabidopsis*, which are grouped into A-, B-, D-, and H-type families (Vandepoele et al. 2002).

RAD6 and RAD17 were induced by UV-B in maize (Casati and Walbot 2004). These proteins are involved in post-replication repair of DNA in yeast. They activate the checkpoints that delay cell cycle progression in yeasts. Moreover, microarray analysis revealed that transcripts for proteins that vary during the cell cycle, including cdc20, ubiquitin, proteasome proteins, and cyclins, are all upregulated by UV-B in maize under UV-B radiation (Casati and Walbot 2004). The delayed induction of CYCD3;1 transcripts under UV-B radiations results in delayed G1-S transition (Jiang et al. 2011). It is also confirmed that the G1-to-S arrest induced by UV-B in root tips was a consequence of DNA damage which has been shown using *uvh1* mutant impaired in removal of CPDs (Jiang et al. 2011).

8.2.4 Photomorphogenesis

UV-B induces a range of morphogenic effects in plants, and these include leaf thickening, cotyledon curling, inhibition of hypocotyls, stem and leaf elongation, axillary branching, and shifts in the root-shoot ratio (Boccalandro et al. 2001). Some of the morphogenic responses show stimulation of specific tissue like axillary

branching and leaf thickening, while others reflect an inhibition of growth like diminished hypocotyls elongation. In general, monocots are morphologically more responsive to UV-B than dicots (Barnes et al. 1990). There is a little consensus on the tolerant and damaging effects of UV-B-induced morphogenic changes. Some of the morphogenic responses can be damaging for the cells or leaves exposed to UV-B. For example, inhibition of hypocotyls elongation has been hypothesized to minimize UV-B exposure of the emerging seedling until it has accumulated UV-B screening pigments (Ballaré et al. 1995). Similarly, leaf thickening is thought to diminish the number of cell layers exposed to ambient UV-B, since these wavelengths penetrate only the upper layers of a leaf (Cen and Bornman 1993). Similarly, the reduction of apical dominance (decreased shoot length with increased axillary branching) protects the plant from UV-B exposure as leaves of short, bushy plants are more likely to be shaded (Barnes et al. 1990). The ecological cost (trade-offs) of these responses is not well known. Clearly, some of the morphogenic responses must have a cost in terms of resources which the plant, in the absence of UV-B, could have allocated elsewhere. Other costs include reduction in the capture of photosynthetic light and photosynthetic capacity because of smaller leaves and shorter stems (Allen et al. 1998; Barnes et al. 1990). Photomorphogenic responses are fluence based (Wilson and Greenberg 1993). Many morphogenic responses are induced by low levels of UV-B which do not impede growth and photosynthesis (Allen et al. 1998).

It is not clear whether UV-B-induced morphogenesis comprises a single molecular mechanism or whether multiple independent mechanisms control distinct components of the morphogenic response. Jansen et al. (2001) found that the *Nicotiana* plants overexpressing the anionic peroxidase are strongly UV sensitive. The coupling between UV tolerance and IAA catabolism has been proposed to be the co-localization in the cell wall of IAA, phenolic substrates, and peroxidase isozymes capable of oxidizing both of these substrates. Thus, peroxidases can be concomitantly involved in two of the acclimation responses: redistribution of UV-B screening phenolics and induction of morphogenic responses by lowering IAA levels (Jansen et al. 2001).

Some of the genes regulated by UVR8 seem to be involved in morphogenesis because the *uvr8* mutant is altered in the UV-B-induced suppression of hypocotyls extension (Favory et al. 2009) and regulation of leaf expansion (Wargent et al. 2009). Several *UV-B light sensitive* (*uli*) mutants have been isolated that are altered in UV-B-mediated inhibition of hypocotyls elongation (Suesslin and Frohnmeyer 2003). These *uli* mutants were specifically insensitive to wavelengths between 300 and 320 nm. The *ULI3* gene encodes an 80 kDa protein with potential domains for heme and diacylglycerol binding. Suesslin and Frohnmeyer (2003) condemned UV-B-mediated DNA damage as the reason behind the hypocotyls response induced by their screening conditions. They checked the hypocotyls response of the photolyase-deficient*uvr2-1* mutant (Landry et al. 1997), which is hypersensitive to UV-B-mediated DNA damage (Kim et al. 1998) and could not find any difference from the wild type. Further, they also observed that high UV-B doses produced similar amounts of DNA dimers in *uli3* mutants and in wild-type plants. Thus, *uli3* mutants were not found to be affected in their capacity to repair

DNA damage. *uli3* is impaired in several UV-B-mediated responses including hypocotyls growth and UV-B-induced expression of CHS, PR1, and NDPK1a. *ULI3* mRNA accumulated in UV-B-irradiated etiolated seedlings and the expression was restricted to hypocotyls and cotyledons.

Endoreduplication is also one of the putative mechanisms that exists in plants which buffer them from the negative effects of UV-B. Endoreduplication is a particular mode of cell cycle where additional rounds of nuclear DNA replication occur in the absence of mitosis, resulting in endoploidy where somatic nuclei contain multiple copies of DNA. Endoploidy has been hypothesized as an adaptive response to UV-B radiation (Vlieghe et al. 2007), possibly via the resultant increased gene copies which could prevent DNA damage. The *Arabidopsis* mutant, *uvi4*, which displays increased levels of ploidy has shown increased resistance to UV-B when grown at high fluxes (Hase et al. 2006). Wargent et al. (2009) showed that UVR8 is required for a UV-B-stimulated compensatory increase in epidermal cell size, while reductions in epidermal cell number in response to UV-B are substantially independent of UVR8, thus demonstrating that UVR8 regulates leaf growth through the control of epidermal cell development. They also reported about the role of UVR8 in the normal progression of endocycle in response to UV-B and has a regulatory role in stomatal differentiation. Hectors et al. (2007), however, reported reduction in the leaf expansion of *Arabidopsis* up to 25 % under chronic, low doses of UV-B.

They further showed that this inhibitory effect of UV-B radiation on leaf expansion occurs in the absence of photosynthetic stress and is not linked to induction of typical stress-responsive genes. Hectors et al. (2010) also reported smaller leaves with shorter petiole under UV-B treatment. As growth results from the formation of cells followed by their expansion and differentiation, UV-B effects should be expected in either process. Contradictory effects of UV-B on cell division were reported (Rousseaux et al. 2004; Lake et al. 2009). Some hold the view that UV-B decreases cell expansion without affecting the cell division in *Solanum lycopersicum* (Ballaré et al. 1995), *Hordeum vulgare* (Liu et al. 1995), and *Lactuca sativa* (Wargent et al. 2009). It was also observed in *Arabidopsis* that UV-B increases cell expansion while inhibiting the cell division process (Wargent et al. 2009). Nogués et al. (1998) in *Pisum sativum* and Hopkins et al. (2002) in *Triticum aestivum* and Hofmann et al. (2003) in *Trifolium repens* concluded that both cell division and cell expansion are negatively affected by UV-B radiation. To understand the contradictory roles of UV-B, it is important to compare the experimental conditions.

Several genes which regulate the cell wall loosening and cell expansion are repressed by short-term UV-B exposure (Favory et al. 2009) and either up- or downregulated under long-term UV-B treatment (Hectors et al. 2007). The expression of cell-wall loosening xyloglucan endotransglucosylase/hydrolase-encoding genes (XTHs; Nishitani and Vissenberg 2007) varies upon the UV-B treatment (Favory et al. 2009). Changes in peroxidase activity, possibly acting on UV-induced phenolics forming cross-links inside the cell walls (Schopfer 1996), could account for the reduced cell expansion.

8.2.5 ROS and Its Scavenging

The equilibrium between the production and scavenging of ROS may be perturbed by UV-B radiation. These disturbances in equilibrium lead to sudden increase in intracellular levels of ROS which can cause significant damage to cell structure and function. ROS like superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radical ($^{\bullet}OH$) are highly reactive and toxic and cause damage to proteins, lipids, carbohydrates, and DNA which ultimately leads to cell death. Stress-induced accumulated ROS is counteracted by enzymatic as well as nonenzymatic antioxidants such as ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione *S*-transferase (GST), catalase (CAT), ascorbic acid (ASH), glutathione (GSH), α-tocopherols, carotenoids, flavonoids, and proline. UV-B-induced oxidative stress on the one hand can lead to disruption of important structure and function and on the other hand can stimulate many protective responses which can develop tolerance in plants against UV-B (Fig. 8.2).

According to the structural functions, UV-B-protectant metabolites can be categorized into three different families: (1) light protection in proportion to aromatic ring, (2) antioxidant activity corresponding to reduction moieties such as phenolic moiety and unsaturated carbon–carbon bonds, and (3) increasing osmotic pressure by compound accumulation in specific location (Tohge et al. 2011).

The production of these ROS can be strongly enhanced by UV-B that inhibits photosynthetic processes and hence cause absorption of light in excess to what can be used in photosynthetic processes. Under these conditions, overreduction of the photosynthetic electron transport chain and accumulation of triplet chlorophyll promote leakage of electron (e^-) or energy to oxygen. It was shown that photooxidative damage to plant leaves is always associated with $^1O_2$-induced lipid

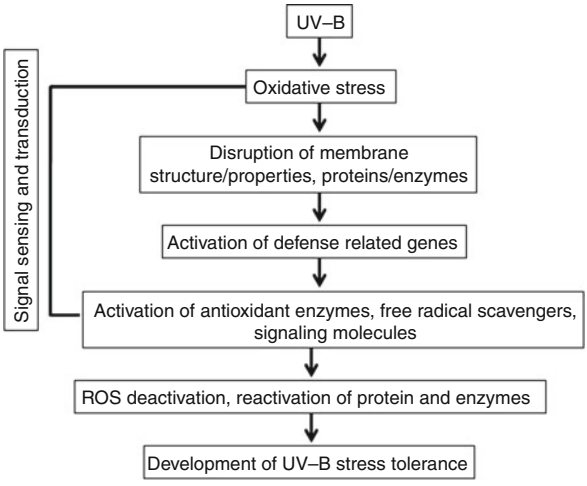

Fig. 8.2 Schematic illustration of UV-B-induced signal transduction mechanism and development of UV-B tolerance in plants

peroxidation (Triantaphylidès et al. 2008). Thus, $^1O_2$ seems to be a major ROS ultimately involved in photooxidative stress-induced cell death. Electrons in the biradical form of O_2 have parallel spin. Absorption of sufficient energy reverses the spin of one of its unpaired electron, leading to the formation of singlet state in which the two electrons have opposite spin. This activation overcomes the spin restriction, and $^1O_2$ can consequently participate in reaction involving the simultaneous transfer of two e^- (s) (Apel and Hirt 2004). In the antennae, insufficient energy dissipation during photosynthesis can lead to the formation of chlorophyll triplet state, whereas in the reaction center, it is via charge recombination of the light-induced charge pair (Krieger-Liszkay2005). $^1O_2$ directly interacts with most of the biological molecules and directly oxidizes nucleic acid, protein, and unsaturated fatty acids (Wagner et al. 2004). $^1O_2$ can be quenched by β-carotene and α-tocopherol or can react with the D1 protein of photosystem II as target (Krieger-Liszkay2005). Ascorbate, carotenoids, tocopherols, and vitamin B6 are also considered as key compounds for protection against UV-B (Harvaux and Kloppstech 2001). Carotenoids constitute the first line of defense against $^1O_2$ toxicity (Triantaphylides and Havaux 2009). They are able to quench this ROS and also directly quench triplet chlorophylls, the major source of $^1O_2$ in plant leaves.

Defense against UV-B implies a metabolic cost to plants. Two main mechanisms are involved in quenching $_1O^2$, which account for the protection of biological systems. The interaction between $_1O^2$ and a quencher leads to the production of an excited complex followed by competing reaction either involving energy transfer or chemical reaction. The mechanism related to energy transfer is denoted as physical quenching. The quencher molecule deactivates $_1O^2$ to the triplet unreactive ground state, gains energy to a triplet excited state, and then losses readily its energy to the environment and returns to its original state. Carotenoids act according to physical quenching (Triantaphylides and Havaux 2009). Ramel et al. (2012) presented results that confirmed the formation of an endoperoxide that appear to be a major oxidation product for both β-carotene and xanthophylls. They also showed that $_1O^2$ is able to cleave every double bond of the β-carotene polyene chain, resulting in a variety of aldehydes with different lengths. Superoxide radicals have also been implied to participate in photoinhibition as products of e^- transport to oxygen by leakage (Miyao 1994) and in-donor side impaired PSII (Chen et al. 1995). Indirect evidence for $O_2^{\bullet-}$ production in *Arabidopsis* under UV-B was reported during long treatment by UV-B (Mackerness et al. 2001).

Dismutation of $O_2^{\bullet-}$ by SOD results in the formation of H_2O_2. The increase in PR-1 transcript and decrease in Lhcb transcript in response to UV-B exposure were shown to be mediated through pathways involving H_2O_2. In contrast, the upregulation of PDF1.2 transcript was directly through $O_2^{\bullet-}$. ROS is involved in

the downregulation of RNA transcripts involved in photosynthetic proteins. H_2O_2 is also a by-product of mitochondrial and peroxisomal metabolism or plasma membrane respiratory burst NADPH oxidases (Cheeseman 2006).

8.2.6 Phenylpropanoid Pathway

UV-B stimulates the synthesis of flavonoids that act in conjunction with other phenolic compounds to provide a UV-B-absorbing sunscreen in epidermal tissues (Bornman et al. 1997). Li et al. (1993) reported that flavonoid-less mutants, tt4 (CHS, chalcone synthase) and tt5 (CHI, chalcone isomerase), revealed hypersensitive phenotype responses to UV-B irradiation. Flavonoids and hydroxycinnamic acids are the two main groups of phenolics that provide protection from UV-B radiation; these compounds have absorption bands in the ranges 240–545 and 227–332 nm, respectively (Cerovic et al. 2008). Plant responses to UV-B radiation depend on the amount of energy and the spectral composition of the radiation (Ibdah et al. 2002). Spectral responses are complex to interpret because there is cross talk between photoreceptors absorbing UV-B radiation and the pathways they activate. The stimulation of flavonoid accumulation is due to increased transcription of various genes encoding flavonoid biosynthesis enzymes in response to UV-B (Jenkins et al. 2001) (Table 8.1). The rapid increase in gene expression for enzymes of the flavonoid biosynthetic pathway in response to UV-B is known to be regulated at the level of transcription (Schulze-Lefert et al. 1989). Several phenylalanine ammonia lyase (PAL) genes as well as genes encoding cinnamate-4-hydroxylase (4CH) and 4-coumarate:CoA ligase (4CL) were upregulated in response to UV-B. In addition to the significant expression of genes encoding structural enzymes, genes involved in lignin biosynthesis such as caffeoyl-CoA-*O*-methyltransferase were also upregulated under low or high UV-B. Flavonoids (including anthocyanins) exert antioxidant activity mainly through three ways: (1) due to their low redox potentials, they are able to reduce highly oxidizing free radicals with redox potential in the range 2.13–1.0 V as $O_2^{\bullet -}$, H_2O_2, and $^{\bullet}OH$; (2) flavonoids may also efficiently chelate trace metals limiting $^{\bullet}OH$ formation; and (3) flavonoids inhibit several enzymes involved in ROS generation.

Table 8.1 List of genes encoding the key enzymes of phenyl propanoid pathway and regulated by UV-B radiation

Gene	Gene annotation	Description
PAL	Phenylalanine ammonia lyase	Encodes for phenylalanine ammonia lyase, the first enzyme of the general phenylpropanoid metabolism. Is involved in plant defense responses, oxidative stress, and biosynthesis of flavonoids
4CL	4-Coumarate:CoA ligase	Encodes for 4-coumarate:CoA ligase, a key enzyme involved in the last step of the general phenylpropanoid
CHS	Chalcone synthase	Encodes chalcone synthase, the first committed enzyme involved in flavonoid biosynthesis. Regulates also the synthesis and accumulation of anthocyanin
CHI	Chalcone isomerase	Encodes chalcone isomerase which is involved in flavonoid biosynthesis. Catalyzes the conversion of chalcones into flavanones
F3H	Flavanone-3-hydroxylase	Encodes for flavanone-3-hydroxylase which catalyzes the formation of dihydroflavonols from flavonones and its expression is also important in the regulation of flavonoid pathway
OMT1	Flavonol-3´-*O*-methyltransferase	Encodes for flavonol-3´-*O*-methyltransferase that is highly active towards the synthesis of quercetin and myricetin
N3D	Naringenin-3-dioxygenase	Encodes flavanone-3-hydroxylase, an enzyme that is coordinately expressed with chalcone synthase and chalcone isomerase. Regulates flavonoid biosynthesis
TT3	Dihydroflavonol reductase	Catalyzes the conversion of dihydroquercetin to leucocyanidin in the biosynthesis of anthocyanins
F5H	Ferulate-5-hydroxylase	Encodes ferulate-5-hydroxylase (F5H) involved in lignin biosynthesis
MYB4	R2R3 MYB protein	Encodes a R2R3 MYB protein which is involved in response to UV-B. It functions as a repressor of target gene expression
HYH	bZIP protein	Encodes a DNA-binding transcription factor that is homologue to hy5 transcription factor. It has important roles in light-regulated process and response to UV-B
TT2	R_2R_2 MYB protein	Encodes a R2R3 MYB domain putative transcription factor that acts as a key determinant in the proanthocyanidin accumulation of developing seed

8.3 Conclusion

Plant response to UV-B depends on wavelength, fluence rate, and duration of UV-B irradiation as well as the extent of adaptation. UV-B has the potential to damage macromolecules including DNA, to generate ROS and to impair cellular processes. Organisms have therefore evolved mechanisms to protect against UV-B and to repair UV-B-induced damage.

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Chapter 9
Metabolic Strategy of Annual Desert Plants: Adaptive Phenomenon of CAM and C_4 Photosynthesis Functioning in a Leaf

Valery Yu. Lyubimov and Karl Ya. Biel

Abstract Three types of autotrophic tissues are shown to be present in the leaves of four species from *Chenopodiaceae* and their anatomo-morphological and physiological characteristics are described. It has been concluded that C_4 photosynthesis can function in two chlorenchyma layers. The potential ability of water storage parenchyma to carry out photosynthesis has been estimated; the dynamics of cell sap pH, the degree of the stomata opening, and starch content in the chloroplasts of a certain tissue during a day have been studied. Rates of CO_2 assimilation over the period of 24 h, the kinetics of radiocarbon incorporation into photosynthates, and the carbon distribution in assimilating tissues as well as the activities of ribulose bisphosphate carboxylase, phosphoenolpyruvate carboxylase, and aspartate and alanine aminotransferases were measured in the leaves of some *Chenopodiaceae* species grown on salt marsh of the Kara-Kum Desert. It was demonstrated that in the leaves of *Suaeda arcuata*, *Suaeda crassifolia*, and *Climacoptera crassa* the C_4 pathway of photosynthesis was operated between the two layers of chlorenchyma cells while in the water storage chlorenchyma, a heterotrophic CO_2 fixation took place with high rates in the night. In ontogenesis of the investigated plants, CO_2 assimilation function of water storage tissue (WST) is found only in the leaves of summer generation during the period of the most severe xerothermic conditions. Anatomy structure and photosynthetic carbon metabolism in plants grown on soils with varying salinity in the Central Kara-Kum Desert were investigated. Annual plants grown on slightly saline soils were mainly presented by C_3 and C_4 xerophytes, but those on heavily saline soils and on salt marsh—by C_4 and C_4-CAM succulents with WSTs assimilating CO_2 by the CAM pathway.

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9.1 Introduction

Environment conditions of growth of terrestrial plants in different climate zones from wet tropics through deserts and temperate zone to the polar regions are very diverse. In the tropical and temperate zones, conditions—temperature, insolation, humidity—are favorable for growth and development of plants, but tundra and hot deserts characterized by the most stringent circumstances, limiting their livelihoods. The main what plants should have for its vitality, is appropriate balance between the temperature and water availability. Both of climatic settings more or less depend on the intensity of insolation. In the tundra in the vegetation period, there is no problem to supply plants with water, but there is a problem of low daily temperatures. The latter reduces the activity of all the enzymes and thus limits the accumulation of biomass. In the tundra, this problem to some extent is solved by longer daylight period. In hot deserts, the main problem for plants is water deficit, which should be optimized on a background of very high temperature and strong insolation. This is especially important for annual plants that need to accumulate sufficient biomass and create fully functional reproductive organs in one growing season. This requires them to have enough energy and metabolic resources, i.e., sufficient integral photosynthesis. The problem of water scarcity in plants is resolved, mainly, through two metabolic strategies. In circumstances of temperate arid zones, C_4 plants are spread. They have very low transpiration coefficient (3–4 times lower than C_3 plants) due to specific leaf structure and metabolic add-ins. In arid hot deserts, there are plants with a CAM type of carbon metabolism, which assimilates most fraction of CO_2 in the dark period. Under conditions of salt stress in addition to water deficit and high temperature, it is acceptable to assume the possibility of the existence of plants that combine the two above-mentioned types of carbon dioxide assimilation, based on one enzyme—PEP carboxylase.

Many plants with C_4 photosynthesis are characterized by a “kranz” type of the leaf anatomy (Laetsch 1974). However, among C_4 plants, there are representatives with another anatomy of photosynthetic apparatus (Carolin et al. 1978; Craig and Goodchild 1977; Crookston and Moss 1973; Olesen 1974; Osmond 1970; Shomer-Ilan et al. 1975). A certain group of these plants, some varieties of *Suaeda* and *Climacoptera* species (*Chenopodiaceae* family), in particular, exists under such severe climatic conditions, when neither typical C_4 plants nor CAM types can grow. Their photosynthetic apparatus is characterized by the presence of the third water storage tissue (WST) in the leaves besides the two adjacent autotrophic layers (Bil’ et al. 1983a, b; Bil’ and Gedemov 1980). This tissue is likely to contribute to the increased drought resistance of halophytes. If in C_4 plants having xeromorphic leaf structure, a thick net of conducting bundles being adjacent to the sheath cells provides intensive water supply to autotrophic tissues under air-drought conditions,

in halophytes this is not likely to occur due to the weak development of the conducting systems and the absence of its direct contact with chlorenchyma layers. It is most likely that in the leaves of these species the photosynthetic tissues are supplied with water by the WST in which the moisture condensed from the atmosphere during the night period is accumulated. This specificity of the supplying of photosynthetic cells with water can happen because in places where these plants grow, a constant drought is not only in the air but also in the soil.

