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Molecular Aspects of Osmotic Stress in Plants

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ABSTRACT: Plant molecular responses to osmotic stress are complex as evidenced by the isolation of numerous OR (osmotic stress-regulated) genes. Although functions including osmolyte biosynthesis, membrane transport, signal transduction, and cellular protection have been predicted for OR genes, few of them have been established. Current efforts toward isolating and analyzing the expression of individual OR genes should be replaced by systematic approaches to analyze all OR genes simultaneously in selected plant species. Both transcriptional and posttranscriptional regulation of OR genes have been described. *Cis*-elements that respond to osmotic stress through abscisic acid (ABA)-dependent as well as ABA-independent pathways have been identified. Functional genetic approaches using yeast and plant model systems are expected to complement current molecular analysis of overexpression of OR genes in transgenic plants. These systems will help to establish functions of OR genes, to dissect osmotic stress-signaling pathways, and to determine critical and rate-limiting cellular processes for osmotic stress tolerance. In this regard, initial results obtained through mutational analysis in *Arabidopsis thaliana* are promising and have identified novel salt-tolerant as well as salt-hypersensitive mutants.

KEY WORDS: osmotic stress, salt stress, osmolytes, signal transduction, osmotic stress-regulated genes, transcriptional regulation, salt-hypersensitive mutant.

I. INTRODUCTION

Land plants evolved from aquatic progenitors of green algae. Because land plants experience constant fluctuations in the availability of water, they have evolved adaptive features to mine and absorb water through the root system, to prevent excessive transpirational water loss using cuticles and stomata on the shoot, and to adjust physiology and metabolism for continued growth and survival in the case of osmotic stress (Levitt, 1972).

Osmotic stress is used broadly here to refer to situations where insufficient water availability limits plant growth and development. It can result from drought or from excessive salt in water. Chilling and freezing may also lead to osmotic stress due to reduced water absorption and cellular dehy-

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dration induced by ice formation. Laboratory research often employs severe osmotic stress in order to easily observe plant responses. In reality, even minor osmotic stress can substantially reduce plant productivity.

Plants respond to osmotic stress at morphological, anatomical, and cellular levels. Particular morphological adaptations may be vital in specific plant species, but are not commonly used in all plants. Basic cellular responses to osmotic stress, however, appear conserved among all plants. Some of the cellular responses, such as osmotic adjustment by synthesizing compatible osmolytes, are common even to all cellular organisms (Csonka and Hanson, 1991). This review summarizes some of the molecular aspects of basic cellular responses to osmotic stress in plants. For previous excellent reviews on this subject, please refer to the following: Sachs and Ho, 1986; Hasegawa et al., 1987, 1994; Skriver and Mundy, 1990; Cushman et al., 1990; Bray, 1993; Serrano and Gaxiola, 1994; Bohnert et al., 1995.

II. OSMOTIC STRESS-REGULATED GENES

In response to osmotic stress, plant cells increase the expression of some genes while at the same time they decrease some others. New genes which are otherwise not expressed in the absence of osmotic stress may also begin to be expressed. For simplicity, we will refer to these new, up-regulated and down-regulated genes as OR (osmotic stress regulated) genes, and their gene products as OR proteins.

After exposure to osmotic stress, plants synthesize and accumulate novel proteins and many such polypeptides were identified by both one- and two-dimensional gel electrophoresis (Hurkman and Tanaka, 1986, 1988; Hurkman et al., 1988; Singh et al., 1985). Eventually, studies identifying differential expression of proteins yielded to various approaches for differential screening of gene libraries made from osmotically treated or adapted plants or plant tissues vs. untreated ones. Much effort in the last decade has been directed at cloning and analyzing OR genes obtained by these screens (Bray, 1993; Winicov, 1994). The aim has been to understand plant osmotic stress at the molecular level, and to find genes that are important for osmotic stress tolerance. The underlying assumption has been that an osmotic stress-regulated gene is important to the ability of a plant to adapt to osmotic stress. Although this notion has not been proven or disproven, there is now a general realization that unless an OR gene has been shown to function in osmotic stress tolerance, it should not be automatically assumed that it does (Bray, 1993). Despite the difficulties in establishing functions for OR proteins in osmotic response, the large number of OR genes identified in the last decade has significantly influenced our understanding of osmotic stress responses. We know now that many OR genes are part of a much more general stress response system because many OR genes also respond to other environmental cues. In addition, developmental factors (age and tissue type) greatly affect expression and environmental responsiveness of OR genes. We also now appreciate the interconnectedness of signaling pathways controlling OR gene expression. Several different categories of OR genes are described below. Their classification is based on their predicted function in osmotic responses, structural features, or expression characteristics.

A. LEA Genes

The largest group of OR genes encode LEA (late embryogenesis abundant) proteins (Baker et al., 1988; Dure et al., 1989). These genes are highly expressed in the seed dur-

ing the dessication stage following maturation, and in vegetative tissues during water deficit. LEA genes have been extensively studied and cloned from many plant species (Baker et al., 1988; King et al., 1992; Close et al., 1989; Hong et al., 1988; Almoguera and Jordano, 1992; Mundy and Chua, 1988; Pla et al., 1989; Cohen et al., 1991; Gilmour et al., 1992; Piatkowski et al., 1990). LEA proteins can be categorized into at least six subgroups based on sequence and expression kinetics (Dure, 1993). Group 2 LEAs are also referred to as dehydrins or RABs (Close et al., 1988; Mundy and Chua, 1988). LEA genes are induced by osmotic stress resulting from drought, high salt content, or cold temperatures. Most LEA genes are known to respond to abscisic acid (ABA; Skriver and Mundy, 1990).

Speculations on the function of LEAs abound, but none has been established. LEAs have been proposed to function in water retention, ion sequestration, and as molecular chaperones (Dure, 1993). This diverse group of proteins likely serves more than a single function. But the extreme hydrophilicity shared by almost all LEAs and their abundant expression during seed maturation and desiccation stress would certainly imply a function in cellular protection. The challenge has been, and still is, how to prove this function, and furthermore, how do LEAs protect cellular structures.

B. Osmolyte Biosynthesis Genes

During osmotic stress, cells accumulate solutes to prevent water loss and to re-establish tugor in order to expand (Rhodes, 1987). The solutes that accumulate during osmotic adjustment include ions such as K^+, Na^+, and Cl^-, or organic solutes such as proline and betaines. Na^+ and most of the Cl^- interfere with cellular activities and have to be compartmentalized to the vacuole (Hasegawa et al., 1987). The organic solutes, however, are compatible with cellular processes, and can accumulate to high levels in the cytosol. Genes encoding enzymes in the biosynthetic pathways of proline and betaine have recently been cloned. For proline biosynthesis, one is delta′-pyrroline-5-carboxylate synthetase, a bifunctional enzyme that has both gamma-glutamyl kinase and glutamic-gamma-semialdehyde dehydrogenase activities (Hu et al., 1992). The other is delta′-pyrroline-5-carboxylate reductase (Delauney and Verma, 1990). These two genes are induced by dehydration and high salt stress (Yoshiba et al., 1995). The gene encoding betaine dehydrogenase which catalyzes the last step in betaine synthesis was cloned from spinach and sugar beets. This gene was shown to be induced by salt stress (Weretilnyk and Hanson, 1990; McCue and Hanson, 1992). A gene encoding myo-inositol *O*-methyl transferase involved in pinitol synthesis was isolated from the ice plant (Vernon and Bohnert, 1992). It was induced almost exclusively by high salt stress.

