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5 Genetic manipulation of plants for increased drought tolerance

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Abstract

On a world basis, water availability is considered the main constraint for crop production. Concerns about water accessibility have always accompanied crop production in dry areas, which are on the other hand the most extensive areas for agriculture on the earth. As a consequence, men had to develop agricultural strategies to cope with water shortage, growing the plants during the short climatic interval of water availability and selecting plants possessing a relatively superior tolerance to water deficiency. Although development of drought tolerant plants by conventional breeding methods has resulted in modest

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achievements, it is expected that genetic transformation contribute to widen still more the water stress tolerance range of plants. A great detail is currently known on the physiology and molecular biology of plants subjected to water stress, from the perception of the stimulus to the response module responsible for reducing the detrimental effects of water deprivation. Part of this deep understanding about the molecular basis of the plant response to water stress has been derived from other model organisms, such as yeast, and it is now being landed into practical grounds to modify the genome of plants for increasing their water stress tolerance. From the existing experimental evidence several conclusions can be drawn. First, there is enormous and complex information concerning the transduction pathways and molecular responses of plants to water deficit, but less is known about the receptors sensing suboptimal osmotic conditions in plants. Second, practically all genetic modifications tested up to now, from the initial perception steps to the final response module, have resulted in an improved response of plants to water stress, with osmolyte pathway alteration being the most studied approach. Third, if a logical scientific framework is to be developed, it is evident the urgency for testing the physiological and productive performance of the genetically modified crops under field conditions in order to define the most robust approach to be adopted in future work. Several issues remain to be addressed in upcoming research: Should we introduce or modify a single pleiotropic gene localized upstream the response module? or Should we alter a gene or set of genes with more defined functions at the response module level? Is a multigenic approach, altering genes at the perception, transduction and/or response module, a more robust strategy for increasing drought tolerance? Can the chloroplast transformation technology assist in improving the effect of the genes already established as conferring water stress tolerance?

Introduction

Arid and semiarid regions are characterized by a very stochastic environment, where biotic and abiotic factors exert determinant influences on ecosystem structure and functioning and restrict distribution and productivity of plants. Among these environmental factors water availability and salinity play major role in determining changes in productivity and community composition (1, 2, 3, and 4). Drought and salinity affect more than 10 percent of crop areas, and land desertification and salinization are rapidly increasing on a global scale decreasing the potential yields for most major crops by more than 50% (5, 6). Droughts affect more people than any other natural hazard (7) and economical losses because of this factor are enormous. The Federal Emergency Management Agency estimates that droughts in the United States cause an average annual economic loss of $6-8 billion dollars (8). This

situation will be seriously aggravated if global warming, as predicted, exerts in the near future a negative effect on agricultural production on some areas, especially in the tropics and subtropics.

In dry areas, rainfall constitutes the major water supply for vegetation survival and growth. This water supply is limited to a brief period of time and shows great year-to-year variation. In some regions such as the North American desert rainfall is concentrated in the summer, so that the favorable conditions for plant growth are restricted to a few months. In addition, this limited period of water accessibility shows a great variation in amount as well as in monthly distribution among years.

This scarce and highly variable water supply imposes a severe selection pressure on wild plants. Facing this erratic water supply plants were naturally selected, through evolutionary time, for specific traits which permitted them to circumvent eventual periods of water shortage. Strategies developed by plants to prevent water deficits are related to avoidance or tolerance mechanisms. Plants evading water tissue deficits develop large roots in order to reach deep water tables, accumulate water in succulent tissues during favorable episodes or complete their life cycle rapidly during the brief period of water availability. In contrast to evaders, 'genuine' tolerant plants can withstand low levels of water in their tissues. This latter category of plants is the subject of this chapter of the book.

Water stress tolerance mechanisms can be visualized as a function of the plant organization level under analysis: cell (osmotic adjustment), tissue (water storage tissues), organ (deep or fast growing roots) and whole plant (water conductance).

At the extreme of the drought stress tolerant plants are those possessing desiccation tolerance. Desiccation tolerance is the ability of an organism to survive the loss of most (>95%) of its cellular water. Plants possessing this capacity can revive from an air-dried state and, therefore, they are sometimes referred as "resurrection plants". This is the severest form of water stress, since most protoplasmic water is lost from the cell. It is considered to be a complex trait that is present in reproductive structures (pollen and seeds) of vascular plants. Desiccation tolerance in vegetative tissues is also relatively common in less complex plants such as bryophytes and lichens but rare in pteridophytes and angiosperms and absent in gymnosperms. Desiccation tolerance operates different in angiosperms and some lower plants such as bryophytes and lichens. In the former, desiccation tolerance is based on induction of several relatively complex protection mechanisms that function during drying, with minimal dependence on repair of desiccation-produced damage during rehydration. Conversely in bryophytes and lichens, desiccation tolerance is constitutive and relies mainly on mechanisms directed to repair cell damage following rehydration (9).

Cultivated crops are generally more sensitive to water stress than wild plants because the former are the result of the anthropogenic selection for specific productive traits which are not always linked to drought tolerance.