However, the WST of halophytes can probably not only provide other leaf tissues with water but also perform the function of CO_2 assimilation. In the representatives of *Chenopodiaceae* species, up to 48 % of the total amount of leaf chloroplasts (up to 33 % by volume) is concentrated in the WST (Bil' and Gedemov 1980). The central position of the WST and its bulk screening from the environmental atmosphere with two layers of chlorenchyma results in the problem of a special mechanism of carbon fixation and metabolism in it.

Biochemical experiments indicate that in the leaves of halophytes containing three types of autotrophic tissues alongside with C_4 photosynthesis, there is a mechanism enabling these plants to assimilate CO_2 efficiently during the night.

In the regions with extreme, contrast climatic conditions, photosynthetic apparatus is more likely to be the mechanism determining the adaptive possibilities of plants. It is known that in a whole plant scale photosynthesis which mainly depends on the structure and function of assimilating organs is the most responsive to a shift of environmental parameters (Mokronosov 1981). Photosynthetic apparatus of Kara-Kum Desert aboriginal plants is subjected to the effects of seasonal and diurnal temperature and relative air humidity changes. Since climatic conditions within one region are the same, the salinity varying in the root-layer from 0.082 in sandy-desert soils to 7 % in a solid residue of salt marsh (Kurbannazarov 1970) is the main factor determining the variety of plant cover in the Kara-Kum Desert.

The aim of this chapter was to review morphological peculiarities, photosynthetic carbon metabolism, and character of plant distribution on three main landscape elements: sandy-desert soils, light-colored gray desert soil, and salt marsh.

9.2 Assessment of Possible Input of Each of the Three Types of Phototrophic Tissues of a Photosynthetic Organ in Diurnal CO_2 Fixation

The experiments were carried out in the vicinity of Kopetdag during summer–autumn periods (Bil' and Gedemov 1980; Bil' et al. 1983a; Lyubimov et al. 1986). To study the structural–functional peculiarities of photosynthetic apparatus, four species of *Chenopodiaceae* family growing on salt marshes have been investigated: *Suaeda arcuata* Bunge, *S. crassifolia* Pall., *S. acuminata* (C.A. Mey) Moq., *Climacoptera crassa* (B.B.) Botsch.

Anatomic–morphological study of leaves was carried out on the cuts obtained from living plants and fixed in 70 % ethanol and on the cuts from the material prepared for the electron microscopy study. In the latter case, the cuts of 1–2 μm thickness obtained on a microtome (LKB) were dyed with 1 % solution of methylene blue buffered by sodium borate (1 %). The cuts were photographed using a film "micrat-300" and a universal optical microscope "Nu." Quantitative characteristics of assimilative tissues were assayed by the method suggested by (Mokronosov and Borzenkova 1978). Diurnal dynamics of starch content in chloroplasts of various tissues were studied on the cuts prepared from the material fixed in 70 % ethanol and dyed by iodine in potassium iodide (Lugol's solution) (Bil' et al. 1983b; Jensen 1965). To determine pH of cell sap of *Amaranthus*, its leaves were ground in a mortar, and then the cell sap was collected from homogenate and its pH was measured. While measuring pH in WST cells of halophytes which occupy up to 85 % of partial volume of a leaf (Bil' et al. 1983b) (Table 9.3) and consist of thin-walled heavily vacuolated cells, the sap was obtained in the following way: after purification of the surface of the assimilating organs from salts, the bottom and top part of the leaf were cut with a blade. The remaining 1.5–2.0 cm pieces were placed between clean glass plates which were slightly pressed. As a result, the cells of the WST were firstly destroyed, and their content by bending the glass plates was collected immediately for pH measuring. The degree of stomata opening has been studied by using celluloid replicas where the areas of stomata slits were measured. The ratio of a stomata area at the moment of replica cutting and an area of a circle with a diameter equal to the longest stomata axis (Bil' et al. 1983a, b) was the final quantitative characteristic.

The majority of representatives of *Chenopodiaceae* growing on salt marshes of the Kara-Kum Desert are characterized by a similar structure of photosynthetic apparatus. As a rule, they have leaves of a cylindrical form in which WST situated in the center occupies a considerable volume. Among the types studied, three representatives of *Chenopodiaceae* have this structure. Epidermis of *Suaeda arcuata* leaves is formed with either cubic or flattened in dorsiventral direction cells which sizes vary within wide limits (Table 9.1). The outer wall of epidermis cells is greatly thickened. Just under the epidermis, there are two concentric layers of chlorophyll-containing cells: the outer and inner ones. The outer layer of chlorenchyma (OLC) consists of cylindrical cells prolonged in the radial direction (Table 9.1). Cells of this which is characterized by big intercellular cavities are in a very close contact with the inner layer of chlorenchyma (ILC) formed by tightly adjoining cells of cubical form. In these species, WST consisting of large cells of irregular shape occupies the central part of a leaf.

Suaeda acuminata leaves are characterized by the absence of WST in them. The conducting bundles are surrounded by parenchyma sheath cells as in typical C_4 plants. However, under epidermis there is a hypodermic layer. It is essential that the cells of this tissue are strongly vacuolated and coincide in form and dimensions with the cells of the WST of other representatives of *Suaeda* species, whereas in some species of *Chenopodiaceae* family which have hypodermis and WST in their leaves, for example, in *Haloxylon persicum* (Gamaley 1985; Hatch and Osmond 1976; Voznesenskaya and Stashehko 1974), hypodermic cells are considerably

Table 9.1 Linear dimensions of cells in various leaf tissues of some desert plants (d_l, d_h, d_w are the length, height, and width of cells, respectively)

	Cell dimensions, μm (variation coefficient ca. 30.2 %)											
	Epidermis			Outer layer of chlorenchyma			Inner layer of chlorenchyma			Water storage tissue		
Species	d_l	d_h	d_w	d_l	d_h	d_w	d_l	d_h	d_w	d_l	d_h	d_w
S. arcuata	52	31	41	73	20	15	44	44	19	80	58	59
S. crassifolia	54	28	68	66	17	15	35	46	29	95	60	75
Cl. crassa	42	12	42	54	11	12	27	18	26	73	95	62
S. acuminata[a]	115	79	104	91	26	27	34	36	40	108	55	80
A. retroflexus[b]	24	17	37	36	11	11	36	25	38	–	–	–
E. turcomanica[b]	51	19	35	53	11	11	31	22	17	–	–	–

[a]In this case instead of WST, there is hypoderma
[b]In this case instead of OLC and ILC, there are mesophyll and bundle tissue

smaller than the cells of the WST. These morphological peculiarities of *S. acuminata* hypodermis give grounds to suggest that in this variety the tissue functions instead of the absent WST.

In contrast to WST of *S. arcuata*, *S. crassifolia*, and *C. crassa*, the cells of which being in the center of a leaf are in a relatively close contact with each other, and the hypodermis of *S. acuminata* as in the other desert edificators having this tissue (Gamaley and Voznesenskaya 1986; Voznesenskaya and Stashehko 1974) is threaded with big intercellular cavities, the presence of which is probably due to the necessity of CO_2 diffusion to chlorenchyma cells.

The conducting system of the leaves of the varieties studied is characterized by branching venation. In the leaf center, smaller conducting bundles are branched from a midrib. The latter ones form a dense net threading the ILC of *S. acuminata*, whereas in the other three species the net threads the WST. In *S. arcuata* and *S. crassifolia*, two chlorenchyma layers form a closed cylinder along the whole length of a leaf, whereas in *C. crassa* this cylinder is open at the adaxial side of a leaf. In this case, the cells of the WST have a contact with epidermis.

The outer and inner chlorenchyma layers present a considerable interest from the point of view of photosynthesis study in these species. By morphological structure, their cells are very similar to the cells of mesophyll and parenchyma sheath of typical C_4 plants. For instance, their linear dimensions and volumes (Tables 9.1 and 9.2) are close to those of C_4 plant cells. In the OLC, plastids are situated mainly along the cell periphery; however, under illumination of the leaves they are often shifted to the walls adjacent to the inner layer. Chloroplasts in the ILC occupy a centrifugal position only in *S. acuminata*, whereas in the other three species they are situated centripetally. Histochemical observations show light-dependent accumulation of starch in the chloroplasts of the ILC, whereas in the chloroplasts of the OLC negligible amounts of this polysaccharide are observed (Table 9.4).

The morphological properties described for two chlorenchyma layers are characteristic for assimilative tissues of typical C_4 plants. Quantitative assessments of photosynthetic structures of two chlorenchyma layers can serve as additional data, which can characterize their metabolic interaction.

Table 9.2 Mesostructural parameters of various leaf tissues of some desert plants

	S. arcuata			*S. crassifolia*			*Cl. crassa*			*S. acuminata*		
Parameter	OLC	ILC	WST	OLC	ILC	WST	OLC	ILC	WST	OLC	ILC	H[a]
Cell volume, 10^3 μm^3	22	37	274	17	47	428	7	13	430	64	49	475
Chloroplast number per cell	27	82	195	39	77	221	24	39	256	47	90	79
Chloroplast volume, μm^3	85	68	46	74	75	35	63	72	35	33	44	20
Chloroplasts volume per cell, 10^3 μm^3	2.3	5.6	9.0	2.9	5.8	7.7	1.5	2.8	9.0	1.6	4.0	1.6
Cell number per 1 mm of leaf	6,035	3,323	1,339	5,474	3,092	1,178	13,558	5,138	2,312	4,018	2,109	393
Cell volume per 1 mm of leaf, 10^6 μm^3	133	123	367	93	145	504	95	67	994	257	103	107
Chloroplast number per 1 mm of leaf, 10^3	163	273	261	214	238	260	325	200	592	189	190	31
Chloroplasts volume per 1 mm of leaf, 10^6 μm^3	14	19	12	16	18	9	21	14	21	6	8	0.6

[a]In this case instead of WST, there is hypoderma

According to the data given in Table 9.2, leaf photosynthetic tissues differ by the amount of chloroplasts contained in them. A cell of the outer layer has at an average 27–50 plastids. Chloroplasts being flattened rotation ellipsoids have a comparatively great length (8.0–8.5 μm) at an insignificant thickness (2 μm). *S. acuminata* is characterized by smaller plastids with an average diameter of 5.9 and 1.8 μm thickness; *S. arcuata*, *S. crassifolia*, and *S. acuminata* have ca. 90 plastids per cell of the inner layer, whereas *C. crassa* has half the value (Table 9.2). Chloroplasts of the ILC differ from plastids of the OLC by smaller diameter (7 μm) and greater thickness (up to 3 μm). The dimensions of *S. acuminata* chloroplasts in the cells of the outer as well as of the inner layers are somewhat less.

According to the data given in Table 9.2, the volume of the plastids in the OLC varies within 60–85 μm^3 in all the plants studied except *S. acuminata* where this value does not exceed 30–35 μm^3. The chloroplasts of the ILC have a volume of 65–75 μm^3 (in *S. acuminata*—ca. 45 μm^3).

At similar individual volumes of plastids in the cells of the outer and inner layers and their less amount in the OLC (at an average, by 2–3 times) the inner chlorenchyma layer seems to have a greater potential photosynthetic activity. However, relative total volumes of chloroplasts of both the tissues differ to a lesser extent (at an average, by 1.3 times) (Table 9.3) which is due to the increase of the ILC number per leaf length unit.

Thus, the complex of structural features of the two chlorenchyma layers in the leaves of the plants studied (namely, a tight contact between two types of cells), close or equal (as in *S. acuminata*) total specific volumes of chloroplasts in these tissues, localization of plastids in the cells of assimilative tissues which is characteristic of C_4 plants, and specialization of chloroplasts in the ILC to light-dependent synthesis of starch indicates that metabolic cooperation of these two autotrophic cell layers is possible by C_4-type photosynthesis. This conclusion is supported by the scheme of ^{14}C distribution among the primary photosynthetic products in *S. arcuata* (Bil' and Gedemov 1980; Bil' et al. 1983a), *S. crassifolia*, and *C. crassa* (Bil' et al. 1981; Lyubimov et al. 1986).

The presence of WST, as it has been already mentioned, is another anatomical peculiarity of the leaves of the plants studied. Table 9.2 shows that in three plant species, one cell of WST contains different amounts of chloroplasts: in *S. crassifolia*, ca. 221; in *S. arcuata*, ca. 195; and in *C. crassa*, ca. 256. Their dimensions are smaller than in chlorenchyma cells, i.e., an average diameter is 5.5–6.0 μm at a 2.0–2.5 μm thickness. In all the plant types, these plastids have granal structure and can accumulate great amounts of starch under illumination (Table 9.4).

In *S. acuminata*, hypodermis cells also contain chloroplasts. However, their dimensions and number in one cell are considerably less than in the cells of WST of the three other plant types (Table 9.2). The role of WST in the leaf photosynthesis can be estimated based on the parameters given in Table 9.3. In spite of the fact that the number of cells of WST is a small percent of the total cell number in a leaf, by the volume this tissue dominates (from 60 to 90 % of the leaf volume). In three plant types, it contains a considerable part of the total chloroplast number (30–50 %). In *S. acuminata*, hypodermis chloroplasts are 8 % of the total amount.

Table 9.3 Relative parameters of assimilative tissues in the leaves of some desert plants

Parameter (%)	*S. arcuata*			*S. crassifolia*			*Cl. crassa*			*S. acuminata*		
	OLC	ILC	WST	OLC	ILC	WST	OLC	ILC	WST	OLC	ILC	H[a]
Chloroplast volume vs. cell volume	10.5	15.1	3.3	17.1	12.3	1.8	21.4	21.5	2.1	2.5	8.2	0.3
Cell number of tissue vs. total cell number	56.0	30.8	13.2	56.0	31.5	12.5	64.4	24.4	11.3	59.0	31.0	18.0
Cell volume of tissue vs. total cell volume	21.4	19.7	58.9	12.6	19.5	67.9	8.2	5.8	86.0	47.0	18.8	34.2
Chloroplast number of tissue vs. total chloroplast number	23.4	39.2	37.5	30.0	33.5	36.5	29.1	17.9	53.0	46.1	46.3	7.2
Chloroplasts volume of tissue vs. total chloroplast volume	31.1	42.2	26.7	37.2	41.9	20.9	37.5	25.0	37.5	41.1	54.8	4.1

[a]In this case instead of WST, there is hypoderma

Table 9.4 Diurnal dynamics of starch accumulation in chloroplasts of three types of autotrophic tissues in annual halophytes

	Starch content, % of the chloroplast volume											
	S. arcuata			*S. crassifolia*			*Cl. crassa*			*S. acuminata*		
Day time	OLC	ILC	WST	OLC	ILC	WST	OLC	ILC	WST	OLC	ILC	H[a]
11 p.m.	0.9 ± 0.09	50.4 ± 4.0	48.9 ± 2.9	0.0	73.2 ± 4.4	52.9 ± 4.2	0.0	56.4 ± 3.4	23.5 ± 1.9	0.8 ± 0.07	42.4 ± 3.6	39.4 ± 3.6
5 a.m.	0.7 ± 0.07	43.1 ± 3.9	33.3 ± 2.7	0.0	58.3 ± 5.2	26.4 ± 2.4	0.0	49.6 ± 3.5	19.1 ± 1.3	0	76.9 ± 7.1	0.8 ± 0.1
10 a.m.	0.0	23.2 ± 2.1	12.1 ± 1.0	0.4 ± 0.03	38.6 ± 3.1	10.8 ± 0.9	0.0	40.4 ± 3.6	22.6 ± 1.8	0.3 ± 0.06	25.4 ± 2.1	4.5 ± 0.3
3 p.m.	1.2 ± 0.08	35.5 ± 2.8	28.2 ± 2.5	0.9 ± 0.06	47.4 ± 3.3	30.6 ± 1.8	0.0	46.1 ± 3.2	47.1 ± 3.3	1.2 ± .02	38.2 ± 3.6	31.6 ± 2.8
11 p.m.	1.1 ± 0.10	46.4 ± 3.2	39.4 ± 2.8	0.3 ± 0.01	66.7 ± 4.0	48.1 ± 3.4	0.0	52.1 ± 3.1	27.9 ± 2.2	0.9 ± 0.07	47.2 ± 4.6	37.8 ± 4.2

[a]Hypoderma

The total volume of WST plastids varies within 20–40 %, in *S. crassifolia*, *S. arcuata*, and *C. crassa*. In *S. acuminata*, the volume of hypodermis chloroplasts does not exceed 4 %. Consequently, in the plants studied (except *S. acuminata*) the water storage parenchyma can play the essential role in total photosynthesis of a leaf.

The pathway of carbon during photosynthesis process in the cells of WST and its interactions with other photosynthetic layers are not clear. However, there is a basis to suggest that these cells can assimilate CO_2 in the night according to the CAM pathway described earlier (Osmond 1978). In fact, when in the light in two chlorenchyma layers there is C_4 photosynthesis during which even small amounts of CO_2 in the intracellular cavities are assimilated (Hatch and Osmond 1976), the concentration of carbon dioxide in the cells of WST being behind the barrier of the outer and inner layers must be rather insignificant. Under such conditions, assimilation of atmospheric CO_2 in this tissue during day time hours is hardly impossible. However, the access of carbon dioxide to the cells' WST can be considerably facilitated in the night when the outer layers do not assimilate CO_2.

The plants metabolizing CO_2 by CAM pathway are characterized by (1) accumulation of C_4-dicarboxylic acids in the dark period, resulting in pH decrease of cell sap; (2) variations in starch content in chloroplasts which are inversely proportional to the changes in the amounts of C_4 acids (Kluge 1976); and (3) diurnal dynamics of stomata state (Osmond 1978).

To verify possible utilization of CO_2 by the cells of WST by CAM or a similar type, the diurnal dynamics of the parameters mentioned above has been determined for the plants studied.

Figures 9.1 and 9.2 present the curves of diurnal changes in cell sap pH of the experimental plants. It is seen that in *S. arcuate*, *S. crassifolia*, and *C. crassa*, the cell sap is maximally acidified at dawn as it occurs in typical CAM plants (Osmond

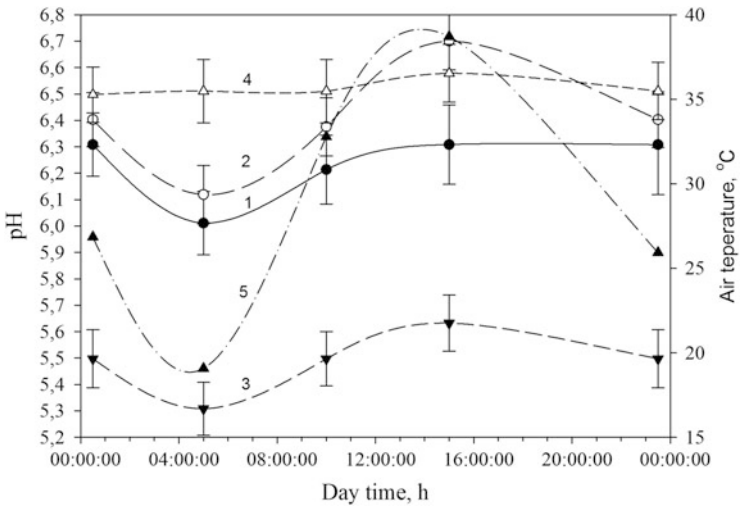

Fig. 9.1 Diurnal dynamics of cell sap pH in the leaves of *Suaeda arcuata* (*1*), *S. crassifolia* (*2*), *Climacoptera crassa* (*3*), *Amaranthus retroflexus* (*4*), and temperature of air (*5*)

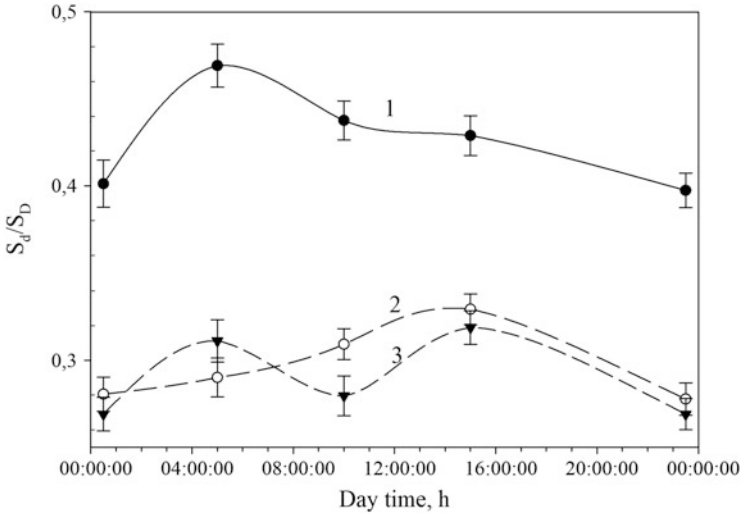

Fig. 9.2 Diurnal dynamics of stomata opening in *S. arcuata* (*1*), *S. crassifolia* (*2*), and *C. crassa* (*3*). S_d, area of a stomata opening at the moment of replica taking; S_D, area of a circle with a diameter equal to the longest stomatal axis

1978). In *S. acuminata*, pH changes during the day are not similar to the pH diurnal dynamics which is characteristic of CAM plants. Minimal pH values of the cell sap in this plant were observed during the second part of the day. Probably, the maximal acidification of the cell sap of *S. acuminata* leaves is due to intensive functioning of C_4 photosynthesis in the afternoon hours, when the level of solar radiation is maximal. As for hypodermis in its leaves, it contributes slightly to total CO_2 assimilation and serves mainly for storing water. Even if in the cells of this tissue there is dark fixation of CO_2, the amount of C_4 acids forming during this process is too small for the diurnal pH dynamics characteristic of typical CAM plants to be manifested when CAM is determined integrally.

While comparing *S. acuminata* with other plant types, it can be concluded that WST but not two layers of chlorenchyma cells carrying out photosynthesis of C_4 type plays the main role in considerable acidification of the cell sap during the night in *S. arcuata*, *S. crassifolia*, and *C. crassa*. This is also supported by the fact that in the leaves of *Amaranthus retroflexus*, typical C_4 plant of aspartate type pH change does not exceed 0.1 per day, whereas pH minimum is observed in the evening (5–8 p.m.) (Fig. 9.1).

