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THE MOLECULAR BASIS OF DEHYDRATION TOLERANCE IN PLANTS

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ABSTRACT

Molecular studies of drought stress in plants use a variety of strategies and include different species subjected to a wide range of water deficits. Initial research has by necessity been largely descriptive, and relevant genes have been identified either by reference to physiological evidence or by differential screening. A large number of genes with a potential role in drought tolerance have been described, and major themes in the molecular response have been established. Particular areas of importance are sugar metabolism and late-embryogenesis-abundant (LEA) proteins. Studies have begun to examine mechanisms that control the gene expression, and putative regulatory pathways have been established. Recent attempts to understand gene function have utilized transgenic plants. These efforts are of clear agronomic importance.

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INTRODUCTION

This review considers molecular mechanisms involved in dehydration tolerance in plants. Most plants encounter at least transient decreases in relative water content at some stage of their life, and many also produce highly desiccation-tolerant structures such as seeds, spores, or pollen. Indeed, physiological drought also occurs during cold and salt stresses, when the main damage caused to the living cell can be related to water deficit (84, 124). Although we are still far from a complete understanding of the damage caused by drought, or the plant's tolerance mechanisms, much molecular data has been collected over the past few years. Current knowledge of the regulatory network governing the drought-stress responses is also fragmentary, with almost no information on signal perception. However, signal transduction, via ABA at least, and the promoter modules of several response genes, are starting to be elucidated.

Some of the most recent efforts to understand gene function have used transgenic plants, and these studies have significant implications for crop development. Plant breeding has already provided an enormous improvement in the drought tolerance of crop plants (1), with selection often allowing desired traits to be transferred from close wild relatives. However, most of the traits are complex, and their molecular basis is frequently not understood. With our rapidly expanding knowledge of the underlying molecular processes involved in dehydration tolerance, together with the technology of gene manipulation, crop improvement can now also be based on genetic material transferred from any organism and used in a directed manner.

RESEARCH STRATEGIES

Dehydration tolerance has been investigated using three main approaches in plants: (*a*) examining tolerant systems, such as seeds and resurrection plants; (*b*) analyzing mutants from genetic model species; and (*c*) analyzing the effects of stress on agriculturally relevant plants.

Tolerant Systems

One approach of physiological research in dehydration tolerance has been to use specific structures or species that can withstand severe desiccation. Most prominent in this category are certain seeds (73, 82), but desiccation-tolerant species such as resurrection plants (angiosperms) (8), mosses (particularly *Tortula ruralis*), and ferns (98) are also included. Both seeds and the resurrection plant *Craterostigma plantagineum* survive severe dehydration; therefore, the detailed molecular analyses of these systems should reveal expressed genes that contain the genetic information for desiccation tolerance.

SEEDS The final maturation stage of the development of seeds is characterized by desiccation, and as much as 90% of the original water is removed in attaining a state of dormancy with unmeasurable metabolism (73). This desiccated state allows survival under extreme environmental conditions and favors wide dispersal. The embryo cannot withstand desiccation at all developmental stages; tolerance is usually acquired well before maturation drying but is lost as germination progresses. The seeds of many species have been used to isolate the mRNA and proteins related to the desiccation-tolerance response, including, in particular, those of *Arabidopsis thaliana* (100) and of crop species such as cotton (*Gossypium* spp.) (6), barley (*Hordeum vulgare*) (9), maize (*Zea mays*) (99), and rice (*Oryza sativa*) (91). However, a significant complication with these studies is the difficulty of separating the pathways leading to desiccation tolerance from those involved with other aspects of development.

The main achievement of molecular studies with seeds has been the identification and characterization of the late-embryogenesis-abundant (LEA) proteins. LEA-protein mRNAs first appear at the onset of desiccation, dominate the mRNA population in dehydrated tissues (111), and gradually fall several hours after embryos begin to imbibe water (see section on Late-Embryogenesis-Abundant Proteins).

RESURRECTION PLANTS Resurrection plants are unique among angiosperms in their ability to survive during drought, when protoplastic desiccation can leave <2% relative water content in the leaves (8). When water is withheld from mature individuals of *C. plantagineum*, changes rapidly occur at the mRNA and protein levels (8), eventually leading to the tolerant state. A particular advantage of these plants in studies at the molecular level is that desiccation tolerance can be investigated in both whole plants and undifferentiated callus cultures (Tolerant callus of *C. plantagineum* is obtained by pretreatment with ABA) (8). In the callus tissue, and to a certain extent in whole *C. plantagineum* plants, the transition to the tolerant state is largely free of the complications of development or other adjustments inherent in seeds or other plant systems. One of the most

striking features of the desiccation-induced genes characterized from vegetative tissues of *C. plantagineum* has been their similarity to the genes expressed in seeds of other species.

Genetic Model Systems

Genetic model systems are a second major approach to the examination of dehydration tolerance. These systems take advantage of detailed genetic information, a wide range of mutants, and the feasibility of positional gene cloning. Progress in understanding the role of ABA in desiccation tolerance has been achieved by characterizing mutants, such as the ABA-deficient mutants *flacca* (tomato, *Lycopersicon esculentum*) (22) and *droopy* (potato, *Solanum tuberosum*) (108). A number of mutations related to ABA action are also available in *A. thaliana*, and their analysis has provided many insights into ABA-mediated drought responses. *A. thaliana* lines that are less sensitive to ABA than the wild-type have mutations at the *abi* loci [43; see also the maize *vp1* mutant (82)]. The detailed genetic information available for *A. thaliana* facilitated the isolation of the *ABI1* and *ABI3* genes by positional cloning (42, 74, 86). *ABI3* is specifically expressed in seeds and probably encodes a transcription factor able to activate *lea*-type genes (100), and *ABI1* encodes a calcium-regulated phosphatase.