Drought results in a suboptimal water status in plants known as osmotic stress. Environmental stresses conducive to osmotic stress include chilling, heat, freezing, drought and salinity. A situation of osmotic stress will arise from suboptimal conditions in the external osmotic pressure: water depletion, flooding or high external solute concentrations. Osmotic stress is most commonly utilized in reference to hyperosmotic stress, a condition resulting from an external water deficiency causing a concomitant increase in concentration of solutes and efflux of water from the cell into the environment. When the extracellular solute concentrations are raised or extracellular ice forms, there is a flux of water from the cells to the surrounding environment, causing a decrease in turgor and an increase in concentration of intracellular solutes. Thus, with the exception of flooding, all major abiotic stresses (drought, freezing, chilling, salinity, heat) result in water deficit stress.

Salt, cold and drought are considered as agents causing an hyperosmostic condition because they all produce a depletion of cellular water. In this regard, it is important to mention that because they produce the same effect in the water status of cells some of the molecular responses to these stresses overlap in part. An additional interconnection of abiotic stress response of plants to pathogens has been sometimes reported (10) because they are assumed to impose an osmotic stress condition on plants.

Genetic engineering of plants has opened the possibility of manipulating the plant genome towards more specific and ambitious objectives, studying gene structure and function as well as determining the relation of gene expression to causal factors. Consequently the application spectrum of molecular technologies is increasing. In this context, technologies such as microarrays and related methodologies, reserve a great potential for the study of genes involved in the response of plants to water stress. Analysis of the genetic expression of plants under water deficit conditions will allow in a future the isolation of genes that for production of transgenic plants possessing an increased by tolerance to drought with the expectation that this technology will result in benefits in crop yield and water saving in areas with severe water restrictions.

Effects of water deficit on physiology of plants

Adaptation to drought is undoubtedly one of the most complex biological processes. It involves numerous changes including reduced growth, transcriptional activation/inactivation of specific genes, transient increases in ABA levels, accumulation of compatible solutes and protective enzymes, increased levels of antioxidants and suppression of energy-consuming pathways.

Drought reduces plant productivity by inhibiting growth and photosynthesis (11). A positive correlation between photosynthesis rate and crop yield is commonly found (12), but factors changing assimilate partitioning and utilization can reduce this association (13). Alteration of growth patterns in plants contributes to survival under water depletion conditions. An increase in root to shoot ratio is found commonly in physiological studies on the effects of drought on plants. Growth arrest can be considered as a medium by which plants can preserve carbohydrates for sustained metabolism, prolong energy supply and recovery faster after stress relief. On the other hand, continuation of root growth increases the exploratory capacity of plants in deeper more humid soil layers.

Reduction of photosynthesis under restricted water supply is caused by stomatal and metabolic effects. Which factor in more important for this reduction has a matter of intense debate since the earliest reports on the effects of drought on photosynthesis (14). Water deficit produces stomatal closure and thereby decreases intercellular CO_2 concentrations, whereas dehydration of the mesophyll cells damages the photosynthetic apparatus. Under conditions in which photosynthesis is impaired and chloroplasts are exposed to excess excitation energy, there is a photoreduction of oxygen that results in a concomitant production of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), the superoxide anion, and hydroxyl radicals (15), which in turn damage membranes and enzymes. In this regard, it is considered that photosystem II (PSII) is more sensitive to drought stress than photosystem I (PSI; 16). Although major ROS production induced by hyperosmotic stress occurs at intracellular sites, it was also shown that a cell wall diamine oxidase (17) and a plasma membrane NADPH oxidase (18) were activated by hyperosmolarity and drought, respectively.

Perception, transduction and response of plants to water deficit

An integral understanding of the stress perception and the resultant signal cascade that finally ends in the generation of a response or a set of responses directed to reduce the impact of the water deficit in plants is complicated given the great variety of elements participating in these processes: ABA, citokinins, auxins, giberelins and ethylene as primary elements in signal transduction events, calcium and IP_3 as secondary messengers in stress response, and compatible solutes, HSP's (Heat Sock Proteins), LEAs (Late Embryogenesis Abundant proteins), osmotin, ROS-protective proteins and aquaporins as response elements for adaptation to osmotic stress.

The regulatory circuits include osmotic stress sensors, signaling pathways comprising a network of protein-protein interactions, transcription factors and

promoters, and the final output proteins or metabolites which are responsible for cell adaptation to osmotic stress.

In order to obtain a clearer picture of the processes of perception-transduction-response of plants to drought stress a compartmentalization into modules can be invoked (19).

Perception (phosphorelay) module

It refers to how the plant cell perceives the environmental stimulus which is converted in an appropriated signal generated to activate the protective cellular mechanisms against the detrimental effects of the water deficit.

In bacteria, histidine kinases function as sensor molecules that transmit external signals to the cytoplasm. This transduction is mediated by phosphotransfer to the associated response regulator. This simple signaling entity is called a two-component system. Commonly, this two-component system is constituted of two types or proteins, a sensory histidine kinase and a response regulator. A typical histidine kinase contains an N-terminal receiver domain with an invariant aspartate residue and a C-terminal output domain. The input domain of the histidine kinase detects suboptimal osmotic conditions and selectively promotes autophosphorylation of a histidine residue within its transmitter domain. After autophosphorylation, the phosphoryl group is transferred to the aspartate residue in the receiver domain of the cognate response regulator.

