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A novel methyl transferase induced by osmotic stress in the facultative halophyte *Mesembryanthemum crystallinum*

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Molecular mechanisms of osmotic stress tolerance were studied in *Mesembryanthemum crystallinum* (ice plant), a facultative halophyte capable of adjusting to and surviving in highly saline conditions. We screened a subtracted cDNA library enriched for salt stress-induced mRNAs to identify transcripts involved in this plant's adaptation to salinity. One mRNA, *Imt1*, was found to be up-regulated in leaves and, transiently, in roots. Nuclear run-on assays indicated that this mRNA is transcriptionally regulated. *Imt1* encoded a predicted polypeptide of M_r 40 250 which exhibited sequence similarity to several hydroxymethyl transferases. Expression of the protein in *Escherichia coli* and subsequent activity assays identified the protein as a novel myo-inositol *O*-methyl transferase which catalyzes the first step in the biosynthesis of the cyclic sugar alcohol pinitol. Pinitol accumulates in salt-stressed *M.crystallinum* and is abundant in a number of salt- and drought-tolerant plants. The presence of high levels of sugar alcohols correlates with osmotolerance in a diverse range of organisms, including bacteria, fungi and algae, as well as higher plants. The stress-initiated transcriptional induction of IMT1 expression in a facultative halophyte provides strong support for the importance of sugar alcohols in establishing tolerance to osmotic stress in higher plants.

Key words: gene expression/myo-inositol *O*-methyl transferase/osmoprotection/salt stress/sugar alcohol

Introduction

For plants, environmental stress is an inescapable and persistent condition. Water stress imparted by drought or high salinity is the most widespread abiotic stress and constitutes the most stringent factor in limiting plant distribution and productivity (Boyer, 1982). A large body of physiological work has described how various species respond to water deficit. A number of salt tolerant plants (halophytes) have evolved strategies that enable them to adjust to salinity, avoid salinity-induced water stress and succeed in environments that will not accommodate salt-sensitive species (glycophytes). Such strategies can include changes in photosynthesis, alterations in membrane structure, exclusion of salts, accumulation and partitioning of ions, increases in intracellular organic solutes and precocious entry into reproductive phase and senescence (Jefferies, 1981).

There have been a number of recent investigations into the molecular responses of plants to water stress. Most of these studies have identified polypeptides that are induced in glycophytic plants during water stress imposed by drought (Gomez *et al.*, 1988; Mundy and Chua, 1988; Mason and Mullet, 1990; Cohen *et al.*, 1991; Plant *et al.*, 1991). Bartels *et al.* (1990, 1991) have identified several mRNAs, including one encoding an aldose reductase, induced during drought in the resurrection plant, *Craterostigma plantagineum*, which can remain dormant yet viable during long periods of complete desiccation. A consistent theme in glycophytic water-stress responses is the expression of polypeptides which are also associated with abscisic acid (ABA)-mediated seed and embryo desiccation (Skriver and Mundy, 1990). Examples include Rab proteins (Mundy and Chua, 1988), Lea proteins (Dure *et al.*, 1989) and the aldose reductase mentioned above, which is induced in barley embryos as well as desiccating resurrection plant leaf tissue (Bartels *et al.*, 1991). The function of many of these proteins (e.g. Rabs and Leas) during stress is not clear. Many share some biochemical characteristics such as high hydrophilicity, and it is thought that their role in maturing seeds and stressed leaf tissue is to preserve the structural integrity of cells during the desiccation process (Skriver and Mundy, 1990).

The effects of salinity on glycophytes have also been investigated. Claes *et al.* (1990) identified a salinity-induced rice mRNA encoding a protein of unknown function. Work with tobacco in tissue culture has established that salt-adapted cell lines exhibit changes in ion compartmentation (Binzel *et al.*, 1988), cell wall chemistry (Iraki *et al.*, 1989) and gene expression (Singh *et al.*, 1989).

Studies with glycophytes have established the importance of gene expression in plant stress responses and defined classes of proteins that are induced by desiccation, but most of these investigations have not been successful in distinguishing pathological from stress-ameliorating responses. We have taken advantage of a unique system, the inducible halophyte *Mesembryanthemum crystallinum* (common ice plant), in order to understand the mechanisms by which some plants avoid salinity-induced water deficit. As a facultative halophyte, the ice plant undergoes a set of stress-inducible physiological and biochemical changes that allow it to adjust and maintain cell viability and turgor, conserve water and continue growth during extreme salt stress. One physiological adaptation, the stress-induced switch from C_3 to Crassulacean acid metabolism (CAM), has been studied in depth (Winter and von Willert, 1972; Bohnert *et al.*, 1988 and references therein). CAM is an alternative photosynthesis pathway that allows night-time carbon fixation, thereby resulting in decreased evaporative water loss (Ting, 1985). The shift to CAM has been shown to require increased expression of mRNAs encoding a number of CAM pathway enzymes (Ostrem *et al.*, 1987; Vernon *et al.*, 1988; Schmitt *et al.*, 1988; Ostrem *et al.*, 1990), establishing *Mesem-*

bryanthemum as an attractive system for the study of salt stress at the molecular level.

CAM is considered to be separate from the ice plant's ability to adjust to and tolerate high salinity at the cellular level. CAM is a complex physiological phenomenon that serves as a long-term water conservation strategy, and is not fully operative until 7–14 days following environmental stress in 6 week-old *M.crystallinum* (Winter and Gademann, 1991; Bohnert *et al.*, 1988). CAM is preceded by a period of transient wilting (2–4 days) followed by recovery and continued growth (Winter and Gademann, 1991; Bohnert *et al.*, 1988; D.Vernon, unpublished). It is likely that there are mechanisms distinct from CAM that are responsible for this observed adjustment to saline conditions. To investigate the molecular basis of salt tolerance, we generated and screened a subtracted cDNA library enriched for stress-induced sequences. Here we show that this facultative halophyte responds to osmotic stress by transcriptional induction of a gene encoding a novel methyl transferase. This methyl transferase is identified by functional assay as a myo-inositol *O*-methyl transferase involved in the biosynthesis of a cyclic sugar alcohol, pinitol. Pinitol is abundant in a number of salt- and drought-tolerant plant species, including salt-stressed *M.crystallinum*, where it can accumulate to >70% of soluble carbohydrate (Paul and Cockburn, 1989). The transcriptional induction of this cyclitol biosynthesis enzyme in salt-stressed *M.crystallinum* indicates that the cyclic sugar alcohol pinitol may play a crucial role during adaptation to osmotic stress in this facultative halophyte.