Thus diurnal variations of cell sap pH in *S. arcuata*, *S. crassifolia*, and *C. crassa* indicate that their WST fix CO_2 along the CAM or similar pathway in the night.

Variations of the starch content in the plastids of the WST in four plant types also resemble the diurnal dynamics of its accumulation and consumption which is typical for CAM plants, i.e., in *S. acuminata* and *C. crassa*, the minimal content of starch in chloroplasts of the WST is observed at 5 a.m., whereas the maximal accumulation takes place at 3–5 p.m., in *S. crassifolia* and *S. arcuata*, the time of maximal starch exhaustion is shifted to 10 a.m. (Table 9.4).

In spite of diurnal variations of the cell sap pH and starch content in chloroplasts, no changes were found in the degree of the stomatal opening. The diurnal dynamics of the stomatal opening state shows (Fig. 9.2) that the variations of S_d/S_D value do not exceed mean quadratic error of measurements, i.e., during 24 h under the following changes: light–dark-light and temperature alternations from 20 to 40–45 °C; the stomata remain open to the same extent in contrast to the experimental C_3, C_4, and CAM plants in which stomatal resistance is considerably changed during a day (Osmond 1978). Consequently, CO_2 access to the leaf intracellular cavities as well as to the assimilating tissues remains constant for 24 h. In this connection, the rates of CO_2 assimilation in the day- and nighttime and their ratio in the plants studied must differ from similar parameters for C_3 or C_4 plants. In fact, the measurements of $^{14}CO_2$ fixation rates showed (Bil' et al. 1981, 1983a) that in plants studied the specific input of night assimilation as compared with the light one is 10–15 times higher than in C_4 plant *Amaranthus retroflexus*. Besides, the rate of photosynthesis in the leaves of *A. retroflexus* was dramatically decreased in the afternoon hours, whereas the photosynthetic activity of our plants was not changed and even increased during this period.

Thus, the results given above indicate that in the leaves of the plants studied besides C_4 photosynthesis there is a possibility of functioning of a mechanism providing their assimilation of atmospheric CO_2 with a high relative efficiency in the night. A positive carbon balance under extreme severe conditions of habitat is probably due to this fact.

The ratio of C_4 and CAM pathways of carbon assimilation is quite an interesting problem. Due to a great biochemical similarity between them (Avadhani et al. 1971), it is not surprising that both the mechanisms are combined in one plant; moreover, the leaves of many types (Gamaley and Voznesenskaya 1986; Ku et al. 1981; Lyubimov et al. 1986; Olesen 1974; Hatch et al. 1971; Shomer-Ilan et al. 1979; Zalensky 1977a, b) (including those discussed in this communication) have anatomical properties as CAM and C_4 plants. However, demonstration of the presence of two metabolic systems in various tissues of one leaf is rather difficult. The above results are only an indirect support of the suggestion given. To solve this principally important problem, it is necessary to study localization and activity of the enzymes participating in dark fixation of carbon dioxide as well as kinetics of carbon distribution among metabolites on both intact leaves and isolated tissues.

9.3 Physio-Biochemical Basis for Adaptation of Phototrophic Tissues to the Day and Night Assimilation of CO_2

The experiments were carried out during the spring–autumn period on the plants of *Chenopodiaceae* species: *Suaeda arcuata* Bunge, *Suaeda crassifolia* Pall, and *Climacoptera crassa* (N.B.) Botschy grown on salt marsh soils of the South-East Kara-Kum Desert. To study the kinetics of photosynthetic products, the leaves

separated from the plants were inserted into a lock chamber in the flow of $^{14}CO_2$ (0.03 %) under natural illumination for 5 or 15 s after that the material was either fixed or additionally exposed to the light in CO_2 for 60 or 300 s. The leaves were fixed in the fumes of boiling ethanol. Two-dimensional paper chromatography was used for separation and identification of low molecular compounds (Tarchevsky and Karpilov 1963). Mixtures of butanol, formic acid, and water (75:13:1) and 80 % water solution of phenol were used as solvents. The radioactivity of spots from chromatograms was determined on a gas flowing counter with a counting efficiency of 30 %.

The activities of RuBisCo and PEP carboxylase were measured radiometrically with ribulose-1,5-bisphosphate and phosphoenolpyruvate as substrates, respectively (Kanai and Edwards 1973); the activities of aspartate and alanine aminotransferase—spectrophotometrically (Hatch and Mau 1973). The protein content was assayed according to Lowry et al. (1951); chlorophyll was calculated by the formula of Arnon (1949).

To measure the rates of photosynthesis, the leaves separated from the illuminated plants were exposed for 1 min in $^{14}CO_2$ (0.03 %) flow with a known specific radioactivity (1–1.5 Cu mol^{-1}). After the exposition, the material was fixed in 5 N HC1. After that, water-acidic extract prepared from it was placed on paper discs and dried; then a Geiger–Muller counter was used to measure its radioactivity. The rate of photosynthesis was expressed in μmol CO_2 (g of dry weight)$^{-1}$ h^{-1}. The plant leaves were exposed in CO_2 flow for 30 min to determine the rate of dark assimilation of carbon dioxide. Further procedures were the same as in case with photosynthesis rate determination. In a number of experiments, the leaf samples after their exposure in CO_2 were fixed simultaneously in 5 N HCl and in 0.5 % solution of 2,4-dinitrophenylhydrazine in 5 N HC1. The radioactivity of the obtained water-acidic extract (A_{DNPH} and A_{HCl}) was given with respect to the leaf fresh weight and to the exposure time in the radioactive carbon dioxide.

To study the distribution of ^{14}C between the leaf tissues, the intact plants in soil were exposed in the flow of $^{14}CO_2$ during the night (12 p.m.–5a.m.) and then kept under natural illumination and usual atmosphere for 5 h (5 a.m.–10 a.m.) and 10 h (5 a.m.–3 p.m.). At 5 a.m., 10 a.m., and 3 p.m., several leaves from each plant were fixed in liquid nitrogen (−196 °C); in the frozen state, the layers of various autotrophic tissues were subsequently scraped and the radioactivity of ethanol–water-soluble fractions was measured and expressed in cpm (g of dry weight)$^{-1}$.

Table 9.5 shows that the kinetics of ^{14}C distribution among photosynthates in the leaves of *S. crassifolia* and *Cl. crassa* is similar to that of typical C_4 plants. For instance, after 5 s photosynthesis in $^{14}CO_2$, the greater part of label (65–95 % of the total radioactivity) is incorporated into C_4-dicarbonic acids. Additional exposure of the leaves to the usual atmosphere results in a gradual transformation of the radioactive carbon into PGA, PES, and then into free sugars. When the leaves which have assimilated $^{14}CO_2$ are illuminated for a long time in the air (up to 300 s), only 3–8 % of the total radioactivity is observed in dicarbonic acids, whereas in free sugars up to 60–75 %.

Table 9.5 Kinetics of photosynthates in the leaves of desert plants of Chenopodiaceae

Time of light exposure(s), in		Radioactivity of ethanol–water-soluble compounds, % of the total					
$^{14}CO_2$	$^{12}CO_2$	PGA + PES	Sugars	Aspartate	Malate	Alanine	Others
Suaeda crassifolia							
5	–	5.8	–	79.2	15.1	–	–
15	–	14.6	–	70.7	9.4	2.2	3.2
15	60	24.6	38.3	6.6	4.4	13.1	13.0
15	300	1.8	76.1	2.9	0.6	10.9	7.9
Climacoptera crassa							
5	–	6.2	3.3	71.7	3.0	–	15.8
15	–	5.9	15.1	32.6	7.9	14.0	24.5
15	60	6.3	63.7	5.2	2.4	9.3	11.1
15	300	1.9	75.0	2.8	–	9.0	11.3

It should be noted that at short light exposures in $^{14}CO_2$, the ratio aspartate/malate in *S. crassifolia* and *Cl. crassa* is 5 and 24, respectively, but there is no malic acid at all in C_4 plants *Amaranthus retroflexus* (usually producing malate at amount equal to ca. 20 % of the sum of dicarbonic acids) cultivated in the places where halophytes grow. Probably, the predominance of aspartate among C_4 acids in the experimental species grown on the saline soils is due to the decrease in the activity of the reducing malate dehydrogenase, since high concentrations of salts are known to have an inhibitory effect on the activity of this enzyme (Kanai and Edwards 1973; Karekar and Joshi 1973). It is interesting that under these conditions, the grana and photosystem II activities are reduced (Bil' and Gedemov 1980) in the chloroplasts of the outer chlorenchyma layer in *S. arcuata* which correspond to mesophyll cells of typical C_4 plants (Bil' et al. 1983a, b).

At short light exposures ^{14}C is also incorporated in alanine in all the plants studied (Table 9.5). Probably, synthesis of considerable amounts of this compound can be accounted for by the fact that these species in their natural places of growth are subjected to the effect of high temperatures, severe drought, and high salt concentrations leading to the appearance of the so-called alanine effect which has been described in (Tarchevsky 1964).

A certain part of the label is also observed in the products of the glycolate pathway: glycolate, glycine, and serine. In this case, the kinetics of radioactivity of the given compounds in the species studied (Fig. 9.3) differs from that of C_3 and CAM plants, but it is similar to the one observed in C_4 plants of aspartate type. A considerable percent of radioactivity in glycolate is observed in the leaves of *S. arcuata* and *Cl. crassa* (Fig. 9.3) after a short-term exposition in CO_2. This can indicate that photorespiration substrate is formed not only during RBP oxygenase reaction in the cells of the inner chlorenchyma layer but also during subsequent oxidative decarboxylation of an oxaloacetic acid in the cells of the outer chlorenchyma layer as in case with mesophyll in the leaves of *Zea mays* (Lyubimov et al. 1978).

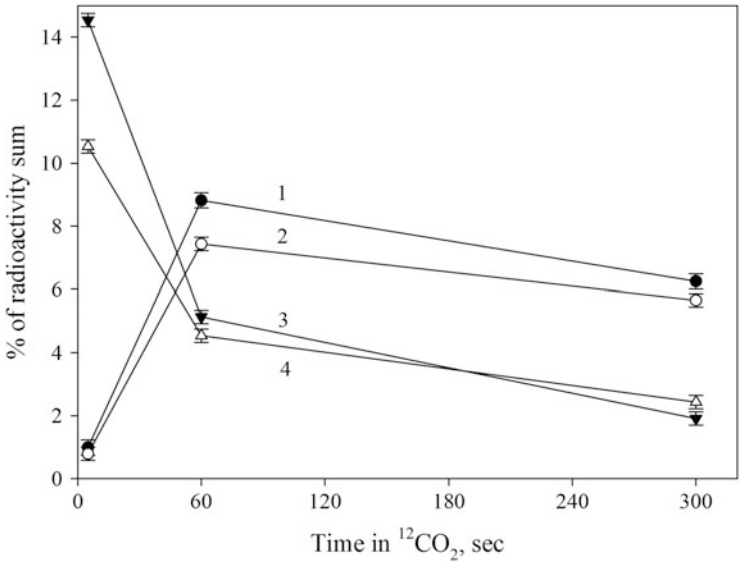

Fig. 9.3 Kinetics of radioactivity of glycolate (*triangles*) and the products of its metabolism (glycine + serine—*circles*) in the leaves of *Suaeda arcuata* (*1*, *3*) and *Climacoptera crassa* (*2*, *4*) after 5 s photosynthesis in $^{14}CO_2$ and additional illumination in the usual atmosphere up to 300 s

The data on the study of activity of some enzymes of carbon metabolism additionally support the functioning of C_4 photosynthesis between two chlorenchyma layers (Table 9.6). High activity of PEP and RBP carboxylases is observed in the species studied. The activity of the first enzyme on 1 mg of chlorophyll basis is several times higher in halophytes than in *Zea mays*, whereas the activity of RBP carboxylase is comparable in all the species. The activity of NADP-malic enzyme is completely absent in the leaves of the plant studied. This is in good agreement with the fact that CO_2 is transferred from the outer into the inner chlorenchyma layer by aspartate but not by malate (Table 9.5). In this connection, it is important to note that the activity of transaminating enzymes, aspartate and alanine aminotransferase, in the leaves of halophytes is considerably higher than in *Zea mays* (Table 9.6). So, the kinetics of ^{14}C distribution among the products of photosynthesis, the activity of a number of key enzymes of carbon metabolism, and the structural peculiarities of cells and their organelles considered by us (Bil' and Gedemov 1980; Bil' et al. 1983b) indicate that in the light between the two layers of chlorenchyma in the halophytes studied, there is a metabolic interaction similar to cooperation of mesophyll and sheath C_4 plants of aspartate type.

Earlier, we have found (Bil' et al. 1983b) that the diurnal dynamics of cell sap pH in the leaves of halophytes differs from that which is characteristic for the typical C_4 plants but it is identical to the changes in the acidity of the cell sap in the plants with CAM (Kanai and Edwards 1973; Osmond 1978). On the other hand, the data in Table 9.6 indicate that in the leaves of the plants studied, PEP carboxylase is

Table 9.6 The activity of enzymes in the leaves of desert plants of *Chenopodiaceae* species and *Zea mays*

Species	Concentration		Enzyme activity, μmol of substrate per min							
	Protein (mg/mL)	Chlorophyll (μg/mL)	PEP-C		RBP-C		AsAT		AlAT	
			1[a]	2	1	2	1	2	1	2
Suaeda crassifolia	1.24 ± 0.06	38.00 ± 1.9	1.10 ± 0.06	35.90 ± 1.8	0.25 ± 0.01	8.20 ± 0.41	0.81 ± 0.04	24.30 ± 1.22	0.40 ± 0.02	12.00 ± 0.60
Climacoptera crassa	0.41 ± 0.02	31.20 ± 1.2	–	–	1.20 ± 0.05	15.50 ± 0.62	0.80 ± 0.04	10.70 ± 0.43	1.33 ± 0.05	17.70 ± 0.71
Suaeda arcuata	0.49 ± 0.02	31.20 ± 1.3	2.12 ± 0.11	33.50 ± 1.3	0.87 ± 0.03	13.80 ± 0.65	2.70 ± 0.11	45.00 ± 1.80	2.20 ± 0.09	36.70 ± 1.84
Zea mays	1.06 ± 0.04	190.80 ± 5.7	1.72 ± 0.07	9.60 ± 0.4	1.66 ± 0.07	9.30 ± 0.37	0.69 ± 0.03	3.40 ± 0.14	0.50 ± 0.02	2.25 ± 0.09

[a]Enzyme activity per 1 mg of protein

highly active. There is a question whether these species can assimilate CO_2 during the night at a high rate.

The rates of carbon dioxide utilization by the leaves at various day times have been measured. It turned out that at the beginning and end of night (Table 9.7) the studied species assimilate CO_2 at the rates characteristic for typical CAM plants (Osmond 1978; Tarchevsky and Karpilov 1963). However, in contrast to succulents, which fix CO_2 during the day time at a less rate than in the night (Osmond 1976), the rate of photosynthesis in the experimental species is relatively high. This bifunctionality of halophytes is due to the presence of three types of autotrophic tissues in the leaves: two layers of chlorenchyma being specific to C_4 photosynthesis and chlorophyll-containing WST capable of CO_2 assimilation in the night (Fig. 9.4). A constant size of the opening of the stomata can also contribute to carbon dioxide assimilation at any time of the day, whereas the stomata resistance

Table 9.7 Rate of $^{14}CO_2$ fixation by the leaves of desert plants of Chenopodiaceae at various time of the day in August

	Day time			
	5 a.m.	10 a.m.	3 p.m.	11 p.m.
Species	μmol/1 g of fresh weight per hour			
Suaeda crassifolia	5.4 ± 0.27	128.36 ± 6.42	177.44 ± 8.87	11.9 ± 0.60
Climacoptera crassa	1.9 ± 0.08	47.9 ± 2.87	30.97 ± 1.86	5.7 ± 0.23
Suaeda arcuata	3.0 ± 0.15	33.19 ± 1.66	38.36 ± 1.92	3.6 ± 0.14

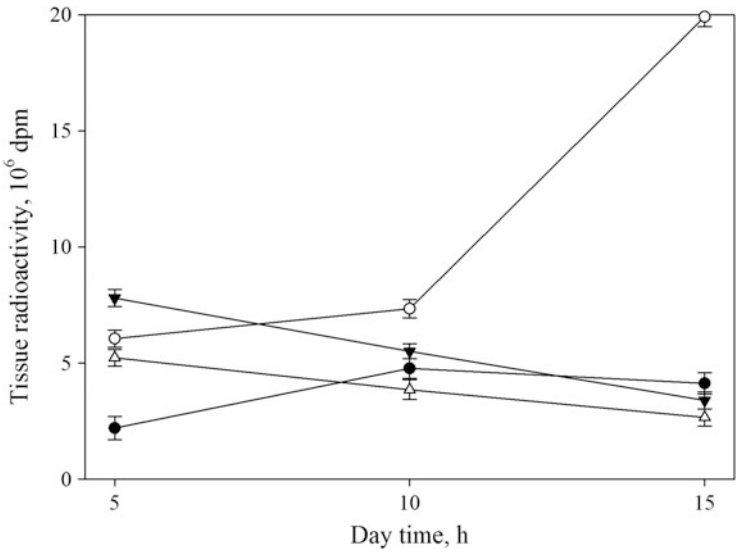

Fig. 9.4 Kinetics of radioactivity of separated tissues of the leaves *Suaeda arcuata* (*circles*) and *Climacoptera crassa* (*triangles*) in the light in the usual atmosphere after 5 h exposure in $^{14}CO_2$ in the darkness. *Closed symbols*, water storage tissue; *opened symbols*, cells of chlorenchyma layers

in the leaves of succulents is dramatically increased in the middle of the day decreasing the rate of photosynthesis (Osmond 1978).

The maximum adaptation of photosynthetic apparatus of halophytes to the extreme conditions of the desert is also proved by the fact that in the afternoon their photosynthetic activity is not changed or is increased (Table 9.7) in contrast to C_4 plant *Amaranthus retroflexus* (growing under the same temperature and insolation conditions but on the irrigated plot) in which photosynthesis is decreased by 50–80 % at 3 p.m.

Since the rate of dark assimilation of CO_2 by the intact leaves cannot help to define localization of the process within the leaf limits, we have attempted to study ^{14}C distribution in the mechanically separated tissues (OLC + ILC and WST) as described above (Fig. 9.4).

In case with $^{14}CO_2$ dark assimilation, in the leaves of *S. arcuata* ^{14}C was mainly localized in the WST, though it could be found in the OLC + ILC fraction. The opposite processes were observed in the leaves of *Cl. crassa*. Further illumination of plants in the usual atmosphere resulted in a simultaneous decrease in radioactivity of tissue fractions in the leaves of *S. arcuata* which was probably due to the efflux of ^{14}C compounds from the leaf and transition of ^{14}C into insoluble substances. It should be noted that in the WST radioactive carbon from alcoholic–water-soluble compounds was more quickly transferred into insoluble ones as compared with the OLC + ILC fraction. It is likely that in this tissue as in CAM plants (Osmond 1978), $^{14}CO_2$ accumulated during the night in the form of C_4 acids is reduced to starch in the light, the amount of which is considerably increased at 3 p.m. in the chloroplasts of the WST (Bil' et al. 1983b).

At first view, the kinetics of radioactivity of tissue fractions in the light obtained on the leaves of *Cl. crassa* (Fig. 9.4) was unclear. During plant illumination in the atmosphere of $^{12}CO_2$, the radioactivity of tissues was not decreased and even did not become stable in the course of time, but it was considerably increased (OLC + ILC—by 3, WST—by 2 times). This dramatic increase of ^{14}C content in the light in the absence of exogenous CO_2 is not likely to be accounted only for by the light-dependent assimilation of radioactive carbon dioxide "weakly" bound during the night exposure. It is most likely that in the leaves of *Cl. crassa* in the dark, $^{14}CO_2$ is assimilated and accumulated largely in the form of an unstable compound, which is destroyed during material fixation. In fact, the kinetics of distribution of radioactivity between the tissues in the light proves it. Figure 9.4 shows that during the first hours of illumination, the amount of ^{14}C is increased in both the fractions, the increase in the WST being more dramatic. Only later stable ^{14}C compound began to flow to chlorenchyma layers resulting in a simultaneous decrease of ^{14}C in the WST and its increase in the OLC + ILC fraction. OAA → malate or OAA → aspartate can be the most likely pair of unstable → stable compound. It is known that OAA in solutions is very quickly subjected to nonenzyme decarboxylation (Karpilov et al. 1978). This acid as well as other α-ketoacids can be stabilized by its condensation with 2,4-dinitrophenylhydrazine (Huber and Edwards 1975; Saltman et al. 1957). Consequently, if one part of the material exposed in $^{14}CO_2$ is fixed by 5 N HCl and the other by 0.5 % solution of DNPH in 5 N HCl, the difference in the radioactivity

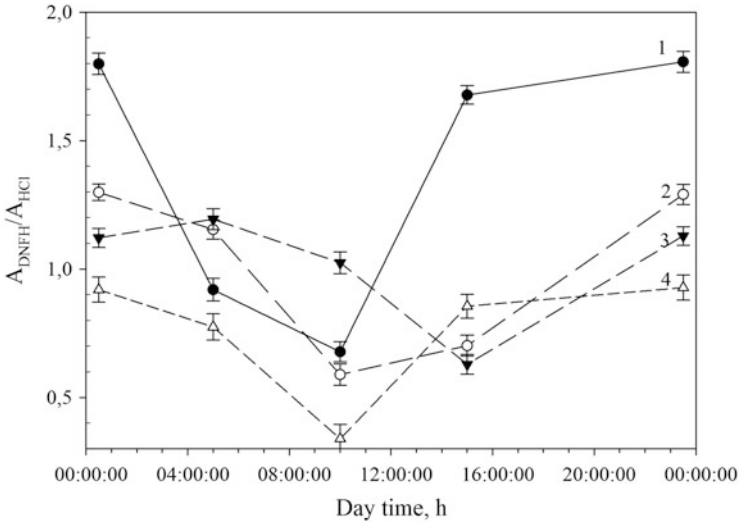

Fig. 9.5 Diurnal dynamics of radioactivity ratio of the leaves fixed in HC1 with and without 2,4-dinitrophenylhydrazine (A_{HCl} and A_{DNPH}, respectively). *S. arcuata* (*1*), *S. crassifolia* (*2*), *Cl. crassa* (*3*) and *A. retroflexus* (*4*)

of the samples obtained can indicate the presence or absence of OAA in the products of $^{14}CO_2$ assimilation.