A two component system is also present in yeast. This perception module consisting of two structurally unrelated membrane-spanning proteins function during conditions of high osmolarity (Sln1 and Sho1). Sln1 is a two-component regulatory system that functions as a phosphorelay between three proteins (Sln1, Ypd1 and Ssk1). When an hyperosmolarity condition presents, these proteins turn on the High Osmolarity Glycerol (HOG1) signaling system. Sln1 and Sho1 regulate a unique branch of the Hog1 pathway which converges on the Mitogen-Activated Protein kinase (MAPK) Pbs2. The yeast osmosensor Sln1 is a fused two-component system with two membrane-spanning domains and a cytoplasmic histidine kinase domain, which is homologous to sensor histidine kinases of bacteria, fungi and plants. It autophosphorylates the histidine residue in the N-terminal sensor domain and then transfers the phosphate group to an aspartate residue in the C-terminal-located response-regulator domain. The phosphate is subsequently transferred to Ypd1, which functions as a second histidine phosphorelay intermediate between the Sln1 and the response regulator Ssk1.

Sln1 is constitutively active in media of constant osmotic pressure, where it inhibits the activity of Ssk1 (20). An increase in external osmotic pressure (i.e., an increase in external solute concentration), results in inactivation (dephosphorylation) of Sln1 and accumulation of dephosphorylated Ssk1,

which is an activator of two redundant MAPKK kinases Ssk2 and Ssk22 (21,22), proteins responsible of activating Pbs2. The second putative high-osmolarity sensor, Sho1, contains four transmembrane domains and an intracellular Src homology 3 (SH3) domain, which binds a proline-rich region of Pbs2 (23). Under conditions of high osmolarity, Sho1 utilizes Ste20 and Ste50 to activate the MAPKK kinase Ste11 (24), which subsequently activates Pbs2.

The physical-chemical mechanism by which plant cells perceive osmotic changes in their environment is not completely known. An increase in external osmotic pressure caused by either water loss or concentrating solutes (e.g. salt) will result in the reduction of and subsequently loss of turgor, and eventually in cell dehydration. Loss of cell turgor is generally considered the signal which turns on the protective apparatus of the cell. However, careful measurements of biochemical responses of cells to osmotic stress indicate that osmosensing is not entirely mediated through turgor changes (25). There is evidence for active interaction between the plasma membrane and the cell wall (26). Changes in cell turgor will affect this interaction. Plasma membrane proteins interacting with the cell wall may subsequently undergo conformational changes and thus relay this signal to the cell response machinery.

In plants, an *Arabidopsis* homolog of Sln1 has been identified, AtHK1, which is able to suppress the salt-sensitive phenotype of the yeast double-mutant *sln1Δ sho1Δ*, which lacks both osmosensors (27). However, because no interaction has been observed between AtHK1 and the response regulators (28), direct evidence for a role of AtHK1 as an osmosensor in plants is still lacking. Another candidates for acting as osmotic receptors in plants are CRE1 (29, 30) and NtC7 (31). CRE1 is a protein with similarity to the cytoplasmic and receiver domains of SLN1, originally identified as a cytokinin receptor that is able to complement the yeast *sln1Δ* mutant. NtC7, a protein originally identified as wounding-responsive, is a membrane-associated receptor-like protein whose conformation may be sensitive to changes in membrane structure to sense hyperosmolarity conditions. Because this protein lacks a kinase catalytic domain it has been suggested that transduction of the osmotic signal to cytoplasmic components is mediated by interaction through its C-terminal tail with protein associates. Thus, several osmosensors would seem to be involved in plant stress signaling perception. Conclusive evidence is still scarce (32, 33). As drought, salinity, cold and other environmental factors that result in hyperosmotic conditions induce specific responses; it is possible that distinct osmosensors (or combinations of them) could be involved in different stress signaling pathways (33).

Transduction (MAP kinase cascade) module

A generic signal transduction pathway starts with stimulus perception, followed by the generation of second messengers (e.g., inositol phosphates and

reactive oxygen species). Second messengers are low-weight diffusible molecules that are used in signal transduction to relay signals within a cell. They are synthesized or released by specific enzymatic reactions, usually as a result of an external signal that was received by a transmembrane receptor and pre-processed by other membrane-associated proteins. There are three basic types of second messenger molecules:

- Hydrophobic molecules like diacylglycerol, IP_3 (inositol triphosphate) and phosphatidylinositols are membrane-associated and diffuse from the plasma membrane into the juxtamembrane space where they can reach and regulate membrane-associated effector proteins.
- Hydrophilic molecules are water-soluble molecules, like cAMP, cGMP, and Ca^{2+}, that are located within the cytosol.
- Gases, nitric oxide (NO) and carbon monoxide (CO) that can diffuse both through cytosol and across cellular membranes.