Results

cDNA isolation

To identify environmentally-induced molecular changes involved in the ice plant's adaptation to salinity, we constructed and differentially screened a subtracted cDNA library enriched for stress-induced sequences. cDNA was generated from poly(A)$^+$ RNA that had been isolated from 7 week-old soil-grown plants stressed with 500 mM NaCl for 10 h. By this time, plants start to recover from stress-induced transient wilting (Winter and Gademann, 1991), but have not yet accumulated large amounts of mRNAs encoding components of the well characterized CAM pathway (Ostrem *et al.*, 1987, 1990). Three cycles of differential screening of ~10^5 plaques with labeled first-strand cDNAs from stressed and unstressed plants yielded eight consistently up-regulated inserts. Cross-hybridization experiments indicated that the inserts represented three distinct clones (data not shown). A 1.6 kb insert, now referred to as *Imt1*, was chosen for further analysis.

Expression of Imt1 mRNA

Expression of the *Imt1* transcript was analyzed on Northern blots of RNA isolated from root and leaf tissue of hydroponically-grown plants (Figure 1). Total RNA was isolated from unstressed plants and plants harvested at several timepoints over the course of a 6 day stress with 400 mM NaCl. The *Imt1* cDNA probe hybridized to a salinity-induced mRNA of ~1.6 kb in both leaf and root RNA. The pattern of induction differed in these two organs. In unstressed leaves, *Imt1* was present at very low levels. The transcript accumulated gradually in stressed leaves (Figure 1A), being detectable after 6 h of stress, but inconspicuous until the second day, after 30 h of stress. Accumulation of the transcript in leaves reached a maximum by the sixth day of salt treatment (126 h). *Imt1* was transiently up-regulated in roots (Figure 1B), rising from undetectable levels to a maximum level of expression during the second day of stress (30 h). Interestingly, the mRNA completely disappeared from roots by the time of maximum expression in leaves. Blots of dilutions of leaf and root RNA indicated that, at the times of maximal expression, the *Imt1* mRNA is >25 times more abundant in leaves than in roots (data not shown). A prolonged exposure of the root RNA Northern blot is shown in Figure 1 to illustrate the complete disappearance of this transcript between the fourth and sixth days of stress.

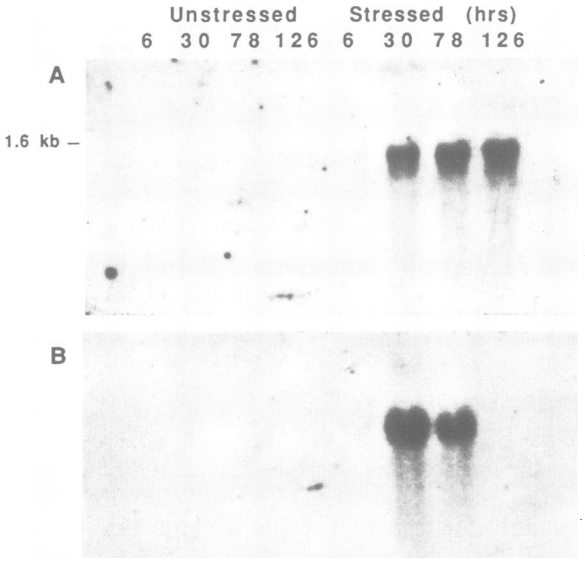

Fig. 1. Hybridization of the *Imt1* cDNA to Northern blots of *M.crystallinum* RNA. Total RNA was isolated from leaves (**A**) or roots (**B**) of hydroponically-grown 6 week-old control plants (unstressed) and plants that had been stressed with 400 mM NaCl for 6, 30, 78 and 126 h. Control plants were harvested at each timepoint. 10 μg of leaf (A) or 20 μg of root (B) RNA from each timepoint were resolved on formaldehyde–agarose gels, transferred to nitrocellulose and probed with ^{32}P-labeled *Imt1* cDNA. Autoradiographs of blots are shown. The root blot was over-exposed to illustrate the disappearance of the transcript in roots between 78 and 126 h of stress. Transcript size is indicated in kb.

Genomic Southern analysis

The *Imt1* transcript induced in leaves and roots is encoded by a single nuclear gene or small gene family. Nuclear genomic DNA digested with various restriction enzymes was resolved by 1% agarose gel and Southern-blotted along with genome copy number equivalents of the cloned cDNA (Figure 2). Blots were probed with ^{32}P-labeled cDNA fragments and signal intensities were quantified using a β-scanner (Betagen Inc., Waltham, MA). Probes specific to either the 5′-coding region (not shown) or the 3′-non-coding end of the cDNA hybridized to single bands of equal intensity in each lane (Figure 2A). High stringency wash conditions were identical to those used for the Northern blots shown in Figure 1. Comparison of band intensities with copy number reconstitutions (Figure 2B) indicated that the bands probably represent a single gene. A blot probed with a 400 bp 3′-end fragment is shown.

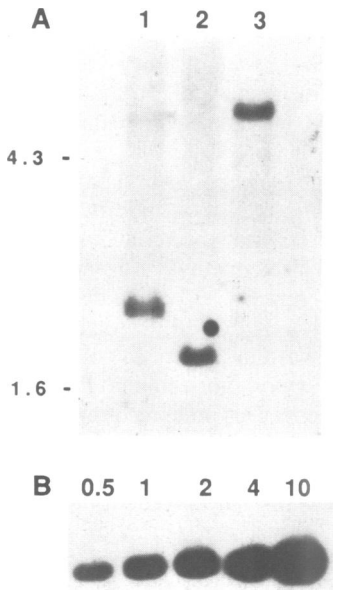

Fig. 2. Southern analysis of *M.crystallinum* genomic DNA. (**A**) 4 μg of nuclear DNA were digested with *Eco*RI (lane 1), *Hin*dIII (lane 2) or *Hin*cII (lane 3), resolved by agarose gel electrophoresis and Southern blotted alongside known amounts of *Imt1* cDNA corresponding to 0.5, 1, 2, 4 and 10 copies per genome (**B**). The blot shown was probed with a ^{32}P-labeled 400 bp 3′-end fragment of the *Imt1* cDNA. Positions of size markers are shown in kb.