Crop Plants

A third approach in researching dehydration tolerance has been to use species important to agriculture to analyze the plant response after drought stress. This type of study is useful because, through intensive breeding or in vitro selection, lines are available with differing degrees of tolerance. Thus, correlative evidence can be sought for genes putatively involved in the drought response. The transient and moderate drought stress represented in studies of crop species probably describes the most common form of dehydration that most plants are likely to encounter. The intensity of research has thus enabled a much more complete picture of the possible factors involved in drought tolerance to emerge.

GENES WITH UPREGULATED EXPRESSION IN RESPONSE TO DEHYDRATION

To establish the basic responses of plants to drought, two of the approaches already outlined—examination of tolerant systems and crop plants—have been most productive. One type of analysis involves targeting genes thought to be important, such as those for the many enzymes in drought-induced metabolic pathways. A second approach uses differential screening to isolate upregulated genes. These experiments have been successful in describing many genes

encoding proteins of known function associated with desiccation (Table 1). Differential screening has also revealed many genes of unknown function, which are included in Tables 1 and 2; the largest group is the array of LEA-protein-related genes (Table 3). Some of the genes may be involved in secondary problems of drought-stressed plants, such as increased susceptibility to pathogens, e.g. *pcht28* (encoding an acidic endochitinase) (Table 1; 17) and SC514 (encoding lipoxygenase) (Table 1; 10). Genes involved in signaling

Table 1 Genes upregulated by drought stress[a] and encoding polypeptides of known function

cDNA	Source	Encoded polypeptide	Ref
GapC-Crat	*Craterostigma plantagineum*	Cytosolic glyceraldehyde 3-phosphate dehydrogenase	129
pSPS1	*C. plantagineum*	Sucrose-phosphate synthase	[b]
pSS1; pSS2	*C. plantagineum*	Sucrose synthases	36
pPPC1	*Mesembryanthemum crystallinum*	Phosphoenolpyruvate carboxylase	130
pBAD	*Hordeum vulgare* (barley)	Betaine aldehyde dehydrogenase	54
cAtP5CS	*Arabidopsis thaliana*	δ^1-pyrroline-5-carboxylate synthetase	145
RD28	*A. thaliana*	Water channel	141
SAM1; SAM3	*Lycopersicon esculentum* (tomato)	*S*-adenosyl-L-methionine synthetases	37
rd19A; rd21A	*A. thaliana*	Cysteine proteases	67
UBQ1	*A. thaliana*	Ubiquitin extension protein	66
pMBM1	*Triticum aestivum* (wheat)	L-isoaspartyl methyltransferase	90
SC514	*Glycine max* (soybean)	Lipoxygenase	10
cATCDPK1; cATCDPK2	*A. thaliana*	Ca^{2+}-dependent, calmodulin-independent protein kinases	127
PKABA1	*T. aestivum*	Protein kinase	4
cAtPLC1	*A. thaliana*	Phosphatidylinositol-specific phospholipase C	53
Apx1 gene	*Pisum sativum* (pea)	Cytosolic ascorbate peroxidase	89
Sod 2 gene	*P. sativum*	Cytosolic copper/zinc superoxide dismutase	135
P31	*L. esculentum*	Cytosolic copper/zinc superoxide dismutase	102
pcht28	*L. chilense*	Acidic endochitinase	17
Atmyb2	*A. thaliana*	MYB-protein-related transcription factor	128
ERD11; ERD13	*A. thaliana*	Glutathione *S*-transferases	63
cAtsEH	*A. thaliana*	Soluble epoxide hydrolase	61

[a]The best-characterized plant genes from which cDNA clones have been demonstrated to show increased mRNA expression levels in response to drought stress have been included. Drought stress has been taken to include quite diverse treatments, ideally where water has been withheld from the plant, but also for example by applying osmotic stress with mannitol solutions or by detaching plant organs.

[b]Ingram & Bartels, unpublished data.

and control processes are considered in the section on Second Messengers and Signaling Molecules.

Metabolism

Changes in primary metabolism are a general response to stress in plants. For example, a cDNA-encoding glyceraldehyde-3-phosphate dehydrogenase, isolated from the resurrection plant *C. plantagineum* (Table 1; 129), shows increased expression during drought and upon ABA treatment. However, increased levels of the enzyme are also associated with other environmental stresses in plants, possibly reflecting increased energy demand. Proteases may also be an important feature of stress metabolism, dispensing with redundant proteins and depolymerizing vacuolar storage polypeptides, thereby releasing amino acids for the massive synthesis of new proteins (Tables 1 and 2; 50).

Enzymes of sugar metabolism are probably critical in desiccation tolerance. It has been demonstrated that certain sugars may be central to the protection of a wide range of organisms against drought (see section on Sugars). In *C. plantagineum*, the overall transcript levels of sucrose-phosphate synthase and sucrose synthase increase immediately in response to drought (36; J Ingram & D Bartels, unpublished data). The expression pattern is complex if the kinetics of individual transcript types are followed over the entire course of dehydration.

Enzymes involved in the synthesis of other compounds that can act as compatible solutes—and whose transcript levels are clearly upregulated during drought—include $\delta\Delta^1$-pyrroline-5-carboxylate synthetase (proline biosynthesis) (Table 1; 145) and betaine aldehyde dehydrogenase (glycine betaine biosynthesis) (Table 1; 54).

The induction of the mRNA encoding phospho*enol*pyruvate carboxylase in *Mesembryanthemum crystallinum* (Table 1; 130) highlights the importance of Crassulacean acid metabolism in enabling carbon fixation with minimal water loss. Such metabolism is a major response in a wide variety of plants to growth in dry conditions (139).