These intracellular messengers have some properties in common. They can be synthesized/released and broken down again in specific reactions by enzymes. Some (like Ca^{2+}) can be stored in special organelles and quickly released when needed. Their production/release and destruction can be localized, enabling the cell to limit space and time of signal activity.

Second messengers can modulate intracellular Ca^{2+} levels, often initiating a protein phosphorylation cascade that finally targets proteins directly involved in cellular adaptation or transcription factors controlling specific sets of osmotic regulated genes. The proteins encoded by these genes may participate in the generation of regulatory molecules like the plant hormones abscisic acid (ABA), ethylene, and salicylic acid (SA). 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. Signal transduction requires a correct spatial and temporal coordination of all signaling molecules. Therefore, there are certain molecules that participate in the modification, delivery, or assembly of signaling components, but do not directly transmit the signal. These proteins include protein modifiers (e.g., enzymes implicated in protein lipidation, methylation, glycosylation, and ubiquitination), scaffolds, and adaptors (34).

In yeast, once the stimulus is perceived and converted in an appropriated signal at Pbs2 in yeast, a cascade of successive phosphorylations, via Pbs2, Pbs2P and $Pbs2P_2$, finally derivates in an activated state of Hog1, $HOG1P_2$. This phosphorylated form of Hog1 enters the nucleus to activate gene expression. Mitogen-activated protein kinase (MAPK) cascades are common signaling modules found in both higher and lower eukaryotic cells. Activation of a MAPK results in modification of a set of target proteins, often

transcription factors, that allow the generation of appropriate cellular responses to an external stimulus. Stress-activated protein kinases (SAPKs) are a subset of MAPKs activated by environmental and genotoxic stresses (35). Hog1 constitutes a prototype of the SAPK family, which specifically responds to increased external osmotic pressure and is required for cell survival under hyperosmotic conditions.

Protein phosphorylation is one of the major mechanisms by which plants control cellular processes in response to external cues. In plants, two classes of stress-activated protein kinases, mitogen-activated protein kinases (MAPK's) and calcium-dependent protein kinases (CDPK's), have so far been reported to integrate multiple environmental stresses and undergo rapid biochemical activation upon exposure to biotic and abiotic stressors.

MAP kinase activation is based on the phosphorylation of conserved threonine and tyrosine residues in the TEY (Thr, Glu, Tyr) activation loop by a specific MAP kinase (MAPKK). A MAPKK kinase (MAPKKK) activates by phosphorylation of conserved threonine and/or serine residues. At the end of the phosphorylations cascade, activation of a cytoplasmic MAPK often results in its translocation into the nucleus where this terminal kinase is able to activate genes through phosphorylation of transcription factors (36). In other cases, these terminal MAPK's remains in the cytoplasma where they may phosphorylate enzymes or cytoskeleton components. MAPK circuits may integrate a variety of upstream signals interacting with other kinases or G proteins (37).

In this regard, the plasma membrane plays an important function in perception and transmission of environmental signals. Osmotic stress commonly leads to an altered membrane fluidity and changes in phospholipids have recently been recognized as important mediating osmotic stress signals in plants (38). It is proposed that phospholipids are cleaved by phospholipases, which produce phopholipid-derived second messengers. According to their cleavage site phospholipases are divided into: phospholipase C (PLC), phospholipase D (PLD) and phospholipase A1 (PLA1) and A2 (PLA2). Phospholipid signaling is regulated by G-proteins and may be closely linked with calcium. The major phospholipid-derived signaling molecules are: inositol 1,4,5-triphosphate (IP_3), diacylglycerol (DAG) and phosphatidic acid (PA).

Signal perception at the plasma membrane leads to the production of second messengers that initiate cascades of signaling events. It has been demonstrated that osmotic stress induces an increase in cytoplasmic calcium. This calcium signal results from external calcium influx and/or calcium release from intracellular sources. Hyperosmotic stress leads to an increase in IP_3 which is blocked by phospholipase C inhibitors (39). As IP_3 is known to trigger vacuolar calcium channels, it has been proposed that calcium is

released from intracellular supplies in response to hyperosmotic stress as a result of the activation of the IP_3 calcium-dependent channels.

Phosphatidic acid accumulates in response to water deficit (40). It can be formed directly by phospholipase D or indirectly by phospholipase D after phosphorylation of diacylglycerol. It has been demonstrated the activation by PA of several kinases, phosphatases and other proteins implicated in signal transduction (41, 42).

One of the best and largely documented physiological responses of plants to drought stress is the increased biosynthesis of the hormone abscisic acid (ABA; 43). Although this hormone induces the expression of several osmotic stress responsive genes, there are, on the other hand, a group of genes which do not respond to ABA. This differential plant response was the basis for proposing the so called ABA-dependent and ABA-independent signaling pathways (44), both involving a series of protein phosphorylations and dephosphorylations events.