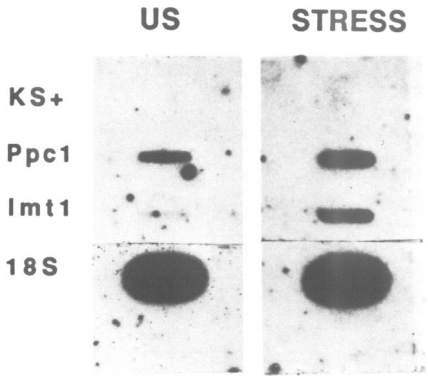

Fig. 3. Nuclear run-on assays of *Imt1* transcription. *Imt1* cDNA was slot-blotted onto nitrocellulose and probed with ^{32}P-labeled transcripts generated by nuclei isolated from unstressed and salt-stressed (102 h) *M.crystallinum*. A PEP carboxylase cDNA clone (*Ppc1*) was included on blots as a positive control for transcriptional induction (Cushman *et al.*, 1989). Bluescript DNA (KS$^+$) served as a control for background. Hybridization to 18S rDNA (18S) was used to normalize total transcription rates of nuclei from stressed and unstressed plants.

Nuclear run-on assays

The *Imt1* mRNA is transcriptionally induced in leaf tissue (Figure 3). Nuclear run-on experiments were performed with nuclei isolated from control and salt-stressed *M.crystallinum*. Transcripts were labeled with [^{32}P]UTP and hybridized to excess *Imt1* cDNA slot-blotted onto nitrocellulose. *Ppc1* DNA, which encodes the transcriptionally induced CAM enzyme PEP carboxylase (Cushman *et al.*, 1989), was included as a positive control. Hybridization to 18S rDNA was used to normalize the overall transcription rates of

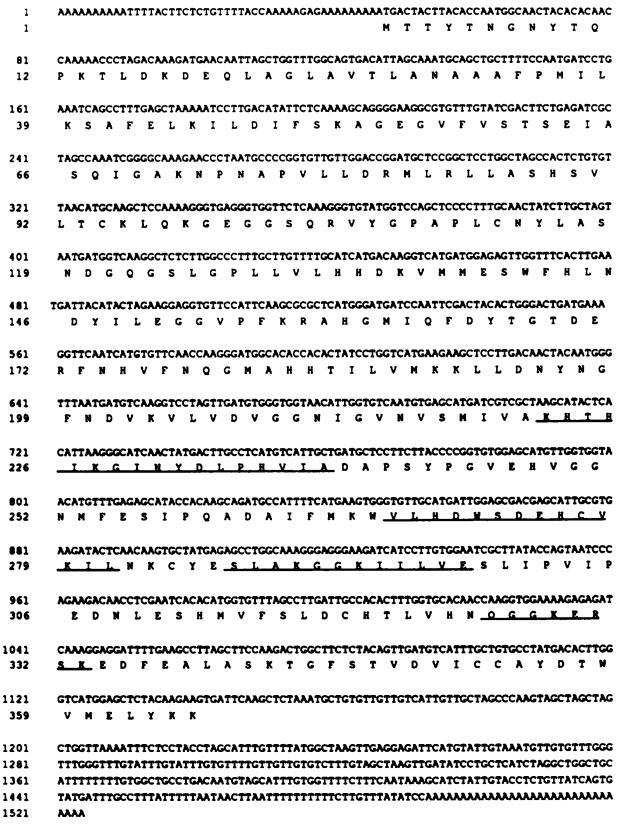

Fig. 4. Nucleotide sequence and predicted amino acid sequence of the *Imt1* cDNA. Underlined regions share homology with hydroxymethyl transferases from plant, bacterial and mammalian sources (Bugos *et al.*, 1991). The sequence data of *Imt*1 have been deposited in the DDBJ/GenBank Nucleotide Sequence Databases under the accession number M87340.

different reactions. β-scan analysis of experiments such as that shown in Figure 3 indicated that transcription of the *Imt1* message is four to five times higher in leaves from salt-stressed plants than in leaves of unstressed control plants of the same age. This increase is comparable to, and perhaps greater than, the stress-induced increase in transcription rate observed for the *Ppc1* transcript (Figure 3, Cushman *et al.*, 1989).

Sequence analysis

To gain insight into the possible biochemical and physiological functions of the IMT1 protein, the sequence of the cDNA was determined (Figure 4). The clone was 1524 bp long. It contained an A+T-rich leader sequence and an ATG start codon followed by an uninterrupted reading frame of 1095 nucleotides. The 3′ end consisted of a long non-coding region of 383 nucleotides, which included two possible polyadenylation recognition sequences (AATAA and AATAAA) located 28 and 74 bases, respectively, upstream of a 31 base poly(A) tail. Analysis of the *Imt1* sequence predicted a hydrophilic polypeptide of 365 amino acids with a molecular mass of 40 250 (Figure 4). A search of the NBRF database revealed similarity to a bovine pineal gland hydroxyindole *O*-methyltransferase (Ishida *et al.*, 1987). Homology (including conservative amino acid replacements) was 55% over the entire length. Significant identity (27%) was confined to 195 amino acids in the C-terminal portion of the protein (alignments not shown). The predicted IMT1

polypeptide was found to be even more closely related to two plant bifunctional hydroxymethyl transferases which methylate the lignin monomers caffeic acid and hydroxyferulic acid (Bugos *et al.*, 1991; Gowri *et al.*, 1991). Comparison with these proteins revealed >50% identity over the entire length of the IMT1 sequence (not shown). Regions of shared homology with *S*-adenosyl-L-methionine (SAM)-dependent hydroxymethyl transferases (OMTs) are underlined in Figure 4 (Bugos *et al.*, 1991).