Osmotic Adjustment

Total water potential can be maintained during mild drought by osmotic adjustment, which involves utilizing sugars or other compatible solutes (12). Both ion and water channels are likely to be important in regulating water flux, and the relevance of these channels to drought-stress has been supported by the isolation of channel protein genes expressed in response to water deficit. The 7a cDNA from pea (*Pisum sativum*) (Table 2; 50) encodes a polypeptide with characteristic features of ion channels, while the RD28 cDNA (*A. thaliana*) (Table 1; 141) and probably also the H2-5 cDNA (*C. plantagineum*)

Table 2 Genes upregulated by drought stress[a] but encoding polypeptides of unknown function

cDNA	Source	Features of encoded polypeptide	Ref
26g	*Pisum sativum* (pea)	Some similarity to aldehyde dehydrogenase	50
7a	*P. sativum*	Similar to channel proteins	50
kin2	*Arabidopsis thaliana*	Similarity to animal antifreeze proteins	69
pcC 37-31	*Craterostigma plantagineum*	Similar to early-light-inducible proteins	7
TSW12	*Lycopersicon esculentum* (tomato)	A lipid transfer protein	125
pLE16	*L. esculentum*	Similar to lipid transfer proteins	107
15a	*P. sativum*	Similarity to proteases	50
pA1494	*A. thaliana*	Similarity to proteases	136
ERD1	*A. thaliana*	Similar to a Clp ATP-dependent protease subunit	64
Ha hsp17.6; Ha hsp17.9	*Helianthus annuus* (sunflower)	Low-molecular-weight heat-shock proteins	21
Athsp70-1	*A. thaliana*	Similar to the HSP70 heat-shock-protein family	66
Athsp81-2	*A. thaliana*	Similar to the HSP81 heat-shock-protein family	66
BLT4	*Hordeum vulgare* (barley)	Similar to protease inhibitors	32
P22	*Raphanus sativus* (radish)	Similar to protease inhibitors	77
BnD22	*Brassica napus* (rape)	Similar to protease inhibitors	31
pMAH9	*Zea mays* (maize)	Similar to RNA-binding proteins	47
MsaciA	*Medicago sativa* (alfalfa)	Similar to pUM90-1 and pSM2075 polypeptides	70
pUM90-1	*M. sativa*	Similar to MsaciA and pSM2075 polypeptides	80
pSM2075	*M. sativa*	Similar to MsaciA and pUM90-1 polypeptides	79
pBN115	*B. napus*	Similar to polypeptides encoded by pBN19 and pBN26 (*B. napus*) , and COR15 (*A. thaliana*)	134
RD22	*A. thaliana*	Similar to an unidentified seed protein from *Vicia faba*	56
salT	*Oryza sativa* (rice)		18
lti65 gene; *lti78* gene	*A. thaliana*		95
pcC 13-62	*C. plantagineum*		104

[a]See Footnote a in Table 1.

(J-B Mariaux & D Bartels, unpublished data) encode putative water-channel proteins (28).

Structural Adjustment

Drought stress has been shown to cause alterations in the chemical composition and physical properties of the cell wall (e.g. wall extensibility), and such changes may involve the genes encoding *S*-adenosylmethionine synthetase (Table 1; 37). Under nonstressful conditions, increased expression of *S*-adenosyl-L-methionine synthetase genes correlates with areas where lignification is occurring (101). Thus, the increased expression in drought-stressed tissue could thus also be due to lignification in the cell wall. Cell elongation stops under prolonged drought stress, and then lignification processes seem to begin (94a). Espartero et al (37) also noted that fungal elicitors cause the coinduction of *S*-adenosyl-L-methionine synthetase transcript with those of other enzymes, e.g. *S*-adenosyl-L-homocystein hydrolase or a methyltransferase, required for cell wall formation.

The *C. plantagineum pcC37-31* cDNA (Table 2; 7) encodes the dsp-22 protein, whose mRNA levels increase in response to various stresses. The cDNA shows significant homology to early light-inducible protein (ELIP) genes (1a). Light is involved in the regulation of the gene expression, and the encoded dsp-22 protein is chloroplastic. ELIPs may play a role in the assembly of the photosystem (1a). During desiccation, *C. plantagineum* chloroplasts undergo morphological changes, and thus the dsp-22 protein could bind pigments or help maintain assembled photosynthetic structures essential for resuming active photosynthesis during resurrection.

Degradation and Repair

Genes encoding proteins with sequence similarity to proteases, and which are induced by drought, have been isolated from both pea (Table 2; 50) and *A. thaliana* (Tables 1 and 2; 64, 67, 136). One of the functions of these enzymes could be to degrade proteins irreparably damaged by the effects of drought (50). During early drought in *A. thaliana*, there is an increase in levels of mRNA encoding ubiquitin extension protein (66), a fusion protein from which active ubiquitin is derived by proteolytic processing. This increase may be significant in terms of protein degradation, because ubiquitin has a role in tagging proteins for destruction. During drought stress, protein residues may be modified by chemical processes such as deamination, isomerization, or oxidation, and it is thus likely that enzymes with functions in protein repair are upregulated in response to drought. Indeed, the response to desiccation in mosses may largely be repair based (98). An example of such repair processes is the observation that L-isoaspartyl methyltransferases may convert modified L-isoaspartyl residues in damaged proteins back to L-aspartyl residues (Table 1; 90).

Mudgett & Clarke (90) have argued that such repair mechanisms could be particularly important during desiccation, when protein turnover rates are low. Although *Escherichia coli* mutants lacking the enzyme grow normally in the logarithmic phase when there is high protein turnover, they survive poorly in the stationary phase when turnover is much lower (75).

The products of two drought-induced genes isolated by differential screening have sequence similarity to heat-shock proteins (Table 2; 66). These encoded proteins are probably chaperonins, involved in protein repair by helping other proteins to recover their native conformation after denaturation or misfolding during water stress. The low-molecular-weight heat-shock proteins (Table 2; 21) may also be chaperonins. This function has been demonstrated for a mammalian low-molecular-weight heat-shock protein (58). An alternative function may be in the sequestration of specific mRNAs in cells subjected to drought (96).