Although several osmotic responsive genes are induced by ABA (e.g. LEA's and some compatible solutes such as proline), the question remains as to how ABA itself is induced by osmotic stress. Some of the components downstream of ABA have been elucidated recently by molecular genetic analysis. *AB11* encodes a 434 amino acid polypeptide with homology to protein phosphatase 2C of yeast (45), which contains an EF-hand domain for binding calcium (46). The relevance of this binding capacity is that it allows for cross talk with calcium signaling turned on by osmotic stress and/or other elicitors.

A role in signaling transduction has been attributed to H_2O_2, a molecule resulting from oxidative stress damage. Accumulation of hydrogen peroxide can, in turn, induce the expression of detoxification and stress protection proteins such as heat shock proteins (HSPs), glutathione-*S*-transferases (GSTs), peroxidases, superoxidases, osmolytes and pathogenesis-related proteins, all of them produced to protect the cell from stress damage (47). The induction of the catalase gene *CAT1* which is responsible for catalyzing the decomposition of H_2O_2 into molecular oxygen and water without the production of free radicals, was shown to be mediated by H_2O_2 itself (48). Thus, despite their toxicity at high concentrations, ROS paradoxically can act as second messengers in signaling pathways mediating their own inactivation.

Gene expression module

Among the genes under the control of the MAPK Hog1 are those implicated in carbohydrate metabolism, general stress protection, protein production, and signal transduction (49). One of the mechanisms by which SAPKs, and MAPKs in general, alter gene expression is by direct modification of transcription activators. In yeast, only four transcription factors, Sko1, Hot1, and the redundant Msn2 and Msn4, have been proposed to be controlled by

Hog1. Hot1, Msn2, and Msn4 activate transcription, whereas Sko1 represses and activates different types of osmotic-inducible and Hog1-regulated genes (50). Sko1 represses genes under non stress conditions by the recruitment of the general co-repressor complex Cyc8-Tup1. In response to osmotic stress, Sko1 is phosphorylated by Hog1, thereby relieving repression (51). Hot1 physically interacts with Hog1, and its binding to DNA and subsequent transactivation activity are regulated by Hog1 kinase activity (52).

Metabolic module

In response to osmotic stress, plant cells increase the expression of some genes while at the same time mRNA levels of others are decreased. New genes which are otherwise not expressed in the absence of osmotic stress may also begin to be expressed. Different groups of genes have been recognized as conferring drought tolerance in plants. According to their function during drought stress they can be grouped into genes encoding or implicated in the synthesis of compatible solutes, antioxidant enzymes, heat shock proteins (HSP's), late embryogenesis abundant proteins (LEA´s), aquaporines, polyamines, membrane stability, and proteins involved in synthesis, processing and degradation.

Compatible solutes

A substantial increase in cellular concentrations of osmotically active compounds, termed compatible solutes, has been observed in a vast number of organisms in response to salinity or drought stress (53, 54, 55). Inorganic solutes such as K^+, Na^+ and Cl^-, can also increase during osmotic stress but Na^+ and Cl^- interfere with cellular activities and have to be compartmentalized to the vacuole (56). Condition for compatibility is that a considerable increase in the concentration of these solutes does not interfere with normal metabolic functions of the cell.

Among the best known compatible solutes are: glycine betaine, amino acids (proline), sugars (sucrose, trehalose), sugar alcohols (manitol, sorbitol) and cyclitols. As much as 6% dry weight mass transfer from protein to proline can occur in some plants such as *Brassica napus* (57).

How these compatible solutes protect cells against damage from osmotic stress is still a matter of debate (58, 59). A first hypothesis was that compatible solutes may help cell conserving remnant water by accomplishing a biophysical function serving as water-attracting or water-conserving molecules that maintain in this way cell turgor. The hydrophilic nature of these compounds has been the support for the proposal that compatible solutes can stabilize proteins and cells structures (53) by replacing water at the surface of proteins, protein complexes or membranes. A further hypothesis is that compatible solutes function as scavengers of reactive oxygen species (60).

Particularly in yeast, after activation of transcription factors by Hog1, a set of genes are transcribed to overcome the detrimental effects of water stress. Two of the genes which are activated by Hog1 encode enzymes Gpd1 and Gpp2, which catalyze the conversion of dihydroxyacetonephosphate via glycerol-3-phosphate to the osmoregulator glycerol.

Antioxidant enzymes

As mentioned above plants under osmotic and other kind of stress produce reactive oxygen species (ROS). Activation of oxygen can occur by two different mechanisms: reversing of the spin of one of the unpaired electrons of oxygen or monovalent reduction. When triplet oxygen absorbs enough energy to reverse the spin of one of its unpaired electrons, a singlet state of oxygen will be produced, in which the two electrons have opposite spins. Singlet oxygen can then participates in reactions involving the simultaneous transfer of two electrons (divalent reduction) to organic molecules. The second mechanism of activation is mediated by stepwise monovalent reduction of oxygen to form superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and finally water. Superoxide can oxidize sulphur, ascorbic acid or NADPH or reduce cytochrome C and metal ions but, in general, ROS can damage lipids, proteins and DNA.

