Molecular aspects of abiotic stress in plants

REVIEW

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ABSTRACT

Drought, salinity and extreme temperature are major adverse environmental factors that limit plant productivity. Sensors initiate a signaling cascade to transmit the signal and activate nuclear transcription factors to induce the expression of specific sets of genes. Ionic and osmotic stress signal transduction triggers the ionic and osmotic homeostasis signaling pathways, detoxification response pathways, and pathways for growth regulation. The ionic stress is signaled via the SOS pathway where an SOS3-SOS2 complex controls the expression and activity of ion transporters. Osmotic stress activates several protein kinases which mediate osmotic homeostasis and/or detoxification responses. Understanding the mechanisms by which plants perceive and transduce the stress signals to initiate adaptive responses is essential for engineering stress-tolerant crop plants. Genetic engineering strategies rely on the transfer of one or several genes that are either involved in signaling and regulatory pathways, or that encode enzymes present in pathways leading to the synthesis of functional and structural protectants, or that encode stress-tolerance-conferring proteins. Certain techniques have been described to identify the gene whose expression is differentially regulated in response to various environmental stresses in higher plants. Such methods include differential display- polymerase chain reaction, suppression subtractive hybridization, serial analysis of gene expression, DNA microarray and cDNA-amplified fragment length polymorphism.

Keywords: abiotic stress, ionic/osmotic stress, gene expression, signal transduction, stress-inducible genes, transcription factor, transgenic plants

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RESUMEN

Aspectos moleculares del estrés abiótico en plantas. Los factores ambientales tales como sequía, salinidad y temperaturas extremas afectan significativamente la productividad de los cultivos. Los receptores inician la cascada de señales para transmitir la señal y activar a los factores de trascripción nucleares e inducir la expresión de grupos específicos de genes. Las señales de transducción del estrés iónico y osmótico conducen a las vías de señalización de la homeostasis iónica y osmótica, vías de destoxificación y vías de regulación del crecimiento. El estrés iónico se señaliza a través de la vía del SOS donde el complejo SOS3-SOS2 controla la expresión y la actividad de los transportadores iónicos. El estrés osmótico activa un conjunto de proteínas kinasas que conducen a la homeostasis osmótica y/o a respuestas de destoxificación. La comprensión de los mecanismos por los cuales las plantas perciben y transducen las señales del estrés para iniciar la respuesta de adaptación es esencial para la obtención de plantas transgénicas tolerantes al estrés. Las estrategias de ingeniería genética se basan en la transferencia de uno o varios genes que están involucrados en las vías de señalización y regulación, que codifican enzimas de las vías que guían la síntesis de protectores funcionales y estructurales o que codifican proteínas que confieren tolerancia al estrés. Se describen algunas técnicas empleadas para identificar los genes cuya expresión es diferencialmente regulada en respuesta a varios estreses en plantas superiores. Tales métodos incluyen expresión diferencial-reacción en cadena de la polimerasa, supresión por hibridación substractiva, análisis seriado de la expresión génica, microarreglos de ADN y polimorfismo de la longitud de los fragmentos de ADNc amplificados.

Palabras claves: estrés abiótico, estrés iónico y osmótico, expresión génica, transducción de señales, genes inducibles por estrés, factores de transcripción, plantas transgénicas

*I*ntroduction

Environmental stresses, such as drought, salinity, cold and heat cause adverse effects on the growth of plants and the productivity of crops. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50%. Among the abiotic factors that have shaped and continue shaping plant evolution, water availability is the most important. Water stress in its broadest sense encompasses both drought and salt stress. Drought and salinity are becoming particularly widespread in many regions, and may cause serious salinization of more than 50% of all arable lands by the year 2050 [1].

Drought and salt stress, together with low temperature, are the major problems for agriculture because these adverse environmental factors prevent plants from realizing their full genetic potential. Abiotic stress leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity [2]. Drought, salinity, extreme temperatures and oxidative stress are often interconnected, and may induce similar cellular damage. They are very complex stimuli that possess many different yet related attributes, each of which may provide the plant cell with quite different information. For example, low temperature may immediately result in mechanical constraints, changes in the activities of macromolecules, and reduced osmotic potential in the cellular milieu [3].

High salt stress disrupts homeostasis in water potential (osmotic homeostasis) and ion distribution (ionic

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homeostasis). This disruption of homeostasis occurs at both the cellular and the whole plant levels. Drastic changes in ion and water homeostasis lead to molecular damage, growth arrest and even death. To achieve salt tolerance, three interconnected aspects of plant activities are important. First, damage must be prevented or alleviated. Second, homeostatic conditions must be re-established in the new, stressful environment. Third, growth must resume, albeit at a reduced rate [4].

Signaling pathways are induced in response to environmental stress and recent molecular and genetic studies have revealed that these pathways involve many components. The multiplicity of information embedded in abiotic stress signals underlies one aspect of the complexity of stress signaling [5]. Nevertheless, most studies on water stress signaling have focused on primarily salt stress because plant responses to salt and drought are closely related and the mechanisms overlap [6, 7]. Responses to stress are not linear pathways, but are complicated integrated circuits involving multiple pathways and specific cellular compartments, tissues, and the interaction of additional cofactors and/or signaling molecules to coordinate a specified response to a given stimulus [8]. Plants respond to these stresses at molecular and cellular levels as well as physiological level. Expression of a variety of genes has been demonstrated to be induced by these stresses. The products of these genes are thought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction in stress response [9, 10].

Salt- and drought-tolerant plants maintain their turgor at low water potentials by increasing the number of solute molecules in the cell. The active transport of solutes depends on the proton gradients established by proton pumps. In plants, three distinct proton pumps generate proton electrochemical gradients across cell membranes. Plant vacuoles constitute 40-90% of the total intracellular volume of a mature plant cell and, in concert with the cytosol, generate the cell turgor responsible for growth and plant rigidity. Cell turgor depends on the activity of vacuolar H^+ pumps that maintain the H^+ electrochemical gradient across the vacuolar membrane, which permits the secondary active transport of inorganic ions, organic acids, sugars, and other compounds. The accumulation of these solutes is required to maintain the internal water balance. In principle, increased vacuolar solute accumulation could confer salt and drought tolerance. The sequestration of ions such as sodium could increase the osmotic pressure of the plant and at the same time reduce the toxic effects of this cation. Exposure to NaCl has been shown to induce the H^+ transport activity of vacuolar pumps in both salt-tolerant and salt-sensitive plants. In principle, enhanced expression of either one of the vacuolar proton pumps should increase the sequestration of ions in the vacuole by increasing the availability of protons [11].