Removal of Toxins

Enzymes concerned with removing toxic intermediates produced during oxygenic metabolism, such as glutathione reductase and superoxide dismutase, increase in response to drought stress and are probably very important in tolerance (89). Decreasing leaf water content and consequent stomatal closure result in reduced CO_2 availability and the production of active oxygen species such as superoxide radicals (117). Increased photorespiratory activity during drought is also accompanied by elevated levels of glycolate-oxidase activity, resulting in H_2O_2 production (89). This could explain why genes encoding enzymes that detoxify active oxygen species such as ascorbate peroxidase (Table 1; 89) and superoxide dismutase (Table 1; 102, 135) have been found upregulated in response to drought.

Late-Embryogenesis-Abundant Proteins

The genes encoding late-embryogenesis-abundant (LEA) proteins are consistently represented in differential screens for transcripts with increased levels during drought. LEA proteins were first described from research into genes abundantly expressed during the final desiccation stage of seed development (see above). Circumstantial evidence for their involvement in dehydration tolerance is strong: The genes are similar to many of those expressed in vegetative tissues of drought-stressed plants (Table 3), and desiccation treatments can often induce precocious expression in seeds. ABA can also induce the *lea* genes in seeds and vegetative tissues.

GENERAL FEATURES Groupings for dividing the LEA proteins originate from a dot matrix analysis with proteins from cotton. A group was assigned on the basis of one cotton LEA protein showing regions of significant homology with

Table 3 Genes upregulated by drought stress[a] that encode polypeptides related to late-embryogenesis-abundant LEA proteins

cDNA	Source	Relationship of encoded polypeptide to LEA proteins	Ref
Ha ds10	*Helianthus annuus* (sunflower)	D19-LEA-protein related	3
Em	*Triticum aestivum* (wheat)	D19-LEA-protein related	76
B19.1; B19.3; B19.4	*Hordeum vulgare* (barley)	D19-LEA-protein related	39
pLE25	*Lycopersicon esculentum* (tomato)	D113-LEA-protein related	23
Ha ds11	*H. annuus*	D113-LEA-protein related	3
pRABAT1	*Arabidopsis thaliana*	D11-LEA-protein related	72
pcC 27-04	*Craterostigma plantagineum*	D11-LEA-protein related	104
M3 (RAB-17)	*Zea mays* (maize)	D11-LEA-protein related	20
B8; B9; B17	*H. vulgare*	D11-LEA-protein related	20
pLE4	*L. esculentum*	D11-LEA-protein related	23
pcC 6-19	*C. plantagineum*	D11-LEA-protein related	104
TAS14	*L. esculentum*	D11-LEA-protein related	46
pLC30-15	*L. chilense*	D11-LEA-protein related	16
H26	*Stellaria longipes*	D11-LEA-protein related	110
pRAB 16A	*Oryza sativa* (rice)	D11-LEA-protein related	91
pcECP40	*Daucus carota* (carrot)	D11-LEA-protein related	62
ERD10; ERD14	*A. thaliana*	D11-LEA-protein related	65
pMA2005	*T. aestivum*	D7-LEA-protein related	26
pMA1949	*T. aestivum*	D7-LEA-protein related	27
pcC 3-06	*C. plantagineum*	D7-LEA-protein related	104
pcC 27-45	*C. plantagineum*	D95-LEA-protein-related	104

[a]See Footnote a in Table 1.

at least one protein from another species (33). The "type" of cotton proteins used for these groupings were LEA D19 (Group 1), LEA D11 [Group 2 (also termed dehydrins)], and LEA D7 (Group 3). The cotton proteins LEA D113 (34, 35) and LEA D95 (40) now define two additional classes. This system will remain useful until clear functions can be assigned.

LEA proteins appear to be located in many cell types and at variable concentrations (19, 34, 35, 45), and within the cell they appear to be predominantly—but not exclusively—cytosolic (19, 45, 91, 114). The concentrations in the cell are characteristically very high. For example, in mature cotton embryo cells, the D7 LEA proteins represent about 4% of nonorganellar cytosolic protein (about 0.34 mM) (111).

A general structural feature of the LEA proteins is their biased amino acid composition, which results in highly hydrophilic polypeptides, with just a few residues providing 20–30% of their total complement. For example, a deduced

D19 protein from cotton contains 13% glycine and 11% glutamic acid (6). Furthermore, most LEA proteins lack cysteine and tryptophan residues.

ROLES We await direct experimental evidence that LEA proteins can protect specific cellular structures or ameliorate the effects of drought stress. Because they are highly hydrophilic, it appears unlikely that they occur in specific cellular structures. Also, their high concentrations in the cell and biased amino acid compositions suggest that they do not function as enzymes (6).

The randomly coiled moieties of some LEA proteins are consistent with a role in binding water. Total desiccation is probably lethal, and therefore such proteins could help maintain the minimum cellular water requirement. McCubbin & Kay (83) have found that the Em protein (D19-group) (Table 3; 76) from wheat is considerably more hydrated than most globular polypeptides because it is over 70% random coil in normal physiological conditions. The random coil tails of the D113 proteins could also bind considerable amounts of water, although the long *N*-terminal helical domain would not share this property (34, 35).

A major problem under severe dehydration is that the loss of water leads to crystallization of cellular components, which in consequence damages cellular structures. This may be counteracted by LEA proteins, and some of the LEA proteins could essentially be considered compatible solutes, which supports the likely role of sugars in maintaining the structure of the cytoplasm in the absence of water. Baker et al (6) have suggested that LEA proteins D11 and D113 could be involved in the "solvation" of cytosolic structures. The random coiling would permit their shape to conform to that of other structures and provide a cohesive layer with possibly greater stability than would be formed by sugars. Their hydroxylated groups would solvate structural surfaces. Furthermore, they could be superior to sucrose as protectants in being less likely to crystallize. However, for the D11-related protein RAB-17, a regulatory role has been postulated (see below).