The function of this group of genes is most obvious because osmotic adjustment is a relatively well-understood process. The importance of osmolyte accumulation was underscored by the finding that transgenic tobacco overexpressing a bacterial mannitol 1-phosphate dehydrogenase gene accumulated mannitol and acquired increased tolerance to high salt stress (Tarczynski et al., 1992, 1993). Since the original report of Tarczynski et al., several other reports of osmotolerant transgenic plants have appeared (Kavi Kishor et al., 1995; Murata et al., 1995; Tapio Palva et al., 1996; Pelon-Smits et al., 1995; Lilius et al., 1996). However, because the engineered levels of these osmolytes were too low to account for a significant osmotic adjustment role, they must protect the engineered plants by other mechanisms. One such mechanism may involve oxygen radical scavenging. Severe osmotic stress can lead to an

excessive production of reactive oxygen species (Kalir and Poljakoff-Mayber, 1981). An OR gene in pea has been found to encode an ascorbate peroxidase which may function in antioxidation (Mittler and Zilinskas, 1994). Obviously, it still cannot be ruled out that transformations leading to osmolyte accumulation trigger other osmoprotective responses.

C. Transporter Genes

A number of genes encoding membrane transporters have been found to be induced by dehydration and salt stress. Plasma membrane as well as tonoplast ATPase genes are induced by high salt stress (Surowy and Boyer, 1991; Niu et al., 1993a,b; Binzel, 1995). The plasma membrane ATPase creates a proton-motive force across the plasma membrane that is required for extrusion of toxic ions such as Na^+ during salt stress. Likewise, the tonoplast ATPase creates a proton-motive-force for compartmentation of Na^+ into the vacuole through perhaps the Na^+/H^+ antiporter (Sze, 1985). Because ATPase genes belong to multigene families, it is not clear which subfamily is the salt-regulated one. It is also not known whether the salt regulation is at the transcriptional level.

A group of OR proteins have been found to share sequence similarities with water channels. They include the up-regulated ones from pea (Guerrero et al., 1990) and Arabidopsis (Yamaguchi-Shinozaki et al., 1992), and several down-regulated ones from the common ice plant (Yamada et al., 1995). The concept of a water channel is relatively new for plant biology and its significance in osmotic stress response and in water physiology in general is under intense investigation. Compared to diffusive water movement across membranes, movement through water channels is much faster and controllable. The ability to control flux through water channels compared with diffusive movement is easy to acknowledge, however, we cannot appreciate the accelerated speed of water movement until we know when cells need to lose or gain water rapidly. During tissue dehydration, is it beneficial for survival if cells lose water as fast as possible, or just the opposite, or maybe it does not matter? What happens during hypo-osmotic stress? Do cells need to gain water faster or slower? The rate of dehydration is known to be an important factor in the resistance of some desiccation-tolerant mosses (see Bewley and Oliver, 1992). Before we have some clues to the above physiological questions, it is difficult to speculate on any adaptive roles for increased or decreased expression of water channels. This problem also represents one of the few examples where physiological studies are lagging behind molecular advances. Some or all of the osmotically induced water channel homologs may function in facilitating the flux of ions or metabolites rather than water in plant cells.

D. OR Genes that Encode Regulatory Proteins

Some OR genes encode putative regulatory proteins such as signal transduction components and transcriptional factors. This characterization is often based on their sequence similarities to other well-characterized signaling proteins and transcriptional factors. Although the osmotic signaling pathway in plant cells has not been identified, several OR proteins were found to have sequence homologies to protein kinases or phosphatidylinositol lipase C (PLC; Hirayama et al., 1995). The induction of PLC by salt stress suggests a role for IP_3 and Ca^{2+} in osmotic signal transduction. An ABA-regulated gene has been isolated from wheat that shows homology to serine/threonine protein kinases (Anderberg and Walker-Simmons, 1992). Several cDNAs corresponding to members of a mitogen-activated protein (MAP) kinase

cascade were identified in Arabidopsis as dehydration and salt inducible (Mizoguchi et al., 1996). These may be similar to components in the osmosensing MAP kinase pathway recently defined in yeast (Maeda et al., 1995). There are probably many signaling components that are not induced by osmotic stress. The inducible ones are likely limiting and thus their increased expression could ensure the efficacy of the entire transduction pathway.

An *myb* homolog was also cloned from dehydrated Arabidopsis plants (Urao et al., 1993). It is regulated at the transcriptional level by dehydration and salt stress. This Myb-related transcriptional factor, if it can bind to the promoter of other OR genes, may regulate their transcription in response to osmotic stress. Genes in plants related to myb appear to belong to a large gene family. Two members in this family, *C1* and *P1* from maize, are known to regulate pigment accumulation by binding to the *cis*-elements of pigment biosynthesis genes (Bodeau and Walbot, 1992; Grotewold et al., 1994).

E. Photosynthetic Genes

In some higher plant species, the plants respond to drought or salt stress by switching from the C3 mode of photosynthesis to crassulacean acid metabolism (CAM; Ting and Rayder, 1982). CAM allows plants to fix CO_2 at night when evaporative water loss is minimal. This type of metabolic response was most successfully analyzed in the common ice plant, *Mesembryanthemum crystallinum*. In *M. crystallinum*, a key enzyme of the CAM pathway, phosphoenolpyruvate (PEP) carboxylase is regulated by osmotic stress at the transcriptional and posttranscriptional levels (Cushman et al., 1989). Two isogenes of PEP caboxylase have been isolated. Only one of the two is regulated by osmotic stress (Cushman et al., 1989). Not surprisingly, genes encoding several other enzymes of the CAM pathway, including pyruvate orthophosphate dikinase and NADP malic enzyme, were also shown to be induced by osmotic stress, along with PEP carboxylase (Cushman et al., 1990; Ostrem et al., 1990).

Photosynthesis-related genes in general, including both nuclear and chloroplast-encoded transcripts, were found to exhibit enhanced mRNA accumulation in salt-adapted cell cultures (Winicov and Button, 1991; Locy et al., 1995). The significance of these changes to the process of salt adaptation of the cultured cells is not known.

F. OR Genes Encoding Proteins Involved in Protein Synthesis, Processing, and Degradation

Protein synthesis is among the cellular processes that are most sensitive to osmotic stress, especially salinity stress (Levitt, 1972). However, how the protein synthesis machinery copes with osmotic stress is not understood. One essential component of protein synthesis, elongation factor 1-alpha, accumulates dramatically in salt-adapted tobacco cells (Zhu et al., 1994) and in salt-adapted fungal cells (N. K. Singh, personal communication). This may indicate a mechanism of osmotic adaptation to protect protein synthesis.

Transcripts encoding a putative cysteine proteinase were induced by dehydration in pea leaves (Guerrero et al., 1990). Transcripts encoding two different cysteine proteinases also accumulate in drought or salt-stressed Arabidopsis plants (Koizumi et al., 1993). These putative cysteine proteinase mRNAs are not induced by ABA treatment. An ubiquitin message also appeared to increase after water deficit (Borkird et al., 1991). It is not known whether cysteine proteinase and ubiquitin levels or activities increase after osmotic

stress. If they do, it may indicate a requirement for metabolizing denatured proteins caused by osmotic stress. It is well established that during and after heat shock, protein degradation accelerates to remove heat-denatured proteins (Burke et al., 1988).

A *Brassica napus* protease inhibitor mRNA was also shown to be induced after prolonged dehydration (Downing et al., 1992). The encoded protein has sequence homology to the Kunitz protease inhibitors. The transcript was inducible in water-stressed leaves, but not in seeds. A plausible function of the induced putative protease inhibitor could be to protect against proteases that may be released after membrane disruption as a result of severe dehydration.