Really, the ratio of specific radioactivity of the leaves fixed by various stop solutions after their exposure in $^{14}CO_2$ shows (Fig. 9.5) that late in the evening, i.e., in the dark in the leaves of all the species, ^{14}C is assimilated at a greater extent, in the form of oxaloacetate (20–45 % of the soluble fraction). In this case, for *Cl. crassa*, this ratio is the greatest and reaches 1.8. In the early morning hours, considerable accumulation of OAA is observed only in the leaves of *S. arcuata* and *S. crassifolia*.

The two species have maximum rates of OAA accumulation at 11–12 p.m., i.e., soon after the darkness fall. A relative content of OAA in *S. crassifolia* is nearly the same at 5 a.m. and 11 p.m. During the light period, this ratio does not exceed 1 in all the species except *Cl. crassa* in which the ratio A_{DNPH}/A_{HCl} begins to increase in the second part of the day. This unlikeness is accounted for by the peculiarities of the leaf anatomy. In contrast to *S. arcuata* and *S. crassifolia*, the WST in *Cl. crassa* is screened from the atmosphere only by a half-cylinder of chlorenchyma tissues (Bil' et al. 1983b). That is why in the leaves of *Cl. crassa* PEP carboxylation with OAA production is carried out not only in chlorenchyma layers but in the WST as well. In this case, PEP can be formed from PGA of Benson–Calvin cycle.

The experiments described above were carried out in different years at the end of July—beginning of August. At this time, the Kara-Kum Desert has characteristic features of strong continental desert climate: there is no precipitation, a day–night temperature drop is 20–25 °C, whereas that of relative air humidity is 70 % (15 % at

day, 85 % at night). During this time, the conditions of their existence are considerably changed. For instance, in April–May a mean monthly temperature is 18–24 °C, a mean monthly relative air humidity is 34–22 %, and there is 26 % annual precipitation rate. For July–August, the corresponding parameters are equal to 30–32 °C and 15–17 % without any precipitation. The concentration of salts in the surface layer of salt marshes (0–10 cm) is increased from 3 to 5.5 g/100 g of soil in the summer period as compared with the spring one.

During the vegetation, the experimental plants constantly changed the leaves. With the increase of the plant age, the period of leaf ripening defined by the criterion of growth cessation (Gamaley and Kulikov 1978) is decreased. Xeromorphogenesis occurring during the vegetation is the most essential anatomical process. The leaf volume is exponentially decreased from 80 to 10 mm^3 from spring till autumn (Fig. 9.6a). The cell volumes of all the three tissues are considerably reduced (Fig. 9.6b), from August till September, the reduction being more dramatic than that from May till August. At the same time, the number of chloroplasts in the spring–summer period is insignificantly decreased in OLC and ILC, whereas in WST it is not practically changed (Fig. 9.6c). Thus, when more severe conditions of existence come, the cell volume of chloroplast (CVCh) is decreased in all the three tissues, but in the water storage parenchyma this decrease is dramatic, i.e., from 1×10^4 to 3.5×10^3 μm^3. CVCh characterizes the provision of cytoplasm with energy equivalents and metabolites produced by one chloroplast (Mokronosov and Bagautdinova 1974) and the intensity of carbon dioxide flow into the chloroplast. Therefore, a considerable decrease of this parameter in the cells of the WST can be interpreted as a morphological provision of the transfer of this tissue to a more intensive level of metabolic activity.

In fact, the comparison of the activity of $^{14}CO_2$ night assimilation of C_4-CAM halophytes during spring and summer period (Tables 9.7 and 9.8) indicates that this parameter is considerably increased by 1–2 orders of magnitude by the time when extra arid condition appears. It should be noted that the rate of dark CO_2 assimilation of halophytes under these conditions reaches the one characteristic of CAM plants.

The dynamics of the cell sap pH in *Cl. crassa* in May differs considerably from this parameter in July–August (Fig. 9.7). If in the summer period pH changes reach 1.0, in the sap of spring leaves they do not exceed 0.1, besides, the absolute values of the cell sap pH of the first generation are at an average by 1.0 higher than those of the second one. The absence of acidification in the sap of the leaves of the first generation is in accord with a low rate of night CO_2 assimilation.

Such considerable increase of metabolic activity of the water storage parenchyma should result from enzymatic reconstruction of the assimilation apparatus. In *S. arcuata* leaves of spring generation, the ratio of the activity of carboxylating enzymes is close to one as in *Zea mays* (Fig. 9.8). In the leaves of summer generation, this ratio is considerably increased in favor of PEP-C. In accord with the experimental measurements carried out on separated tissues, this increase occurs mainly in WST. During the vegetation period from August till September–October, the number of chloroplasts is dramatically decreased in the cells of the

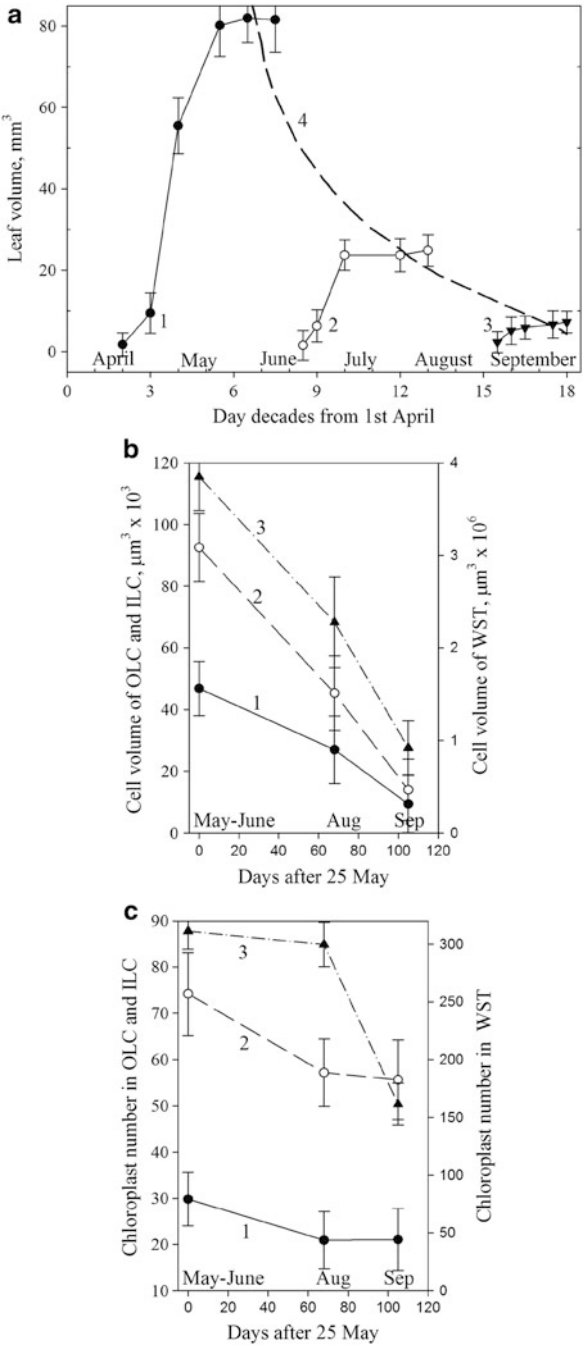

Fig. 9.6 Xeromorphogenesis of desert plants. Ontogenesis (**a**) of *Climacoptera crassa* leaves of the first (*1*), second (*2*), and third (*3*) generations and changes of maximal leaf volume (*4*) during xeromorphogenesis. Cell volumes (**b**) and number of chloroplasts (**c**) per cell in the autotrophic tissues of *Suaeda arcuata* leaves of the first (*1*), second (*2*), and third (*3*) generations. Leaf samples were harvested: (1) 20–25 May, (2) 1–5 August, (3) 3–10 September

Table 9.8 Rate of $^{14}CO_2$ fixation by the leaves of desert plants of *Chenopodiaceae* at various time of the day in May

	Day time			
	5 a.m.	10 a.m.	3 p.m.	11 p.m.
Species	μmol/1 g of fresh weight per hour			
Suaeda crassifolia	0.26 ± 0.01	135.9 ± 6.80	–	0.12 ± 0.006
Climacoptera crassa	0.01 ± 0.005	10.3 ± 0.41	–	0.007 ± 0.0004
Suaeda arcuata	0.02 ± 0.008	12.4 ± 0.62	–	0.007 ± 0.0003

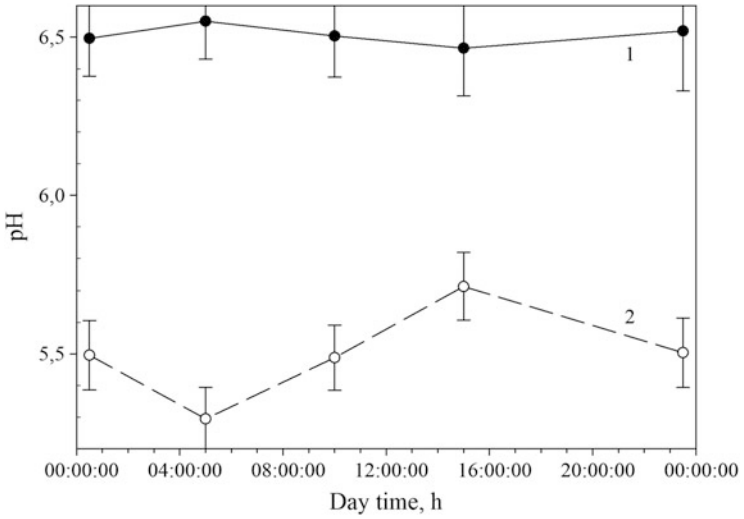

Fig. 9.7 Diurnal dynamics of the cell sap pH in the spring (1) and summer (2) generation leaves of *Climacoptera crassa*

water storage parenchyma (Fig. 9.6b), whereas the content of plastids in OLC and ILC is not changed. Thus, it can be suggested that when the xerothermal period is over, CO_2-assimilating metabolic activity of the WST is considerably decreased, and the plant continues to exist mainly due to C_4 photosynthesis which functions in two chlorenchyma layers.

No notable accumulation of OAA is observed in *Amaranthus retroflexus* leaves at any part of the day (Fig. 9.5). Since two types of tissues in the leaves of this C_4 plant and two chlorenchyma layers in the leaves of desert plants perform CO_2 assimilation by C_4 pathway, the absence of notable accumulation of OAA in *Amaranthus retroflexus* leaves can indirectly indicate that this process is characteristic only for the assimilating apparatus of the WST of the experimental plants. It is still unclear why the ratio A_{DNPH}/A_{HCl} is less than 1 in halophytes in the afternoon and in *Amaranthus retroflexus* all day round.

Thus, the complex of structural and biochemical studies allows to describe at a tissue level a system of the primary CO_2 assimilation which have not been found

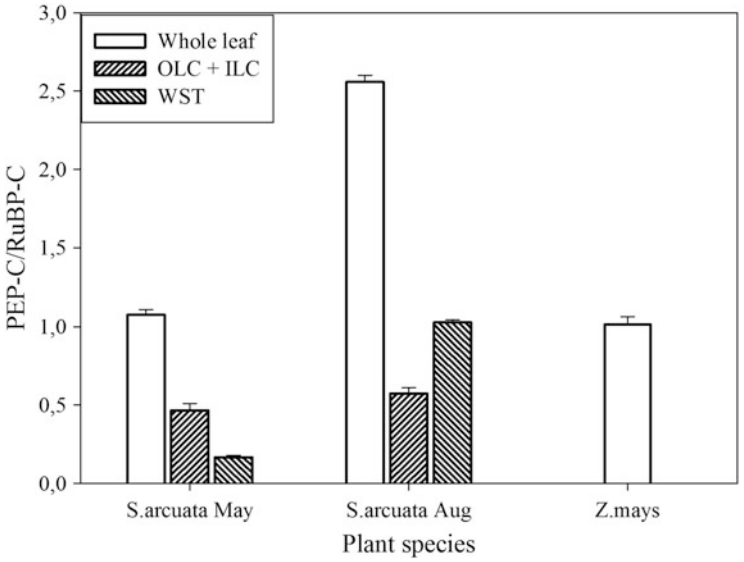

Fig. 9.8 Ratio of the carboxylase activities in the leaves of *S. arcuata* and *Z. mays* and in the separated tissues of *S. arcuata* at different vegetation periods

earlier in other plants. In the light period, C_4 photosynthesis functions in two out of the three autotrophic tissues in the leaves of the experimental plants. The third tissue, namely, the water storage chlorenchyma is not only enriched in water but also performs heterotrophic fixation of carbon dioxide under the decrease of air temperature from 40–45 °C in the day to 18–20 °C at night and the increase of relative humidity content from 15 to 85 %. Phosphoenolpyruvate resulting from glycolysis can serve as a CO_2 acceptor in this case.

However, the role of water storage chlorenchyma in the diurnal dynamics in various species is different and depends on its position in a leaf. In *S. arcuata* and *S. crassifolia*, the WST can assimilate atmospheric carbon dioxide only at night, since it is tightly surrounded by two layers of chlorenchyma cells consuming CO_2 in the light. When the water storage chlorenchyma has a direct contact with the atmosphere as in case with *Cl. crassa*, it can assimilate carbon dioxide in the day time as well. In this case, the primary CO_2 assimilation occurs by means of PEP carboxylase as well as directly in pentose phosphate reducing cycle in chloroplasts of the water storage chlorenchyma. The results given in Table 9.5 indicate that at short light exposures of *Cl. crassa* leaves in the atmosphere of radioactive carbon dioxide, a considerable percent of ^{14}C is incorporated into 3-PGA, PES, and free sugars. This is not characteristic for halophytes, in which WST is completely protected against the atmosphere by chlorenchyma layers. It is also possible that in the light a part of carbon dioxide released in ILC after aspartate decarboxylation can be reduced not only by this tissue but also in Benson–Calvin cycle of water storage chlorenchyma chloroplasts.

9.4 Adaptive Possibilities of Photosynthetic Apparatus of Plants Growing on the Main Landscape Components of the Kara-Kum Desert (Methodological Aspects)

The plants studied grow on the boundary of the southern outskirts of the Kara-Kum Desert and on the plain near Kopetdag mountain. The last one is characterized by severe continental climate. The annual temperature oscillations are 70–80 °C (Nechaeva et al. 1973; Shuravin 1970, and the diurnal difference of temperature and relative humidity according to our observations in July–August are 25–30 °C and 60 %, respectively. The maximum air temperature fixed by us in August 1979 was 49°C, whereas the surface layer of light-colored gray desert soil and sands was heated to 65 and 75 °C, respectively.

The experiments were carried out on the leaves and green segmented shoots of the following edifactors of the desert: (a)*Euphorbia turcomanica* Botsch., *E. cheirolepis* Fisch. Mey ex Zedeb (Euphorbiaceae), *Amaranthus retroflexus* (Amaranthaceae), *Vexibia pachycarpa* Schrenk ex C.A. Mey (Fabaceae), *Agriophyllum latifolium* Fisch. et Mey, *Atriplex tatarica* L., *A. turcomanica* (Mog). Detach., *Chenopodium glaucum* L. (Chenopodiaceae), and *Aeluropus littoralis* (Gowan) Parl. (Gramineae) grown on weakly salinized areas on the boundary of sands and light-colored gray desert soil; (b)*Haloxylon aphyllum* (Minkw) Jljin, *Halocnemum strobilaceum* (Pall.) Bieb, and *Aellenia subaphylla* (C.A. Mey) Aell. (*Chenopodiaceae*) grown on salt marsh of the Central Kara-Kum Desert; and (c)*Bassia hyssopifolia* (Pall.) O. Kuntze, *Climacoptera crassa* (N.B.) Botsch., *C. lanata* (Pall.) Botsch., *Salsola incanescens*(C.A. Mey), *S. dendroides* Pall., *S. australis* R.Br., *Suaeda arcuata* Bunge, *S. acuminata*(C.A. Mey) Mog., *S. crassifolia* Pall., and *Halocharis hispida* (Schrenk) (Chenopodiaceae) from salt marsh on the boundary of the Kopetdag foot plain and the Kara-Kum Desert. Typical CAM plants such as *Bryophyllum tubiflorum* Harvey, *B. daigremontiana* (Crassulaceae), *Stapelia semota* N.E. Brown (Asclepiadaceae), *Aloe arborescens* Mill. (Liliaceae), and *Rhipsalis capilliformis* Web. (Cactaceae) which do not grow in the study area have been cultivated in phytotron chambers under illumination of 400 W m^{-2}, day and night temperatures of 35 and 15 °C, and relative air humidity of 25 and 85 %, respectively.

Anatomo-morphological characteristics of photosynthetic organs were studied by the method described in (Bil' et al. 1983b). Quantitative measurements and their statistic processing were carried out by the methods (Mokronosov and Borzenkova (1978) and Stefanov (1974a, b).

Protein content was assayed in 50 mg of dry matter by using a well-known technique described in (Lowry et al. 1951). Soluble protein was sedimented from water homogenate by 5 % TCAA, whereas insoluble protein fraction after isolation from carbohydrates and lipids by boiling in 0.6 N $HClO_4$ was hydrolyzed for 10 min at 100 °C in 1 N NaOH.

The kinetics of photosynthesis and primary products of $^{14}CO_2$ assimilation was studied by the complex of techniques described in (Bil' et al. 1981).

Three–five plots with an area of 4 m^2 were selected at random to determine the density of plants having different functional and morphological characteristics and growing on each soil type under study. After counting, the number of plants of a certain type was averaged for the plots and was given on 1 m^2 basis. Since in all the cases, a mean square root error of the averaged values did not exceed ± 0.02; whole numbers are used in figures.

Among the plants studied in the present work, there is a great variety of anatomic structure of assimilating organs.

The leaves of the majority of species collected in the boundary zone of sands and light-colored gray desert soil contain one–two layers of column and one layer of spongy chlorenchyma round conducting sheaths. The plants with the leaves having several layers of column tissue only are more seldom used. In the same area, there is a great variety of species in the leaves of which assimilating tissues are differentiated in mesophyll and parenchyma bundle sheath cells. It should be noted that the plants numerated above belong to a relatively large circle of such families as Euphorbiaceae, Amaranthaceae, Gramineae, Chenopodiaceae, and Fabaceae.

When the light-colored gray desert soils are changed into the Kara-Kum Desert salt marsh and salt marsh at the boundary of the Kopetdag foot plain, the plants with a new type of anatomy structure of cylindrical assimilating organs are more often met (a quantitative characteristic of this phenomenon will be given below). Under the epidermis of these organs (real leaves or segmented green shoots), there are two layers of autotrophic cells which surround WST and differ in the form and packing density. Slight variations characterize this type of leaf anatomy. For instance, in some species both concentric cell layers can be open on the adaxial leaf side (*Cl. crassa*) or as in *Bassia hyssopifolia*, the outer and inner chlorenchyma layers form a semicircle round the conducting sheaths immersed into the WST. The species in the shoots of which the chlorenchyma round the water storage tissue has a form of fence-like cells are more seldom met. Among the plants with the anatomy of succulent leaves *Chenopodiaceae* species prevail.

It is known that a certain type of photosynthetic carbon metabolism corresponds to a certain leaf anatomy. For instance, C_3 plants are characterized by the leaves with column and spongy tissues or homogeneous mesophyll. C_4 photosynthesis is always provided by the presence of two types of autotrophic tissues, namely, by mesophyll and BSC (Karpilov 1974) or two concentric cell layers (Bil' et al. 1983b).

In the leaves with column and spongy chlorenchyma—*Chenopodium glaucum*, *Agriophyllum latifolium*, *Euphorbia cheirolepis*, and *Vexibia pachycarpa*—the transformation kinetics of ^{14}C compounds is typical for C_3 plants, i.e., carbon for 1 min after exposure in the atmosphere with $^{14}CO_2$ is transformed from phosphorus compounds into free sugars. The content of ^{14}C in C_4 acids is slightly changed. In the leaves of *Atriplex turcomanica*, *A. tatarica*, and *Euphorbia turcomanica* as it should be expected, the label in the first 15 s of photosynthesis is accumulated in C_4-dicarbonic acids and then via PES is transferred to free sugars at the end of the first min. Consequently, these plants both by their anatomic structure and photosynthetic carbon metabolism belong to typical C_4 plants (Welkia and Caldwell 1970).

Succulent shoots and leaves in which WST is surrounded by layers of column chlorenchyma (as in *Halocnemum strobilaceum*) as well as typical C_3 plants at first fix carbon from the atmosphere with the formation of PGA. Then the label is transferred to free sugars. Special attention should be paid to a very low level of C_4 acids and rather high content of ^{14}C in photorespiratory metabolites.

Finally, the succulent leaves with the WST surrounded by two layers of differentiated tissues (e.g., *Aellenia subaphylla*, *Climacoptera crassa*, *Bassia hyssopifolia*) have typical kinetics of C_4 photosynthesis.

Microscopic studies have revealed that in a number of plants of *Chenopodiaceae* family the WST occupies more than half of a leaf. In many species this tissue presents an aggregate of tightly packed vacuolated cells containing no chloroplasts. But as it has been found in some species, the plastid content in the WST reaches 30–50 % of their total amount in a leaf. Naturally, there appears a question on the function of these chloroplasts screened from atmospheric CO_2 by two dense layers of actively assimilating cells. According to our results (see above), chloroplasts of WST fix CO_2 at night forming a closed cycle of diurnal assimilation.

Besides, a comparative anatomical analysis of annual halophytes and typical CAM plants shows that WST in the first ones and assimilating tissue in the second are formed by homogeneous cells which volumes considerably exceed those of halophyte autotrophic tissues and have the same order of magnitude though with great variability (430–3,187 μm^3) (Table 9.9). It is important that the plastid number per cell of WST in halophytes is at an average 1.5 times greater than in the cells of CAM plants.

Thus, the majority of plants studied can be divided into five distinct functionally morphological types: (1) C_3 xerophytes (fence-like + spongy chlorenchyma), (2) C_4 xerophytes (mesophyll + BSC), (3) C_3 succulents (fence-like cells round WST), (4) C_4 succulents (two types of chlorenchyma tissues round WST), and (5)C_4-CAM succulents (the same but with chloroplasts in WST).