Baker et al (6) have hypothesized that the 11-amino-acid motif (T/A A/T Q/E A/T A/T K/R Q/ED K/R A/T X ED/Q) (34) of LEA protein D-29 (which is also present in D7 LEA proteins) could counteract the irreversibly damaging effects of increasing ionic strength in the cytosol during desiccation. Such problems could be mitigated by the formation of salt bridges with amino acid residues of highly charged proteins. The repeating elements most likely exist as amphiphilic helices (34), which means that hydrophobic and hydrophilic amino acids are contained in particular sectors of the helix. The helices probably form intramolecular bundles, which would present a surface capable of binding both anions and cations. Further analyses of the D7-group molecules have allowed precise structural predictions to be made: The intersurface edges

of the interacting helical regions of the (putative) dimer reveal periodically spaced binding sites for suitably charged ions.

SUGARS

The involvement of soluble sugars in desiccation tolerance in plants is suggested by studies in which the presence of particular soluble sugars can be correlated with the acquisition of desiccation tolerance (73). Such studies have followed work with animals, fungi, yeast, and bacteria, in which a high level of the disaccharide trehalose has been established as important in surviving desiccation. Trehalose is the most effective osmoprotectant sugar in terms of minimum concentration required (25). Whereas trehalose is extremely rare in plants, sucrose—together with other sugars—appears able to substitute. Although sugar accumulation is not the only way in which plants deal with desiccation (12), it is considered an important factor in tolerance.

Many studies with seeds have demonstrated the accumulation of soluble sugars during the acquisition of desiccation tolerance (73); similar results have been demonstrated in resurrection plants. A common theme has emerged. Various soluble carbohydrates may be present in fully hydrated tissues, but sucrose usually accumulates in the dried state. For example, desiccation in the leaves of *C. plantagineum* is accompanied by conversion of the C8-sugar 2-octulose (90% of the total sugar in hydrated leaves) into sucrose, which then comprises about 40% of the dry weight (11).

Total water potential can be maintained during mild drought by osmotic adjustment. Sugars may serve as compatible solutes permitting such osmotic adjustment, although many other compounds usually associated with salt stress are also active, such as proline, glycine betaine, and pinitol (54, 84, 145). Increasing sucrose synthesis and sucrose-phosphate synthase activity is not only a drought-response of desiccation-tolerant plants such as *C. plantagineum* (36) but also of plants that cannot withstand extreme drying, such as spinach (109).

One way sugars may protect the cell during severe desiccation is by glass formation: Rather than solutes crystallizing, through the presence of sugars a supersaturated liquid is produced with the mechanical properties of a solid (68). Glass formation has been demonstrated in viable maize seeds and has been associated with their viability (137). Differential scanning calorimetry has been used to examine the effect of termperature on glass formation by sugar mixtures; only sugar mixtures equivalent in concentration and composition to those in desiccation-tolerant embryos are able to form glass at ambient temperatures (68). It seems likely that sugar composition, rather than just concentration, is related to glass formation. During desiccation, glass would fill space, thus preventing cellular collapse, and in restricting the molecular

diffusion required by chemical reactions would permit a stable quiescent state (68).

Phosphofructokinase is a tetrameric enzyme that usually dissociates irreversibly into inactive dimers during dehydration (14). However, it was found that in vitro the disaccharides sucrose, maltose, and trehalose stabilize the activity of the enzyme during drying.

Crowe et al (24) have shown that, in vitro, drying and rehydration of the model-membrane sarcoplasmic reticulum usually results in the fusion of vesicles and loss of the ability to transport calcium. However, when the sugar trehalose was present at concentrations equivalent to those in desiccation-tolerant organisms, functional vesicles were preserved. Many other studies show that sugars can protect membranes in vitro (25); it is suggested that sugars alter physical properties of dry membranes so that they resemble those of fully hydrated biomolecules.

The mechanism by which proteins are stabilized by sugars is better understood than the situation with membranes. Infrared spectroscopy has shown that trehalose probably forms hydrogen bonds between its hydroxyl groups and polar residues in proteins (25). Hydrogen bonding between the hydroxyl group of trehalose and the phosphate head group of phospholipids can be inferred from comparisons of changes in the infrared spectrum of the molecules during dehydration. Strauss & Hauser (120) used the cation Eu^{3+}, which is known to form a specific ionic bridge to the phosphate of phospholipids, to show that sucrose is probably bound between phosphate sites in dry membranes. This was inferred from experiments in which Eu^{3+} ions were added to preparations of sucrose and phosphatidylcholine vesicles; the stabilization of liposomes by sucrose during freeze drying decreased as the Eu^{3+} ions were added, which suggests competitive binding of sucrose and Eu^{3+} at the phosphate sites of the phospholipids.

REGULATION OF GENE EXPRESSION DURING DEHYDRATION

The machinery leading to the expression of drought-stress genes conforms to the general cellular model, with a complex signal transduction cascade that can be divided into the following basic steps: (*a*) perception of stimulus; (*b*) processing, including amplification and integration of the signal; and (*c*) a response reaction in the form of de novo gene expression. No molecular data are available on the perception of drought stress, although turgor change has been suggested as a possible physical signal. An attractive model for the activation of a transduction pathway by a stress signal has been derived from studying the heat-shock response in yeast (60). Kamada et al (60) suggest that heat-induced activation of a particular pathway is in response to increased

membrane fluidity in the cell wall. The cell detects this weakness in the cell wall by sensing stretch in the plasma membrane. Examples such as this from simple systems may provide the conceptual framework for devising experiments in plants.

The drought-activated signal transmission process has begun to be dissected at the molecular level, mostly on the basis of studies of isolated drought-responsive genes. Endogenous ABA levels have been reported to increase as a result of water deficit in many physiological studies, and therefore ABA is thought to be involved in the signal transduction (15, 43). Many of the drought-related genes can be induced by exogenous ABA; however, this does not necessarily imply that all these genes are also regulated by ABA in vivo.

We now discuss promoter studies, signaling molecules, and both posttranscriptional and posttranslational modifications in the context of drought-regulated gene expression.