G. HSP Genes

Heat shock proteins (HSPs) are proteins that accumulate during and after heat shock (Vierling, 1991). Genes encoding HSP-cognates are also induced by osmotic stress. An HSP70 gene accumulates in rice cells during osmotic stress adaptation (Borkird et al., 1991). A low molecular weight HSP was shown to be induced by osmotic stress in sunflower (Almoguera and Jordano, 1992). Transcripts of ANJ1, a gene in the DnaJ family of HSPs, was induced by high salt stress in *Atriplex numularia* cells that had been adapted to salinity, but was not induced in normal unadapted cells (Zhu et al., 1993a). HSPs are among the most conserved of all proteins and are found in heat-stressed bacteria, fungi, animals, and lower and higher plants. Constitutively expressed HSP homologs exist and function in normal plant growth and development. Most of the HSPs probably function as molecular chaperones that assist in protein folding and prevent protein denaturation (Zhu et al., 1993a). Protein aggregation and denaturation are aggravated during heat stress, necessitating more HSPs. Severe osmotic stress may also render cellular proteins more susceptible to aggregation and denaturation and thus increased synthesis of HSPs may help protect the proteins. One interesting observation from physiological studies is that there exists a mechanism for cross protection between heat and osmotic stress in plant cells, i.e., sublethal heat stress will increase tolerance to a subsequent osmotic stress and vice versa (Harrington and Alm, 1988). The inducibility of some HSPs by both heat and osmotic stress probably contributes to this cross-protection mechanism.

H. Osmotin and Other PR Genes

Osmotin was originally identified by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as the most abundant protein in salt-adapted tobacco cells (Singh et al., 1985). It also accumulated in polyethylene glycol-adapted cells, but not in cells under osmotic shock. The accumulation of osmotin was found to be correlated with osmotic adaptation (LaRosa et al., 1989). In tobacco, there are at least three isoforms of osmotin distributed in various compartments including the vacuole, cytoplasm, and extracellular space (LaRosa et al., 1992). mRNAs encoding proteins with homology to osmotin have been isolated from many plant species (see review by Kononowicz et al., 1994). Not all of these mRNAs are induced by osmotic stress. For example, only one of the two Atriplex osmotin homologs cloned shows induction by high salt (Casas et al., 1992).

Despite its correlation with osmotic adaptation, it is still not understood whether or how osmotin, which is a *p*athogenesis-*re*lated type 5 PR gene, functions in osmotic stress adaptation. At least some isoforms of other PR genes, that is, PR1 (Pierpoint et al., 1981; Okashi and Matsuoka, 1985) and chiti-

nases (PR3; Yun et al., 1996), are also induced by osmotic stress. It is clear that these genes function in defense against pathogens (Broglie et al., 1991; Alexander et al., 1993; Ponstein et al., 1994; Liu et al., 1994). Osmotin was found to be an antifungal protein both by *in vitro* assays and *in vivo* in transgenic potato overexpressing the tobacco osmotin (Woloshuk et al., 1991; Liu et al., 1994). Although the exact mode of action of osmotin as an antifungal protein is not well understood, it is known that it causes lysis of fungal hyphae and maximal activity requires the presence of the fungal cell wall (Abad et al., 1996). The osmotin gene is induced by fungal and viral pathogens in addition to osmotic stress as a result of dehydration, high salt, and cold stress (Kononowicz et al., 1992; LaRosa et al., 1992). Is there any possible connection between the pathogen induction and osmotic stress induction? Kononowicz et al. (1992) have pointed out that many plant pathogens themselves induce symptoms of osmotic stress and therefore defense genes may have evolved osmotic responsiveness to increase their efficacy against pathogen invasion. If this is found indeed to be the case, it would be interesting to know whether this defense gene has evolved any function in osmotic stress protection besides its osmotic stress inducibility.

I. Other OR Genes

Other OR genes identified include those encoding an RNA-binding protein (Ludevid et al., 1992) and some putative lipid transfer proteins (Plant et al., 1991), RD29A, RD29B (Yamaguchi-Shinozaki et al., 1993), kin1, and kin2 (Kurkela and Borg-Franck, 1992). The latter four consist of two pairs of homologous genes from Arabidopsis with no significant homology to others in the databank. One distinct feature is that they are highly induced by cold stress and their encoded proteins are hydrophilic.

III. REGULATION OF OR GENE EXPRESSION

Studies on the regulation of OR genes aim to understand how plant cells perceive the osmotic stress signal and transduce this signal to produce changes in OR gene expression. Because of the existence of ample natural variability among plants for both biotic and abiotic stress resistance, 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 (pathogens and pests) has been demonstrated to be the result of single genes. Recently, several of these genes have been isolated and found to encode specific proteins involved in signal transduction (Staskawicz et al., 1995). Interestingly, specific genes controlling resistance to biotic (pathogens) stress are very specific and control resistance to only a particular pathogen because that pathogen triggers a defense response in the plant involving its corresponding "R" resistance gene. In contrast to the situation with biotic stress resistance, abiotic stress resistance in nature, although clearly genetic, has unfortunately afforded us very few specific mendelian genes. A hallmark of abiotic stress resistance is its marked "multigenic" characteristic. Nevertheless, over the past two decades scientists have begun to understand that abiotic stress tolerance, like its more manageable counterpart, biotic stress tolerance, is the result of coordinate gene regulation through an appropriately responsive signal transduction system that perceives a hostile abiotic environment (Niu et al., 1995). Because there have been virtually no specific "mendelian" tolerance genes to identify by a plausible cloning strategy, most of the molecular studies of abiotic stress

tolerance have focused on the identification of genes whose expression is regulated by exposure to stress. This approach was adopted by several investigators primarily because of the observation that many plants exhibit a facultative resistance to stress in that they are able to respond phenotypically to stress imposition and become tolerant. Because the stress tolerance phenotype is induced by the imposition of stress, an attractive hypothesis developed that essentially embraced the theme that stress-induced genes were responsible for tolerance. This then became the central theme forming the underlying basis for OR gene importance.

In fact, early in the beginning of the application of modern molecular gene cloning technology to research in plant osmotic stress, it was thought that genes controlling abiotic stress tolerance would be quickly identified by various techniques of differential screening and that the "secret" genes that controlled tolerance would be rapidly revealed. Indeed, several differentially regulated genes have been identified and we have discussed the numerous OR genes that have been found by several experimental approaches. Even though many of these OR genes may be involved in osmotic tolerance, it has become increasingly clear with time that such genes have only very small effects on tolerance, and that strategies to identify the regulatory genes that control them would be needed. Strategies such as identification of *trans*-acting regulatory factors are being attempted (see Figure 1). They hold much promise for identifying stress regulatory genes, but the complexity of regulatory elements on most stress-responsive genes indicates that this will be a difficult task (Bohnert et al., 1995). We have also begun to realize that it may be necessary to identify genes much closer to the point of stress signal perception to be able to impact tolerance in a major way. A few years ago Ramon Serrano and others (Haro et al., 1993; Serrano and Gaxiola, 1994) began to advocate a new approach toward identifying genes that play major roles in salinity stress tolerance. This approach was to use model systems (such as yeast) to produce salt-sensitive mutants that could be complemented by gene transformation, or to overexpress large numbers of genes in such model systems and screen for enhanced tolerance. Recently, Wu et al. (1996) have been able to identify salt-sensitive mutants of Arabidopsis where map-based cloning of the affected plant genes is possible. Also, Prieto and co-workers have isolated marker-tagged salt-sensitive mutants of *Chlamydomonas* (Prieto et al., 1996). Because the approach of using mutants involves a screen for genes that can "function" (or complement) in stress tolerance, and not a screen for genes whose "expression responds" to stress, the likelihood of finding genes that play limiting key roles in transducing a signal from the perception of stress to the multigenic adaptive response is very high.

A. Osmotic Signal Sensing and Transduction

The physical-chemical mechanisms by which plant cells or any cells perceive osmotic changes in their environment are not known. What constitutes an osmotic stress signal? A decrease in extracellular water potential caused by either water loss or high salt will result in the reduction and subsequent loss of turgor, and eventually intracellular dehydration. Loss of turgor is generally considered a likely mechanism to perceive osmotic stress. However, careful measurements of biochemical responses of plant cells to osmotic stress indicated that osmosensing is not entirely through turgor changes (Handa et al., 1986). Alternatively, intracellular dehydration or cytosolic solute concentration could be sensed by induced conformational changes in certain proteins.