Changes in the electrical potential difference across the plasma membrane of higher plants have been shown to be among the most rapid alterations induced by abiotic and biotic stresses. The sensitivity of the electrical membrane potential to different stimuli suggests that the electrogenic exchange of ions across the plasma membrane could serve for the transduction of signals perceived at the plasma membrane. Thus a hyperpolarization-activated influx of Ca^{2+} into the host cell could provide a pathway for the elevation of cytosolic free Ca^{2+} concentrations that mediate the induction of several biochemical pathways that are part of the plant defense response. Biochemical responses associated with plant defense mechanisms are inhibited by the depletion of extracellular Ca^{2+} or stimulated in the presence of ionophores that permitted the entry of Ca^{2+} into the cells suggesting that fluctuations in cytosolic Ca^{2+} are required for an effective defense response by the cell [12].

The objective of this review is to report recent advances in the stress-response mechanisms and their biotechnological applications in plants. The main mechanisms such as signal transduction pathways, regulation of gene expression, ion transport, and detoxification mechanisms are described. Emphasis is given to transgenic plants that were engineered for stress tolerance, based on different mechanisms of stress response.

Multiplicity of signaling pathways

Sensors

Sensors are molecules that perceive the initial stress signal. Sensors will initiate (or suppress) a cascade to transmit the signal intracellularly and in many cases, activate nuclear transcription factors to induce the expression of specific sets of genes. A single sensor might only regulate branches of the signaling cascade that are initiated by one aspect of the stress condition. For example, a sensor detecting low temperature would initiate a signaling cascade responsive to membrane fluidity but would not necessarily control signaling initiated by an intracellular protein whose conformation/activity is directly altered by low temperature [3].

Drought, salt and cold stresses have been shown to induce transient Ca^{2+} influx into the cell cytoplasm derived from either influx from the apoplastic space or release from internal stores. Channels responsible for the Ca^{2+} influx represent one type of sensor for the stress signals. Internal Ca^{2+} release is controlled by ligand-sensitive Ca^{2+} channels. These ligands are second messengers. An important feature of the role of Ca^{2+} as a signal is the presence of repetitive Ca^{2+} transients. These transients may be generated both by first round second messengers and by signaling molecules such as abscisic acid (ABA) that may themselves be produced as a result of cascades of early Ca^{2+} signals.

Receptor-like kinases (RLKs) are found in both animals and plants. Structurally, they consist of an extracellular domain that may function in ligand binding or protein-protein interactions, a transmembrane domain and an intracellular kinase domain. The two-component sensor-response regulator systems involving histidine kinases that were initially found in prokaryotes for the perception of various environmental signals also exist in eukaryotes, including plants. When the extracellular sensor domain perceives a signal, the cytoplasmic histidine residue is autophosphorylated and the phosphoryl moiety is then passed to an aspartate receiver in a response regulator, which may constitute part of the sensor protein or a separate protein. The sensors may couple with a downstream mitogen-activated protein kinase (MAPK) cascade or directly phos-

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phorylate specific targets to initiate cellular responses. Upon receiving a signal from membrane receptors, cells often utilize multiple phosphoprotein cascades to transduce and amplify the information. Protein phosphorylation and dephosphorylation are perhaps the most common intracellular signaling modes. They regulate a wide range of cellular processes such as enzyme activation, assembly of macromolecules, protein localization and degradation. Secondary signals [i.e., hormones and second messengers: inositol phosphates and reactive oxygen species (ROS)] can initiate another cascade of signaling events, which can differ from the primary signaling in time and space [13].

Signal transduction pathways

Signal transduction networks for cold, drought, and salt stress can be divided into three major signaling types (Figure 1): (I) osmotic/oxidative stress signaling that uses MAPK modules, involves the generation of ROS scavenging enzymes and antioxidant compounds as well as osmolytes; (II) Ca^{2+} dependent signaling that lead to the activation of late embryogenesis abundant (LEA)-type genes (such as the DRE/CRT class of genes), involves the production of stress-responsive proteins mostly of undefined functions and (III) Ca^{2+} dependent salt overlay sensitive (SOS) signaling that regulates ion homeostasis. It involves the SOS pathway which is specific to ionic stress [14].

Osmotic/oxidative stress signaling

Salt, drought, heat, cold stress and oxidative stress are accompanied by the formation of ROS such as superoxide, hydrogen peroxide, and hydroxyl radicals, causing extensive cellular damage and inhibition of pho-

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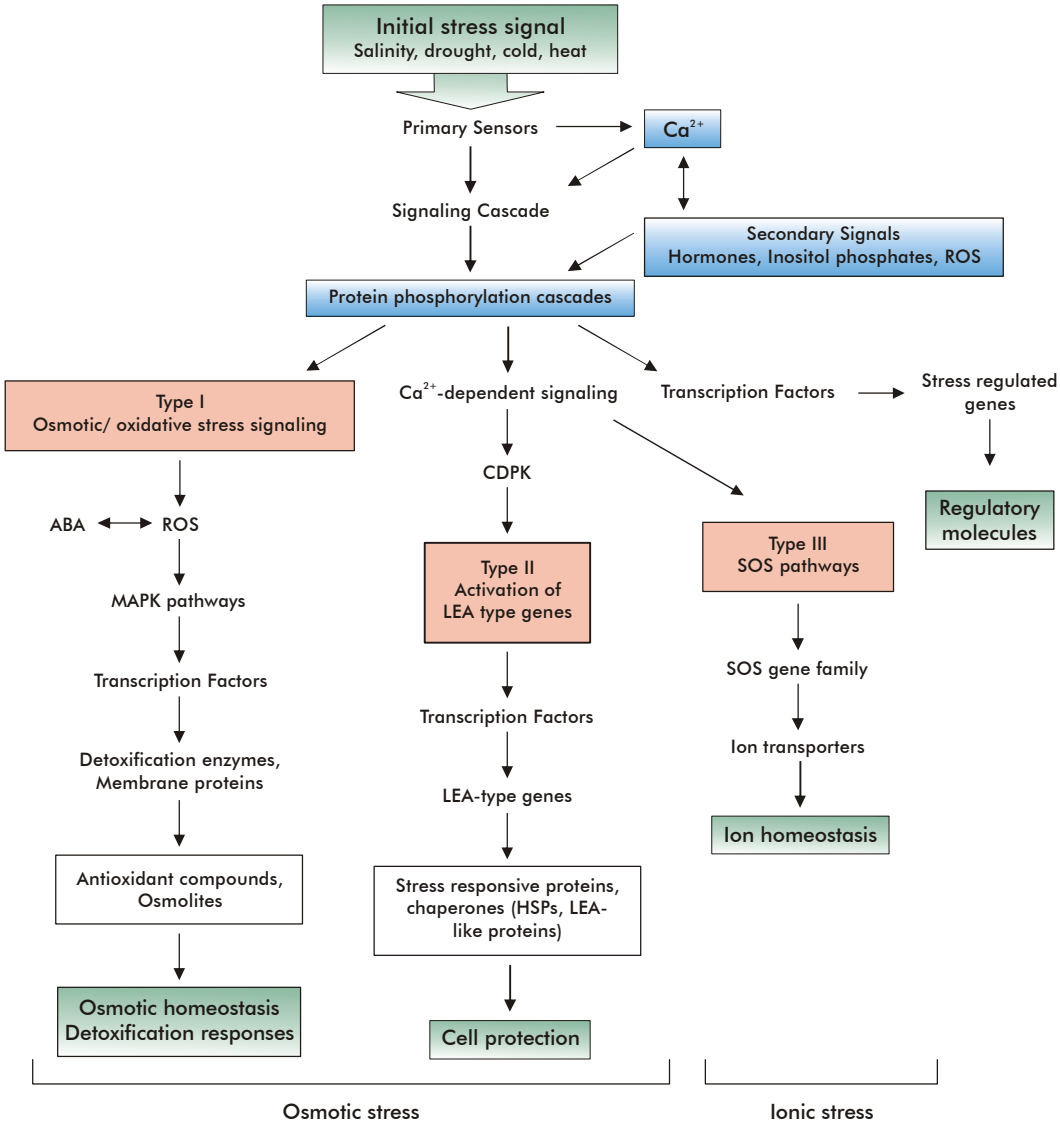