Promoter Studies

CIS- AND *TRANS*-ACTING ELEMENTS Many of the changes in mRNA levels observed during drought reflect transcriptional activation. Treatment with ABA can also induce these changes, and this treatment has been utilized for setting up experimental systems to define *cis*- and *trans*-acting elements. *cis*- and *trans*-acting elements involved in ABA-induced gene expression have been analyzed extensively (Tables 4 and 5; 43).

Table 4 *cis*-acting promoter elements relevant to ABA or drought

Gene	Element	Sequence[a]	Ref
Rab16A (*Oryza sativa*)	ABRE (Motif I)	GT*ACGT*GGCGC	119
EM (*Triticum aestivum*)	Em1A	GGAC*ACGT*GGC	51
Hex3 (synthetic tetramer) (derived from *Nicotiana tabacum*)		GGTGACGTGGC	71
rab28 (*Zea mays*)	ABRE	CC*ACGT*GG	106
Cat1 (*Zea mays*)		CCAAGAAGTC-C*ACGT*GGAGGTGGAAGAG	138
HVA22 (*Hordeum vulgare*)	ABRE3 and CE1	GCC*ACGT*ACA and TGCCACCGG	118
CDeT27-45 (*Craterostigma plantagineum*)		AAGCCCAAATTTCA-CAGCCCGATAACCG	93
rd29 (*Arabidopsis thaliana*)	DRE	TACCGACAT	144

[a]The G-box core elements ACGT are in italic.

The best-characterized *cis*-element in the context of drought stress is the ABA-responsive element (ABRE), which contains the palindromic motif CACGTG with the G-box ACGT core element (44). ACGT elements have been observed in a multitude of plant genes regulated by diverse environmental and physiological factors. Systematic DNA-binding studies have shown that nucleotides flanking the ACGT core specify the DNA-protein interactions and subsequent gene activation (57). G-box-related ABREs have been observed in many ABA-responsive genes, although their functions have not always been proven experimentally. The best-studied examples of these ABRE promoter elements are Em1a from wheat and Motif I from the rice *rab 16A* gene (Table 4; 81, 92). Multiple copies of the elements fused to a minimal 35S promoter confer an ABA response to a reporter gene (51, 119), which supports the hypothesis that ABREs are critical for the ABA induction of relevant genes (although it is difficult to explain why single copies are not

Table 5 Characterization of promoters in transgenic plants

Gene	Native gene activity	Reporter gene activity	Ref
Rab 16B	Embryos of Oryza sativa	*Nicotiana tabacum* embryos	142
Em	Embryos of Triticum aestivum	*Nicotiana tabacum* embryos	81
Rab 17	Embryos of Zea mays	The embryos and endosperm of Arabidopsis thaliana	131
Hex3 (synthetic tetramer) (derived from *Nicotiana tabacum*)		Mature seeds of *N. tabacum;* inducible in seedlings by desiccation, salt, and ABA	71
Rd 22	Dehydrated A. thaliana plants	Constitutive in flowers and stems of *A. thaliana;* inducible in *N. tabacum* by ABA or dehydration	56
Rd 29A	Dehydrated A. thaliana plants	Inducible by dehydration in most vegetative parts of *A. thaliana;* inducible in *N. tabacum* by cold, ABA, and salt	143, 144
CDeT27-45	C. plantagineum dehydrated or ABA-treated vegetative tissues	In embryos and mature pollen of both *A. thaliana* and *N. tabacum*	39a, 88
CDeT6-19	C. plantagineum dehydrated or ABA-treated vegetative tissues	In developing embryos and mature pollen of both *A. thaliana* and *N. tabacum* also inducible in their leaves and guard cells	87, 39a, 123
CDeT11-24	C. plantagineum dehydrated or ABA-treated vegetative tissues	Embryos of both *A. thaliana* and *N. tabacum;* inducible in *A. thaliana* leaves by dehydration	[a]
DC8	Embryos of *Daucus carota*	D. carota seed tissues	49
DC3	Embryos of Daucus carota	N. tabacum seedlings; also inducible in the leaves by either drying or ABA treatment	132

[a]R Velasco, F Salamini & D Bartels, unpublished data.

sufficient for this response). The ABA effect on transcription was orientation independent in both the wheat and rice elements, which suggests that they possibly function as enhancer elements in their native genes. Electrophoretic-mobility-shift assays and methylation-interference footprinting have shown that both Em1a and Motif1 interact with nuclear proteins; these DNA-binding proteins are constitutively expressed in an ABA-independent manner (51, 92). cDNAs encoding ABRE-binding proteins (wheat EMBP-1 and tobacco TAF-1) have been cloned and shown to contain a basic region adjacent to a leucine-zipper motif that is characteristic of transcription factors (51, 97). Despite the fact that both proteins exhibit specific and distinct binding properties, their roles in vivo are not understood. It seems possible that they are not directly involved in ABA-responsive gene expression but that they cooperate with other regulatory factors.

Recently, two different elements have been described that must be present to allow a single copy of the ABRE to mediate transcriptional activation in response to ABA, and thus define an ABA response complex. An ABRE element in the barley *Amy32b* α-amylase promoter has been shown to allow ABA-stimulated transcription to increase only in the presence of an O2S element that interacts with the ABRE within tight positional constraints. A second coupling element has been identified during promoter analysis of the ABA-induced barley *HVA22* promoter (118). The coupling element (CE1) acts together with a G-box-type ABRE (GCCACGTACA) in conferring high ABA induction, whereas the ABRE alone is not sufficient for transcriptional activation. CE1-like elements have been found in many other ABA-regulated promoters, but their function remains to be demonstrated (118). The specific sequence of a coupling element may profoundly affect the specificity of ABA-driven gene expression and may explain differences between functional and nonfunctional ABREs.

In promoters such as *CDeT27–45* or *CDeT6–19*, isolated from *C. plantagineum*, G-box-related ABREs do not appear to be major determinants of the ABA or drought response (87, 88). The *CDeT27–45* promoter contains an element that specifically binds nuclear proteins from ABA-treated tissue; this promoter fragment is essential but not sufficient for conferring a response to ABA on a reporter gene (93).