Expression of IMT1 in Escherichia coli and identification of activity

Although sequence alignments suggested a general biochemical function for the IMT1 protein, amino acid identities were not sufficient for the identification of the enzyme's substrate specificity. We hypothesized that one possible role for a methyl transferase in the salt stress response in *M.crystallinum* was in the biosynthesis of pinitol (1D-3-*O*-methyl *chiro*-inositol). Pinitol is a methylated cyclic sugar alcohol (cyclitol) that accumulates to high levels in salt-stressed *M.crystallinum* (Paul and Cockburn, 1989). Its appearance and accumulation parallel that of the *Imt1* mRNA (Figure 1) during the initial days of salt stress (Paul and Cockburn, 1989). Pinitol has potentially osmoprotective properties and is thought to serve as an intracellular osmolyte (possibly in the chloroplasts) during osmotic adjustment in this inducible halophyte (see Discussion). Pinitol is derived from the six-carbon cyclic sugar alcohol myo-inositol (Figure 5). Its synthesis involves an initial methylation of myo-inositol by a SAM-dependent hydroxymethyl transferase, followed by an enzyme-catalyzed epimerization via a *keto* intermediate. In angiosperms, the pathway proceeds via ononitol (1D-4-*O*-methyl myo-inositol; Figure 5). An isomer of ononitol, sequoyitol (1D-5-*O*-methyl myo-inositol), is the methylated intermediate in gymnosperms (Dittrich and Brandl, 1987).

To substantiate the hypothesized physiological role of the IMT1 protein in pinitol biosynthesis, the polypeptide was expressed in *E.coli* and bacterial lysates were tested for myo-inositol hydroxymethyl transferase activity. As is evident from Figure 6A, *E.coli* containing the clone in the proper orientation expressed a protein of molecular mass ~40 kDa that was not present in control cells (containing the clone in the opposite orientation) that were grown under identical conditions. This protein co-migrated on SDS–polyacrylamide gels with the reticulocyte system *in vitro* translation product of the *in vitro*-transcribed *Imt1* clone (Figure 6B). Expression ranged from ~1% to >10% of *E.coli* protein, depending on growth conditions.

We tested *E.coli* extracts from control cells and cells expressing the IMT1 protein for myo-inositol-dependent *O*-methyl transferase activity in an assay using $^{14}CH_3$-labeled SAM as a methyl group donor. Carbohydrates from post-assay extracts were separated by HPLC and radioactive fractions identified by scintillation counting. A radioactive product with a retention time of ~11.1 min was generated by extracts from cells expressing the IMT1 protein, but not by control extracts (Figure 7). The appearance of this peak was dependent on the addition of myo-inositol to the assays.

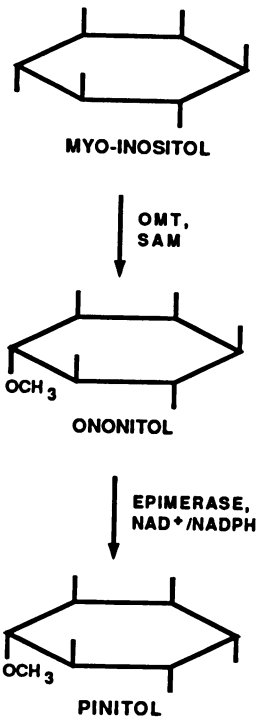

Fig. 5. Pathway of pinitol biosynthesis in angiosperms. Pinitol (1D-3-*O*-methyl *chiro*-inositol) is synthesized from myo-inositol, a six-carbon ring polyol derived from glucose-6-phosphate. The synthesis pathway consists of a methylation step catalyzed by a position-specific *S*-adenosyl-L-methionine (SAM)-dependent *O*-methyl transferase (OMT), followed by an enzyme-catalyzed epimerization which may require NAD^+ and/or NADPH (Loewus and Dickinson, 1982). The methylated intermediate is ononitol. Vertical bars on ring structures represent hydroxyl groups.

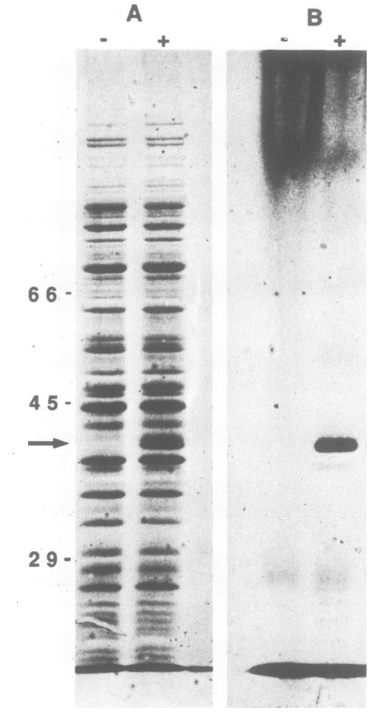

Fig. 6. Expression of IMT1 in *E.coli*. The *Imt1* cDNA expression constructs were transformed into *E.coli* BL21(DE3) cells as described in Materials and methods. (**A**) SDS–PAGE of proteins from *E.coli* transformed with *Imt1* cloned 3′–5′ (−) or 5′–3′ (+) behind the bluescript T7 promoter. (**B**) SDS–PAGE of *in vitro* translation products of unprogrammed reticulocyte lysate translation system (−) or lysate programmed with *in vitro*-transcribed *Imt1* RNA (+). All samples in panels A and B were run on the same gel, which was cut and either Coomassie stained (A) or fluorographed (B). Positions of size markers are labeled in kDa. Arrow indicates position of IMT1.

Two less prominent peaks were generated by all assays (regardless of myo-inositol addition or IMT1 expression), suggesting the presence of endogenous SAM-dependent OMTs in *E.coli*. The activity was sensitive to both heat (70°C, 15 min) and proteinase K treatment, and ^{14}C incorporation into the product was linear during the course of the assay (data not shown). These assays indicated that *Imt1* encodes a SAM-dependent myo-inositol *O*-methyl transferase.