Figure 1. Schematic pathway for the transduction of osmotic and ionic stress in plants. A signal transduction pathway starts with signal perception, followed by the generation of second messengers (e.g., inositol phosphates and reactive oxygen species [ROS]). Second messengers can modulate intracellular Ca^{2+} levels, often initiating a protein phosphorylation cascade that finally targets proteins directly involved in cellular protection or transcription factors controlling specific sets of stress-regulated genes.

tosynthesis. This phenomenon is called oxidative stress and is known as one of the major causes of plant damage as a result of environmental stresses [15].

These ROS may be signals inducing ROS scavengers and other protective mechanisms, as well as damaging agents contributing to stress injury in plants. Plants have developed several antioxidation strategies to scavenge these toxic compounds. Enhancement of antioxidant defense in plants can thus increase tolerance to different stress factor antioxidants (ROS scavengers) and include enzymes such as catalase, superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase, as well as non-enzyme molecules such as ascorbate, glutathione, carotenoids, and anthocyanins. Additional compounds, such as osmolytes, proteins and amphiphylic molecules (e.g. tocopherol), can also function as ROS scavengers [14].

Osmotic/oxidative stress signaling is a phosphoprotein module used in plants for abiotic stress signaling. MAPK pathways also mediate ROS signaling. The MAPK pathway is activated by receptors/sensors such as protein tyrosine kinases, G-protein-coupled receptors, and two-component histidine kinases in response to osmotic stress and is responsible for increased production of osmolytes that are important for osmotic adjustment. The primary function of osmolytes is to maintain cell turgor and thus the driving gradient for water uptake. Recent studies indicate that compatible solutes can also act as free-radical scavengers or chemical chaperones by directly stabilizing membranes and/or proteins Compatible solutes fall into three major groups: amino acids (e.g. proline), quaternary amines (e.g. glycine betaine, dimethylsulfoniopropionate) and polyol/sugars (e.g. mannitol, trehalose) [16].

Parts of several MAPK modules that may be involved in osmotic stress signaling have been identified in alfalfa and tobacco. Salt stress can activate different MAPKs at different times after the onset of stress, and the activities of these MAPKs also last for different time periods. A common observation both in plants and in other organisms is that one MAPK module can be used for the transmission of multiple signals. The MAP kinase pathways are intracellular signal modules that mediate signal transduction from the cell surface to the nucleus. MAPK cascades are likely conserved in all eukaryotes. They seem to be widely used as osmolarity signaling modules. The core MAPK cascades consist of 3 kinases that are activated sequentially by an upstream kinase. The MAP kinase kinase kinase (MAPKKK), upon activation, phosphorylates a MAP kinase kinase (MAPKK) on serine and threonine residues. This dual-specificity MAPKK in turn phosphorylates a MAP kinase (MAPK) on conserved tyrosine and threonine residues. The activated MAPK can then either migrate to the nucleus to activate the transcription factor directly, or activate additional signal components to regulate gene expression, cytoskeleton-associated proteins or enzyme activities, or target certain signal proteins for degradation [14].

Osmotic stress activates several protein kinases including mitogen-activated kinases, which may mediate osmotic homeostasis and/or detoxification responses. A number of phospholipid systems are activated by osmotic stress, generating a diverse array of messenger molecules, some of which may function upstream of the osmotic stress-activated protein kinases. Abscisic acid biosynthesis is regulated by osmotic stress at multiple steps. Both ABA -dependent and -independent osmotic stress signaling first modify constitutively expressed transcription factors, leading to the expression of early response transcriptional activators, which then activate downstream stress tolerance effector genes [6].

Ca^{2+}-dependent signaling that lead to the activation of LEA-type genes

Ca^{2+} is involved in various intracellular signaling processes, both in animals and plants. As such, the concentration of intracellular Ca^{2+} is carefully tuned. Ca^{2+} concentration in the cytosol is low, and upon stimulation, Ca^{2+} is released from intracellular storage or enters the cell via various Ca^{2+} channels. Cold, drought and salinity have been shown to induce transient Ca^{2+} influx into the cell cytoplasm. Channels responsible for this Ca^{2+} influx represent one type of sensor for these stress signals. Calcium-dependent protein kinases (CDPKs) are implicated as important sensors of Ca^{2+} influx in plants in response to these stresses. CDPKs are serine/threonine protein kinases with a C-terminal calmodulin-like domain with up to 4 EF-hand motifs that can directly bind Ca^{2+}. CDPKs are encoded by multigene families, and the expression levels of these genes are spatially and temporally controlled throughout development. In addition, a subset of CDPK genes responds to external stimuli. The CDPK pathway seems more connected to increasing the expression of LEA proteins for anti-desiccation protection [17].