Besides the ABA-mediated gene expression, the investigation of drought-induced genes in *A. thaliana* has also revealed ABA-independent signal transduction pathways (144). The *A. thaliana* genes *rd29A* and *rd29B* are differentially induced under conditions of dehydration, salt or cold stress, and ABA treatment. The *rd29A* gene has at least two *cis*-acting elements. 1. The 9-bp direct repeat sequence, TACCGACAT, termed the dehydration-responsive element (DRE), functions in the initial rapid response of *rd29A* to drought, salt, or low temperature (144). 2. The slower ABA response is medi-

ated by another fragment that contains an ABRE (143). It will be interesting to see whether the same *cis* elements function in other *A. thaliana* genes that are induced during progressive drought; besides ABA, at least two other different signals are involved in this induction (48). The existence of ABA-dependent and -independent pathways is corroborated by studies on the accumulation of three distinct *Lea* transcripts in barley embryos. Selected transcripts increased in response to osmotic stress without requiring ABA, whereas induction by salt did require ABA (38).

A different class of potential transcription factors with relevance to drought stress is represented by the *A. thaliana* gene *Atmyb2.* This gene encodes an MYB-related protein and is induced by dehydration or salt stress and by ABA (128). Plant *myb*-related genes comprise a large family that may play various roles in gene regulation. The ATMYB2 protein expressed in *E. coli* has been shown to bind the MYB-recognition sequence, PyAACTG, which supports its role as a DNA-binding protein. Another *A. thaliana* drought stress–induced gene, *rd22* (56), has a promoter with no ABRE but with two recognition sites for the transcription factors MYC and MYB. Binding of the ATMYB2 protein appears likely but has not been proven experimentally.

ASSESSMENT OF PROMOTERS IN TRANSGENIC PLANTS Promoter analysis using transient expression assays has resulted in the characterization of several distinct *cis*-acting elements and the cloning of related transcription factors. However, tests with a range of promoters derived from drought- or ABA-inducible structural genes in transgenic plants have shown that the promoter activities defined in transient assays are not always correlated with the expression patterns of their corresponding structural genes. A summary of results is given in Table 5. A problem with the approach could be the use of heterologous plant expression systems. Although the genes are always active in seeds, expression in vegetative tissues is not always induced upon drought or ABA treatment, which points to an incomplete activation of the transcriptional machinery. It is interesting to note that ectopic expression of the otherwise seed-specific *abi-3* gene product (42) allows the ABA-mediated activation of *Lea* genes in vegetative tissues of *A. thaliana* (100). Similarly, the *CDeT27–45* promoter from *C. plantagineum* was only fully responsive to ABA in *A. thaliana* in the presence of the ABI3 product (39a). These experiments suggest that the ABI3 gene product can functionally interact with different promoters.

Second Messengers and Signaling Molecules

Protein phosphorylation and dephosphorylation (via kinases and phosphorylases, respectively) are major mechanisms of signal integration in eukaryotic cells. Two *A. thaliana* genes encoding calcium-dependent kinases are induced by dehydration (Table 1; 127), which suggests that they may participate in

phosphorylation processes occurring in response to drought. A serine-threonine-type protein kinase has also been isolated from wheat and shows accumulation in ABA-treated embryos and in dehydrated shoots (Table 1; 4). However, the phosphorylation targets of these kinases are not yet known, and their exact roles are obscure.

A role for protein phosphorylation in the drought-stress response is also suggested on the basis of functional studies of the ABA-responsive RAB17 protein from maize (45). This protein is highly phosphorylated in vivo, probably via catalysis by casein kinase 2. The RAB17 protein has been found to be distributed between the cytoplasm and the nucleus of maize embryos, in different states of phosphorylation (5, 45). Biochemical studies showed that RAB17 binds peptides with nuclear localization signals and that the binding is dependent on phosphorylation. It has been suggested that RAB17 mediates the transport of specific nuclear-targeted proteins during stress (45).

Cytoplasmic calcium acts as a second messenger in many cellular processes and may also be involved in the signaling pathways mediating the expression of drought-related genes (13). Stomatal closure is an early plant response to drought, and increases in the cytosolic concentration of free calcium, together with pH changes, are considered to be primary events in the ABA-mediated reduction of stomatal turgor (115). However, it is likely that calcium, together with phosphorylation processes, plays a more general role in the mechanisms associated with drought-stress perception. For example, the *A. thaliana ABI1* gene product is thought to be a calcium-activated phosphoprotein phosphatase (74, 86). Furthermore, a transcript encoding a phosphatidylinositol-specific phospholipase C, an enzyme involved in catalyzing the synthesis of inositol 1,4,5-triphosphate, increases during dehydration (Table 1; 53); inositol-triphosphate stimulates the release of Ca^{2+} from intracellular stores.

Posttranscriptional Control

Much of the effort to understand gene regulation during drought has been devoted to transcriptional mechanisms, but it has become clear that other potential control points include mRNA processing, transcript stability, translation efficiency, and protein modification or turnover. General posttranscriptional mechanisms in plants have recently been reviewed (41, 121). Evidence is emerging that these mechanisms also play a role during stress responses. In *C. plantagineum,* drought stress induces some proteins that are synthesized in a light-dependent manner (see above); for some of these proteins the levels of the mRNA do not parallel those of the proteins, which suggests posttranscriptional regulation (2). A more detailed analysis of alfalfa (*Medicago sativa*) suggests that increased mRNA stability is involved in the accumulation of the MsPRP2 transcript (30). The maize *pMAH9* cDNA clone encodes a transcript that is upregulated by drought. The corresponding protein has RNA-binding

characteristics, which suggests that it may play a role in the selective stabilization of mRNAs (Table 2; 78).