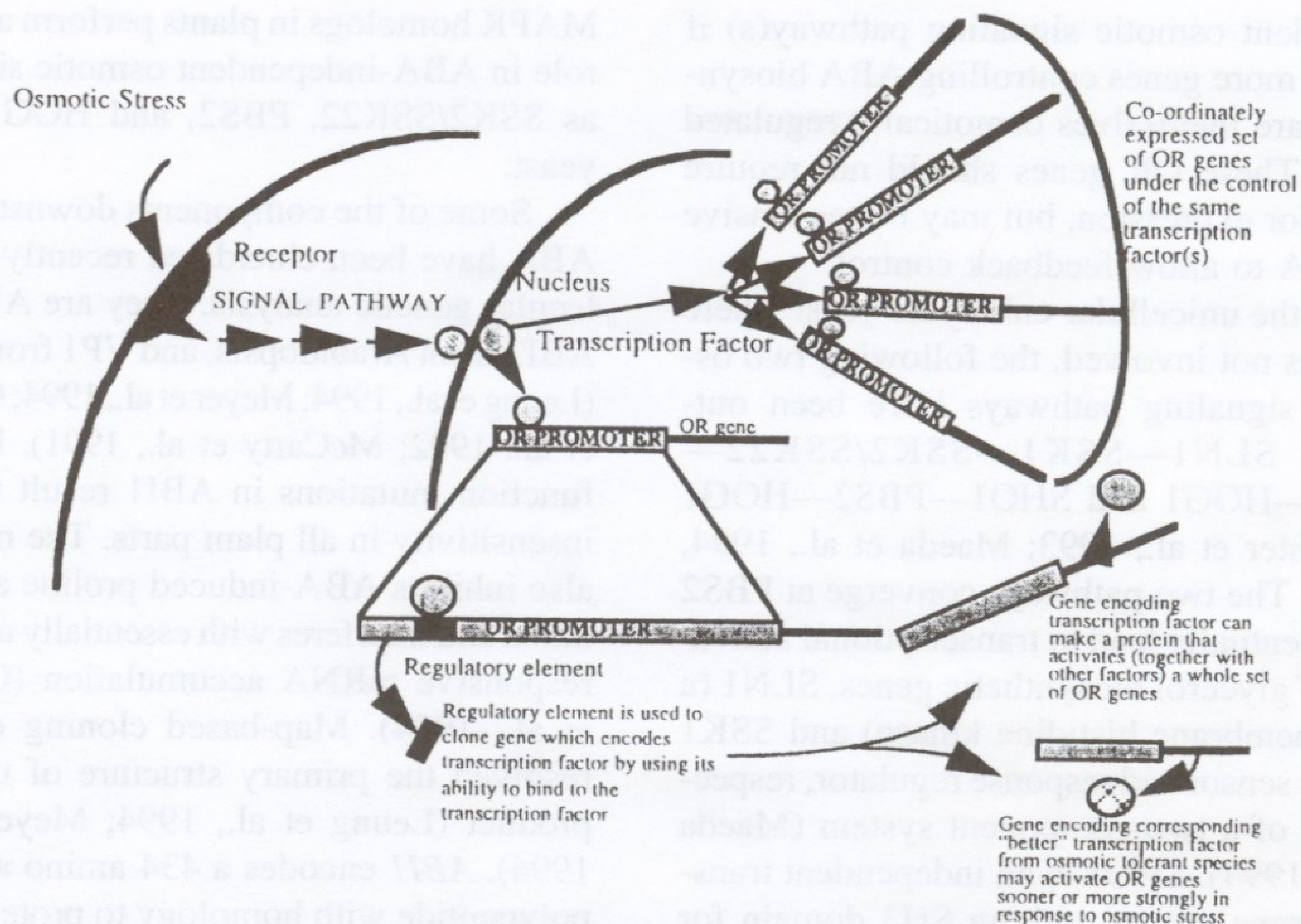

FIGURE 1. Molecular approach toward isolating genes encoding transcription factor proteins that control the activity of OR genes by interacting with OR promoters. The DNA sequence of an OR promoter that is responsible for controlling the response of the gene to osmotic change (osmotic regulatory element) is first identified by a series of deletion and mutation experiments. This regulatory element is a small piece of DNA sequence that is then used as a probe to identify proteins that bind specifically to this DNA sequence. The probing is performed on a cDNA expression library allowing the isolation of a cDNA clone of any protein (transcription factor) that can bind to the osmotic regulatory element and regulate activity of the OR promoter. Such transcription factors represent the final step in signal cascades that allow perception of osmotic changes (also see Figure 2).

There is evidence for active interaction between the plasma membrane and the cell wall (Zhu et al., 1993b). Changes in turgor will impact this interaction. Plasma membrane proteins that interact with the wall may then undergo conformational changes and thus relay this information to the cell interior. Alternatively, turgor loss could lead to protoplast volume reduction and could activate stretch-activated ion channels (Ding and Pickard, 1993).

Osmotic stress results in increased biosynthesis of the phytohormone, ABA (Zeevaart and Creelman, 1988). ABA in turn induces the expression of some OR genes (Skriver and Mundy, 1990). There are, however, OR genes that are not responsive to ABA (Guerrero et al., 1990; Casas et al., 1992). Even some of the ABA-responsive OR genes can be activated by osmotic stress through an ABA-independent pathway (Yamaguchi-Shinozaki and Shinozaki, 1994). This has been convincingly demonstrated in the ABA-deficient mutants of Arabidopsis and tomato (Nordin et al., 1991; Grillo et al., 1995).

Although several OR genes are induced via the ABA-dependent osmotic signaling pathway, a critical question remains as to how ABA is induced by osmotic stress. The signal transduction leading to ABA accumulation may be connected with the ABA-in-

dependent osmotic signaling pathway(s) if one or more genes controlling ABA biosynthesis are themselves osmotically regulated (OR). These OR genes should not require ABA for expression, but may be responsive to ABA to allow feedback control.

In the unicellular eukaryote yeast where ABA is not involved, the following two osmotic signaling pathways have been outlined: SLN1—SSK1—SSK2/SSK22—PBS2—HOG1 and SHO1—PBS2—HOG1 (Brewster et al., 1993; Maeda et al., 1994, 1995). The two pathways converge at PBS2 and eventually lead to transcriptional activation of glycerol biosynthetic genes. SLN1 (a transmembrane histidine kinase) and SSK1 are the sensor and response regulator, respectively, of a two-component system (Maeda et al., 1994). SHO1 is an independent transmembrane sensor with an SH3 domain for interaction with the SH3-binding motif of a downstream component, PBS2 (Maeda et al., 1995). It remains to be seen whether and how SLN1 and SHO1 sense osmotic stress signal directly. SSK2/SSK22, PBS2, and HOG1 are homologs of MAPKKK, MAPKK, and MAPK, respectively, of a MAP kinase pathway. Several homologs of MAPKKK and MAPK have been isolated from plants, some of which are up-regulated by osmotic stress (Mizoguchi et al., 1996). In addition, yeast appears to have a third signal system that functions to regulate ion homeostasis and involves a key regulatory protein phosphatase, calcineurin (see Figure 2). A higher plant two-component system, ETR1, was discovered. It functions as an ethylene receptor, but it is unlikely to be involved in osmotic signaling (Chang et al., 1993). ETR1 contains both a response sensor and a regulator. It is possible that another ETR1-related protein could be involved in osmosensing in plants. Because a mammalian MAPK homolog was able to complement the salt-sensitive *hog1* mutation in yeast (Galcheva-Gargova et al., 1994), perhaps the osmotic stress-regulated MAPKKK, MAPKK, and MAPK homologs in plants perform a similar role in ABA-independent osmotic signaling as SSK2/SSK22, PBS2, and HOG1 do in yeast.