Figure 9.9 shows plant growth density of soil cover of this or that of functionally morphological type depending on salinity degree. C_3 and C_4 xerophytes prevail on weakly saline soils, though C_4 and C_4-CAM succulents also grow there. With the enhancement of salinity, the density of C_4-CAM and C_4 succulents is dramatically increased, whereas xerophyte types are seldom met. And only on salt marsh small amounts of C_3 succulents appear, but C_4 and C_4-CAM succulents prevail.

C_4-CAM plants contain approximately the same protein amount per 1 g of dry leaf weight as the other metabolic types of succulents growing on salt marsh. At the same time in xerophyte plants, this parameter is 1.7–2.0 higher. However, the dry weight of the aboveground part of an annual halophyte exceeds that of the species vegetating during winter–spring and spring–summer periods by 25–30 times (Nechaeva et al. 1973). Taking into account that various plant types grow on the soils studied, it can be concluded that C4-CAM succulents produce protein 20 and 3–4 times as much as C_3 xerophytes and C_4 succulents, respectively, per salt marsh area unit.

Table 9.9 Cytomorphometry of water storage and assimilation tissues in photosynthetic organs of some species of Chenopodiaceae family and CAM plants

Species	Cell volume, 10^3 μm^3		Chloroplast number in a cell	
	WST	Mesophyll	WST	Mesophyll
C_4-CAM				
Climacoptera crassa	3,187	–	279	–
Halocharis hispida	679	–	219	–
Suaeda arcuata	1,533	–	301	–
Salsola incanescens	2,303	–	221	–
CAM				
Rhipsalis capilliformis	–	430	–	92
Bryophyllum daigremontiana	–	2,834	–	198
Bryophyllum tubiflorum	–	2,467	–	131
Stapelia semota	–	2,378	–	128
Aloe arborescens	–	1,359	–	130

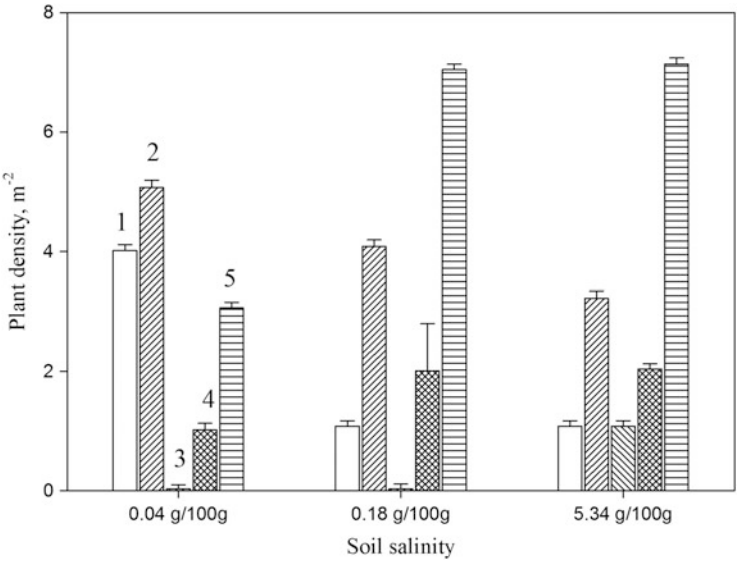

Fig. 9.9 Growth density of plants with various functional–morphological characteristics on soils with different salinity. (*1*) C_3 xerophytes, (*2*) C_4 xerophytes, (*3*) C_3 succulents, (*4*) C_4 succulents, (*5*) C_4-CAM succulents

Thus, the experiments with plants in various ecological areas which differ mainly in soil salinity have revealed that C_4 succulents and especially C_4-CAM succulents assimilating CO_2 24 h a day have the highest adaptability to salinity. Probably, the increased salt resistance of these plants is due to their two main properties. Firstly, CO_2 fixation in cells of outer layer and WST via PEP carboxylase reaction is not inhibited by salts (Kuramoto and Brest 1979). Secondly, WST

separating autotrophic tissues from conducting bundles can play the role of buffer capacity for the salts getting into assimilating organs via xylem (Storey and Jones 1979).

9.5 Conclusion

Tolerance of plants in relation to adverse damaging environment is based on the energy, metabolic, and genetic mechanisms. There are two kinds of tolerance. One of them is quickly emerging. In response to the many adverse effects—the drought, heavy metals, ultraviolet light, and heat (Qiaoa et al. 2014; Kreslavski et al. 2009). Already in tens of minutes or 2–3 h, the activation of synthesis of enzymes and other components of cells necessary for protection from stress occurs. The purpose of such quick changes is to restore the homeostasis of cells, tissues, and the organism as a whole.

The other type of sustainability of higher plants to adverse environment is evolutionarily acquired. Very stress sensitive is the process of photosynthesis. Depending on the environment, the predominance of plants with one or another type of organization of this process is seen. There are a lot of types of leaf structure (Edwards et al. 2001; Pyankov et al. 1998), energy reactions distribution, and enzyme composition (Voznesenskaja et al. 1999).

In our chapters, we have given experimental and theoretical explanations of existence of plants under the most unfavorable conditions. In the South-East Kara-Kum Desert and foot plain of Kopetdag in July–August, there are simultaneously a few strong environmental features: drought, high temperature, insolation, and soil salinity. In these conditions, the most population of annual plants is C_4-CAM succulents with three types of photoautotrophic tissues in leaves.

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Chapter 10
Salicylic Acid and Drought Stress Response: Biochemical to Molecular Crosstalk

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Abstract Salicylic acid, a naturally occurring phenolic compound, is a multifaceted plant growth modulator and activates the systemic acquired defence in plants as a response to pathogen effect. In recent years in addition to the activation of SAR, SA is reported to play a major role in the modulation of plant responses to biotic and abiotic stresses like drought, salinity, heat, heavy metal stress, osmotic stress, defence against pathogenic elicitors and effectors and symbiotic relationships. Additionally, SA has well laid out physiological roles in growth and development of plants. Several of the targets of SA have been recognized, and the molecular mode of action elucidating the complex signal transduction and involving crosstalk of multiple metabolic pathways is being unravelled. This chapter deals with recent findings on the improvement of drought tolerance vis-à-vis salicylic acid-induced modulation of metabolic pathways and signalling mechanisms.

Keywords Salicylic acid • Drought stress • Reactive oxygen species • Antioxidant enzymes

10.1 Introduction

Plants are constantly exposed to various kinds of biotic and abiotic stresses. Stress is an adverse force or condition which prohibits the normal performance of living organisms (Jones and Jones 1989). While biotic stresses in plants is caused by living organisms such as virus, bacteria and fungi, abiotic stresses occur due to non-living surroundings and environment such as drought, exposure to ultraviolet light, salt, ozone, heat, cold, oxidative stress and heavy metal toxicity. Among the various environmental stresses that adversely affect crop productivity and yield, drought stress is the most severe and widespread (Hasanuzzaman et al. 2013).

Drought is a brief deviation, frequent character of climate defined as an extended period—a season, a year or more of deficient rainfall. Drought has significant and

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harmful impact on land, water, vegetation quality, wildlife environments, agronomy and ecosystem especially for arid and semi-arid areas where high evapotranspiration rates expose plants to further adaptive pressure. Plant growth, yield, membrane integrity, pigment content, osmotic adjustment, photosynthetic activity, etc. are affected by drought stress. Several defences and signalling mechanisms are adapted by plants to protect themselves from drought, but these can only ensure survival to a certain extent and are not enough to maintain productivity under drought stress.

The role of abscisic acid (ABA) has been extensively studied in drought response, although several reports indicate an ABA-independent pathway involving an array of plant growth regulators and a crosstalk involving several stress response pathways (Valliyodan and Nguyen 2006; Huang et al. 2008; Nakashima et al. 2009). Increasing evidences indicate that one of the most potent regulators of such a crosstalk is salicylic acid (SA), a phenolic compound and a key component of defence signal transduction, inducing cascade of genes involved in systemic acquired resistance (SAR). SA is present in the plant system generally in a free state or in the form of glycosylated, methylated, glucose-ester or amino acid conjugates (Lee et al. 1995). SA signalling is known to be activated in several biotic and abiotic stresses, viz. heat stress (Dat et al. 1998), chilling damage (Kang and Saltveit 2001), heavy metal stress (Metwally et al. 2003), drought stress (Bezrukova et al. 2001) and biotic stress (Kundu et al. 2011, 2012, 2013a). Proteomic study of *V. mungo* plants has revealed an array of interacting metabolic pathways that are involved in conferring tolerance to MYMIV (Mungbean Yellow Mosaic India Virus) and most importantly indicate presence of extensive crosstalk between biotic and abiotic stress responses (Kundu et al. 2011, 2013b).

'Salicylic acid' (SA), ubiquitously distributed in the plant kingdom (Raskin et al. 1990), derives its name from the word 'Salix', meaning willow tree in Latin. The Greeks have been using the leaves and bark of willow trees as painkiller and antipyretic since ancient times. Salicyl alcohol glucoside (salicin) was isolated from willow bark by Johann Andreas Buchner (1828) and later on converted to salicylic acid (SA) by Raffaele Piria. Acetyl salicylic acid, popular as a drug known as aspirin, is among the best known 'antistress compounds' (Raskin 1992a). Recent findings indicate SA to be an 'effective therapeutic agent' for plants as well. SA has diverse regulatory roles in plant metabolism and is involved in crosstalk between several pathways related mostly to biotic and abiotic stress response and a myriad of interaction related to growth and development, a property which places it at par with plant growth regulators (Raskin 1992b; Popova et al. 1997; Vicente and Plasencia 2011).

SA is reported to play a major role in the modulation of plant responses to biotic and abiotic stresses like drought, salinity, heat, heavy metal stress, osmotic stress, defence against pathogenic elicitors and effectors and symbiotic relationships. However, SA is more than just a stress response in plants as it has well laid out physiological roles in growth and development of plants starting with but not limited to seed germination, vegetative growth, photosynthesis, respiration, stomatal response and transpiration, thermogenesis, floral meristem initiation,

senescence, etc. (Durner and Klessig 1996; Yan and Dong 2014). The biochemical and molecular actions modulated by SA are still being elucidated with more research focused on the modulation of antioxidant enzymes with implications on maintaining 'cellular redox homeostasis', induction of alternative respiratory pathway (Moore et al. 2002) and regulating gene expression through RNA-dependent RNA polymerase with major impact on post-transcriptional gene silencing (Xie et al. 2001).

Therefore, a comprehensive review may help to understand drought sensitivity which would help improve drought tolerance vis-à-vis salicylic acid-induced modulation of metabolic pathways and signalling mechanisms.

10.2 Plant Response to Drought Stress

10.2.1 Morphological and Physiological Responses

The susceptibility of any plant to drought mainly depends on the degree of stress, different accompanying stress factors, plant species and their developmental stages (Demirevska et al. 2009). Drought stress in comparison to other stresses widely affects agricultural crops and finally reduces fresh and dry biomass (Zhao et al. 2006; Farooq et al. 2009; Shao et al. 2009). Physiological and biochemical processes of plants such as photosynthesis, respiration, translocation, ion uptake and nutrient metabolism are affected by drought (Jaleel et al. 2008a, b, c, d, e, f; Farooq et al. 2008). Decreased water content, diminished leaf water potential, turgor loss, closure of stomata and decreased cell enlargement and growth (Hussain et al. 2008) are the main characters of drought-affected plants. Severe water stress may lead to plant death (Jaleel et al. 2008d). Reduced productivity is reported in several plants, viz. water-stressed soya bean (Specht et al. 2001), *Poncirus trifoliata* seedlings (Wu et al. 2008), common bean, green gram (Webber et al. 2006) and *Petroselinum cripsum* (Petropoulos et al. 2008). The first and most important effect of drought is impaired germination and poor stand establishment (Harris et al. 2002). Decreased water content affects elongation and expansion of plants (Anjum et al. 2003a; Bhatt and Srinivasa Rao 2005; Kusaka et al. 2005; Shao et al. 2008). It is an important limiting factor at the initial phase of plant growth and establishment. Some crops are more susceptible to drought in comparison to others such as rice (a submerged crop). Decreased stem length is observed in soya bean under drought stress (Specht et al. 2001). This occurs due to decline in the cell enlargement and leaf senescence (Bhatt and Srinivasa Rao 2005). Optimal leaf area is very important to increase photosynthesis and yield. The effect of drought on plants is the reduced leaf growth and size in many species like in *Populus* (Wullschleger et al. 2005) and in soya bean (Zhang et al. 2004). In comparison to root growth, leaf growth is more sensitive to drought. Reduction in leaf area causes decreased transpiration and supports the plant to tolerate drought stress. Similarly,

leaf area adjustment is observed by increased senescence and abscission of the older leaves under drought stress.

Relative root growth may undergo enhancement under drought stress and supports plant growth by extracting water from deeper soil layers. A prolific root system accelerates plant growth during the early crop growth stage by extracting the water from shallow soil layers which is easily lost by evaporation (Johansen et al. 1992). No significant reduction is observed in root growth of certain crop plants like maize and wheat under drought stress condition (Tahir et al. 2002). Increased root growth is reported in sunflower and in *Catharanthus roseus* under drought stress (Sacks et al. 1997; Tahir et al. 2002; Jaleel et al. 2008a). Generally the ratio of root and shoot increases during drought stress because roots are less sensitive than shoots to growth inhibition by low water potentials (Wu and Cosgrove 2000). It is reported that cell size is reduced under drought stress such as in cassava plants and in common beans (Alves and Setter 2004; Martínez et al. 2007). Drought stress at bud initiation stage in comparison to seed-filling stage is more harmful for plants like sunflower which finally reduces yield (Prabhudeva et al. 1998). In accordance, drought reduces seed yield in soya bean when compared to control plants (Specht et al. 2001).

Generally, the first response of all plants to drought is the closure of stomata which prevents further water loss due to decrease in transpiration (MansWeld and Atkinson 1990). There are two processes metabolic dependent and metabolic independent for the closure of stomata. When stomata close due to direct evaporation of water from guard cells without any metabolic involvement, this is called hydro-passive closure. On the other hand, hydro-active closure of stomata is metabolically dependent (Lake et al. 2002) and requires ions and metabolites. ABA is an important regulator of this process. ABA as well as cytokinin and ethylene are involved in root–shoot signalling.

10.2.2 Biochemical Responses

Generally decreased content of chlorophyll *a*, *b* and carotenoids are observed in plants during drought stress (Anjum et al. 2003b; Farooq et al. 2009) such as in cotton and in *Catharanthus roseus* (Massacci et al. 2008). Oxidative stress decreases photosynthesis activity due to stomatal or non-stomatal mechanisms (Ahmadi 1998; Del Blanco et al. 2000; Samarah et al. 2009). When leaves are subjected to drought, leaves exhibit large reduction in relative water content and water potential (Nayyar and Gupta 2006). In severe drought stress, decreased Rubisco activity is observed that ultimately results in limited photosynthesis (Bota et al. 2004). Studies suggest that decrease in photosynthetic activity primarily occurs due to CO_2 deficiency. Reduced intracellular CO_2 level accelerates overproduction of components within the electron transport chain, and electron gets transferred to oxygen at photo-system I which ultimately leads to the generation of reactive oxygen species or ROS.

ROS is generated in plant cells during normal metabolic processes, but in most plants its production increases due to disruption of metabolic processes by different abiotic stress conditions such as drought (Farooq et al. 2009), salinity, flooding, heat and cold (Mittler 2002). Organelles with a highly oxidizing metabolic activity such as chloroplast, mitochondria or microbodies are sites of ROS production (Mittler et al. 2004; Foyer and Noctor 2003). ROS is mainly divided into two classes: free radical forms (O^{-}_2, $OH^{\cdot}$, OH_2) and non-radical forms (H_2O_2, etc.). It plays dual role in plant systems as an important signal transduction molecule and as toxic by-product of aerobic metabolism that accumulate in cells during different stress conditions.

Because ROS are detrimental to plants, there is an elaborate and efficient network of scavenging mechanisms to overcome the effect of ROS that use some of these toxic molecules as signal transduction mediators (Mittler et al. 2004; Bailey-Serres and Mittler 2006). ROS gene network is a large network of genes that balance between ROS production and scavenging and includes more than 152 genes in *Arabidopsis* (Mittler et al. 2004).

10.2.3 Tolerance and Avoidance of Drought Stress

Different types of physiological and biochemical responses are exhibited by plants at cellular and organism level to alleviate the effect of drought. Some important mechanisms are drought escape, drought avoidance and drought tolerance (Turner 1986). In drought escape, under drought stress plants show dormancy and when the conditions are favourable they complete their life/reproductive cycle. In drought avoidance, plants show their ability to establish themselves in period of significant rainfall. Drought tolerance is achieved by turgor maintenance and dehydration tolerance (Turner 1986). It is achieved either by avoiding water loss through stomatal closure (Jones 1998) and leaf drop (Turner 1986) or by increasing water uptake through enhanced root growth (Turner 1986; Subbarao et al. 1995). Plants try to restrict damages caused by drought by initiating a signal transduction that encompasses the following parameters:

- Restores the osmotic as well as ionic equilibrium of the cell which maintain cellular homeostasis under different stress conditions
- Control and repair of stress damage by detoxification signalling
- Provides signal for cell division to maintain the demands of the plant under stress conditions (Liu and Zhu 1998)

Glycine betaine, proline, other amino acids, organic acids and polyols are different types of low molecular weight osmolytes necessary for plants to maintain drought stress. Salicylic acid, auxins, gibberellins, cytokinin and ABA also act as antioxidant substances and signalling compounds.

The antioxidant defence system of plants consists of enzymatic and non-enzymatic molecules (Apel and Hirt 2004). This system controls the oxidative

damages caused by the excess amount of ROS and plays an important role in destroying active oxygen species (Foyer and Noctor 2003). As the production of ROS increases during drought stress, response of antioxidative defence system is also increased simultaneously (Gressel and Salun 1994). Non-enzymatic antioxidative defence system includes lipid-soluble membrane-associated antioxidants (e.g. α-tocopherol and β-carotene) and water-soluble reductants (e.g. glutathione, ascorbate and phenolics). Superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), catalase (CAT) and peroxidase (POX) are enzymes of antioxidative systems. These are capable of scavenging H_2O_2, neutralizing free radicals and oxy-intermediates (Sang et al. 2007). Since plants have limited mechanisms of stress avoidance, they require flexible means of adaptation to changing environmental conditions.

10.3 Drought Stress and Salicylic Acid

10.3.1 Salicylic Acid: Diverse Roles in Plants

10.3.1.1 Origin and Biosynthesis

It is well documented that phenolic compounds exert their influence on physiological and biochemical processes including photosynthesis, ion uptake, membrane permeability, enzyme activities, flowering, heat production and growth and development of plants. Among the phenolic compounds, the role of salicylic acid and its analogues such as acetylsalicylic acid (ASA) and gentisic acid (GTA) are increasingly being evident as plant growth regulator to increase tolerance to biotic and abiotic stresses. Salicylic acid (SA) is a naturally occurring phenolic compound. While being a moderately water-soluble crystalline powder, SA is highly soluble in polar organic solvents and has a melting point of 157–159 °C with pH, pK and Kow values being 2.4, 2.98 and 2.26, respectively, in aqueous solution (Raskin 1992a).

Salicylic acid is an endogenous growth regulator and also exhibits antioxidative characteristics and is established to have a major role in SAR in plants under biotic stress (Durrant and Dong 2004). The biosynthesis of salicylic acid in plants starts from shikimate–phenylpropenoid pathway (El-Basyouni et al. 1964) involving the PAL (phenylalanine ammonia lyase) enzyme that converts phenylalanine into trans-cinnamic acid (tCA), the gateway from primary to secondary metabolism. This tCA is either hydroxylated to o-coumaric acid before oxidation of the side chain to form salicylic acid or the tCA side chain is shortened to benzoic acid (BA) which is finally hydroxylated into SA (Sticher et al. 1997). SA is present in plants either in a free state or in the form of glycosylated, methylated, glucose-ester or amino acid conjugates (Lee et al. 1995).

10.3.1.2 Role of SA in Drought Stress Tolerance

Involvement of salicylic acid in plant defence mechanism was first determined by injecting SA into tobacco leaves. It increased PR protein accumulation and enhanced resistance towards TMV in tobacco leaves. Studies suggested that exogenous application of salicylic acid on plants increases the plant tolerance to several abiotic stresses such as osmotic stress (Wang et al. 2010), drought stress (Azooz and Youssef 2010) and heavy metal stress (Moussa and EI-Gamel 2010). Seed germination, stomatal closure, ion uptake and transport, membrane permeability, fruit maturity and flowering, etc. are also influenced by salicylic acid (Aftab et al. 2010 ; Vicente and Plasencia 2011). It increases yield by increasing the pod number in mung bean (Singh and Kaur 1980). Enhanced potato productivity is also reported (Koda et al. 1992). It is observed that genes that are required for drought tolerance have higher response in the presence of salicylic acid (Chini et al. 2004). Depending upon the plant species, the concentration of salicylic acid varies, such as in rice, crab grass, green foxtail, barley and soya bean, and the level of salicylic acid is about 1 μg g^{-1} fresh weight. The level of salicylic acid is largest at flowering in thermogenic flowers and after pathogenic infection (Raskin et al. 1989; Raskin 1992a, b).

Although the role of SA in plant pathogen relationship is broadly known and extensively investigated (Loake and Grant 2007; Kundu et al. 2011, 2012), its contribution to alleviate plants from drought stress is still relatively unexplored. The cascade of signals initiated on pathogenic stress involving SA is of prime importance to the SAR (Volt et al. 2009), and SA may induce similar signalling response in plants under drought stress.