Pathways leading to the activation of LEA-type genes including the dehydration-responsive element (DRE)/C-repeat (CRT) class of stress-responsive genes may be different from the pathways regulating osmolyte production. The activation of LEA-type genes may actually represent damage repair pathways [18]. During development and maturation, when natural desiccation takes place, seeds accumulate transcripts and proteins at a relatively high concentration; for this reason, when first found, proteins were named LEA proteins. LEA proteins accumulate under conditions of extreme desiccation in higher plants [19]. Water deficit, high osmolarity, and low temperature stress results in the accumulation of a group of LEA proteins. Such proteins may preserve protein structure and membrane integrity by binding water, preventing protein denaturation or renaturing unfolded proteins, and sequestering ions in stressed tissues. LEA proteins and chaperones have shown to be involved in protecting macromolecules like enzymes, lipids and mRNAs from dehydration [9]. LEA proteins have been grouped into at least six families on the basis of sequence similarity [20, 21].

Three biological systems seem to act in concert to achieve desiccation tolerance: enzymes involved in osmolyte synthesis; proteins specialized in desiccation protection of membranes and proteins (LEA proteins), and antioxidant enzymes and molecules. Both osmolytes and LEA proteins contribute to stabilization of membrane and protein structures by conferring preferential hydration at moderate desiccation

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and replacing water at extreme desiccation. Osmolytes also contribute to osmotic adjustment and act as hydroxyl radical scavengers [22].

Ca^{2+} dependent SOS signaling that regulates ion homeostasis

SOS signaling appears to be relatively specific for the ionic aspect of salt stress and is calcium-dependent. The targets of this type of signaling are ion transporters that control ion homeostasis under salt stress.

The input of the SOS pathway is likely excess extracellular or intracellular Na^+, which somehow triggers a cytoplasmic Ca^{2+} signal. The outputs are expression and activity changes of transporters for ions such as Na^+, K^+, and H^+. The input for osmotic stress signaling is likely a change in turgor. Salt stress signal transduction consists of ionic and osmotic homeostasis signaling pathways, detoxification (e.g. damage control and repair) response pathways, and pathways for growth regulation. The ionic aspect of salt stress is signaled via the SOS pathway where a calcium-responsive SOS3-SOS2 protein kinase complex controls the expression and activity of ion transporters such as SOS1 [6].

Genetic analysis indicated that SOS1, SOS2 and SOS3 function in a common pathway in controlling salt tolerance. An important group of Ca^{2+} sensors in plants is the SOS3 family of Ca^{2+} binding proteins. The amino acid sequence of SOS3 is most closely related to the regulatory subunit of yeast calcineurin (CNB) and animal neuronal calcium sensors. A loss of function mutation in the *Arabidopsis* SOS3 gene renders the mutant plants hypersensitive to NaCl. Interestingly, the salt-hypersensitive phenotype of SOS3 mutant plants can be partially rescued by increased concentrations of Ca^{2+} in growth media. Thus, SOS3 may underlie part of the molecular basis for the long-observed phenomenon that higher external Ca^{2+} can alleviate salt toxicity in plants. There is genetic evidence for regulation of the *Arabidopsis* SOS1 transporter by a calcium-activated protein kinase complex composed of the SOS2 kinase subunit and of the SOS3 calcium-binding subunit. This signaling pathway mediates salt induction of the SOS1 gene in *Arabidopsis*. In addition, the SOS2-SOS3 kinase directly phosphorylates and activates the SOS1 transporter [14, 17].

Studies comparing the growth of wild-type and mutant plants in response to NaCl, and sequence analysis of the predicted SOS1 protein suggested that SOS1 encodes a Na^+/H^+ exchanger (antiporter) on the plasma membrane [23]. Because the SOS pathway operates during ionic stress, it is thought that homologs of SOS3 and SOS2 may also function in the transduction of other stress or hormonal signals. Including SOS2 and SOS3, *Arabidopsis* has eight SOS3-like Ca^{2+} binding proteins and 22 SOS2-like protein kinases [24].

Transient increases in cytosolic Ca^{2+} are perceived by various Ca^{2+} binding proteins. In the case of abiotic stress signaling, evidence suggests that Ca^{2+}-dependent protein kinases (CDPKs) and the SOS3 family of Ca^{2+} sensors are major players in coupling this universal inorganic signal to specific protein phosphorylation cascades. It seems that calcium signaling is crucial for salt tolerance in plants [14].

Second messengers can modulate intracellular Ca^{+2} levels, often initiating a protein phosphorylation cascade that finally targets proteins directly involved in cellular protection or transcription factors controlling specific sets of stress-regulated genes. The products of these genes may participate in the generation of regulatory molecules like the plant hormones ABA, ethylene, and salicylic acid (SA).

During biotic or abiotic stress, plants produce increased amounts of hormones such as ABA and ethylene. In addition, SA and perhaps jasmonic acid may be involved in some parts of stress responses. These hormones may interact with one another in regulating stress signaling and plant stress tolerance. Salt, drought, and to some extent, cold stress cause an increased biosynthesis and accumulation of ABA by activating genes coding for ABA biosynthetic enzymes, which can be rapidly catabolized following the relief of stress Many stress-responsive genes are upregulated by ABA. In addition, ABA can feedback stimulate the expression of ABA biosynthetic genes, also likely through a Ca^{2+}-dependent phosphoprotein cascade. The observation that ROS may mediate both ABA signaling and ABA biosynthesis suggests that the feedback regulation of ABA biosynthetic genes by ABA may be mediated in part by ROS through a protein phosphorylation cascade [25].

These regulatory molecules can, in turn, initiate a second round of signaling that may follow the above generic pathway, although different components are often involved. The secondary signals may also differ in specificity from primary stimuli, may be shared by different stress pathways, and may underlie the interaction among signaling pathways for different stresses and stress cross-protection. Therefore, one primary stress condition may activate multiple signaling pathways differing in time, space, and outputs. These pathways may connect or interact with one another using shared components generating intertwined networks [3, 16, 26, 27].

Possible outputs of osmotic signaling pathways include gene expression and/or activation of osmolyte biosynthesis enzymes as well as water and osmolyte transport systems. Most of the other changes induced by salt or drought stress can be considered as part of detoxification signaling. These include (a) phospholipid hydrolysis; (b) changes in the expression of LEA/dehydrin-type genes, molecular chaperones, and proteinases that remove denatured proteins; and (c) activation of enzymes involved in the generation and removal of reactive oxygen species and other detoxification proteins [6].