Chloroplasts are considered to be a focal point of ROS metabolism (61). ROS are mainly generated in this organelle by direct transfer of excess excitation energy from chlorophyll to produce singlet oxygen, or by univalent reduction of oxygen at photosystem I, in the Mehler reaction (62) and to some extent in mitochondria. Increased concentration of ROS inhibits the synthesis of protein D1, a component of the reaction center of photosystem II. This effect is probably mediated by translational inhibition of *psbA* gene, which encodes the precursor for D1 protein (63).

Complex systems for scavenging activated-oxygen exist in plant cells with complementary and interdependent mechanisms. In *Arabidopsis*, a network of least 152 genes control ROS metabolism (64). This network is thought to regulate the rates of ROS production and ROS scavenging in the different cellular compartments and to modulate the steady state level of ROS for signaling as well as defense purposes.

ROS detoxification systems can be broadly divided into enzymatic and nonenzymatic. Major nonenzymatic antioxidants include ascorbate (vitamin C), glutathione, tocopherol (vitamin E), flavonoids, alkaloids and carotenoids. Enzymatic antioxidants comprise superoxide dismutase, peroxidases and catalase. Additional enzymatic systems include aldehyde dehydrogenases, aldose/aldehyde reductases, peroxiredoxins, thioredoxins and peptide-methionine sulfoxide reductases (33).

In leaf cells, an intricate balance exists between H_2O_2 and O_2-production in the chloroplast and peroxisome during photosynthesis and the activities of the ROS-scavenging enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (65,66,67).

Some components such as carotenoids prevent the formation of activated oxygen by competing for the energy leaked from the photosystems. Other components are lipid soluble and reside in the membrane bilayer to terminate the lipid peroxidation chain reactions. Even others, such as ascorbate and glutathione, are aqueous scavengers that detoxify activated oxygen directly or serve to recycle other protective components back to their reduced state. The enzymes involved in catalyzing the synthesis, degradation and recycling of these antioxidants are essential for cell viability.

Heat shock proteins (HSP's)

HSP's have been reported to serve as molecular chaperones that participate in ATP-dependent protein unfolding or assembly/disassembly reactions and prevent protein denaturation during stress (68). Correlations between expression of HSPs and thermotolerance have been found in maize, tomato, and creeping bentgrass (69,70,71).

Late Embryogenesis Abundant Proteins (LEA´s)

Late embryogenesis abundant proteins are expressed in the seed following the desiccation stage after embryo maturation in all angiosperms and in vegetative tissues during osmotic stress provoked by cold, salt or drought. LEA´s include at least six subgroups based on sequence and expression profiles (72). Group 2 LEA's are also referred to as dehydrins or RAB's (73). Most LEA genes are responsive to ABA (74). As with compatible solutes a definitive the role of LEA´s in drought stress response has not been established. LEA´s have been proposed to function in water retention, ion sequestration and as molecular chaperones (72).

Aquaporins

These proteins facilitate the water movement through plasma membrane by creating water-specific pores within the lipid bilayer, increasing in this way the rate flow of the water. Aquaporins are members of a large family of membrane spanning proteins, the major intrinsic proteins (MIP's), and have very conserved structures. In general, MIP's are most abundantly expressed in rapidly growing tissues and in cells characterized by an intense water flux such as cells in the root. It seems that these protein channels can be facultatively expressed in response to the extracellular water potential. Smith-Espinoza et al. (75) demonstrated that a specific aquaporine gene was up-regulated by dehydration but down-regulated by salinity. The notion of water channels is

relatively new in plant biology and presently is the subject of intense investigation focused to elucidate the ecological and physiological significance of these proteins in the water relations of plants.

Polyamines

The polyamines - such as putrescine, cadaverine, spermidine, and spermine - are organic compounds having two or more primary amino groups that are growth factors synthesized in cells via highly-regulated pathways in both eucaryotic and procaryotic organisms. Polyamines have been involved in a variety of physiological processes such as growth and development in plants, and a role for these growth regulators in stress response of plants has been proposed. Biosynthesis of polyamines in plants is controlled by the enzymes ornithine decarboxylase and arginine decarboxylase which are required for production of putrescine, and S-adenosyl-L-methionine (SAM) decarboxylase that is necessary for the formation of spermidine and spermine. Cumulative direct and indirect experimental evidence supports a role for these growth regulators in stress tolerance of plants (76,77,78,79). Although a function as ROS scavengers was initially proposed (80), recent data have probed the interaction of these compounds with bent adenine tracts in double-stranded DNA suggesting a direct role of polyamines in differential gene transcriptional activity (81).

Membrane stability

Other attractive approaches for genetic engineering of drought tolerance of plants, such as cell membrane stability, merit to be evaluated. A correlation of membrane stability with drought tolerance at the whole plant level as been reported (82,83). Membranes are main targets for degradative processes induced by drought and it has been shown that, under water stress, a decrease in membrane lipid content (84,85) is correlated to an inhibition of lipid biosynthesis (86,87) and a stimulation of lipolytic and peroxidative activities (88). A relation has been found between the capacity of a plant to maintain (increase) its polyunsaturated fatty acids content and tolerance to drought stress and salinity (89,90).