Both endogenous and exogenous SA have been shown to have various effects on plants (Raskin 1992a, b), but this is not always beneficial. Lower concentration of salicylic acid is generally beneficial, but the higher concentration of salicylic acid is either inhibitory or of no additional benefit. Application of SA and other phenolic compounds influence the uptake of numerous ions. In barley, phosphate (Glass 1973) and potassium ion (Glass 1974) uptake are inhibited by SA. It is experimentally proved that treatment of bean leaves with 10 mM SA decreases the transpiration rate (Larque-Saavedra1978, 1979). If salicylic acid is applied for a long period, it reduces the quantity of the Rubisco enzyme that finally inhibits the photosynthetic activity in barley (Pancheva and Popova 1998). In an experiment wheat plants were treated with salicylic acid for 7 days in which low (0.05 mM) concentration of SA promoted photosynthesis while higher (0.5–1.0 mM) inhibited it. The inhibitory effect of paraquat on photosynthesis is reduced by the application of 0.5 mM SA for 1 day in the dark. Reduction in the H_2O_2 production, lipid peroxidation and membrane damage are also observed at this concentration (Ananieva et al. 2002). Higher concentration of SA also increases the level of stress. On soaking of wheat seeds in acetyl SA, the plants show better resistance to drought stress (Hamada 1998; Hamada and Al-Hakimi2001). In accordance, soaking of seeds in 100 ppm acetyl SA for 6 h, before sowing, not only provides

tolerance to drought but also increases dry matter and transpiration rate. Application of SA is able to stimulate the adventitious root primordia of bean plants (Kling and Meyer 1983). SA and acetyl SA at concentrations of 0.1 and 0.5 mM protect plants against drought stress (Senaratna et al. 2000). Thus, the effect of SA is influenced by concentration and by the developmental stage of the plant. The level of salicylic acid is decreased under the recovery period, but it is higher than that observed before drought. Thus, it may be possible that endogenous SA induces the protective mechanism under drought stress. SA stimulates the activity of Cu and Zn SOD and finally increases the level of H_2O_2 (Rao et al. 1997; Azevedo et al. 2004). Higher H_2O_2 level and lipid peroxidation are involved in the signal transduction process and provide SA-dependent resistance (Anderson et al. 1998). In barley plants, water deficit increased the SA content in the roots, whereas the SA content in the leaves did not change (Bandurska and Stroinski 2005). The probabilities of higher damaging effects are reduced if plants are pretreated with physiologically active concentrations of SA. Salicylic acid plays important role in induction of resistance against both biotic and abiotic stresses. The role of SA in the signal transduction processes of biotic stress tolerance has been widely studied. It is involved in the development of the hypersensitive reaction (HR): in tobacco leaves infected with tobacco mosaic virus and there is an increase in the level of endogenous SA in the necrotic lesion and surrounding tissues (Enyedi et al. 1992). The external application of SA induces the expression of pathogenesis-related (PR) proteins in tobacco (Malamy et al. 1990; Yalpani et al. 1991) and in rice (Rakwal et al. 2001). A large body of evidences indicate that SA is also required for the development of SAR. The level of endogenous SA increased in cucumber plants during development of acquired resistance (Métraux et al. 1990).

10.3.2 Mode of Action

10.3.2.1 Antioxidant Mechanism

SA affects the activity of antioxidant enzymes and increases the level of H_2O_2 (Klessig et al. 2000; Ganesan and Thomas 2001). Salicylic acid has the ability to bind and inhibit the activity of catalase (Chen et al. 1993a, b). If SA is applied for a long period, the activity of APX and catalase is reduced in *Arabidopsis* and leading to cell death (Rao et al. 1997). Similar results are observed in *Astragalus adsurgens* callus culture (Luo et al. 2001). SA-mediated catalase inhibition is studied in *Arabidopsis*, tomato and cucumber (Sánchez-Casas and Klessig 1994; Horváth et al. 2002). SABP (salicylic acid-binding protein) is a catalase protein found in tobacco. It is the first protein shown to reversibly bind (Kd 14/~M) with salicylic acid. The SABP is a 240–280 kDa complex which appears to be composed of four 57 kDa subunits. SA is found to block SABP's catalase activity; it also inhibited the activity of catalases in crude tobacco leaf extracts. Functional analogues of salicylic acid, i.e. 2,6-dichloroisonicotinic acid (INA), benzothiadiazole (BTH), also inhibit

the activity of catalase (Chen et al. 1993a, b; Conrath et al. 1995; Wendehenne et al. 1998). Generation of salicylic acid free radicals also inhibit the activity of catalase and ascorbate peroxidise. For the inhibition of catalase activity, SA donates its electron (Durner and Klessig 1996) and initiates lipid peroxidation such as in tobacco suspension cells (Anderson et al. 1998). It is observed that ascorbate peroxidase was inhibited by SA analogues while inactive analogues of salicylic acid did not (Wendehenne et al. 1998; Durner and Klessig 1996). The increased level of H_2O_2 induced by SA may facilitate development of SAR (Kundu et al. 2011) and protect against abiotic stresses (Chen et al. 1993a).

Induced resistance by SA-mediated catalase inhibition is still unclear because binding between catalase and SA is not specific. It is reported that aconitase, an iron containing protein, also binds with SA (Rüffer et al. 1995). It is also observed that SA-mediated catalase inhibition is not common to all plants. SA-mediated catalase inhibition is studied in tobacco (Durner and Klessig 1996), but the same results are not found in maize and rice (Sánchez-Casas and Klessig 1994). Further studies in maize and rice showed differences between the catalase isoenzymes in their sensitivity to SA. In maize plants treated with 2mM SA; the activity of CAT1 isoenzyme is inhibited, but in case of CAT2 the inhibition is competitive and weak (Horváth et al. 2002). When inhibitory effect of salicylic acid is studied in rice, it is found that CATb isoenzyme is inhibited while CATa is not inhibited (Chen et al. 1997). It is observed that CAT1 isoenzyme of maize and the CATb isoenzyme of rice showed sequence homology with tobacco catalase and sensitive to SA.

Although the role of SA in increasing drought tolerance is still debatable and is broadly concentration dependent, whereby lower concentrations are favourable, but higher concentrations injurious, it is interesting to note that SA treatment also increases ABA content in barley leaves (Bandurska and Stroinski 2005). It remains to be seen whether SA-induced ABA production may be implicated in increased drought tolerance.

Recent proteomic studies indicate differential regulation of a wide range of proteins involved in varied metabolic pathways on SA pretreatment, many of which are linked to plant defence pathways. There is underlying similarity and uniqueness in the proteomic responses of plants treated with SA under both pathogenic and drought stress (Kundu et al. 2011; Kang et al. 2012).

10.3.2.2 Salicylic Acid-Induced Gene Expression and Its Implications in Drought Tolerance

Several reports indicate involvement of candidate genes in drought tolerance. A majority of drought responses are thought to be triggered by the production of ABA which induces H_2O_2 and nitric oxide (NO) activating MAPK cascades resulting in up-regulation of several downstream products especially antioxidant enzymes (Hao et al. 2008; Xing et al. 2008). However, recent evidences indicate a definite role of

Table 10.1 Genes modulated on SA treatment specifically on drought stress

S. No.	Gene/ genetic element	Features	Induced (+)/repressed (−)	Reference
1	*BcDh2*	Dehydrin-like gene	(+) Salinity, heat shock, drought, SA, Me jasmonate, ABA	Shen et al. (2004)
			(−) low temperature stress	
2	*BnBDC1*	Encodes a protein containing the BURP domain	(+) Drought, mannitol, NaCl, ABA)	Shunwu et al. (2004)
			(−) UV irradiation, SA	
3	*BoWS*	95 Amino acid protein	(+) Salt stress, drought, ABA, mannitol, SA, H_2O_2	Li et al. (2004)
4	*CPRD46*	Reported in dehydrated cowpea	(+) High salinity, drought and heat stress, ABA, methyl JA, SA	Iuchi et al. (1996)
5	*OsGGT*	Defensive response to various environmental stresses	(+) Salt, drought. SA, benzyl adenine, ethylene, gibberellins, ABA	Qi et al. (2005)
6	*Ppsrk11*	A serine/threonine kinase receptor-like protein kinase gene	(+) Drought, SA	Bassett et al. (2005)
			(−) Light	
7	*PvSR1*	Maintains cellular integrity during stress conditions	(+) Alfalfa mosaic virus infection, wounding, heat shock, UV, drought, salt stress, SA, H_2O_2	Chai and Zhang (1999)
8	*SbPRP*	Encodes a soybean proline-rich protein	(+) Virus infection, circadian rhythm, salt stress, drought stress, plant hormones SA	He et al. (2002)
9	*TaLTP1*	Facilitate transfer of phospholipids between membranes in vitro	(+) Wounding, salt and drought stress, SA, ethephon, H_2O_2	Kader (1996), Jang et al. (2004)
10	*TOP 2*	Encodes topoisomerase II	(+) Salt and drought stress, low temperature, ABA, SA	Hettiarachchi et al. (2005)
11	*GST 1*, *GST 2*	Glutathione cycle	(+) Drought	Kang et al. (2013)
12	*AtZAT6*	Transcription regulator	(+) Drought, SA accumulation	Shi et al. (2014)

SA in modulation of gene behaviour for drought stress response with considerable crosstalk (Table 10.1).

A water stress-induced gene in *Brassica oleracea* (BoWS) encoding a 95 amino acid protein is up-regulated by ABA, mannitol, NaCl, drought, SA and H_2O_2 (Li et al. 2004). BcDh2 dehydrin-like gene is determined from *Boea crassifolia* highly expressed by drought, salt stress, ABA and heat stress and slightly when induced by methyl jasmonate and SA (Shen et al. 2004). Drought treatment of *Brassica napus*-expressed BnBDC1 gene that encodes a protein containing the BuRP domain (Shunwu et al. 2004). It is up-regulated by mannitol, NaCl and ABA and downregulated by UV irradiation and SA. BnBDC1 might be involved

in multiple cell signalling pathways, plant pathogen infection and may play important role in response to osmotic stress. TaLTP1 gene encodes plant lipid transfer proteins (LTPs), which has a role in in vitro transfer of phospholipids and facilitates the formation and reinforcement of plant surface layers, embryogenesis and defence against pathogens, symbiosis and abiotic stress tolerance (Kader 1996). Transcripts of the TaLTP1 are increased by drought, salinity, wounding and application of SA, ethephon and H_2O_2 (Jang et al. 2004). OsGGT (a - submergence-induced gene) is determined in submergence-tolerant cultivar of rice (*Oryza sativa* L.) (Qi et al. 2005). Its expression is induced by SA and benzyl adenine, ethylene, gibberellins, ABA, drought and salt treatment. Thus, the involvement of OsGGT gene might be related to submergence stress and general defensive response to environmental stresses. CPRD46 gene found in dehydrated cowpea (*Vigna unguiculata*) is responsive to salt stress, ABA, heat stress, methyl JA and SA (Iuchi et al. 1996). PvSR1 (*Phaseolus vulgaris*stress-related protein) gene encodes a proline-rich protein in the leaves of *Phaseolus vulgaris*. PvSR1 is greatly expressed by alfalfa mosaic virus infection, wounding, heat shock, UV, salinity, drought and also by exogenously applied SA and H_2O_2. PvSR1 may be involved in maintaining cellular integrity during stress conditions (Chai and Zhang 1999). Ppsrk11 (a serine/threonine kinase receptor-like protein kinase gene) is studied in bark of *Prunus persica* (peach) (Bassett et al. 2005). It is supposed that the expression of this gene may be responsive to low temperatures, short-day photoperiod or water limitation. This gene is up-regulated by SA treatment in fruit and by drought stress in bark and roots and downregulated by light.

A gene that encodes chloroplast translocation elongation factor (EF-Tu) is differentially expressed under abiotic stresses in pea. This indicates that it is an important gene in plant adaptation under stress conditions (Singh et al. 2004). Its expression is downregulated by salinity and ABA while up-regulated by SA and low temperature. TOP2 gene encodes topoisomerase II. The expression level of TOP2 gene is up-regulated by salt stress, low temperature, ABA and also by salicylic acid (Hettiarachchi et al. 2005). SAR 8.2 gene is modulated both under biotic and abiotic stress in *Capsicum annuum* (Lee and Hwang 2003). Results of in situ hybridization showed that AtPLAs is a family of ten genes in *Arabidopsis* comprised by cytosolic patatin-related phospholipase A enzymes. It might be involved in auxin and pathogen signalling (Holk et al. 2002) and iron stress (Rietz et al. 2004). Its expression is up-regulated by SA, wounding, ACC and jasmonic acid. PhOSM which encodes osmotin is reported from petal protoplast cultures of *Petunia hybrida* (Kim et al. 2002). Osmotin showed increased tolerance to NaCl in tobacco cells (Singh et al. 1987). Expression level of PhOSM increased on pathogen infection, wounding and by the treatment with aspirin or salicylic acid. PhOSM may be involved in stress signal transduction. SbPRP gene is reported from leaves and epicotyls of soya bean seedlings encoding a soya bean proline-rich protein (He et al. 2002). Its expression is increased by salt stress, drought stress, plant hormones, virus infection and SA. SbPRP gene may be involved in responses to different internal and external factors in plants. It is studied that the expression of many cytochrome P450 genes is induced by environmental stresses and also by hormone treatments such as SA (Narusaka et al. 2004). These genes contained the

recognition sites of MYB and MYC, ACGT core sequence, TGA box and W-box for WRKY transcription factors in their promoters. These cis-acting elements are known to participate in the regulation of plant defence.

Experiments have revealed both SA-dependent drought tolerance and disease resistant by studies on SA-accumulating *Arabidopsis* mutants *adr1*, *myb96-1d*, *siz1*, *acd6 and cpr5* (Chini et al. 2004; Miura and Tada 2014). Microarray studies also reveal up-regulation of 27 genes in two clusters in the SA-accumulating mutants on drought stress.

10.4 Conclusions

Despite the evident involvement of SA in plant responses to biotic and stress, the drought or salt stress signal transduction mechanisms downstream SA as well as the genetic modulation remain obscure. Drought tolerance in plants involves multiple interactive pathways, and gene expression studies indicate the modulation and coordinated expression of a network of genes under drought stress (Talame et al. 2006; Xiao et al. 2007). An understanding of the genetic and molecular mechanisms underlying drought resistance is essential to speed up the development of new drought-resistant cultivars. Additionally, magnitude and duration of stress, stages of plant growth also affect expression levels (Drame et al. 2007). The genetic determinants within plant genomes that confer drought tolerance are increasingly being elucidated by improvements in quantitative trait loci mapping technology which has benefited marker-assisted selection in breeding programs albeit with the several limitations (Tuberosa and Salvi 2006; Pathan et al. 2007; Muchero et al. 2010). Increased genome sequence information has made it possible to identify and map candidate genes onto identified QTL regions, some specific to drought tolerance (Tondelli et al. 2006; Salentijn et al. 2007).

Several reports indicate involvement of candidate genes in drought tolerance. A majority of drought responses are thought to be triggered by the production of ABA which induces H_2O_2 and nitric oxide (NO) activating MAPK cascades resulting in up-regulation of several downstream products especially antioxidant enzymes (Hao et al. 2008; Xing et al. 2008). However, studies in ABA-insensitive (*abi*) mutants suggest an alternative ABA-independent regulation of gene expression during stress responses (Nakashima et al. 2009). Thus, the role of other signalling pathways especially those triggered by SA needs to be further studied to understand the molecular responses to drought stress.

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Chapter 11
Drought Resistance in Crops: Physiological and Genetic Basis of Traits for Crop Productivity

Renu Khanna-Chopra and Kalpana Singh

Abstract Drought is the most important environmental stress affecting agricultural productivity worldwide. Breeding of drought-tolerant crops is important in order to meet demands of food security in the face of an ever increasing world population, global warming and water shortage. Drought resistance (DR) is defined as the mechanism causing minimum loss of yield in a water deficit environment relative to the maximum yield in a water constraint free management of the crop. Plants have evolved several mechanisms to cope with water deficit stress which includes drought escape and drought tolerance. Plant breeders and physiologists have identified some important traits associated with DR in crop plants. Many of these traits relate to making appropriate use of water when it is available, often with the aim of ensuring reproductive success and grain yield. Traits associated with DR serve as important breeding tools in identifying stress-resistant genotypes and in introgressing the resistance traits into high yielding genotypes. Marker-assisted selection based around screening for desirable alleles at QTL for DR is an important approach for improving DR in crops. Dissecting these complex phenotypic traits into simpler heritable traits has led to the identification of genes associated with some QTLs for DR. Breeding for DR has met with limited success following either empirical or marker-assisted selection approach. It is essential to integrate crop physiology, functional genomics and breeding approaches to dissect complex DR traits, understand the molecular basis of DR and develop improved cultivars to adapt to the changing climate. This chapter focuses on the DR traits important for agricultural productivity in major crops, i.e. wheat and rice. The physiological and genetic basis of traits is discussed to highlight the complexity of the quantitative traits and the need to integrate this information in breeding drought-resistant crops.

Keywords Crop yield • Drought resistance • Molecular markers • QTL

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11.1 Introduction

Drought is the most important environmental stress affecting agricultural productivity worldwide. There is need to breed drought-tolerant crops in order to meet the demands of food security in the face of an ever increasing world population, global warming and water shortage. Breeding of drought-tolerant crops is an important objective in both national and international institutes.

Drought has been defined as the inadequacy of water availability, including precipitation and soil moisture shortage capacity, in quantity and distribution during the life cycle of the crop to restrict expression of its full genetic yield potential (Sinha et al. 1986; Khanna-Chopra and Sinha 1998). Drought may occur at any stage during the life cycle of a crop. Drought stress is often variable in intensity and duration across years and location (Beltrano and Marta 2008). Different combinations of drought pattern led to the selection of numerous types of resistance mechanisms at various levels of plant organization. The study of these mechanisms can provide important information for crop breeding programmes (Araus et al. 2008).

DR is defined as the mechanism causing minimum loss of yield in a water deficit environment relative to the maximum yield in a water constraint free management of the crop (Blum 2005). DR of a crop is essentially linked to its ability to access water from soil and to use it most productively (Richards et al. 2010). Plants have evolved several mechanisms to cope with water deficit stress. DR is a complex trait and can broadly be achieved through drought escape and drought tolerance. Drought escape can be defined as the ability of a plant to complete its life cycle before serious soil and plant water deficit develop. This mechanism involves rapid phenological development, developmental plasticity and remobilization of pre-anthesis assimilates to the grain. Escape strategies rely on successful reproduction before the onset of severe stress (Chaves et al. 2003). Breeding for shortening the crop duration has been a very successful strategy worldwide (Araus et al. 2002). However, early flowering may increase the risk of other abiotic stresses and in better than average seasons, determinate crops such as wheat and barley have little potential to take advantage of late rain. This has led to the search for cultivars that can tolerate drought and still produce an economic yield.

Drought tolerance can be achieved with high water potential or low water potential. DR with high water potential can be achieved by (1) reduction in water loss by increase in stomatal and cuticular resistance, (2) reduction in radiation absorbed, (3) reduction in leaf area, (4) maintenance of water uptake by increase in root density and depth and (5) increased liquid phase conductance. DR with low water potential can be achieved by maintaining turgor pressure through osmotic adjustment (OA) and increase in cellular elasticity. OA maintains cell water contents by increasing the osmotic force that can be exerted by cells on their surroundings and thus increasing water uptake. The adjustment results from compatible organic solutes accumulating in the cytoplasm which decrease the osmotic

potential of the cytosol. The increase in OA allows sustaining cellular hydration and thus support continued photosynthesis and growth at slow rate under stress.

DR is a complex trait and drought stress is often accompanied by heat or other stresses. Plants use multiple strategies to respond to drought stress and have evolved to adapt to drought via morphological and physiological changes through diverse signalling cascades and OA. Plants have also evolved tolerance mechanisms to survive severe drought stress through accumulation of osmoprotectants, antioxidants and reactive oxygen species (ROS) scavengers. The processes of the plant response to drought stress include stress signal perception, signal transduction and amplification and adaptation at various levels of organization. In these processes, many proteins including transcription factors, protein kinases and diverse stress-related proteins function to enhance DR via outputs such as growth delay, transpiration reduction, OA and ROS scavenging. Hundreds of genes in these pathways that control the key processes of the plant response to drought stress have been identified by genetic, genomic and transgenic approaches. Some of them have been confirmed to have potential for improving the DR of crops in field trials (Hu and Xiong 2014). However, the biochemical and molecular basis for drought perception, signal transduction and stress adaptation remains largely unclear, which continues to be a major challenge for the genetic improvement of DR.

This chapter focuses on the DR traits important for agricultural productivity in major crops, i.e. wheat and rice. The physiological and genetic basis of traits is discussed to highlight the complexity of the quantitative traits and the need to integrate this information in breeding drought-resistant crops.

11.2 Traits Associated with Drought Resistance in Cereals

Plant breeders and physiologists have identified through long-term research some important traits associated with DR in crop plants. Different traits have importance at different stages of crop development. Many of these relate to making appropriate use of water when it is available, often with the aim of ensuring that adequate water is available during the sensitive times of floral development, flowering and grain growth.

The traits important for DR in cereals are:

1. Grain yield and yield components
2. Delayed senescence
3. Phenology
4. Root traits
5. Stem reserve mobilization
6. Coleoptile length

11.2.1 Grain Yield and Yield Components

Grain yield (GY) is an integrative trait. It can be dissected into components such as biomass, harvest index, plant number per unit area, spike number per plant, grain number per spike, 1,000 grain weight and spikelet number per spike. GY has low heritability while its component traits such as grain weight and grain number have high heritability (McIntyre et al. 2010). Many researchers have studied GY and related traits under water deficit stress environments through QTL analysis. In the recent past, QTLs have been identified for GY and related traits on chromosomes 1A, 1B, 2A, 2B, 2D, 3B, 3D, 4A, 5A, 5B, 7A and 7B under water deficit stress in wheat (Mir et al. 2012). However, majority of these studies have been done under terminal water deficit stress or rain-fed environments. Some of the major QTLs explaining phenotypic variation (PV) of yield and yield-related traits in wheat and rice are highlighted in Table 11.1 and a few are discussed also.