Abiotic stress-inducible genes

The complex plant response to abiotic stress involves many genes and biochemical-molecular mechanisms. Various genes respond to drought-stress in various species, and functions of their gene products have been predicted from sequence homology with known proteins. Many drought-inducible genes are also induced by salt stress and low temperature, which suggests the existence of similar mechanisms of stress responses. Genes induced during drought-stress conditions are thought to function not only in protecting cells from water deficit by the production of impor-

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tant metabolic proteins but also in the regulation of genes for signal transduction in the drought stress response [9, 10, 28]. Thus, these gene products are classified into three major groups. (1) those that encode products that directly protect plant cells against stresses such as heat stress proteins (HSPs) or chaperones, LEA proteins, osmoprotec-tants, antifreeze proteins, detoxification enzymes and free-radical scavengers [1, 26]; (2) those that are involved in signaling cascades and in transcriptional control, such as MAPK, CDPK [29] and SOS kinase [30], phospholipases [31], and transcriptional factors [32, 33]; (3) those that are involved in water and ion uptake and transport such as aquaporins and ion transporters [34].

Stress-inducible genes have been used to improve the stress tolerance of plants by gene transfer. It is important to analyze the functions of stress-inducible genes not only to understand the molecular mechanisms of stress tolerance and the responses of higher plants, but also to improve the stress tolerance of crops by gene manipulation. Hundreds of genes are thought to be involved in abiotic stress responses [35].

Regulation of gene expression by transcription factors

Transcription factors (TFs) are small molecules that attach to specific sites on a DNA molecule in order to activate or deactivate the expression of certain genes. Plant genomes contain a large number of transcription factors; for example, *Arabidopsis* dedicates about 5.9% of its genome coding for more than 1 500 TFs [36]. Most of these TFs belong to a few large multigene families. Individual members of the same family often respond differently to various stress stimuli; on the other hand, some stress responsive genes may share the same TFs, as indicated by the significant overlap of the gene-expression profiles that are induced in response to different stresses [37-40]. As a consequence, these diverse environmental stresses often activate similar cell signaling pathways and cellular responses, such as the production of stress proteins, up-regulation of anti-oxidants and accumulation of compatible solutes [41].

Transcription-factor genes were found among the stress-inducible genes, suggesting that various transcriptional regulatory mechanisms function in the drought, cold or high-salinity stress signal transduction pathways. Molecular and genomic analyses have shown that several different transcriptional regulatory systems are involved in stress-responsive gene induction. Several different sets of cis- and trans-acting factors are known to be involved in stress-responsive transcription. Some of them are controlled by ABA but others are not, indicating the involvement of both ABA-dependent and -independent regulatory systems for stress-responsive gene expression [6, 42].

An understanding of gene regulation is particularly important in the case of a multigenic trait like desiccation tolerance because different regulatory pathways determine the expression of a whole set of genes. Knowledge of regulatory circuits is scarce; individual factors have been characterized, but their interaction with other molecules within the network is for the most part unknown. Several experimental approaches have been followed to identify molecules involved in the activation of gene expression in response to stress. Most information is derived from promoter analyses and from differential screening procedures [32]. The regulation of gene expression by dehydration and ABA involves several signaling pathways and different cis-acting elements in the stress-responsive genes. In genes regulated by ABA and osmotic stress, one or more ABA-response elements (ABREs) play a key role in promoter activity. The ABREs have a core ACGT-containing G-box motif. Proteins binding to this ABRE contain basic region leucine zipper (bZIP) motifs [13, 43, 44]. The ABRE and the dehydration response element (DRE) are promoters that play an important role in regulating gene expression in response to drought stress. The DRE is also involved in low-temperature and salt responsive gene expression [14, 45].

These stress-inducible transcription factors include members of the DRE-binding protein (DREB) family, the ethylene-responsive element binding factor (ERF) family, the zinc-finger family, the basic helix-loop-helix (bHLH) family, the basic-domain leucine zipper (bZIP) family and the homeodomain transcription factor family. These transcription factors could regulate various stress inducible genes cooperatively or separately, and may constitute gene networks. The transcription factor DREB1A specifically interacts with the DRE and induces the expression of stress tolerance genes. Overexpression of the cDNA encoding DREB1A in transgenic *Arabidopsis* plants activated the expression of stress tolerance genes under normal growing conditions and resulted in improved tolerance to drought, salt loading, and freezing [9, 46]. Functional analysis of these stress-inducible transcription factors should provide more information on the complex regulatory gene networks that are involved in responses to drought, cold and high salinity stresses. ABRE is a major cis-acting element in ABA-responsive gene expression [47]. Similar transcription factors DREB2A and DREB2B are activated by osmotic stress and may confer osmotic stress induction of target stress-responsive genes [14].

Plants also have some transcriptional factors with unique DNA binding domains such as the AP2: EREBP domain. In the activation of abiotic stress-responsive genes, it seems that there is no general rule regarding which class of transcriptional factors activates which class of stress-responsive genes. Instead, there could be several kinds of transcriptional factors regulating one group of stress-responsive genes, or even several transcriptional factors that can cooperatively activate the same gene. Proteins binding to this ABA-responsive complex contain bZIP motifs. These bZIP proteins include, for example, wheat EmBP1, the tobacco TAF-1, rice OSBZ8 and osZIP-1a. Some of these transcriptional activators are themselves induced at the transcriptional level by ABA or stress treatments [13].

All organisms respond to supraoptimal temperatures by synthesizing a specific set of HSP. They are needed to protect cells from heat damage, and they assist in the normalization of functions during recovery. Heat stress proteins can be assigned to families of proteins conserved among bacteria, plants and animals.

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HSPs are molecular chaperones essential for maintenance and/or restoration of protein homeostasis. As molecular chaperones they assist in folding, intracellular distribution, assembly and degradation of proteins, mainly by stabilizing partially unfolded states. Denaturation of proteins and problems in the processing of newly synthesized proteins du-ring stress are assumed to result in a decrease of the pool of free chaperones. The transcription of HSP encoding genes is controlled by regulatory proteins called heat stress transcription factors (Hsfs). They exist as inactive proteins mostly found in the cytoplasm.

The ability to examine the expression of many genes simultaneously has led to major advances in the understanding of gene regulatory networks. More than half of the drought inducible genes are also induced by high salinity and/or by ABA but only 10% are induced by cold stress. The control of gene expression by TFs is being dissected using a genomic approach. There are various possibilities for decreasing TF expression: using knockout mutants or RNAi constructs, or for increasing TF levels by overexpression, either constitutively or with the use of an inducible promoter. Changing the expression of a number of TFs affects signaling, particularly the responses to disease and abiotic stress [48].