Some of the components downstream of ABA have been elucidated recently by molecular genetic analysis. They are ABI1 and ABI3 from Arabidopsis, and VP1 from maize (Leung et al., 1994; Meyer et al., 1994; Giraudat et al., 1992; McCarty et al., 1991). Loss-of-function mutations in ABI1 result in ABA insensitivity in all plant parts. The mutation also inhibits ABA-induced proline accumulation and interferes with essentially all ABA-responsive mRNA accumulation (Giraudat et al., 1994). Map-based cloning of *ABI1* resolved the primary structure of the gene product (Leung et al., 1994; Meyer et al., 1994). *ABI1* encodes a 434 amino acid (aa) polypeptide with homology to protein phosphatase 2C of yeast (Maeda et al., 1993) and animals (Tamura et al., 1989). Thus ABA signaling involves cascades of protein phosphorylation and dephosphorylation which is the theme of almost all cell signaling. In plants, protein phosphorylation is known to participate in everything from light to hormonal and defense responses. What is really unique about ABI1 is that, in addition to the protein phosphatase 2C structure, it contains an EF hand domain for binding calcium (Meyer et al., 1994). This will certainly allow for cross talk with calcium signaling elicited by osmotic stress and/or other effectors. All osmotic responses take place in the context of other developmental, hormonal, and environmental factors, many of which work through the second messenger, Ca^{2+}. Therefore, through ABI1, plants may coordinate osmotic responses with other cellular activities.

ABI3 and *VP1* encode a transcriptional factor that functions specifically in the seed to prevent precocious germination (Giraudat et al., 1992; McCarty et al., 1991). Several LEA-type mRNAs have reduced levels in *abi3* and *vp1* mutants (Finkelstein, 1993;

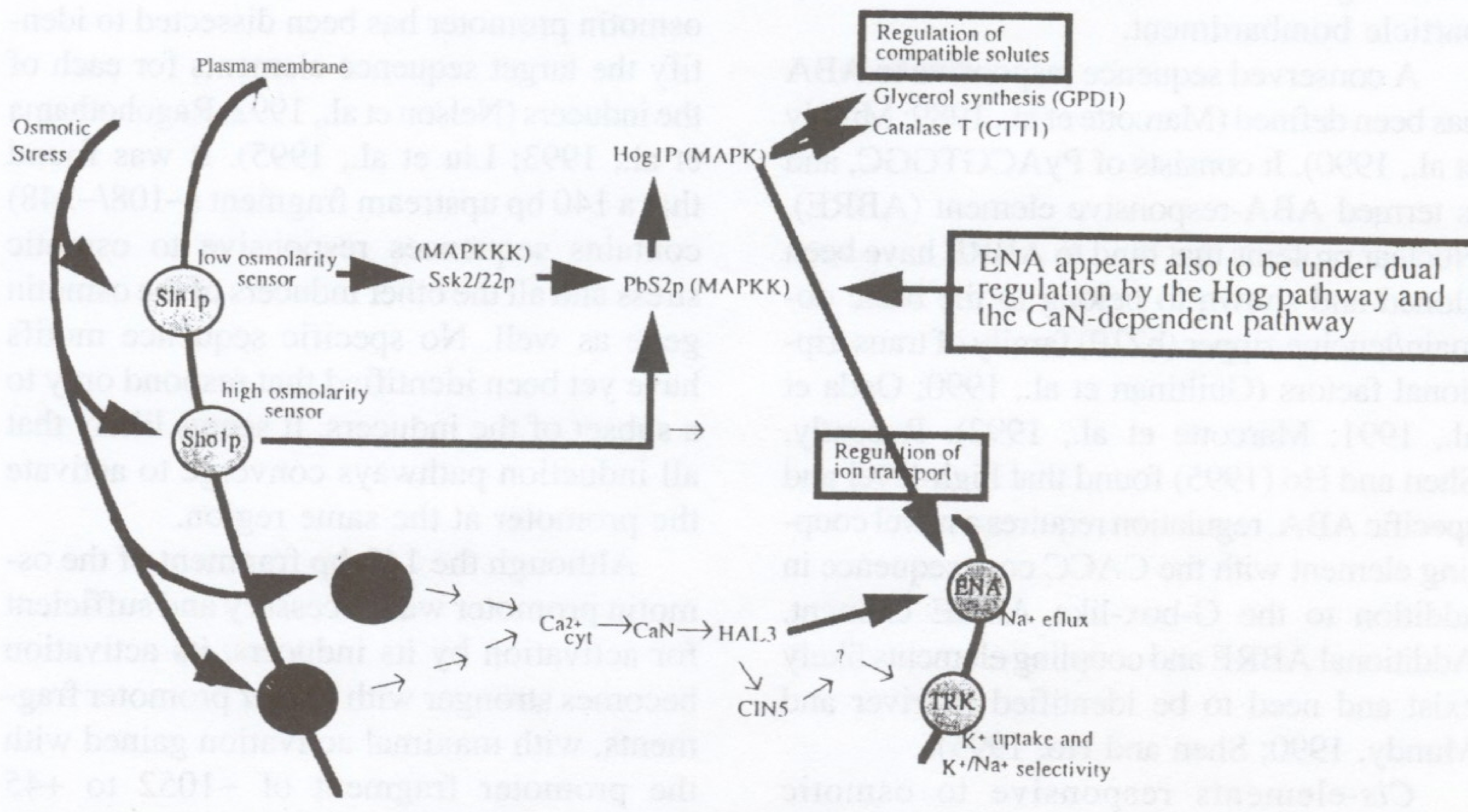

FIGURE 2. The signal transduction pathway for osmotic responsive (OR) genes in yeast. Two membrane sensors (receptors) are known to sense osmotic changes in the environment, Sln 1P and Sho1p. The Sln1p senses low osmolarity changes and interacts through a kinase cascade with SsK2/22p, PbS2p, and Hog1p, all components of a MAP kinase pathway referred to as the Hog1 pathway. This pathway transcriptionally activates glycerol phosphate dehydrogenase (GPD1) and catalase T (CTT1). Transcriptional activation of GPD1 results in glycerol accumulation and subsequent osmotic adjustment of the cell to an osmotic homeostatic situation. When cells are exposed to elevated NaCl levels resulting in osmotic stress, ion transport functions are activated to establish ion homeostasis. The sensor or receptor for this pathway is unknown, but it is mediated by the Ca^{2+}/calmodulin-activated protein phosphatase, calcineurin, which acts through HAL3. The pathway then apparently branches to regulate ion efflux through activation of the Na^{+}-ATPase, ENA. A separate branch involves a transcription factor CIN5 (J. Pardo et al., unpublished results) that participates in the regulation of ion influx/selectivity through the K^{+} transporter, TRK.

Williams and Tsang, 1991). Therefore, ABI3/VP1 seems to function only in determining developmental specificity of ABA responses, and is not involved in OR gene expression in vegetative tissues.

B. Transcriptional Regulation of OR Genes

A goal of studies on transcriptional regulation of OR genes is to identify regulatory *cis*-acting sequences and *trans*-acting factors that participate in the osmotic stress regulation of transcription. Transcriptional regulatory sequences are located within the promoter region 5′ to the transcribed sequence. The promoters can be identified from appropriate genomic clones. The transcriptional start site is determined by primer extension. The promoter consists of sequences that are as many as several kilobases 5′ to the transcription start site. Promoter activity is usually demonstrated by fusing the 5′ upstream sequence with a reporter gene such as GUS. The chimeric gene construct is then evaluated by transient assays such as protoplast transformation or bombardment into tissues or cultured cells. For detailed studies on the pattern of regulation, stable plant transformants are necessary. This is achieved by

either *Agrobacterium* transformation or by particle bombardment.