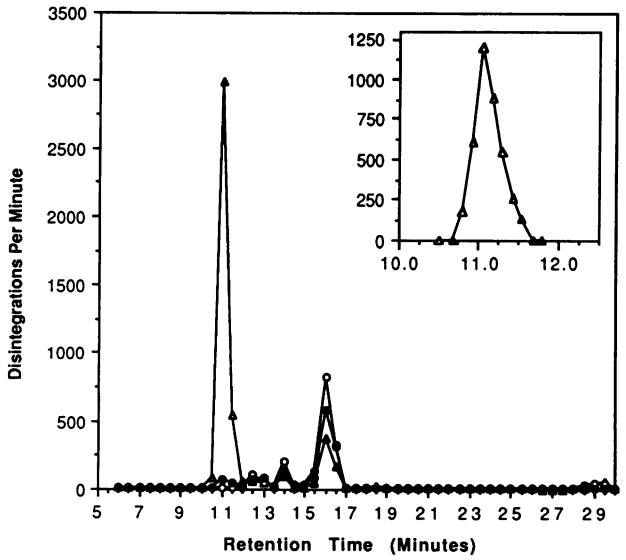

Fig. 7. Myo-inositol *O*-methyl transferase assays of *E.coli* extracts. Equal amounts of total soluble protein from cells expressing IMT1 were assayed with (△) or without (●) myo-inositol. Control extracts from cells transformed with only Bluescript (○) were assayed with myo-inositol. [^{14}C]methyl-SAM was included as a methyl group donor. A portion (8%) of post-assay carbohydrates were resolved by HPLC as described in Materials and methods. ^{14}C d.p.m. of 0.5 min fractions from representative HPLC runs are shown. Inset: to define more precisely the retention time of the IMT1 product, 7.5 s fractions with retention times between 10.5 and 12.0 min were collected and analyzed.

The ^{14}C-labeled product of the assays on *E.coli* extracts was visible on HPLC traces as a distinct peak with a retention time of slightly under 11.1 min (Figure 8, panel 3) that was not present in assays from control extracts (Figure 8, panels 1 and 2). To establish that the methylated myo-inositol generated by the IMT1 protein was ononitol, the methylated intermediate in pinitol biosynthesis, its retention time was compared with that of methyl-myo-inositol standards. There are four methyl-myo-inositol isomers: sequoyitol, ononitol and D- and L-bornesitol (1D- or 1L-1-*O*-methyl myo-inositol) (Loewus and Loewus, 1980). Only ononitol and sequoyitol are possible precursors for pinitol (Dittrich and Brandl, 1987; Figure 5), and only ononitol has been documented as the precursor to pinitol in angiosperms (Dittrich and Brandl, 1987). Extracts from control *E.coli* (such as that shown in Figure 8, panel 1) were spiked with standards, processed as assays (see Materials and methods) and analyzed by HPLC. The retention times of sequoyitol and bornesitol were 11.5 and 12.2 min respectively (data not shown). Ononitol, however, displayed a retention time identical to that of the reaction product (Figure 8, panel 4), indicating that the myo-inositol-dependent *O*-methyl transferase encoded by the *Imt1* mRNA does indeed generate the methylated intermediate in the biosynthesis of pinitol.

IMT1 activity in plant extracts

To verify that the induction of the *Imt1* mRNA in stressed *M.crystallinum* is accompanied by an increase in IMT1 expression, extracts from leaves of salt stressed (102 h) and unstressed *M.crystallinum* were assayed for IMT1 enzyme activity (Figure 9). Carbohydrates from post-assay plant extracts were resolved by HPLC, fractions were collected

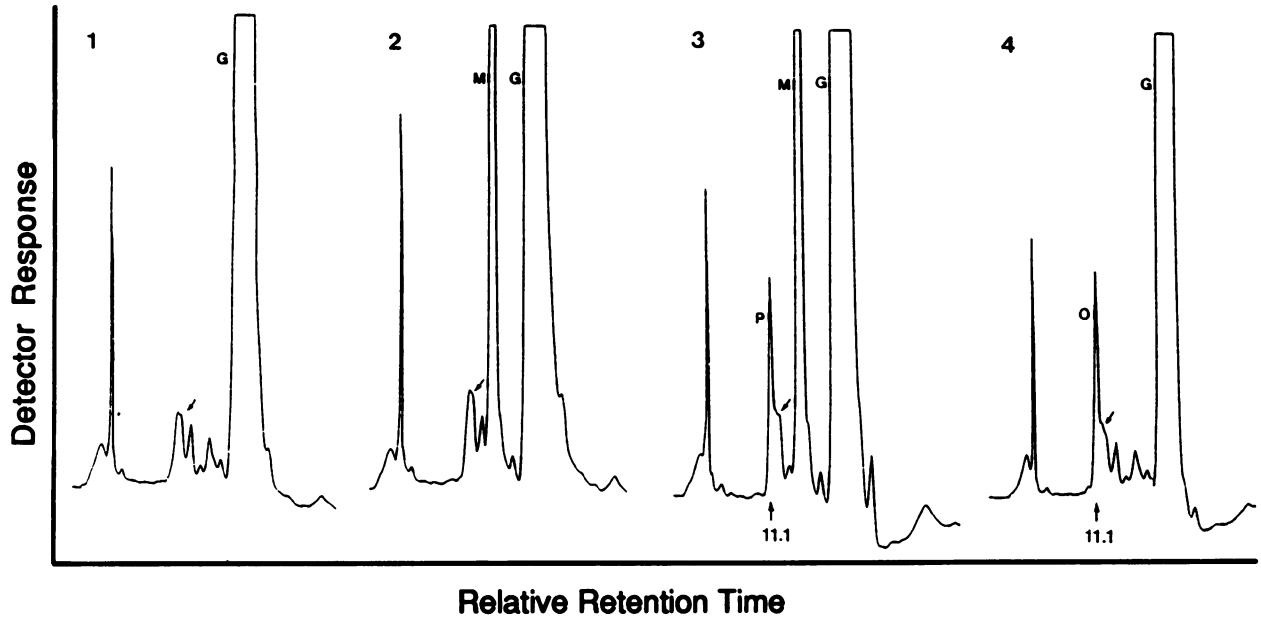

Fig. 8. Identification of the methyl-myo-inositol product of IMT1. Representative HPLC traces of carbohydrates from post-assay *E.coli* extracts are shown: **Panel 1**: assay of extract containing IMT1 but no myo-inositol. **Panel 2**: assay (with myo-inositol) of extract from control cells transformed with vector only. **Panel 3**: assay of extract containing IMT1 protein plus myo-inositol. **Panel 4**: same extract as panel 1, but spiked with 1.5 nmol of ononitol standard. Peak labels: G, glycerol added to protein extracts for storage; M, myo-inositol added to extracts for assay; P, assay product; O, ononitol spike. Tilted arrows indicate the position of an endogenous *E.coli* peak present in all extracts, useful here as a marker. Retention times of product and of ononitol standard are indicated.