Proteins involved in synthesis and degradation

Protein synthesis is one of cellular processes most sensitive to osmotic stress and proteolysis is, on the other hand, required for maintenance of an appropriated protein and nitrogen homeostasis.

One essential component of protein synthesis, elongation factor 1-alpha, significantly accumulates in salt-adapted tobacco cells (91). Increased protein degradation in response to drought and salt stress is frequently observed (92,93) and can be interpreted as a mechanism to discard damaged proteins or to remobilize nitrogen (94). Thus, it is not surprising that ubiquitinases and

cysteine proteinases show an increased production under drought and salinity conditions (95, 96, 97, 98). An *Arabidopsis DegP2* gene encoding a novel chloroplast homologue of the prokaryotic trypsin was shown to increase in response to salt, desiccation and high light (99). The protein encoded by this gene probably has a role in primary cleavage of the photo-damaged D1 protein of PSII prior to its elimination by secondary proteolysis (33). Thus, a subtle equilibrium should to be maintained between the protection and destructive cell machinery. Probably after reaching certain stress threshold the destructive mechanisms preponderate over the protective ones.

Developing drought tolerant plants by traditional breeding

In light of critical global scenarios related to water availability for human consumption and crop production anticipated to arise in the near future, intensive research is currently being conducted on basic and applied issues, from molecular to ecological approaches. Considering that up to 70-80% of the fresh water is utilized for irrigation of field crops (100), development of plants with less water requirements can contribute much to alleviate the problem of excessive water consumption in agriculture.

Because of the existence of ample natural variability among organisms for both biotic and abiotic tolerance, the existence of genes that control the response of plants to environmental stress has been long accepted. In fact, genetic resistance to many biotic stresses has been demonstrated to be the result of single (Mendelian) genes.

Classical breeding approaches have demonstrated, on the other hand, that traits conferring stress tolerance are controlled by a great variety of genes acting additive and synergistically (101,102,103,104), which makes genetic manipulation of plants for increased drought tolerance a difficult task.

Conventional breeding methods based on crosses and selection schemes have made some contributions towards stress-tolerance crop improvement (105), though attempts to generate plant varieties with improved salinity or drought tolerance using this approach have proved largely unsuccessful (106,104).

Although it may be possible to improve abiotic stress tolerance using whole plant phenotypic or physiological strategies and pyramiding breeding schemes, such approaches, even those based on marker-assisted selection, are costly, slow, require massive screening labors to identify specific quantitative traits, while linkage of agronomically important QTL´s to undesirable traits can sometimes occur. For example, selection for glycine betaine content could result in increased incidence of some insects (aphids; 107) and microbial diseases such as *Fusarium* (108). It is expected that selection supported on

genetic molecular markers help resolving some drawbacks of the conventional breedings methods.

Meanwhile, efforts to engineer improved tolerance using single or multigene transfer of genes by genetic transformation offer far more rapid and promising improvements in stress tolerance (109).

Genetic manipulation for increased drought tolerance in plants

Because of the occurrence of common responses (e.g. increased production of compatible solutes) to drought and salinity, some authors have proposed that these two factors can be treated simultaneously (110,111), even though some caution should be taken into account because some injuries are stress-specific. For example, the toxicity caused by sodium deserves to be treated separately (112).

Plant response to drought stress has been analyzed at the ecological, cellular, physiological and molecular levels. The knowledge gained by studies in these research areas have settled the technological basis now utilized for increasing the drought tolerance of plants by genetic manipulation. The limiting factor is the availability of structural genes and regulatory elements directly involved in tolerance to drought stress. If molecular biology is to really contribute to improvement of water stress tolerance of plants a logical framework for revealing basic parameters of stress tolerance should be followed. Firstly, water stress responsive genes can be identified by means of all the expression analysis techniques now available. Latter, isolation of these genes can be accomplished by differential hybridization techniques and their function evaluated in transgenic plants using model species as receptor organisms. Transgenic crop performance can be finally evaluated under field conditions or the identified genes used for marker assisted selection (MAS).

Research designed to identify, characterize and determine the function of genes and their products that are directly involved in the ability of certain plants to survive and fully recover from desiccation in currently underway. The hypothesis subjacent to much work on genetic improvement of stress tolerance that at least part of the genetic background required for tolerance is also present in non-tolerant plants is supported by the observation that gradual acclimation of sensitive plants or plant cells leads to acquisition of certain degree of tolerance (113). However, it is clear the existence of highly specific and specialized genes in some tolerant plants (114). Because some stress-relevant genes are ubiquitously present in the plant kingdom, an alternative hypothesis is proposed that stress tolerance is more the result of global expression patterns than of genome composition (33).

Another assumption in molecular work related to stress tolerance has been that an osmotic-stress regulated gene is important to the ability of a plant to

adapt to osmotic stress. The other point of view is that not necessarily genes expressed in response to a particular stress are involved in adaptive mechanisms permitting an organism to tolerate the osmotic stress condition. Although these notions have not been proved or disapproved, there is now a general realization that function of genes should be appropriately tested for conferring them a real participation in drought tolerance of plants (115). From the achievements reached in molecular work we have learnt for example that many osmotic responsive genes are part of a more general stress response system because many osmotic responsive genes also respond to other environmental factors. Thus, it has been realized a lack of specificity in plant responses to stress, as demonstrated by the osmotic induction of heat shock proteins, antioxidative damage enzymes, antifungal proteins and inhibitors of insect digestion (116).