A conserved sequence responsive to ABA has been defined (Marcotte et al., 1989; Mundy et al., 1990). It consists of PyACGTGGC, and is termed ABA-responsive element (ABRE). Nuclear proteins that bind to ABRE have been cloned and shown to belong to the basic domain/leucine zipper (bZIP) family of transcriptional factors (Guiltinan et al., 1990; Oeda et al., 1991; Marcotte et al., 1992). Recently, Shen and Ho (1995) found that high-level and specific ABA, regulation requires a novel coupling element with the CACC core sequence in addition to the G-box-like ABRE element. Additional ABRE and coupling elements likely exist and need to be identified (Skriver and Mundy, 1990; Shen and Ho, 1995).

Cis-elements responsive to osmotic stress, but not ABA, are of great interest because they are the terminal target of ABA-independent osmotic signal transduction pathway(s). One such element, the dehydration-responsive element (DRE), has been uncovered in the OR gene *RD29A* of Arabidopsis (Yamaguchi-Shinozaki and Shinozaki, 1994). This 9-bp *cis*-acting element (TACCGACAT) can confer ABA-independent induction of the GUS reporter gene by dehydration, high salt concentration, or low temperature. Nuclear proteins that bind specifically to DRE exist but have not been cloned.

It is interesting that in addition to DRE, an ABRE is also present in the RD29A promoter. Yamaguchi-Shinozaki and Shinozaki (1994) have proposed that the presence of these two *cis*-elements explains the two-step induction of *RD29A*, that is, a rapid induction before ABA accumulation and a strong slower second phase of induction after ABA accumulation.

The osmotin gene is activated by a large number of environmental and chemical signals such as drought, high salt, cold, ABA, ethylene, cytokinin, salicylic acid, jasmonic acid, UV light, wounding, and viral and fungal pathogens (Kononowicz et al., 1994). The osmotin promoter has been dissected to identify the target sequence elements for each of the inducers (Nelson et al., 1992; Ragohothama et al., 1993; Liu et al., 1995). It was found that a 140 bp upstream fragment (−108/−248) contains sequences responsive to osmotic stress and all the other inducers of the osmotin gene as well. No specific sequence motifs have yet been identified that respond only to a subset of the inducers. It seems likely that all induction pathways converge to activate the promoter at the same region.

Although the 140-bp fragment of the osmotin promoter was necessary and sufficient for activation by its inducers, its activation becomes stronger with longer promoter fragments, with maximal activation gained with the promoter fragment of −1052 to +45 (Raghothama et al., 1992). Further increases in the promoter size resulted in decreased activation, suggesting the presence of negative or silencer-like element(s) in the upstream region of the promoter.

Specific protein factors have been identified that interact either directly or indirectly with the osmotin promoter (Liu et al., 1995). DNaseI footprinting revealed that three conserved promoter elements are involved in DNA-protein interactions on the 140-bp fragment. These elements are: (1) a cluster of three overlapped G-box-like sequences (G sequence); (2) an AT-1 box-like sequence, 5′-GTATTTTATTAA-3′ (AT sequence); and (3) a sequence highly conserved in ethylene-induced PR gene promoters, 5′-TAAGTGCCGCC-3′ (PR sequence). Transient expression assays indicated that osmotin promoter activity correlated with the protein binding activity of these elements. Protein complex bound to the 140-bp fragment consists of at least four polypeptides of 28, 29, 40, and 42 kDa (Liu et al., 1995). The 28- and 29-kDa proteins are associated with the G sequence, and the 40- and 42-kDa proteins were associated with the AT and PR elements, respectively. One of the proteins

associated with the G sequence is a 14-3-3 homolog. 14-3-3 is a multifunctional regulatory protein originally identified in the animal brain (Lu et al., 1992). The tobacco homolog probably interacts indirectly with the promoter sequence and modulates the activities of its associated proteins in the transcriptional complex. A 42-kDa protein that binds to the PR element has been cloned and designated PR-6 (R. A. Bressan, unpublished). This factor is encoded by a homeotic-like gene with sequence homology to a number of other transcriptional factors such as the *MASTERMIND* gene of *Drosophila*.

C. Posttranscriptional Regulation

Regulation of gene expression can be transcriptional or posttranscriptional. Posttranscriptional regulation in the broadest sense can occur at any one of the following steps: RNA splicing, processing and transport, mRNA stablility and translation efficiency, posttranslational modification of proteins, enzyme activation, and protein degradation rate. For many OR genes, their transcriptional activation may not completely explain their mRNA accumulation, thus making mRNA stability a potentially important factor. But this is often difficult to assess due to imprecise quantitation. Use of transcription inhibitors should help in such cases. Initial observations on posttranscriptional regulation were made because of discrepancies in cellular protein profiles vs. profiles of *in vitro* translated proteins (Hurkman, 1989). Subsequently, it was found that the OR protein levels may not always correlate with OR mRNA levels determined by Northern analysis (LaRosa et al., 1992). LaRosa et al. (1992) found that among all the inducers of osmotin mRNA which include drought, high salt, cold, ABA, ethylene, cytokinin, wounding, pathogen infection, etc., only drought, high salt, and ethylene significantly induced osmotin protein accumulation as determined by immunoblot analysis. Cushman et al. (1989) also noted discrepancies between transcript and protein accumulation of the stress-induced PEP carboxylase 1 gene. These examples showed that studies on gene expression that consider the mRNA abundances only, and not the accumulation of the protein, do not necessarily reflect gene expression at the protein level. Therefore, the significance of changes in the level of mRNA in the absence of protein measurements should be viewed with caution. Unfortunately, antibodies are not available for the majority of OR proteins reported which makes it almost impossible to quantitate their protein levels. In the few cases where posttranslational regulation has been demonstrated to be involved in OR gene expression, no mechanistic information has yet been gained.

IV. MOLECULAR GENETICS OF OSMOTIC STRESS RESPONSES

As Serrano and Gaxiola (1994) pointed out, the major problem with most studies on OR genes is that the research is not able to address easily the possibility that the identified genes are able or unable to function in critical or limiting roles in osmotic stress tolerance. Although the OR gene studies have advanced our understanding of plant osmotic stress responses, we still do not know the function of OR genes in plant osmotic responses except in a few cases. We also do not know what cellular processes are most critical or rate limiting, nor do we know what genes are essential for osmotic stress tolerance. These questions are best answered and sometimes can only be answered by functional genetic studies.

Availability of a model system is a requisite for systematic genetic analysis. In the absence of an ideal higher plant genetic model system for osmotic stress responses, many

researchers have turned to microbial or algal systems. Various bacterial mutants with increased sensitivity to osmotic stress were identified over a decade ago (Csonka, 1989; Csonka and Hanson, 1991). Analysis of these mutants has established the essential roles of osmolytes (e.g., proline, betaine, trehalose, etc.), biosynthetic genes, solute transporter genes, and their regulators in osmotic stress tolerance in prokaryotes. In eukaryotes, studies on salt-sensitive mutants of the budding yeast *Saccharomyces cerevisiae* have been very successful. Many salt-sensitive yeast mutants have been identified in recent years, and the cloning of the mutated genes has illuminated the nature of many genes essential for osmotic stress tolerance. These genes range from those involved in the biosynthesis of the compatible osmolyte glycerol or those involved in ion homeostasis to those that function in signal transduction (Brewster et al., 1993; Mendoza et al., 1994). Through molecular genetic analyses, a scheme has emerged where an osmotic stress signal is directly or indirectly perceived by a two-component system or an independent membrane receptor. The signal is then transduced through a series of protein kinases via cascades of protein phosphorylation and eventually arrives at the nuclear transcriptional machinery to turn on glycerol biosynthetic genes (Maeda et al., 1995). In a separate pathway for ionic signaling and response, the protein phosphatase 2B (i.e., calcineurin) plays an essential role in activating the Na^+-ATPase gene and high-affinity potassium transport (Mendoza et al., 1994).