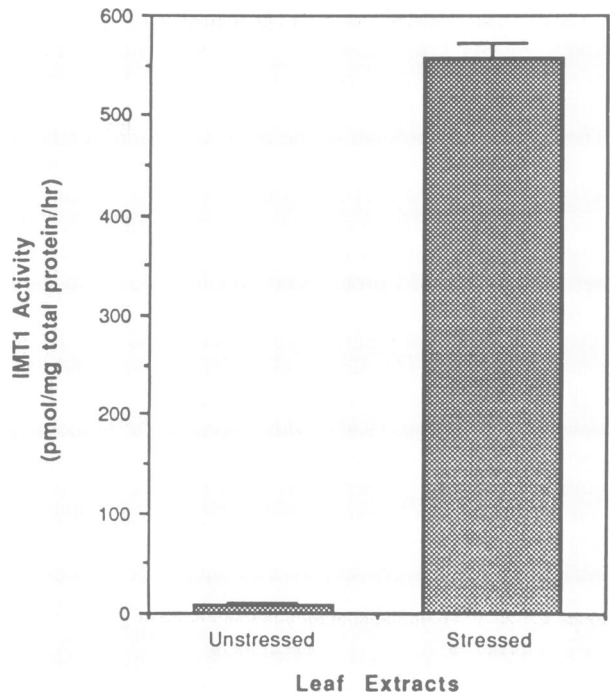

Fig. 9. IMT1 activity in stressed and unstressed *M.crystallinum*. Crude soluble protein extracts from leaves of unstressed plants (unstressed) or plants stressed for 102 h (stressed) were assayed for myo-inositol OMT activity in assays similar to those run on *E.coli* extracts (Figure 7). Assays were run for 2 h, during which time ^{14}C incorporation into product was linear (data not shown). Proteins were extracted from leaf material pooled from three stressed or three unstressed plants. Activity values are the mean of three separate assays; standard deviations are shown. No activity was detected in assays carried out without myo-inositol (data not shown).

and c.p.m. determined by scintillation counting. ^{14}C c.p.m. were detected in a single peak with a retention time of ~11.1 min and the appearance of ^{14}C in carbohydrate fractions was dependent on the addition of myo-inositol to assays (data not shown). Stressed plants exhibited a large (~60-fold) increase in IMT1 activity over unstressed plants, which contained an almost undetectable level of activity (Figure 9). ^{14}C incorporation into product was linear during the course of the assay (data not shown). These assays indicated that *Imt1* mRNA induction results in increased IMT1 activity in stressed-plant extracts, suggesting that the increased expression of this transcript is, at least in part, responsible for the concurrent accumulation of pinitol observed in salt-stressed *M.crystallinum*.

Discussion

Halophytic plants have developed mechanisms of osmoregulation that allow them to avoid salinity-induced water stress. We have used a facultative halophyte, *M.crystallinum*, to investigate the molecular basis of salt tolerance. When subjected to salinity stress, *M.crystallinum* undergoes a brief period of wilting followed by recovery and continued growth (Bohnert *et al.*, 1988; Winter and Gademann, 1991). In an attempt to elucidate the biochemical mechanisms responsible for this observed adjustment to stress, we generated a cDNA library enriched for transcripts induced during the initial hours of stress. Differential screening of this library yielded a dramatically up-regulated mRNA, *Imt1*, encoding a polypeptide with homology to several methyl transferases. Expression of the protein in *E.coli* and activity assays identified the enzyme as a myo-inositol *O*-methyl transferase. This enzyme methylates myo-inositol to produce ononitol, which is subsequently epimerized by a different enzyme to produce pinitol, a cyclic sugar alcohol known to accumulate to high levels in salt-stressed *M.crystallinum* (Paul and Cockburn, 1989). Enzyme assays performed on plant extracts established that the induction of *Imt1* mRNA is accompanied by the appearance of myo-inositol *O*-methyl transferase activity in salt-stressed plants. To our knowledge, this study is the first example of differential screening of a cDNA library leading to the biochemical identification of a novel plant enzyme with a defined physiological function.

Pinitol is one of a larger class of compounds, sugar alcohols, whose presence correlates with osmotic stress tolerance in a wide range of organisms. Cyclic sugar alcohols such as pinitol and straight-chain polyols, such as mannitol and sorbitol, are found in high levels in desiccation- and salt-tolerant bacteria, fungi and algae as well as in halophytic higher plants (Csonka and Hanson, 1991; Bieleski, 1982; Hellebust, 1976). Pinitol is the most common of the various myo-inositol-derived cyclitols found in plants (Dittrich and Korak, 1984). It is found at high levels in a diverse set of salt-tolerant plant species, including maritime pine (Nguyen and Lamant, 1988), several legumes (Gorham *et al.*, 1988), halophytic mangroves (Popp, 1984) and both dicots and monocots from maritime habitats (Gorham *et al.*, 1981). In *M.crystallinum*, pinitol can accumulate to >70% of total soluble carbohydrate (up to 10% of plant dry weight) following NaCl stress (Paul and Cockburn, 1989). It may reach intracellular concentrations of up to 300 mM. Localization studies by Paul and Cockburn (1989) suggest that >60% of the pinitol in stressed *M.crystallinum* is in the cytoplasm, with the rest residing in the chloroplasts.