Using a RIL mapping population derived from Seri/Babex cross, consistent QTLs on chromosome 3BL were detected for GY and related traits under six field trials in North-West Mexico and explained up to 15 % of PV under drought stress condition (Pinto et al. 2010, Table 11.1). This QTL region also co-located with several traits such as flowering time, amylase content and grain weight (Marza et al. 2006; Kirigwi et al. 2007; Kuchel et al. 2007). A QTL for GY was identified on chromosome 4AL explaining 20 % of PV under drought stress in wheat RIL mapping population derived from Dharwar Dry/Sitta (Kirigwi et al. 2007). Quarrie et al. (2006) identified GY QTL on 7AL explaining 18 % of PV in 11 trials out of 21 trials under drought stress in a wheat double haploid (DH) population derived from Chinese Spring/SQ1 (Table 11.1). On comparison with rice chromosome 6, candidate genes such as *AINTEGUMENTA* and *G-protein* subunit affecting lateral cell division have been identified underlying this QTL region. However, the role of these genes in GY formation is not clear. Foulkes et al. (2004) identified major QTLs for GY on chromosome 2D corresponding to photoperiod response gene (*Ppd-D1*) which is known to affect yield. Another stable QTL for GY on chromosome 2DS has been identified in four out of six environments including irrigated and rain-fed condition (Edae et al. 2014). Two major QTLs on chromosomes 2BL and 3BS explained up to 22 % of PV and showed consistent additive and epistatic effects on 1,000-kernel weight, peduncle length and GY in eight environments (Graziani et al. 2014, Table 11.1). These QTLs are good candidates for positional cloning in order to gain a better understanding of the functional basis of their effect on the plasticity of grain weight and GY.

QTLs for GY and related traits in rice were reported much earlier than wheat (Babu 2010). Major QTLs identified for GY on chromosome 1 of rice explained up to 32 % of PV under drought stress condition by both Kumar et al. (2007) and Ghimire et al. (2012) (Table 11.1). Rice chromosome 1 has been reported to be linked with several DR traits such as GY, biomass, deep root mass, leaf drying, plant height, RWC, OA and canopy temperature under drought stress (Kanagaraj et al. 2010; Trijatmiko et al. 2014). The major gene controlling semi-dwarf stature

Table 11.1 Important QTLs for drought resistance-related traits in wheat and rice

Crops	QTL/ marker	Chromosome	Phenotypic variation (%)	QTL function under drought	References
Wheat	*Qyld.csdh.7AL*	7A	18.3	Grain yield	Quarrie et al. (2006)
Wheat	*Xwmc420*	4A	20.0	Grain yield	Kirigwi et al. (2007)
Wheat	*wPt-1804*	3B	15.1	Grain yield	Pinto et al. (2010)
Wheat	*Xbarc133–Xgwm493*	3B	22.7	Thousand kernel weight, peduncle length, test weight	Graziani et al. 2014
Rice	*EM11_11–RG109*	1	32.0	Grain yield	Kumar et al. (2007)
Rice	$qtl_{12.1}$	12	51.0	Grain yield	Bernier et al. (2007)
Rice	$qDTY_{1.1}$	1	32.0	Grain yield	Ghimire et al. (2012)
Rice	*SPP1*	1	51.1	Spikelet per plant and grain yield	Liu et al. (2009)
Rice	*qGW5*	5	24.3	Controls grain width and grain length–width ratio	Wan et al. (2008)
Rice	*Gn1a*	1	34.0	Cytokinin accumulation in inflorescence meristems and increases the number of spikelets	Ashikari et al. (2005), Li et al. (2013)
Rice	*qFLL 6.2*	6	52.7	Number of spikelets per panicle, number of filled grains per panicle and grain weight per panicle	Shen et al. (2012)
Wheat	*Qsg.bhu.1A*	1A	24.8	Stay green	Kumar et al. (2010)
Rice	*ccfs3*	3	36.4	Stay green	Yoo et al. (2007)
Wheat	*QHd.pser-2DS*	2D	40.5	Heading date	Xu et al. (2005)
Wheat	*gwm263*	7B	42.4	Heading date	Peleg et al. (2009)
Wheat	*QSRA.cgb-3B*	3B	24.3	Seminal root angle	Liu et al. (2013)
Rice	*DRO1*	9	67	Deep root angle	Uga et al. (2013)
Wheat	*XcsME1*	4B	49.0	Coleoptile length	Rebetzke et al. (2007)

sd-1 is also located on chromosome 1 and is known to affect many aspects of plant morphology, i.e. plant height, tillering, panicle length, biomass, harvest index, root traits and responsiveness to fertilizers (Vikram et al. 2012). A major QTL for number of spikelets per panicle, *SPP1* was located on rice chromosome 1 explaining 51 % of PV. A candidate gene which encodes indole acetic acid (IAA) synthase has been identified through fine mapping of this QTL region (Liu et al. 2009, Table 11.1). IAA may play an important role in regulating reproductive growth and the development of inflorescence meristem in rice. Bernier et al. (2007) identified a large effect QTL for GY, *qtl12.1* on chromosome 12 explaining 51 % of PV under drought in F3 lines derived from a cross between upland drought-resistant rice variety Vandana and drought-sensitive variety WayRarem over 2 years of field evaluation at IRRI (Table 11.1). The WayRarem-derived allele of this QTL has been shown to improve GY under moderate or severe upland drought conditions in 9 out of 10 trials (Bernier et al. 2009).

Many studies have reported genes for GY productivity either through fine mapping of QTLs or mutants in wheat and rice which are enumerated in Table 11.2. These include genes such as *GS3* for grain size, *GW5* for grain width and grain length–width ratio, *GW2* for grain weight, *GW8* for grain size and shape and *CKX2*, *GIF1* and *SPL14* for grain number in rice (Valluru et al. 2014). *GS3* gene for grain weight and length was identified through positional cloning and transformation of QTL for grain weight *qGW3.1*. The study revealed that C to A mutation has occurred in the second exon of *GS3* gene which was associated with enhanced grain size. This substitution played a critical role in defining the seed morphologies of modern subpopulations of *Oryza sativa* in rice evolution (Takano-Kai et al. 2009, Table 11.2). A major stable QTL *qGW5* for grain width and length–width ratio was identified and explained 24.3 % of PV in Asominori/IR24 RIL population and Chromosome segment substitution lines (CSSLs). QTL *qGW5* was fine mapped to a 49.7-kb genomic region with high recombination frequencies on chromosome 5 using 6781 BC4F2 individuals and 10 newly developed simple sequence repeat markers into single recessive gene *gw5* which controlled both grain width and grain length–width ratio (Wan et al. 2008, Table 11.1). QTL *GW2*, influences grain width and weight and encodes RING-type protein with E3 ubiquitin ligase activity, which is known to be involved in the degradation processes of the ubiquitin-proteasome pathway (Song et al. 2007, Table 11.2). The loss-of-function allele of *GW2* increased cell number resulting in a larger (wider) spikelet hull and accelerated grain filling rate, resulting in enhanced grain width, weight and yield. Two homologues of *GW2*, *ZmGW2-CHR4* and *ZmGW2-CHR5* have also been identified in maize. Linkage analysis has demonstrated that *ZmGW2-CHR4* is located within a consistent QTL of 1,000-kernel weight in maize (Li et al. 2010). QTL *GW8* for grain size and shape encodes *SQUAMOSA PROMOTER-BINDING PROTEIN LIKE 16* (*OsSPL16*), a protein that is positive regulator of cell proliferation. Over-expression of *OsSPL16* promoted cell division, grain filling, enhanced grain size, shape quality and GY (Wang et al. 2012, Table 11.2).

QTL for Grain number, *Gn1a* has been identified on chromosome 1 which explained 34 % of PV (Ashikari et al. 2005, Table 11.1). QTL *Gn1a* increases

Table 11.2 Genes important for grain yield and physiological traits for drought resistance identified through QTL analysis under drought stress in wheat and rice

Traits	QTL	Gene	Function	Crop	References
Grain yield	*WFP* (WEALTHY FARMER'S PANICLE)	*OsSPL14* (SQUAMOSA PROMOTER-BINDING PROTEIN LIKE 14)	Over-expression of *OsSPL14* in the reproductive stages promoted panicle branching and increased grain yield	Rice	Miura et al. (2010)
Grain number	*Gn1a* (Grain number 1a)	*OsCKX2* (Cytokinin oxidase/ dehydrogenase)	Reduced expression of *OsCKX2* caused cytokinin accumulation in inflorescence meristem, increased the number of spikelets resulting in enhanced grain yield in transgenics	Rice	Ashikari et al. (2005)
		TaCKX6-D1 (Cytokinin oxidase/ dehydrogenase)	*TaCKX6-D1* was cloned and its haplotype variants showed significant association with 1,000-grain weight	Wheat	Zhang et al. (2012)
	gif1 (Grain incomplete filling)	*CIN4* (Cell wall invertase 4)	*GIF1* is mainly expressed in seed vascular tissues and controls sucrose unloading for starch synthesis at the early grain-filling stage	Rice	Wang et al. (2010)
	DEP1 (Dense and erect panicle)	PEBP (Phosphatidylethanolamine-binding protein-like domain protein)	Dominant allele at the *DEP1* locus is a gain-of-function mutation causes truncation of PEBP by which plants showed dense panicle, high grain number per panicle and erect panicle resulting in increased grain yield	Rice	Huang et al. (2009)
Grain weight/size	*gw3.1*	*GS3* (Grain size)	Improved grain length and weight	Rice	Takano-Kai et al. (2009)
	GW2	RING-type protein with E3 ubiquitin ligase	*GW2* was cloned and its loss of function increased cell number, resulting in a larger (wider) spikelet hull, accelerated the grain milk filling rate, resulting in enhanced grain width, weight and yield	Rice	Song et al. (2007)

(continued)

Table 11.2 (continued)

Traits	QTL	Gene	Function	Crop	References
	GW8	*OsSPL16* (SQUAMOSA PROMOTER-BINDING PROTEIN LIKE 16)	Over-expression of *OsSPL16* promoted cell division, grain-filling and enhanced grain size, shape, quality and grain yield	Rice	Wang et al. (2012)
Delayed senescence	*7A* (Flag leaf senescence)	*6-SFT*(6-sucrose fructan fructosyltransferase)	*6-SFT* gene is induced by drought stress and increases fructan synthesis which serves as carbon source for storage	Wheat	Livaja et al. (2011), Khoshro et al. (2014)
	Gpc-B1 (grain protein content)	NAC domain protein	Associated with increased grain protein, zinc and iron content and played important role in auxin signalling and leaf senescence	wheat	Uauy et al. (2006), Distelfeld et al. (2014)
Flowering date	*Ghd7* (Grain number, heading date and plant height)	CCT domain protein and a putative HAP3 subunit of the CCAAT box-binding transcription factor	Delayed heading date under long-day conditions and increased plant height and panicle size	Rice	Xue et al. (2008)
	Ghd8	Encodes the *OsHAP3* subunit of a CCAAT box-binding protein	Delayed flowering under long-day condition but down-regulation promoted flowering under short-day conditions	Rice	Yan et al. (2011)
	Ehd1 (Early heading date)	Encodes B-type response regulator	Promoted heading by inducing *FT-like* gene expression under short-day conditions	Rice	Doi et al. (2004)
Water soluble carbohydrate	*rg5* (ratio of filled grains)		Showed high sink activity, increased assimilate partitioning to particularly inferior caryopses in the late stage and improved the total ratio of filled grains	Rice	Ishimaru et al. (2005)
Root traits	*DRO1* (deep rooting 1)		Increased deep-rooting pattern and maintained grain yield	Rice	Uga et al. (2013)

grain productivity in rice and encode a cytokinin oxidase/dehydrogenase enzyme previously named *OsCKX2* which degrades the phytohormone cytokinin. The reduced expression of *Gn1a* causes cytokinin accumulation in inflorescence meristem and increases the number of spikelets, thus enhancing GY (Li et al. 2013, Table 11.2). Zhang et al. (2012) reported, *TaCKX6-D1* gene, a wheat ortholog of rice *OsCKX2* gene which was cloned and its haplotype variants were determined to be significantly associated with 1,000-grain weight on the basis of linkage mapping association and gene expression analysis. A major QTL for flag leaf length, *qFLL6.2* on chromosome 6 has been identified from Indica rice cross Zhenshan 97/Milyang 46 and explained 52.7 % of PV. QTL *qFLL6.2* was shown to have major effects on the number of spikelets per panicle, number of filled grains per panicle and grain weight per panicle (Shen et al. 2012, Table 11.1). QTL *WEALTHY FARMER'S PANICLE* (*WFP*) encodes *SQUAMOSA PROMOTER-BINDING PROTEIN LIKE 14*, *OsSPL14* when over-expressed, promoted panicle branching and higher GY in rice (Miura et al. 2010, Table 11.2). QTL for *DENSE AND ERECT PANICLE1*, *DEP1* was identified from two F_2 populations derived from Japonica/Indica crosses. The mutation allele of *DEP1* encoding phosphatidylethanolamine-binding protein like domain protein (*PEBP*) showed dense panicle, higher grain number per panicle and erect panicle, resulting in increased GY (Huang et al. 2009, Table 11.2). The loss-of-function mutant for *gif1*, a grain filling and grain weight gene in rice, showed lower levels of glucose, fructose and sucrose in the grains than wild-type*GIF1* ascertaining the role of cell-wall invertases in carbon partitioning during early grain filling in rice (Wang et al. 2010, Table 11.2).

11.2.2 Delayed Senescence

Senescence is an internally programmed cell death and is affected by environmental factors like abiotic and biotic stresses (Khanna-Chopra2012; Gan 2014). Senescence includes loss of chlorophyll and decline in photosynthetic capability of the leaf. The ability to maintain green leaf area during grain filling is one of the important physiological traits that has an implication on yield potential related to increase assimilate availability. Hence, selection for stay green types which maintain photosynthetic ability during grain filling under irrigated and water stress environment is a desirable character (Thomas and Ougham 2014).

Genetic variability for green flag leaf area (GFLA) has been reported in both bread (Verma et al. 2004) and durum wheat (Hafsi et al. 2000). There are very few studies on the inheritance of GFLA in wheat, which exhibits moderate heritability. QTLs for chlorophyll content were identified on chromosomes 1A, 1B, 2B, 2D, 3B, 4A, 5A, 5B, 6A, 7A and 7D in durum wheat and wild emmer wheat (Peleg et al. 2009; Kumar et al. 2010, Table 11.1). In another study, QTL for green flag leaf duration (GFLD) were identified on chromosome 2A, 3A, 3B, 6A, 6B and 7A and both the parents contributed favourable alleles for most of the senescence-

related traits (Vijayalakshmi et al. 2010). In winter wheat, QTL for GFLA percentage detected on chromosome 2D was found to control 22 % of PV at 35 days after anthesis and showed association with *Ppd-D1* gene (Verma et al. 2004). Recently, three QTLs for GFLA on chromosome 1A, 3B and 7D were reported using a RIL population derived from a cross between the parent Chirya 3 and Sonalika (Kumar et al. 2010). The QTL on chromosome 1A was stable, explained up to 24.8 % of PV and alleles for higher GFLA were derived from Chirya 3 (Table 11.1). Another major QTL for flag leaf senescence was detected on the short arm of chromosome 7A and is associated with the candidate gene 6-sucrose fructan fructosyltransferase (*6-SFT*) which was successfully mapped on wheat DH population derived from Kerubino/Reiner (Livaja et al. 2011, Table 11.2). *6SFT* is involved in fructan biosynthesis, induced by drought stress and the produced fructan serves as a carbon source for storage (Table 11.2, Khoshro et al. 2014).

QTLs for stay green trait have been identified on chromosome 2, 3 6 and 9 and explained up to 36.45 % of PV in rice (Yoo et al. 2007, Table 11.1). In sorghum, four major QTLs namely *stg1*, *stg2*, *stg3* and *stg4* were consistent in different genetic and environmental backgrounds and accounted for 53.5 % of PV. The *stg1*, *stg2* and *stg3* QTLs were significantly correlated with the chlorophyll content at physiological maturity (Subudhi et al. 2000). Other studies showed that the four *stg* QTLs regulate canopy size by (1) reducing tillering via increased size of lower leaves, (2) constraining the size of the upper leaves, (3) in some cases, decreasing the number of leaves per culm. In addition, *stg* QTLs variously affect leaf anatomy and root growth. The multiple pathways by which *stg* QTLs modulate canopy development can result in considerable developmental plasticity. The reduction in canopy size associated with *stg* QTLs reduced pre-flowering water demand, thereby increasing water availability during grain filling and ultimately GY (Borrell et al. 2014).

Many advances in the understanding of leaf senescence at the molecular level have been achieved through the identification and characterization of hundreds of SAGs and senescence-related mutants (Liu et al. 2011). Guo et al. (2004) have provided comprehensive transcriptome study in *Arabidopsis thaliana* leaves with 6,200 of senescence associated expressed sequence tags (ESTs), representing approximately 2,500 genes. Transcription factor *WRKY* (*WRKY 2*, *WRKY19*) plays an important role in leaf senescence process of *Arabidopsis thaliana* and *WRKY53* is one of the transcription factors involved in early senescence process (Penfold and Buchanan-Wollaston 2014; Semwal et al. 2014). Uauy et al. (2006) reported positional cloning of *Gpc-B1*, a wheat QTL associated with increased grain protein, zinc and iron content. The ancestral wild wheat allele encodes an *NAC* transcription factor (*NAM-B1*) that accelerates senescence and increases nutrient remobilization from leaves to developing grains, whereas modern wheat varieties carry a non-functional *NAM-B1* allele (Distelfeld et al. 2014, Table 11.2). NAC transcription factors are networked to ROS and pathogen signalling pathways. NACLIKE, ACTIVATED BY AP3/PI (AtNAP/ANAC029), is a key senescence-regulating NAC transcription factor in Arabidopsis. Inducible over-expression of *AtNAP* in young leaves triggers precocious senescence, whereas a knockout mutant

of *AtNAP* exhibits retarded leaf senescence. NAP is thought to regulate leaf senescence partially through its direct binding to the promoter of *sag113*, a negative regulator of the abscisic acid (ABA) pathway that inhibits stomatal closure, which in turn triggers leaf senescence (Woo et al. 2013). The phenotype of *sag113* mutant plants induced to senesce by ABA treatment is stay-green. Thus, high degree of interactivity between nodes of transcriptional regulation, hormone and ROS mediated signalling pathways and sensors of environmental cues and stress regulates senescence process in plants (Hickman et al. 2013).

A wheat mutant *tasg1* with delayed leaf senescence was identified from HS2, a common wheat cultivar using ethyl methane sulfonate (EMS) as a mutagen. Natural senescence in *tasg1* was distinctly delayed in the field, as indicated by the slower progression of chlorophyll degradation and decline in net photosynthetic rate than its wild type. Improved water balance and effective antioxidative system may also be involved in the drought resistance of *tasg1* (Tian et al. 2013). Similarly, Luo et al. (2013) characterized a strong functional stay-green wheat variety, CN17 which exhibited significantly higher maximal photochemical efficiency for photo system II (PSII) and Fv/Fm at 21 days post-anthesis thus extending the period of grain-filling and thereby increasing GY.

Most transgenic approaches aimed at enhancing productivity of crop plants by delay of leaf senescence have employed the *IPT* gene from *Agrobacterium tumefaciens* under control of a senescence-inducible promoter (Gregersen et al. 2013). The *IPT* gene encodes the isopentenyl transferase, an enzyme catalyzing the rate-limiting step in cytokinin biosynthesis. In wheat, senescence-induced expression of the *IPT* gene was shown to significantly delay senescence as measured by chlorophyll content of flag leaves 10 days after anthesis when grown under limited nitrogen supply (Sýkorová et al. 2008). The authors proposed that the delay in senescence interferes with the wheat reproductive strategy based on fast programmed translocation of metabolites from senescing leaves to the reproductive sinks shortly after anthesis. Functional characterization of these transporter genes will help us to understand the nutrient recycling processes which will lead to higher GY and better quality nutrition.

11.2.3 Phenology

Plant phenology, i.e. timing of the various developmental stages of the plant, is a most widely used trait for breeding for DR (Araus et al. 2008). Crop plants show a certain degree of developmental plasticity in order to survive under drought conditions. If the pattern of water deficit is predictable in the target region, selection of a flowering date which ensures satisfactory grain development before the occurrence of water deficit is an effective way to improve adaptation to drought (Distelfeld et al. 2009).

Many QTL studies have been reported for phenological parameters. QTLs for flowering time were mapped on chromosomes 1B, 2B, 2D, 3A, 3B, 4A, 4B, 5A, 5B,

5D, 6D, 7A, 7B and 7D in wheat (Peleg et al. 2009). Peleg et al. (2009) identified QTL for days from planting to heading on chromosome 7B explaining 42.4 % of PV in a durum wheat population (Table 11.1). A major consistent QTL for heading date was identified on 2DS explaining 40 % of PV in wheat (Xu et al. 2005, Table 11.1).

Two major photoperiod-sensitive QTL explaining up to 31 % of PV were mapped on 2B and 2D at the same position as the two major genes *Ppd-B1* and *Ppd-D1* (Hanocq et al. 2004). The pleiotropic effects of *Ppd* genes have been reported on various agronomic and morpho-physiological traits of wheat in different studies (Kamran et al. 2013). Dominant *Ppd* genes are known to reduce plant height (Worland 1996), final leaf number (Dyck et al. 2004), spikelet primordial initiation (Rawson and Richards 1993), tillering, spikelet per spike (Dyck et al. 2004) and green leaf area index (Foulkes et al. 2004). The *Ppd-D1* locus of wheat is collinear with barley locus *Ppd-H1* on chromosome 2H (Börner et al. 1998). The barley gene has been cloned and identified as a member of pseudo-response regulator (*PRR*) family (Turner et al. 2005). *PRR* genes are distantly related to the well-understood *CONSTANS*-like family of transcription factors of Arabidopsis and rice. *CONSTANS* (CO) is a central regulator of photoperiod pathway. It coordinates light and clock inputs in leaves to trigger the expression of mobile florigen hormone *FLOWERING LOCUS T* (*FT*) that induces flower differentiation (Chen et al. 2014). In Arabidopsis, *CO* promotes the expression of *FT* under inducing long days. In rice, a short day plant, *CO* acts as a repressor in non-inductive long days (Hayama et al. 2003).