Isolation of strees-responsive genes

Expression profiling has become an important tool to investigate how an organism responds to environmental changes. Plants have the ability to alter their gene expression patterns in response to environmental changes such as temperature, water availability or the presence of deleterious levels of ions. Sometimes these transcriptional changes are successful adaptations leading to tolerance while at other times the plant ultimately fails to adapt to the new environment and is considered to be sensitive to that condition. Expression profiling can define both tolerant and sensitive responses. These profiles of plant response to environmental extremes are expected to lead to regulators that will be useful in biotechnological approaches to improve stress tolerance as well as to new tools for studying regulatory genetic circuitry [49].

Gene isolation and cloning through molecular biology research can be based on RNA or protein expression, differential screening, differential display technique, DNA insertions such as transposon or T-DNA insertions, map based cloning and methods of random cDNA sequencing and genome sequencing. The recent upsurge in activities concerned with identifying genes with unknown functions through research on expressed sequence tags (ESTs) and sequencing of total genomes is a boon for stress work. However, genes identified, isolated and cloned by such approaches would need to be functionally-characterized [50].

Certain techniques have been employed to identify the gene whose expression is differentially regulated in response to various environmental stresses in higher plants. Such methods include differential display polymerase chain reaction (DDPCR), suppression subtractive hybridization (SSH), serial analysis of gene expression (SAGE), DNA-chip and microarray, and cDNA-amplified fragment length polymorphism (AFLP).

Differential display-polymerase chain reaction (DD-PCR) is one of several methods designed to identify differentially induced or expressed genes and has been used successfully in many studies to identify new genes in various tissues or cells. Differential display polymerase chain reaction is a simple, sensitive and powerful method for screening cDNAs, and is useful in characterizing tissue-, organ- or development-specific cDNAs. Differential display was developed as a method to identify differences in gene expression among eukaryotic cells. Different primer combinations are used in a reverse transcriptase (RT)-PCR to generate cDNAs from mRNAs expressed in a given cell. By comparing the cDNAs derived from multiple cell types, or from a single cell type under different conditions, it is possible to detect differences in transcription products derived from the diverse conditions. These differentially expressed products are identified on an acrylamide gel, excised, eluted, re-amplified, and eventually sequenced. Liu and Baird [51] reported that 17 cDNA clones were isolated from sunflower by means of DD-PCR. Genes corresponding to 13 of these cDNAs were confirmed by quantitative RT-PCR to be expressed differentially in response to osmotic stress. Their expression patterns were analyzed in leaves of drought-stressed plants, and in roots and shoots of drought- or salinity- stressed seedlings.

To understand the molecular regulation of the stress response, the relevant subsets of differentially expressed genes of interest must be identified, cloned, and studied in detail. Suppression subtractive hybridization (SSH) has been a powerful approach to identify and isolate cDNAs of differentially expressed genes. In general, they involve hybridization of cDNA from one population (tester) to an excess of mRNA (cDNA) from other population (driver) and then the separation of the unhybridized fraction (target) from hybridized common sequences. These subtraction techniques often require more than 20 mg of poly(A)RNA, involve multiple or repeated subtraction steps, and are labor intensive [52].

Serial analysis of gene expression (SAGE) is a powerful tool that allows the analysis of overall gene expression patterns with digital analysis. Because SAGE does not require a preexisting clone, it can be used to identify and quantitate new, as well as known genes. SAGE is a method for the comprehensive analysis of gene expression patterns. Three principles underlie the SAGE methodology: a short sequence tag (10-14bp) that contains sufficient information to uniquely identify a transcript provided that the tag is obtained from a unique position within each transcript; sequence tags can be linked together to form long serial molecules that can be cloned and sequenced; and the quantitation of the number of times a particular tag is observed provides the expression level of the corresponding transcript. Large-scale identification of genes expressed in roots of the model plant *Arabidopsis* was performed by SAGE, on a total of 144 083 sequenced tags, representing at least 15 964 different mRNAs. This new resource enabled the characterization of the expression of more than 3 000 genes [53].

The use of DNA microarrays provides insight into tissue- and developmental-specific expression of

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genes and the response of gene expression to environmental stimuli. DNA microarray technology has given rise to the study of functional genomics. The entire set of genes of an organism can be microarrayed on an area as small as a fingernail and the expression levels of thousands of genes are simultaneously studied in a single experiment. DNA microarray technology allows comparisons of gene expression levels on a genomic scale in all kinds of combinations of samples derived from normal and diseased tissues, treated and non-treated time courses, and different stages of differentiation or development. Further computational analysis of microarray data provides the classification of known or unknown genes by their mRNA expression patterns. Gene expression profiling using cDNA microarrays or gene chips is a useful approach for analyzing the expression pat-terns of genes under conditions of drought, cold and high salinity [54-56].

The cDNA-AFLP is a RNA finger printing technique used to analyze genes that are differentially expressed in genotypes with contrasting stress tolerance grown under normal and stress conditions. In this technology, double stranded cDNA is digested with two restriction enzymes, adapter molecules are ligated to the cDNA's and PCR amplification is performed with a primer that is complementary to the adapter, but has an additional 1-3 selective bases. The products are separated on a sequencing gel [57, 58].

cDNA-AFLP was used to analyze differentially expressed genes in wheat RH8706-49, a salt-stress resistant line (SR) and H8706-34, a salt-stress sensitive line (SS), with or without NaCl stress. A large number of gene fragments related to salt stress were found. Among them, a cDNA encoding glycogen synthase kinase-shaggy kinase (TaGSK1) showed to be induced by NaCl stress, and expressed more strongly in SR than in SS. These results suggest that TaGSK1 is involved in wheat response to salt stress as a part of the signal transduction component [59].

Functional genomics

In the development of genomic technologies that provide structural and functional information, gene characterization has received a significant boost during the last few years. The recent discovery of promoter regulatory elements, like DRE or ABRE involved in both dehydration- and low-temperature-induced gene expression in *Arabidopsis*, as well as the identification of transcriptional factors interacting with those promoters, are exciting developments. The characterization of the genes involved in the initiation phase of the stress response should be a logical priority, since they represent the "upstream keys" to global genomic responses that might involve hundreds of genes. Moreover, once they have been identified, the expression of these key genes should serve as a "timing reference" to identify expression products from downstream genes involved in stress responses [60].

The new field of functional genomics will provide useful information through profiling experiments and/or through the candidate gene approach based on the genomic location or function of interest. The candidate gene approach is facilitated by looking at the large number of sequences and freely available gene information found in plant genetic databases to identify potential candidate genes and pathways involved in drought tolerance [38].