The exact physiological role of sugar alcohols during osmotic stress has not been determined. One view is that sugar alcohols are involved in osmoregulation: they accumulate to high levels to act as intracellular osmolytes that balance external osmotic pressure. In many osmotolerant fungi and algae, sugar alcohol levels are variable and correlate with the osmotic potential of the extracellular environment (Bieleski, 1982; Hellebust, 1976). In halophytic higher plants, sugar alcohols are thought to accumulate in the cytoplasm (and/or chloroplasts) to counteract the high osmotic potential of the vacuoles, which selectively accumulate salt to maintain turgor and sequester ions away from the cytosol, where they would be harmful to enzyme activities (McCue and Hanson, 1990; Pollard and Wyn Jones, 1979; Hellebust, 1976). A similar role has been proposed for other low molecular weight metabolites such as proline and glycine-betaine, which accumulate in many environmentally-stressed plants (McCue and Hanson, 1990; Pollard and Wyn Jones, 1979). Compounds that accumulate to high levels to serve as osmolytes in the cytoplasm must be compatible with cellular function. They must be non-disruptive to enzyme structure and activity even when present in high concentration, and they must be non-reactive (Yancey *et al.*, 1982; Gorham *et al.*, 1981; Hellebust, 1976). Also, osmolytes that accumulate to high intracellular concentration must be removed from the flux through major metabolic pathways. Metabolically inert, non-reactive cyclitols such as pinitol fit all of these criteria.

Osmoregulation is only one of the roles suggested for sugar

alcohols in stress-tolerant organisms. It has also been proposed that their primary purpose is one of macromolecular osmoprotection (Schobert, 1977). *In vitro* studies indicate that these compounds may have hydrogen bonding characteristics that allow them to protect macromolecules from the adverse effects of excessive water loss, high temperature and increased ionic strength in the surrounding medium (Laurie and Stewart, 1990; Bieleski, 1982; Ahmad *et al.*, 1979), presumably by tightly associating with proteins and membrane components to replace water lost during desiccation (Yancey *et al.*, 1982; Schobert, 1977). For this purpose, sugar alcohols are thought to be important in desiccation-tolerant organisms such as yeast (Coutinho *et al.*, 1988) and, possibly, the resurrection plant *Craterostigma plantagineum* (Bartels *et al.*, 1991). It is likely that sugar alcohols can carry out either osmoregulatory or osmoprotective roles in the various organisms where they are found, depending on the level to which they accumulate, their intracellular location and the environmental stress being encountered.

Although polyol accumulation has been correlated with osmotolerance in a number of plants, the mechanisms of accumulation have not been clear (Loewus and Dickinson, 1982). It has been noted that the higher levels of cyclitols and other putative intracellular osmolytes seen in stressed or salt-tolerant plants could, rather than being a beneficial response, be a consequence of 'impaired metabolism' during stress (Wyn Jones and Gorham, 1983). The transcriptional induction of the myo-inositol OMT (Figure 3) during adjustment to saline conditions indicates that sugar alcohol accumulation in the ice plant is not an artifact of disturbed metabolism. Rather, it appears to be part of a genetically determined, adaptive response that enables *M.crystallinum* to adjust physiologically to extreme osmotic stress.

The IMT enzyme is one of four distinct *O*-methyl transferases that methylate myo-inositol at specific hydroxyl positions to create ononitol, sequoyitol, or L- or D-bornesitol (Loewus and Loewus, 1980; Hoffmann-Ostenhoff *et al.*, 1978). Although two of these enzymes (those that methylate myo-inositol to make L- and D-bornesitol) have been purified (Hoffman-Ostenhoff, 1978), none have been extensively biochemically characterized and no myo-inositol OMT sequences have been determined prior to this study. That these enzymes are highly specific for their site of methylation is supported by the work of Hoffmann-Ostenhoff (1978), as well as by our assay results, which generated only ononitol (Figures 7 and 8; and data not shown). Cloning and sequence analysis of other myo-inositol OMTs, as well as more in-depth biochemical analysis of the ice plant OMT described here, would provide information on the comparative biochemistry and specificity of these enzymes. The overexpression of IMT1 in *E.coli* (Figure 6) is a first step for both of these endeavors.

The transient expression of *Imt1* in roots (Figure 1) provided the first indication that the *Imt1* transcript was involved in a non-organ-specific stress response distinct from the leaf-specific switch to CAM photosynthesis that occurs in salt-stressed *M.crystallinum*. However, the different patterns of *Imt1* expression in leaves and roots (Figure 1) and the much greater abundance of the transcript in leaf tissue may indicate that the cells in these organs have distinct mechanisms of osmoregulation or that the roots play a different role in the response to stress at the whole-plant level. Indeed, it has been noted in physiological studies that the root cortical cells of *M.crystallinum* do not fully regain turgor following NaCl stress, while leaves recover turgor after 4 days of stress (Winter and Gademann, 1991). The greater accumulation of *Imt1* mRNA in leaves reflects what has previously been observed for pinitol levels in the ice plant (Paul and Cockburn, 1989). Higher expression of *Imt1* in leaves may be indicative of a role for pinitol in chloroplast osmoprotection, although pinitol is not localized solely to these organelles (Paul and Cockburn, 1989).

The salinity-induced accumulation of *Imt1* is due to transcriptional activation (Figure 3). Based on a number of genes whose patterns of expression have been studied in the ice plant, increased transcription appears to be the primary mechanism of regulation for stress-induced genes (Figure 3; Vernon *et al.*, 1991; Cushman *et al.*, 1989). This is true for both CAM-related mRNAs and those more directly involved in salt tolerance, such as *Imt1*. An understanding of how the plant's transcriptional machinery is cued by the environment is central to an understanding of environmental stress tolerance, and this topic is currently being investigated by the in-depth analysis of selected promoters.

The transcriptional induction of a myo-inositol hydroxymethyl transferase in this facultative halophyte suggests that sugar alcohols play an important role in osmotic stress tolerance. Whether the exact function of these compounds is to serve as non-disruptive cytoplasmic osmolytes or to act as 'osmoprotectants' of protein and membrane structure remains to be elucidated. Experiments utilizing transformation of glycophytes with the *Imt1* gene will address the question of whether production of cyclitols is sufficient for higher plant osmotolerance. If so, the ability of this methyl transferase to create a non-reducing, metabolically inert sugar alcohol from a ubiquitous plant substrate makes it an attractive enzyme for the creation of stress-resistant transgenic plants.