In an alternative strategy, Serrano's group isolated halotolerance genes by their capability to improve salt tolerance upon overexpression. They have successfully identified two such genes this way, *Hal1* and *Hal2*. Hal1 is involved in the selective accumulation of potassium when cells are challenged with high sodium (Gaxiola et al., 1992). Hal2 is identical to the methionine biosynthetic enzyme, MET22, which is very sensitive to sodium and lithium ions (Murguia et al., 1995). The results demonstrated that potassium homeostasis and certain salt-sensitive enzymes are rate limiting for yeast salt tolerance.

The success of the mutational approach in microbes is encouraging, but the knowledge may not always be directly applicable to higher plants. Therefore, a higher plant model system has to be developed. *Arabidopsis thaliana* has become the model of choice for plant molecular genetic studies in recent years because of its small genome size, short generation time, the absence of extensive repetitive DNA, the ease of obtaining mutations by chemical and molecular means, as well as the availability of standardized cloning and mapping techniques and transformation systems (Meyerowitz, 1989). Due to the very salt-sensitive nature of Arabidopsis, initial efforts have been to isolate salt-tolerant mutants. Saleki et al (1993) have isolated several Arabidopsis mutants capable of germination under high salt stress. Similarly, Werner and Finkelstein (1995) have also identified mutants that tolerate high salt at the germination stage. The mechanism for the increased salt tolerance is currently not known. These mutants are not more salt tolerant than the wild type at developmental stages other than at seed germination (Saleki et al., 1993).

Wu et al. (1996) have isolated mutants that are more sensitive to salt stress than the wild type (Figure 3). These *sos* (*s*alt *o*verly-*s*ensitive) mutants are hypersensitive to salt stress at all developmental stages. Loss of function mutations in one of the first loci defined by the mutants, *sos1*, resulted in over a 20-fold greater sensitivity to NaCl inhibition than the wild type. *sos1* was found to be defective in high-affinity potassium uptake (Wu et al., 1996). The results illustrate the essential role of the high-affinity potassium uptake system in Na^+ tolerance in higher plants. This high-affinity potassium uptake system also appears important for osmotic stress tolerance. *sos1* is hypersensitive to

A. 0 mM NaCl

B. 100 mM NaCl

FIGURE 3. Phenotype of salt-hypersensitive mutants, *sos1* and *sos2. SOS1* and *SOS2* represent two genetic loci controlling salt tolerance in *Arabidopsis thaliana. sos1* and *sos2* mutations affect plant growth only in the presence of NaCl (B), while growth in the absence of NaCl (A) is not affected.

osmotic stress imposed by mannitol only at low levels of the stress (Figure 4). The biphasic response of *sos1* to osmotic stress indicates perhaps a dual mechanism of plant osmotic stress response. Under low to moderate levels of osmotic stress, mechanism 1 plays a predominant role, whereas under high stress, mechanism 2 becomes important. Thus, mechanism 1 possibly involves potassium accumulation for osmotic adjustment and mechanism 2 could involve the synthesis of organic osmolytes such as proline. Under osmotic stress, *sos1* was shown to accumulate more proline than the wild type (J.-K. Zhu, unpublished), consistent with it not being hypersensitive to high levels of mannitol stress. While this hypothesis of a dual mechanism of osmotic stress response is currently very speculative for plants, it is well established that in bacteria, potassium accumulation is most important for tolerance to low osmotic stress, but under high stress, potassium accumulation is not sufficient for adequate osmotic adjustment and proline accumulation becomes critical (Epstein, 1986).

Besides *sos1*, several other types of *sos* mutants have been identified (J.-K. Zhu, unpublished). These mutants are expected to define other essential genes for salt tolerance which may be involved in ion homeostasis, osmolyte biosynthesis, as well as in osmotic and ionic sensing and signal transduction (Wu et al., 1996). There are several avenues for cloning the *SOS* genes. Some of the *SOS* genes have been mapped to initiate positional cloning. Alleles of the *sos* mutants have also been identified from T-DNA transformation lines (Feldman, 1991) which will allow more rapid progress using tagged cloning procedures.

V. PERSPECTIVES

Much of the effort in osmotic stress studies has been on the identification and analysis of OR genes. These OR genes have been isolated from various plant species. In the future, blind isolation of individual OR genes should yield to studies where all OR genes can be identified and analyzed simultaneously and systematically to gain a global and integrated picture of plant molecular osmotic responses. Recent technological advances in systematic gene expression analysis and in wholesale cDNA sequencing make this a practical goal (Nowak, 1995). The microarray method of gene expression analysis (Schena et al., 1995) is able to perform micro-Northerns on hundreds or even thousands of cloned genes simultaneously, thus making it possible to compare the expression of all known OR genes with each other and with non-OR genes in

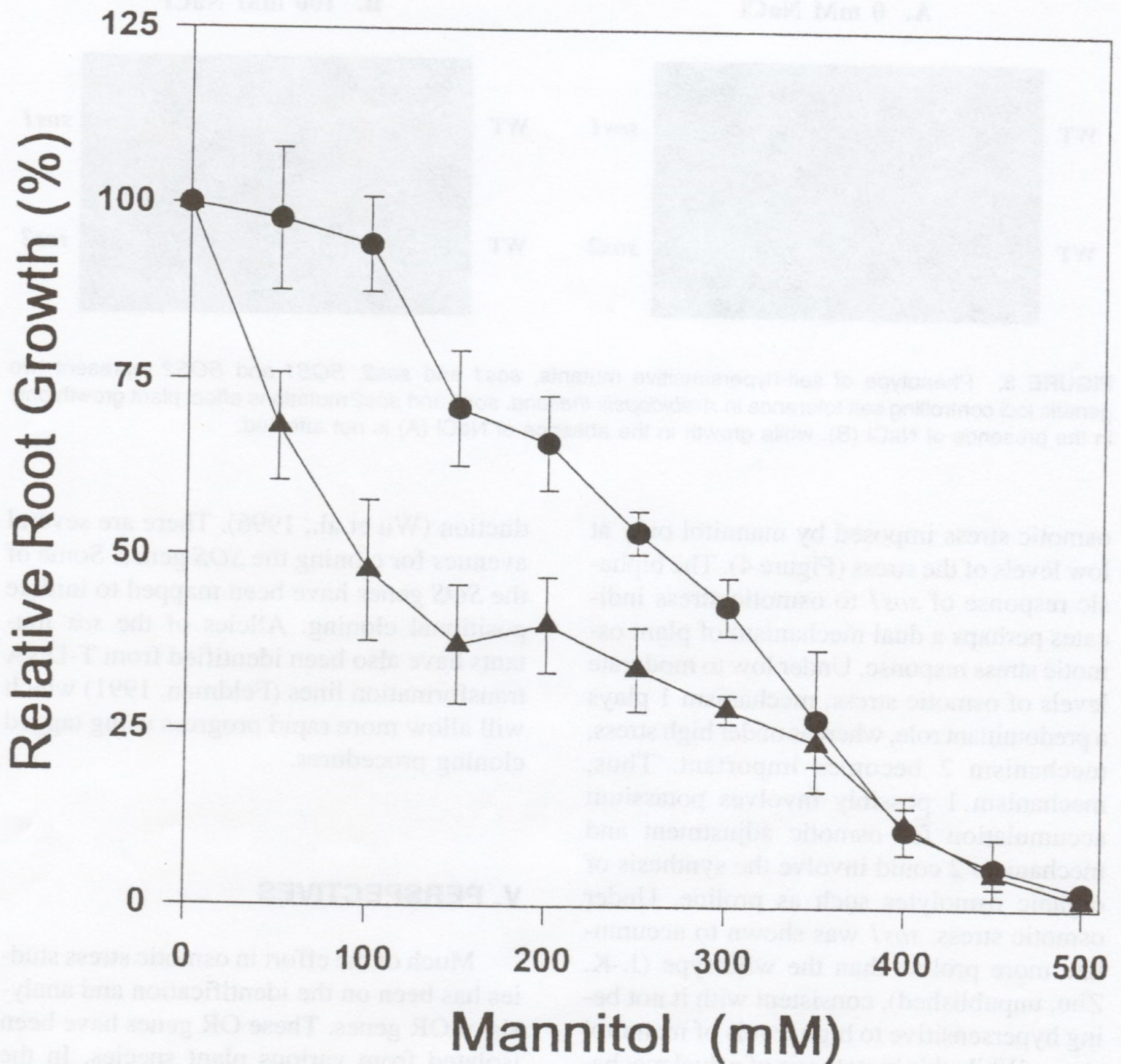

FIGURE 4. The salt-hypersensitive mutant *sos1* exhibits a biphasic response to osmotic stress. *sos1* is hypersensitive to low (<150 m*M* mannitol) but not high (>150 m*M* mannitol) osmotic stress.