A second major control point appears to be the posttranslational modification of proteins, in which phosphorylation is a key mechanism. For example, phosphorylation is involved in the modification of the fructose-1,6-bisphosphatase in drought-stressed leaves of sugar beet (*Beta vulgaris*) (52). Some of the proteases induced by drought stress (Tables 1 and 2) may also have a function in posttranslational modification. Schaffer & Fischer (113) have hypothesized that a thiol protease, the mRNA of which is cold-induced in tomato, could proteolytically activate certain proteins. This mechanism could also operate during drought stress. It has also been suggested that putative protease inhibitors induced during drought (Table 2) have a role in controlling the activity of endogenous proteases (31).

Downregulation of Genes

Until now, most research has focused on understanding how relevant genes are upregulated during drought stress. However, the response to drought also involves the downregulation of several genes. For example, studies of *C. plantagineum* have revealed that transcripts encoding proteins relevant to photosynthesis are downregulated during the dehydration process and thus possibly reduce photooxidative stress (C Bockel & D Bartels, unpublished data). Jiang et al (59) have also shown that the promoter regions of storage protein genes contain the information for their downregulation during seed desiccation. Furthermore, it has recently been reported that histone H1 transcripts accumulate in response to drought stress in vegetative tissues of tomato, and it was suggested that H1 histones are implicated in the repression of gene expression (E Bray, personal communication).

TRANSGENIC PLANTS ASSESSING GENE FUNCTION

Transgenic plants allow the targeted expression of drought-related genes in vivo and are therefore an excellent system to assess the function and tolerance conferred by the encoded proteins. With ectopic expression of genes involved in controlling ABA biosynthesis, it should also be possible to alter the hormonal balance in vivo and thus to clarify the role of ABA in the drought response. Another purpose for using transgenic plants is to improve drought tolerance in agronomically valuable plants. However, despite extensive research, examples of transgenic plants with improved stress tolerance are scarce (see also 12). A reason for this is that stress tolerance is likely to involve the expression of gene products from several pathways.

The accumulation of low–molecular weight metabolites that act as osmoprotectants is a widespread adaptation to dry, saline, and low-temperature

conditions in many organisms. In engineering plants that synthesize protective osmolytes, microorganisms appear to be useful sources for genes. Transgenic tobacco plants that synthesize and accumulate the sugar alcohol mannitol have been obtained by introducing a bacterial gene that encodes mannitol 1-phosphate dehydrogenase. Plants producing mannitol showed increased salt tolerance (122). Similarly, a freshwater cyanobacterium that was transformed with *E. coli bet* genes produced significant amounts of glycine betaine; this stabilized photosynthetic activity in the presence of sodium chloride, allowing improved growth (94). Tobacco plants that accumulate the polyfructose molecule fructan have been engineered using microbial (*Bacillus subtilis* or *Streptococcus mutans*) fructosyltransferase genes. These plants showed improved growth under polyethylene-mediated drought stress (105), with a positive correlation observed between the level of accumulated fructans and degree of tolerance. The mechanism by which fructans confer tolerance is not known, although a mere osmotic effect seems unlikely.

One consequence of drought and many other stresses is the production of activated oxygen molecules that cause cellular injury, and therefore plants with increased concentrations of oxygen scavengers should show improved performances under nonlethal stress conditions. When tobacco Mn-superoxide dismutase was overexpressed in alfalfa, the plants showed an increased growth rate after freezing stress (85).

Although *Lea*-related genes are upregulated abundantly in most plants during all types of osmotic stress, separate ectopic expression of three different representatives in tobacco did not yield an obvious drought-tolerant phenotype (55). However, this result is perhaps less surprising considering that drought stress does induce an array of different LEA-related proteins in plants. It is also likely that other factors are required for the expression of tolerance where LEA-type proteins are involved.

FUTURE PERSPECTIVES

Despite the many genes that have been identified in association with drought stress, much of the data is descriptive, with the functions of only a few of the encoded proteins established. The production of mutants using an antisense-RNA approach is a powerful technique that should continue to elucidate certain aspects of stress tolerance, but it has been most successful only with well-characterized areas of plant metabolism. It is also difficult to devise screening procedures for useful dehydration-tolerance mutants, because of the array of processes simultaneously affected by drought. Resurrection plants would be an excellent source for mutants with decreased tolerance, but *C. plantagineum*, as well as many other resurrection-plant species, has a polyploid genome and is thus unsuitable. Mutant analyses so far exploited for

drought stress have been with ABA-related mutations, and the power of the approach is shown in the cloning of *Abi1* and *Abi3* (43), which has provided new perspectives. Another valuable approach may be to identify those metabolic steps that are most sensitive to drought stress (a technique used to genetically dissect salt stress in yeast) (116). Such an approach can at least begin to elucidate which gene products are of primary importance.

The plant hormone ABA regulates different aspects of the drought-stress response, and thus the synthesis of pure active ABA analogues (103) may help in the development of probes for ABA-binding proteins, which could then shed some light on primary signals. In contrast with the situation with signal perception, some information is available on *cis*- and *trans*-regulatory factors. Several elements in a promoter need to cooperate with multiple DNA-binding proteins to mediate gene expression. The recently described coupling elements (118) are probably only a beginning in resolving the regulatory network. Little progress has been made with the cloning and analysis of drought-related transcription factors, although a biochemical approach and use of the recently established yeast one- and two-hybrid systems (133) should produce new insights. Regulation at stages beyond transcription must also be further considered, because this could make a major contribution to the final gene expression pattern.

The complexity of drought tolerance apparent throughout this review points to control by multiple genes, and thus the identification of quantitative-trait-loci (QTLs) for drought resistance may well be an effective analytical tool. The approach has just begun to be applied to the environmental-stress responses of plants (126) and is particularly promising considering that saturated DNA–marker maps are now available for both genetic model plants and crop plants.

The molecular analysis of the drought response has arrived at a stage where research can build upon a large collection of characterized genes. The use of novel approaches combining genetic, biochemical, and molecular techniques should provide exciting results in the near future.

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