The flowering habit of wheat is determined primarily by complex group of genes for vernalization (*Vrn*), photoperiod (*Ppd*) and earliness per se (*Eps*) that interact with the environment to regulate the rate and development of floral primordia (Gomez et al. 2014). The requirement of vernalization is particularly important for winter cereals to avoid cold injury of the sensitive floral organs during the winter. Vernalization affects time of floral initiation, number of leaves and timing of other growth stages up to emergence of the flag leaf and tiller number. Natural variation of wheat vernalization requirement is mainly controlled by the *Vrn-1*, *Vrn-2*, *Vrn-3* and *Vrn-4* gene. The first three genes have been identified using map-based cloning and validated using mutants and transgenic plants and less is known about the *Vrn-4* gene. *VRN-1* gene encodes a MADS-box transcription factor with very high similarity to Arabidopsis meristem identity genes *APETALA1*, *CAULIFLOWER* and *FRUITFUL* which is essential for the transition from vegetative to reproductive stage in wheat (Distelfeld et al. 2009). *VRN-2*, a dominant repressor of flowering, is down-regulated by vernalization. The *VRN-2* region includes two similar *ZCCT* genes, i.e. *ZCCT1* and *ZCCT2* encoding proteins with a putative zinc finger and a CCT domain that is associated with alleles for spring growth habit (Distelfeld et al. 2009). *Vrn-3* gene encodes a RAF kinase inhibitor-like protein with high homology to Arabidopsis protein *FT* and promotes the transcription of *Vrn-1* and accelerate flowering. It is evident that response pathways to vernalization and photoperiodism integrate a variety of other environmental cues. *Eps* genes can accelerate developmental rate at any particular growth phase. *Eps* genes can induce early flowering by initiating floral primordia with a minimum vegetative growth

and independent of photoperiodism. The presence of *Eps* genes have been demonstrated by QTL mapping studies in barley and wheat since a long time (Worland 1996). *Eps* genes have been fine mapped in diploid or hexaploid wheat on chromosomes 1A and 3A (Faricelli et al. 2010; Gawroński and Schnurbusch 2012). Recently, the molecular identification of two *EARLY MATURITY* genes, *eam8* and *eam10*, has been reported in barley (Zakhrabekova et al. 2012; Campoli et al. 2013). These genes cause circadian defects and interact with the *Ppd-H1* to accelerate flowering under long and short days in barley.

Heading date is one of the crucial agronomic traits in rice for ecological adaptation to different cultivation areas and cropping seasons and changes in heading date greatly impact rice yield. Heading date is a complex trait that is mainly affected by photoperiod sensitivity, basic vegetative growth and temperature sensitivity in rice. Photoperiod sensitivity is the most important factor influencing flowering and most of the heading date-related genes are sensitive to photoperiod. Many studies have shown that advanced heading date shortens the vegetative growth period of rice and results in a reduction of GY, while delayed heading provides the crop with sufficient vegetative growth lead to better GY and/or biomass (Liu et al. 2012). Several QTLs for rice heading date such as *Hd1*, *Hd3a*, *Hd6*, *Ehd1*, *Ehd2*, *Ehd3*, *Ghd7*, *Ghd8*, *Hd16*, *Hd17*, *LH1* and *LH2* have been identified and characterized by map-based cloning. Major QTLs for heading date *Hd1*, *Hd3a* and *Hd6* have been identified in a population derived from rice cultivars Nipponbare and Kasalath (Yano et al. 2000; Kojima et al. 2002; Takahashi et al. 2001). Molecular and physiological analysis of rice *Hd1* suggested that this QTL promoted flowering under short day and repressed flowering under long-day conditions. The mechanism involves action of the red-light photo-receptor phytochrome B (PHYB) as mutations in *phyB* or phytochrome chromophore synthesis attenuate this conversion and maintain *Hd1* as an activator during any photoperiod. In *Hd3a*, a candidate gene was identified with high similarity to the *FT* gene, which promotes flowering in Arabidopsis. The tri-protein complex composed of *Hd3a*, 14-3-3 protein and *OsFD1* has been named as florigen activation complex (FAC) which is essential for flowering mechanism (Tsuji et al. 2013). FAC acts as an ABA receptor and interacts with RNA metabolism to delay the onset of flowering in Arabidopsis. This suggests a possible mechanism between the increases in ABA levels induced by water deficits and delayed onset of flowering in crops. The manipulation of the activity of such proteins complex may help to accelerate the onset of flowering to minimize the loss of GY under water deficit stress conditions. *Hd6* was isolated to a 26.4-kb region and identified as a gene encoding the α-subunit of protein kinase *CK2* (*CK2α*). Further research indicated that premature stop codon was present in the Nipponbare *CK2α* allele, and the resulting truncated product was non-functional, whereas the Kasalath *CK2α* allele, which increased days to heading, was functional (Takahashi et al. 2001).

QTL for grain number, plant height and heading date 7 (*Ghd7*) encodes a CCT domain protein and a putative HAP3 subunit of the CCAAT box-binding transcription factor. The functional alleles of this gene delay heading date under long-day conditions and increase plant height and panicle size. Over-expression of *Ghd7*

increased drought sensitivity, whereas knockout of *Ghd7* enhanced DR. Gene-chip analysis of expression profiles revealed that *Ghd7* was involved in regulation of flowering time (Table 11.2, Xue et al. 2008). A QTL for grain yield, heading date, and plant height *Ghd8* on chromosome 8 in rice has been cloned and characterized. *Ghd8* was narrowed down to a 20-kb region containing two putative genes, of which one encodes the *OsHAP3* subunit of a CCAAT box-binding protein (HAP complex). *Ghd8* up-regulated the expression of *Ehd1*, *RICE FLOWERING LOCUS T1* (*RFT1*), and *Hd3a* genes under long-day conditions resulting in delayed flowering, but down-regulation promoted flowering under short-day conditions (Table 11.2). *Ghd8*up-regulated *MOC1*, a key gene controlling tillering, branching and increased the number of tillers, primary and secondary branches, thus producing 50 % more grains per plant (Yan et al. 2011). QTLs *Hd16* and *Hd17* have been mapped on chromosome 3 and 6, respectively, and are involved in photoperiod response, as revealed by observation of heading date in near isogenic lines (NILs) derived from cv. Nipponbare and Koshihikari in rice under short- and long-day conditions (Matsubara et al. 2014). Doi et al. (2004) identified heading date QTL, Early heading date 1 (*Ehd1*), using a RIL mapping population derived from T65 and Nipponbare in rice. QTL *Ehd1* encoded a B-type response regulator and promoted heading by inducing *FT-like* gene expression only under short-day conditions, and this promotion of heading was independent of *Hd1*. *Ehd1* expression was suppressed under long-day conditions (Table 11.2). QTL *Ehd3*up-regulated *Ehd1* expression to promote flowering in long-day condition (Matsubara et al. 2011). A set of dominant complementary genes for late heading *LH1* and *LH2* were identified by molecular marker analysis. Expression analysis showed that the epistatic effects of *LH1* and *LH2* act on the circadian clock-related genes which are upstream of *Hd1*, *Ehd1* and *Hd3a* in photoperiod flowering pathway (Liu et al. 2012).

Hence, it appears that natural variation of flowering time is shaped by the combination of small numbers of genes with large effect and several QTLs with small effects.

11.2.4 Root Traits

Root plays a crucial role for the uptake of nutrients and water from deeper layers of the soil. Deeper and profuse roots were found to increase plant access to water from deeper layers of the soil and support greater crop growth under water stress condition (Mir et al. 2012; Narayanan et al. 2014). The root system is complex and comprises traits such as root volume, root length, root penetration, root thickness and root/shoot ratio which are controlled by many genes and have generally been considered to be contributors to DR (Qu et al. 2008). The plant root system architecture is plastic and dynamic, allowing plants to respond to environmental changes, which then promotes root growth and development to avoid water deficit in the early stages of drought stress. However, measuring root traits in soil is difficult to perform and has low heritability. Hence, selection for

root-related traits has rarely been attempted in cereals under field conditions (Manschadi et al. 2008).

Very few QTLs have been reported for root traits in wheat in response to drought stress. QTLs for root traits have been reported on chromosomes 1B, 2A, 3B, 3D, 4A, 4B, 5D, 6A and 6B explaining up to 24.3 % of PV in wheat (Bai et al. 2013; Liu et al. 2013, Table 11.1). QTLs on 4BS explaining up to 14.5 % of PV have been identified in WL711/C306 RIL population for root traits and co-located with GY under drought stress during post-anthesis stage (Kadam et al. 2012). Total of 9 QTLs for four root traits namely maximum root length, root biomass above 30 cm length, root biomass below 30 cm length and root: shoot ratio have been reported under irrigated and water stress condition. Transcriptome profiling of bulk tolerant and susceptible RILs revealed five differentially expressed candidate genes such as glutathione transferase, proline-rich protein, putative transporters of sucrose/fructan transferase, zinc finger and MYB protein and 2OG-Fe oxygenases involved in GA biosynthesis were identified underlying the genomic region on chromosome 4BS in wheat.

There are several studies reporting QTLs for root traits in rice. Price and his co-workers invested many years of work in studying rice roots in relation to DR with the main focus for root QTL identification. Seven QTLs on chromosomes 1, 2, 4, 7, 9 and 11 which explained up to 18.2 % of PV were identified from rice RIL population derived from Bala/Azucena (Price et al. 2002). Steele et al. (2006) used marker-assisted backcrossing to introduce four QTLs, i.e. *QTL2*, *QTL7*, *QTL9* and *QTL11* for root penetration, deep root weight/thickness and root length from japonica upland cultivar Azucena into Indian upland rice cultivar Kalinga III. Twenty two NILs with the target QTLs were evaluated in five fields experiments and one NIL containing the target segment on chromosome 9 from Azucena, showed increased root length under well watered and water deficit stress condition. The strategy was used to breed a novel upland rice cultivar that has been released in India as BirsaVikasDhan 111 (Steele et al. 2013). Recently, Uga et al. (2013) cloned and characterized a QTL, *DRO1* for deep rooting. QTL *DRO1* ison chromosome 9 accounts up to 67 % of PV and controls root growth angle in rice (Table 11.1). *DRO1* is negatively regulated by auxin and is involved in cell elongation in the root tip that causes asymmetric root growth and downward bending of the root in response to gravity. *DRO1* was introduced into a shallow-rooting rice cultivar by backcrossing and the NILs exhibited enhanced DR by increasing deep rooting and maintained high GY performance under drought (Table 11.2).

Wild wheat relatives are important source of genetic variation for root traits such as deep rooting, shallow rooting, total root biomass and root length. Placido et al. (2013) introduced an alien chromosome segment (7DL) from a wild wheat relative species (*Agropyron elongatum*) into cultivated wheat (*Triticum aestivum*). The wheat translocation line (TL) showed improved water stress adaptation and higher root and shoot biomass compared with the control genotype, which showed significant drop in root and shoot biomass during stress. Enhanced access to water due to higher root biomass enabled the translocation line to maintain more

favourable gas-exchange and carbon assimilation levels relative to the wild-type wheat genotype during water stress. Transcriptome analysis of roots identified candidate genes associated with root development. Six candidate genes that are either induced by brass inosteroids (BRs) or directly involved in BR signalling were identified. BR signalling promoted cell wall loosening, root elongation and lateral root development (Wolf et al. 2012). Two candidate genes *KNAT3*, *SERK1* and a transcription factor *E2F* mapped to the site of translocation on chromosome 7DL based on single-feature polymorphism analysis. A wheat ortholog of the *KNOTTED-LIKE3* (*KNAT3*) homeobox gene in Arabidopsis was down-regulated in roots and shoots of TL compared with control under water-limited conditions. In Arabidopsis, *KNAT3* has been proposed to act as a negative regulator of lateral root development. The expression of *SOMATIC EMBRYOGENESIS RECEPTOR KINASE1* (*SERK1*), a member of the Leu-rich repeat, receptor-like kinase protein family, was up-regulated in roots and shoots of TL under water limitation compared with control in the array experiment. *SERK1* plays a critical role in root differentiation in response to auxin in addition to being involved in somatic embryogenesis and gamete development. A wheat *E2F-RELATED* (*E2F*) transcription factor is down-regulated in roots and shoots of TL under water-limited conditions. *E2F* proteins are a family of transcription factors that regulate cell cycle progression in plants and animals. In Arabidopsis, *E2Fc* is known to play an antagonistic role in cell division and is a negative regulator of lateral root formation. Jeong et al. (2010) have shown that root-specific expression of the *OsNAC10*, a rice NAC for No Apical Meristem *ATAF1-2*, and *CUC2* for Cup-Shaped Cotyledon gene, using the root-specific promoter RCc3, resulted in thicker roots (1.25-fold increase in root diameter) due to the enlarged stele, cortex and epidermis. These transgenic rice plants also had an increased GY of 5–14 % and 25–42 % over the controls under normal and drought conditions, respectively. These results clearly demonstrate that thick roots can enhance the yield potential of rice under drought.

11.2.5 Stem Reserve Mobilization

Grain-filling is a crucial time during the developmental stage of a crop. In wheat, most often this is the time when the temperatures are increasing and soil moisture levels are decreasing. Water deficit stress at this stage can be detrimental for GY and related traits. Grain-filling in cereal crops is dependent on both current photosynthesis and on a temporary pool of carbohydrates stored primarily in the stems around the time of anthesis (Shukla and Khanna-Chopra 2010; McIntyre et al. 2012). Water-soluble carbohydrates (WSC) are stored in the form of glucose, fructose, sucrose, starch and mainly fructan (Halford et al. 2011).

It has been estimated that in wheat around 10–20 % of the stem reserves are mobilized towards grain-filling under irrigated conditions and up to 50 % or more under severe stress condition (Rattey et al. 2009). Broad sense heritability of WSC has been reported to be high ($h^2 = 0.9$) in wheat (Ruuska et al. 2006). Rebetzke

et al. (2008) reported that WSC is negatively and significantly correlated to days to anthesis, number of tillers per square metre, number of grains per square meter and biomass and positively correlated with grain weight and GY under water stress condition. They established a relationship between stem morphological properties viz. stem length, diameter and solidness, WSC and GY under drought stress conditions and this reinforces the potential use of stem morphology in breeding for high WSC and ultimately higher GY under water stress environment.

In wheat, QTLs for WSC were identified on chromosomes 1A, 1BL/1RS, 1D, 2B, 2D, 3D, 4A, 4B, 6A, 6B, 7A, 7B and 7D explaining up to 4 % of PV (Yang et al. 2007; McIntyre et al. 2010). Yang et al. (2007) identified QTLs in wheat on 6AS and 6BS regions for 1,000-grain weight, grain-filling efficiency at early and late stage and stem WSC at the maturity stage. Since fructan 1-exohydrolase (*1FEH-6A*) and (*1FEH-6B*) were mapped to 6AS and 6BS, respectively. The high gene product of *FEH* genes in stem and sheath indicate that it is the major gene contributing to *FEH* activity to facilitate fructan degradation and later grain-filling in wheat. In wheat, QTL located on 7A were found to be co-located with sucrose synthase gene which is the strong candidate for explaining WSC. Sucrose synthase, an important enzyme in carbohydrate metabolism, catalyzes the reversible conversion of sucrose and UDP to UDP-glucose and fructose in vitro (McIntyre et al. 2010).

Fructans are the major component of temporary carbon reserve in the stem of temperate cereals, which is used for grain-filling. Fructan is synthesized from sucrose by the action of a group of enzymes called fructosyltransferases. The activity of this enzyme is dependent on sucrose concentration and hence fructan accumulation is high if sucrose content is high. Three families of fructosyltransferases are directly involved in fructan synthesis in the vacuole of *Triticum aestivum*. The regulatory network of the fructan synthetic pathway is largely unknown. Recently, a sucrose up-regulated wheat MYB transcription factor (TaMYB13-1) was shown to be capable of activating the promoter activities of *sucrose*:*sucrose 1-fructosyltransferase* (*1-SST*) and *sucrose*:*fructan 6-fructosyltransferase* (*6-SFT*) in transient transactivation assays (Khoshro et al. 2014). *TaMYB13-1* over-expression resulted in up-regulation of all three families of fructosyltransferases including *fructan*:*fructan 1-fructosyltransferase* (*1-FFT*). A *γ-vacuolar processing enzyme* (*γ-VPE1*), potentially involved in processing the maturation of fructosyltransferases in the vacuole, was also up-regulated by *TaMYB13-1* over-expression. Multiple *TaMYB13* DNA-binding motifs were identified in the *Ta1-FFT1* and *Taγ-VPE1* promoters and were bound strongly by *TaMYB13-1*. The expression profiles of these target genes and *TaMYB13-1* were highly correlated in recombinant inbred lines during stem development as well as the transgenic and non-transgenic wheat dataset, further supporting a direct regulation of these genes by *TaMYB13-1*. Over-expression of *TaMYB13-1* in wheat led to enhanced fructan accumulation in the leaves and stems and also increased spike weight and grain weight per spike in transgenic plants under water-limited conditions.

QTLs for WSC have been detected in rice on chromosome 4 and 5 (Wang et al. 2008; Ishimaru et al. 2005). In rice, QTL *GIF1*, *GRAIN INCOMPLETE FILLING 1* that encodes a cell-wall invertase gene required for carbon partitioning during early grain-filling on chromosome 4 (Wang et al. 2008; Huang et al. 2013, Table 11.2) and QTL *rg5* for ratio of filled grains were mapped on chromosome 5 (Ishimaru et al. 2005, Table 11.2). The cultivated *GIF1* gene shows a restricted expression pattern during grain-filling compared to the wild rice allele (Table 11.2). The near-isogenic rice line carrying *rg5* gene showed high sink activity and increased assimilate partitioning to particularly inferior caryopses in the late stage and thus showed higher ratio of filled grains (Ishimaru et al. 2005). Several transcription factors such as *Carbon Starved Anther* (*CSA*; Zhang et al. 2010) and *Rice Starch Regulator1* (*RSR1*; Fu and Xue 2010) were shown to be linked to nutrient and carbon partitioning. These genes could be useful to increase assimilate partitioning and grain architecture in crops.

Together this information suggests that increasing WSC via marker-assisted selection (MAS) in breeding programmes should be possible as many of these QTL loci appear to be conserved across diverse populations.

11.2.6 Coleoptile Length

Selection of wheat cultivars with long coleoptile is an important component of improving emergence under dry environments. Wheat with long coleoptiles emerges with higher frequency than those with short coleoptiles especially when sown deep. Wheat seedlings with short coleoptile do emerge but much later and lack seedling vigour (Rebetzke et al. 2007). Deep sowing allows growers to use soil moisture lying below the drying topsoil and is an option considered by growers in Australia and wheat planted in stored soil moisture in water-limited environments in India. Temperature and dwarfing genes (*Rht-B1b* and *Rht-D1b*) influence coleoptile length (CL). In order to combine dwarf phenotype with greater CL GA-sensitive *Rht* genes could be used to select shorter height wheat cultivar to retain the yield advantages (Richards et al. 2010).

In wheat, QTLs for CL were identified on chromosomes 1A, 1B, 4B, 4D, 5A, 5B and 6A, 6B, 7D and these are repeatable across temperatures and populations (Singh and Khanna-Chopra 2010). Rebetzke et al. (2001) have identified a major QTL coinciding with *Rht-B1* on chromosome arm 4BS, which accounts for up to 42 % of PV in CL across a range of temperatures. They also identified major CL QTL, located close to the marker locus *XksuC2* on chromosome arm 4BL, which accounted for up to 27 % of PV. Two major QTLs were mapped to the *Rht-B1b* locus flanked by the marker *XcsME1* on chromosome arm 4BS and the *Rht-D1b* locus on chromosome arm 4DS and explained up to 49 % of PV for CL in four wheat cultivars (Rebetzke et al. 2007, Table 11.1). Landjeva et al. (2008) identified five QTLs for CL on 1A, 1B, 6B and 7D which explaining up to 51 % of PV in wheat RIL population derived from Opata and W7984. Recently, two major novel

and consistent QTLs for CL on chromosomes 4BS and 3BS has been identified in WL711/C306 RIL population in wheat. Two candidate genes namely *gibberellin C-20 oxidase 1* and *α-Expansin* underlying genomic regions were identified which play a role in cell wall expansion in the young expanding tissue in the coleoptile (Singh et al. 2014). Expansins are encoded by a superfamily of genes that are organized into four families. These proteins loosen the cell wall in a pH-dependent manner and are hypothesized to break hydrogen bonds between hemicelluloses and cellulose microfibrils, thereby allowing turgor-driven cell enlargement. Previous reports have provided evidences that expansins are associated with environmental stress tolerance in plants. Over-expression of an expansin gene, *RhEXPA4*, in Arabidopsis confers strong DR to transgenic plants (Han et al. 2014). Xing et al. (2009) isolated *β-Expansin* gene (*TaEXPB23*) from wheat coleoptiles and transformed to tobacco. Over-expression of *TaEXPB23* in tobacco resulted in accelerating growth of leaves and internodes at the earlier developmental stages and also involved in regulating plant development.

There is dearth in information of QTLs for CL in crops and hence there is need to identify the more genomic region and associated candidate gene for CL in crop plants for better establishment and improve crop productivity.

11.3 Conclusion

Drought resistance is a quantitative trait with complex phenotype and genetic control (MacWillium 1989). There is need to understand the genetic basis of drought resistance in crops in order to develop superior genotypes through conventional breeding. Marker-assisted selection has not contributed significantly to cultivar improvement for dry environments because of the complexity of genetic control of drought resistance and breeding has relied on direct phenotypic selection. The genetic control of drought resistance is complex as it is multigenic, has low heritability and high $G \times E$ interactions. The empirical selection for yield under water-limited condition has yielded modern cultivars with good performance under stress environments (Tester and Langridge 2010). However, these selections are suited to specific environments and have less wider adaptability due to variable nature of drought stress environments.

Marker-assisted selection based around screening for desirable alleles at QTL for drought resistance is an important approach for improving drought resistance in crops. Hundreds of QTLs for the diverse traits related to drought resistance have been mapped but only a small portion of them can be repeatedly detected in different environments and populations and a few have been verified and cloned (Hu and Xiong 2014). A recent survey of molecular markers being deployed in wheat breeding programmes failed to identify a single case, where a drought or drought-related marker was being implemented (Gupta et al. 2010). This strongly implies that the previous drought QTL studies have failed to identify loci of value to wheat breeding programmes. Accurate and high-throughput phenotyping of

drought resistance traits especially in field conditions is currently the major limiting factor for both genetic dissection and breeding of drought resistance in crops.

Successful cloning of QTLs for drought resistance traits will enable us to better understand the genetic basis of the traits and more effectively manipulate the traits for drought resistance breeding. Newly emerging breeding approaches such as marker-assisted backcrossing, marker-assisted recurrent selection and genome-wide selection provide more powerful tools for pyramiding multiple QTLs or integrating multiple traits for drought resistance. More integrative studies that link the genetics, genomics, physiology, system biology and agronomics of drought resistance will advance our knowledge of drought resistance and assist the breeding programme for drought resistance in crops.

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