one plant species. The SAGE (*s*erial *a*nalysis of *g*ene *e*xpression) method (Velculescu et al., 1995), which relies on short (9 to 12 bp) unique sequence tags from cDNAs and identifies the cDNAs by searching databanks, reveals the expression pattern of all mRNAs by the frequency of encounter of their cDNA tags. Using this method, one can obtain the expression profile of all sequenced cDNAs in the course of osmotic stress and at the same time discover previously not sequenced cDNAs that are osmotic stress regulated.

Obviously, both the microarray method and the SAGE strategy are most appropriate for plant species such as Arabidopsis and rice where large numbers of cDNAs have been sequenced. Perhaps the most valuable aspect of such comprehensive and systematic approaches toward OR gene identification will be an increased ability to see the patterns of genes that are coregulated by different environmental cues. With enough genes identified, the patterns may eventually allow the assignment of functional subsets of genes

based on their coregulatory properties. Such information should allow the consideration of theoretically new functions for genes which might eventually be tested by genetic modification of coordinate regulatory mechanisms.

Transcriptional regulation studies on OR genes are expected to continue in order to define more thoroughly the osmotic stress-regulated binding sites on OR gene promoters and the transcriptional factors that interact with these sites (see Figure 1). Eventually, this has to be connected with signal transduction analyses. The availability of well-characterized OR promoters should facilitate signal transduction studies where promoter-reporter gene fusions are used to identify *trans*-signaling mutants in which OR genes respond abnormally to osmotic stress (become constitutive or fail to be induced) (see Figure 5). These type of signaling studies have been successfully reported for salicylate signaling (Bowling et al., 1994; Cao et al., 1994) and phototransduction (Li et al., 1994) in Arabidopsis.

The need for molecular genetic analysis of osmotic stress tolerance using model plants can not be overemphasized. Initial results from such studies are encouraging. But more

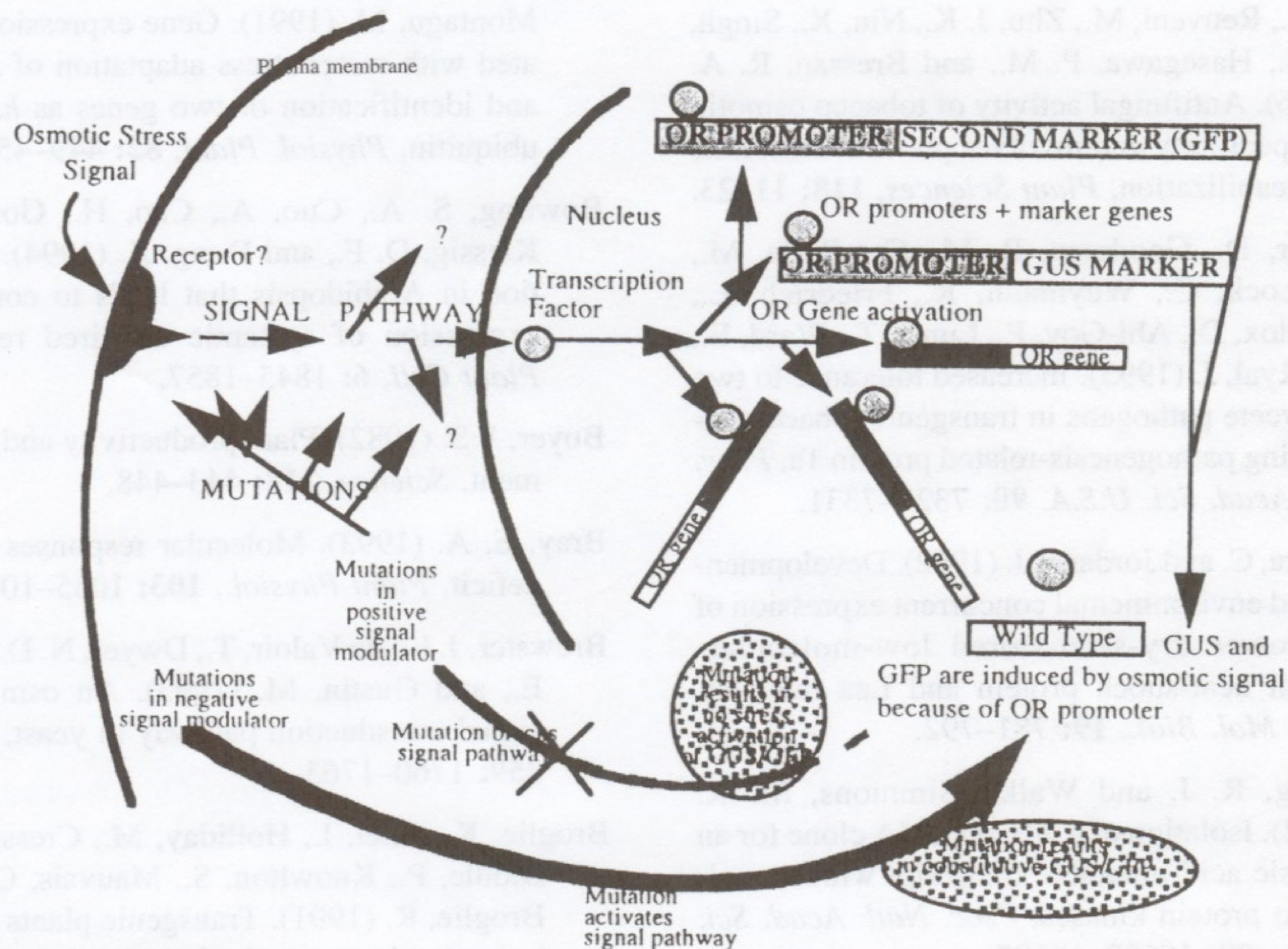

FIGURE 5. Molecular approach to isolate mutations in genes controlling osmotic stress signal transduction. An osmotically responsive (OR) gene promoter is fused to an easily recognized marker gene such as β-glucuronidase (GUS). This allows the detection of the activation of the stress signal pathway because the OR promoter responds to the activation and directs the synthesis of the enzyme β-glucuronidase. In the presence of its substrate (X-gluc), this enzyme produces a blue-colored product which indicates gene activation. Mutations in genes encoding proteins that participate in the signal pathway leading to this OR promoter disrupt (mutations in positive regulatory factors) or permanently activate (mutations in negative regulatory factors) the appearance of the blue color. Therefore, mutants can be identified by searching for permanently colored tissue or tissue that remains colorless after appropriate application of osmotic stress. The fusion of the same OR promoter to a second marker gene such as the green fluorescent protein (GFP) allows the elimination of mutations in the OR promoter or marker gene.

mutants with various levels of sensitivity or tolerance to osmotic stress agents (salt, mannitol, PEG, drought, etc.) are necessary. Integrated genetic, physiological, biochemical, and molecular analysis of these mutants will eventually provide us with insights into the cellular processes that are critical or rate limiting, and which genes are essential or sufficient for osmotic stress tolerance in plants. Only with these insights will we be able to intelligently engineer plants with increased osmotic stress tolerance.

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