Although modern and high throughput technologies such as expression profile analysis by DNA microarray technology and analysis of protein profiles by one- and two-dimensional gel electrophoresis are permitting us to know the genes (and proteins) that are up-regulated, down-regulated or newly expressed in response to stress, and in consequence determining those which are central to drought tolerance and genes that are unique to particular strategies for water deficit tolerance, the function of only a limited number of genes products have been established (117,118,33).

Because some traits conferring drought tolerance can be, at least theoretically, altered by manipulation of single enzymatic reactions, such as those implicated in osmotic adjustment, it has been theorized on the possibility of generating plants tolerant to water stress by transferring, through genetic transformation technology, only one or a few genes (119). The isolation of single genes and the possibility of testing these genes in a new genetic context can be achieved by gene scrutiny, recombination and transformation technologies currently available.

Current strategies for developing drought tolerant plants by transgenic approaches

Part of the possibility of generating drought tolerant plants by means of the transference of only one or few genes derives from the fact that, in order to reduce the impact of the water stress, some plants accumulate certain compounds known as compatible solutes, osmolytes or osmoregulators, and that the alteration or incorporation of the routes conducive to their biosynthesis might redound in an increased tolerance to water deficit.

By the knowledge gained through analysis of the perception and transduction modules, it was clear from the commencement of genetic manipulation of plants for increased stress tolerance the convenience of

manipulating a "master" pleiotropic gene with multiple effects on the plant response to stress (120). This "master" gene would be located upstream of the response module. However, current strategies for improving tolerance of sodium stress rely primarily on the production of low relative molecular mass (Low-M_r) solutes and on enhancing radical-scavenging enzymes systems (58).

From recent reviews concerning the performance of transgenic plants developed for increased drought stress, it is evident that every aspect of the perception-transduction-response modules has been approached (33; Aguado-Santacruz in preparation). In this regard, it is very interesting to note that practically all adopted manipulating strategies resulted in improved performance of the transformants. However, it is also important to highlight that most of these studies were conducted under laboratory or greenhouse conditions. Therefore, it is evident the urgency of testing these modified crops under field conditions. In addition, the importance of collaborative research between laboratories in different areas (physiology, biochemistry, ecology, functional genomics) is emphasized, because contradictory results have sometimes been obtained (121,122,123). These situations would prevent harvesting the fruits of this technology in benefit of the farmers.

As with other transgenic organisms, some aspects of genetic manipulation for increased tolerance of plants should be taken into account. It has consistently been found that individual transformants to an unique construct show extensive variability in expression levels, unusual developmental patterns of expression, transgene silencing and occasional phenotypic instability (124,125,126). The molecular basis of this variability has been attributed to "positional effects" or transgene rearrangements which have not been adequately analyzed because the available technological framework have permitted to disregard this situation while identifying the transgenic organisms expressing the transgene in a more "convenient" way. In this connection, the chloroplast transformation technology is expected to solve part of the drawbacks of the nuclear transformation (127). The major progress in the field of plant genetic engineering was the transition from the insertion of a single gene to the introduction of multiple genes in a single transformation event. Chloroplast transformation offers the advantage of introducing multiple trangenes in a single transformation event because of the chloroplast´s capacity to transcribe the operons into polycistronic mRNA and translate this mRNA with or without further processing. The high polyploidy of the chloroplast leads to an exceptionally high transcripts levels and accumulation of abundant translated products, up to 46% of total leaf protein (128). Furthermore, the positional effects observed in nuclear transformation are not observed in chloroplast transgenic expression due to site-specific integration of transgenes into the spacer region of the chloroplast genome through homologous recombination. Additionally, it has been observed shown

that there is no gene silencing at the transcriptional or translational levels in chloroplast transformation (128,129). Chloroplasts also offer a medium to compartmentalize toxic foreign proteins, thereby preventing any adverse effects of the gene products (130). In most angiosperms, plastid genes are inherited uniparentally in a rigorously maternal fashion. Even supposing transgenic chloroplast could be present in pollen, plastid DNA is eliminated from the male germ line at different points during sperm cell development (131). Thus, a minimum risk of transgene dispersion in the nature is achieved through chloroplast transformation. Chloroplast genetic engineering has been applied to improve agronomic traits of plants with successful results in development of insect-resistant plants (132), herbicide resistance (133), disease-resistant plants (134) and drought (130) and salt tolerance (135). Alteration of these latter two traits was approached by increasing the cellular concentration of two important compatible solutes: trehalose and glycine betaine.

Finally, If we are to properly manipulate the stress tolerance of plants, it is necessary to increase our knowledge of plant responses at each level of organization, analyzing, for example, the effect of water stress on the photosynthetic behavior of plants and the contribution of stomatal *vs*. metabolic effects to the reduction in photosynthesis rate and, consequently, in crop productivity (136).

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