There are two different approaches to study the function of stress proteins. One, called the inducible approach, considers stress proteins as those induced in cells during stress. The other, the functional approach, considers stress proteins as those crucial for cellular defense against a particular stress. The inducible approach is based on differential screening, differential display, and modern microarray methodologies. The functional approach implies that mutational gain or loss of function of genes encoding functional stress proteins should result in altered stress tolerance. Functional stress proteins may be constitutive and become activated during stress by modification instead of synthesis. Therefore, it is the functional approach that may provide the tools for genetic engineering of improved stress tolerance. The genes encoding proteins which act as positive crucial factors in salt tolerance are called halotolerance genes and are recognized by their capability to improve salt tolerance upon overexpression [17].

Genetic engineering

The application of transgenic technology, through either over- or under-expression of the transgenes, is a powerful way of finding the role(s) of these discovered genes. Molecular analysis of transgenic plants should provide information on the relationship of different gene products with stress tolerance and it will be interesting to see how these transgenic plants perform under real stress situations in the field, and thus contribute to sustainable agriculture.

The genetically complex responses to abiotic stress conditions are very difficult to control and engineer. Present engineering strategies rely on the transfer of one or several genes that are either involved in signaling and regulatory pathways, or that encode enzymes present in pathways leading to the synthesis of functional and structural protectants, such as osmolytes and antioxidants, or that encode stress-tolerance-conferring proteins. The current efforts to improve plant stress tolerance by gene transformation have resulted in important achievements; however, the nature of the genetically complex mechanisms of abiotic stress tolerance, and the potential detrimental side effects, make this task extremely difficult [16].

The use of transgene as a reporter in genetic analysis signal transduction has many advantages, especially where visible phenotypes fall short. A chimeric gene consisting of the promoter of a stress-responsive gene and a convenient reporter gene is introduced into plants and transgenics are used as starting material for mutagenesis. The main objective is to generate transgenic plants that synthesize a high level of an osmoprotectant or a protein only under stress conditions [13, 30].

Strong and constitutive promoters are beneficial for a high-level expression of selectable marker genes, which is necessary for efficient selection and generation of transgenic plants. However, constitutively active promoters are not always desirable for plant genetic engineering because the constitutive overexpression of a transgene may compete for energy and building blocks

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for synthesis of proteins, RNA, etc, which are also required for plant growth under normal conditions [47].

Bioengineering stress-signaling pathways to produce stress-tolerant crops is one of the major goals of agricultural research. Recent developments in designing transgenic rice plants with genes such as choline oxidase (*codA*), D-pyrroline-5-corboxylate synthase (P5CS), LEA protein group 3 (HVA1), alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) have shown that abiotic stress tolerance can be improved in rice [61, 62].

Transgenic *Arabidopsis* carrying the *codA* gene showed tolerance for light stress and increased the accumulation of H_2O_2 and several other stress related chemicals. Rice does not accumulate glycine betaine unlike other plants, but Sakamoto, *et al*. [63] reported transgenic rice expressing the *codA* gene in the chloroplast and the cytosol recovered to normal growth at a faster rate than the wild type after an initial growth inhibition under salt and low-temperature stress.

A novel strategy was reported by Wu and Garg [11] which use the stress-inducible promoter to drive the overexpression of *Escherichia coli* trehalose biosynthetic genes (*otsA* and *otsB)* as a fusion gene (TPSP) for providing abiotic stress tolerance in rice. The TPSP fusion gene has the dual advantages of needing only a single transformation event to introduce both genes simultaneously into the rice genome, while at the same time increasing the catalytic efficiency for trehalose formation by the bifunctional enzyme [64, 65].

Transgenic tobacco plants overexpressing chloroplastic Cu/Zn-SOD showed increased resistance to oxidative stress caused by high light and low temperatures. Transgenic alfalfa (*Medicago sativa*) plants expressing Mn-SOD evidenced reduced injury from water-deficit stress, as determined by chlorophyll fluorescence, electrolyte leakage and re-growth. In *Arabidopsis*, overexpression of the chyB gene that encodes b-carotene hydroxylase (an enzyme active in the zeaxanthin biosynthetic pathway) resulted in a 2-fold increase in the pool of the xanthophyll cycle. These transgenic plants showed greater tolerance to high light and increased temperatures, and it was suggested that stress protection was most likely due to the action of zeaxanthin in preventing oxidative damage to membranes. Transgenic tobacco plants expressing alfalfa aldosealdehyde reductase, a stress-activated enzyme, showed reduced damage when exposed to oxidative stress and increased tolerance to heavy metals, salt and dehydration stress. Targeting detoxification pathways are an appropriate approach for obtaining plants with multiple stress-tolerance traits. Accumulation of compatible solutes may also protect plants against damage by scavenging reactive oxygen species, and by their chaperone-like activities in maintaining protein structures and functions. Engineered overproduction of these compatible solutes provides an opportunity to generate more tolerant plants [26, 66-68].

MAPK cascades play an important role in mediating stress responses in eukaryotic organisms. OsMAPK5 is a single-copy gene but can generate at least two differentially spliced transcripts. The OsMAPK5 gene, its protein, and kinase activity were inducible by abscisic acid as well as various biotic (pathogen infection) and abiotic (wounding, drought, salt, and cold) stresses. Interestingly, suppression of OsMAPK5 expression and its kinase activity resulted in the constitutive expression of pathogenesis-related (PR) genes such as PR1 and PR10 in the dsRNAi transgenic plants and significantly enhanced resistance to fungal and bacterial pathogens. However, these same dsRNAi lines showed significant reductions in drought, salt, and cold tolerance. In contrast, overexpression lines exhibited increased OsMAPK5 kinase activity and increased tolerance to drought, salt, and cold stresses. These results strongly suggest that OsMAPK5 can positively regulate drought, salt, and cold tolerance and negatively modulate PR gene expression and a broad-spectrum disease resistance. Such an opposite effect could be explained by the potential antagonism between distinct MAPK cascades [69, 70].

The transcription factors play an important role in the acquisition of stress tolerance, which may ultimately contribute to agricultural and environmental practices. Although plant transformation with stress responsive TFs permits overexpression of downstream stress-associated multiple genes, it may also activate additional non-stress genes that adversely affect the normal agronomic characteristics of a crop. One common negative effect of TF-modified plants is the growth retardation in transgenic plants that constitutively express TFs. These negative effects can be partially prevented by the use of stress-inducible promoters that control the expression of the TF [71, 72].

Engineering salt tolerance in plants

Plant halotolerance genes have been identified by a gain of functions in transgenic plants and by loss of functions in *Arabidopsis* mutant collections, followed by the identification of the mutated gene by either map-based cloning or tagging with T-DNA or transposons. Overexpression in transgenic plants of osmolyte synthesis enzymes, LEA proteins, and antioxidant defenses has resulted in improved salt tolerance [27].

Genetic engineering of salt tolerance has been achieved via different strategies. Transgenic improvements through the detoxification strategy triggers transgenic plants overexpressing enzymes involved in oxidative protection, such as glutathione peroxidase, superoxide dismutase, ascorbate peroxidases and glutathione reductases. More recent engineering with the regulatory protein NPK1, a mitogen-activated protein (MAP) kinase, is another good example; this protein kinase appears to mediate oxidative stress responses. Engineering with osmolytes such as mannitol, fructans, trehalose, ononitol, proline, glycinebetaine and ectoine also works through oxidative detoxification. These osmolytes are active in scavenging ROS. In addition, targeted production of the osmolytes in the chloroplast by placing a signal sequence in front of the engineered enzymes, results in better protection. The osmolyte-producing transgenic plants are improved in their tolerance not only to salts but also to various other stresses such as chilling, freezing, heat and drought, which also generate ROS. This is demonstrated clearly in glycinebetaine-producing plants [4].

The protecting mechanism of osmolytes engineered in transgenic plants cannot be explained by osmotic adjustment because the concentrations achieved are in the modest range of 1-50mM. More subtle effects of

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the osmolytes such as the scavenging of hydroxyl radicals and the protection of membrane and protein structures during desiccation may be involved. Water stress increases the formation of ROS through membrane perturbation of electron transport chains. The loss of catalase the function and the gain of the glutathione S-transferase/peroxidase function implicate defenses against oxidative damage as crucial factors in plant salt tolerance [17].

Another strategy for achieving greater tolerance is to help plants re-establish homeostasis in stressful environments. Both ionic and osmotic homeostasis must be restored. Various ion transporters are the terminal determinants of ionic homeostasis. Because Na^+ inhibits many enzymes, it is important to prevent Na^+ accumulating to a high level in the cytoplasm or in organelles other than the vacuole [4].

Concerning plant ion transporters involved in salt tolerance, none of them were isolated as halotolerance genes upon their expression in yeast. However, gain or loss of functions in plants demonstrated their crucial role in ion homeostasis [9]. Increased expression of the vacuolar H^+/Na^+ antiporter encoded by the NHX1 gene has improved salt tolerance in *Arabidopsis*, tomato [73], and *Brassica* [74] plants. Another vacuolar transporter, the AVP1 H^+-pumping pyrophosphatase, also improves salt tolerance upon overexpression in *Arabidopsis*. Presumably, both NHX1 and AVP1 mediate sequestration of sodium in the vacuole, reducing its toxic effect at the cytosol [17].

The SOS1 gene encodes a putative plasma membrane Na^+-H^+ antiporter. Mutations in SOS1 render *Arabidopsis* plants extremely sensitive to Na^+ stress. Overexpression of SOS1 lowers shoot Na^+ content and improves salt tolerance in *Arabidopsis* plants and callus tissues. As this antiporter is mostly expressed in parenchyma cells at the xylem/symplast boundary of roots, it has been proposed that it functions in retrieving sodium from the xylem stream, providing the molecular basis of «sodium exclusion» from the shoots [4, 9, 17].

A transport protein called AtNHX1 of *Arabidopsis thaliana* is located in membranes of cell vacuoles where it can protect the plant from the drying effect of salt by transporting sodium ions from the cell cytoplasm into the vacuole. Vacuoles function as large storage compartments of sodium. In the vacuoles, sodium has no toxic effect. Overexpression of the AtNHX1 gene (increased production of AtNHX1) resulted in enhanced salt-tolerance of *Arabidopsis thaliana***.** Other finding also showed that overexpression of the AtNHX1 gene also protects greenhouse-grown tomatoes from high salt concentrations. Fruit sodium content of the genetically modified tomatoes was low because the plants store the sodium in the leaf vacuoles. This trait prevents the plants from a quality loss as well as from toxic effects of sodium in cells and the salt impaired nutrient acquisition. Fruits of the transgenic plants were somewhat smaller but in other respects no significant differences to non-transgenic plants were observed [73].

Ruiz and Blumwald [75] showed that S-assimilation and the synthesis of cysteine and reduced glutathione (GSH) increased significantly when wild-type *Brassica napus* was exposed to salt stress. On the other hand, these changes were minimal in transgenic salt tolerant *B. napus* plants, overexpressing a vacuolar Na^+/H^+ antiporter. These results suggest that the processes leading to the biosynthesis of GSH are salt-stress-elicited and that the active accumulation of excess Na^+ in the vacuole of the transgenic plants minimizes the stress response. The authors concluded that in wild-type *Brassica* plants, salt stress induced an increase in the assimilation of S and the biosynthesis of cysteine, and GSH was aimed to mitigate the salt-induced oxidative stress. The small changes seen in the salt-tolerant transgenic *Brassica* plants overexpressing the vacuolar Na^+/H^+ antiporter suggest that the accumulation of excess Na^+ in the vacuoles greatly diminished the salt-induced oxidative stress, highlighting the important role of Na^+ homeostasis during salt stress.

The halotolerance genes already identified indicate that the crucial factors for salt tolerance in plants include Na^+ transport and Na^+ toxicity targets. In addition, plant halotolerance genes include defenses against osmotic and oxidative stresses. The genetic analysis indicates that enzymes involved in osmolyte synthesis, osmoprotecting LEA proteins and antioxidant enzymes such as catalases, and glutathione S-transferase/peroxidades are crucial factors for plant salt tolerance [17].

Conclusions

Signaling pathways have to be regarded as complex networks. The signal transduction network is characterized by multiple points of convergence and divergence that enable signal integration at different levels, and provide the molecular basis for the appropriate downstream responses that characterize them.

Molecular analyses of the signaling factors provide a better understanding of the signal-transduction cascades during abiotic stress. Growth is limited predominantly by osmotic stress, but in species that have a high rate of salt uptake, or cannot compartmentalize salt effectively in vacuoles, salt-specific effects that develop with time, impose an additional stress on the plant and give rise to the categories of 'salt-sensitive' and 'salt-tolerant' plants. This implies that any improvement in drought resistance would make a plant more adapted to saline soil. However, the processes that adapt a plant specifically to saline soil involve the regulation of the uptake and how they compartmentalize salt, to delay as long as possible the time it takes to accumulate toxic levels in leaves that are actively photosynthesizing. Breeding or genetic engineering of plants better adapted to saline soils should focus on these processes.

Transgenic plants have been analyzed to reveal the function of stress-responsive loci in plants. The reverse genetic approach will be even more important in extending the understanding of regulatory factors in stress signaling.

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