Materials and methods

Plant material

M.crystallinum were grown from seed as described (Ostrem *et al.*, 1987) and either kept in soil or transferred 14 days after seeding to Hoagland's solution in aerated, light-tight containers for hydroponic growth. Plants were salt stressed when 6 weeks old (unless otherwise noted) with 500 mM NaCl (Ostrem *et al.*, 1987) or, for hydroponics, by addition of 400 mM NaCl to Hoagland's solution. Unstressed controls for each experiment were grown alongside stressed plants and were harvested at the same time. To avoid potential experimental variation due to diurnal fluctuation of RNA and protein levels, all harvests were carried out at the same time during the dark/light cycle (4 h before the end of the light period). Harvested material for RNA extraction was frozen in liquid N_2 and stored at −70°C.

Subtracted cDNA library construction and screening

Leaf RNA was extracted and poly(A)$^+$ mRNA prepared as described by Ostrem *et al.* (1987). First-strand cDNAs were generated from poly(A)$^+$ RNA from stressed plants using a kit (BRL, Gaithersburg, MD). Following first-strand synthesis, RNA was removed by base hydrolysis with 0.1 M NaOH (70°C, 60 min) and single stranded (ss) cDNA was hybridized to a 5.5-fold excess of poly(A)$^+$ RNA from control plants using the protocol and conditions described (Briehl *et al.*, 1990). Hybridizations were carried out for 18 h. Single-stranded cDNA remaining after hybridization was isolated by differential elution from hydroxyapatite (Davis, 1986) and made double-stranded using cDNA synthesis kit materials (BRL, Gaithersburg, MD) with poly(dN)$_6$ random primer (Pharmacia Inc., Piscataway, NJ). Double-stranded cDNA was ligated to hemi-phosphorylated *Eco*RI/*Not*I adaptors (Invitrogen Inc., San Diego, CA), ligated into λZapII (Stratagene Inc., La Jolla, CA) and packaged using Gigapack *in vitro* packaging extracts (Stratagene Inc., La Jolla, CA). Phage were plated and screened by three

cycles of standard differential plaque hybridization (Maniatis *et al.*, 1982). ^{32}P-labeled ss cDNA probes used for screening were generated from poly(A)$^+$ RNA isolated from unstressed *M.crystallinum* and plants salt stressed for 10 h. DNA from selected plaques was rescued as Bluescript SK$^-$ phagemids (Stratagene Inc., La Jolla, CA) and used to transfect *E.coli* XL1-Blue cells for future DNA analysis and manipulation. Cloned insert DNA was isolated (Maniatis *et al.*, 1982), excised by *Eco*RI digestion and analyzed on agarose gels.

RNA isolation, Northern analysis and transcription assays

Total RNA from leaves and roots of hydroponically-grown plants was isolated as previously described (Ostrem *et al.*, 1987), except that the buffer used for root RNA extractions contained additional detergent: 2% SDS and 2% Triton X-100. Total RNA was resolved on formaldehyde–agarose gels and Northern blotted (Thomas, 1983). Blots were probed, washed at high stringency and visualized as previously described (Vernon *et al.*, 1988) using ^{32}P-labeled, gel-purified *Imt1* insert DNA as a probe. Nuclei isolations from fresh leaf tissue, *in vitro* run-on experiments, DNA slot blotting and hybridization of transcripts to DNA were carried out as described previously (Cushman *et al.*, 1989). The c.p.m. hybridized to filters were directly quantified using a β-scanner (Betagen Inc., Waltham, MA).

Gene copy number determination

Nuclear genomic DNA was prepared as described by Bedbrook (1981). 4 μg of nuclear DNA was digested to completion with either *Eco*RI, *Hin*dIII or *Hin*cII, resolved on 1% agarose gels and Southern blotted (Southern, 1975). Blots were processed as described (Vernon *et al.*, 1988). ^{32}P-labeled probes were generated from gel-purified 5′- or 3′-end restriction fragments of *Imt1* or from full-length insert. Genome copy-number equivalents were based on an estimated *M.crystallinum* haploid genome size of 390 Mbp (De Rocher *et al.*, 1990). Hybridization signals were quantified using a β-scope (Betagen, Inc., Waltham, MA).

Sequence analysis

The nucleotide sequence of both strands of the *Imt1* cDNA was obtained by dideoxy sequencing (Sanger *et al.*, 1977) of suitable overlapping *Imt1* restriction fragments and full-length insert cloned into Bluescript KS$^+$ and KS$^-$ cloning vectors (Stratagene Inc., La Jolla, CA). IMT1 amino acid sequence and restriction sites were identified using the Mount–Conrad–Meyers program (Williams, 1988). Sequence comparisons to the NBRF protein sequence database were performed using the FASTA program (Pearson and Lipman, 1988).

Expression of IMT1 in E.coli

The full IMT1 reading frame was cloned into Bluescript KS$^+$ as a transcriptional fusion in both orientations behind the T7 polymerase promoter. Constructs were transformed into BL21(DE3) cells (Studier *et al.*, 1987), which contain the gene encoding the T7 polymerase under the control of an isopropyl-β-D-thiogalactoside (IPTG)-inducible promoter. A fortuitously located AAGAG sequence in the 5′ leader of the cDNA was predicted to act as an *E.coli* ribosome binding site. IPTG was added to cultures at a final concentration of 0.5 mM 4 h before harvest. Protein expression was analyzed by SDS–PAGE on 10% acrylamide gels (Ostrem *et al.*, 1987). Samples were prepared by boiling aliquots of *E.coli* cultures for 3 min in an equal volume of SDS extraction buffer.

In vitro transcription of the *Imt1* cDNA was carried out with T7 RNA polymerase (Stratagene Inc., La Jolla, CA). *In vitro* translation of the resulting transcript was accomplished using a reticulocyte lysate system (BRL Inc., Gaithersburg, MD). [^{35}S]methionine-labeled translation product was resolved by SDS–PAGE alongside *E.coli* protein samples and visualized by fluorography.

Soluble protein extraction and activity determination

Soluble protein from *E.coli* transformed with the IMT1 expression construct or native Bluescript KS$^+$ was extracted from 20–200 ml cultures pelleted at 2500 *g* for 10 min and resuspended in methyl transferase extraction buffer (MTEB): 100 mM Tris–Cl pH 8, 10 mM EDTA and β-mercaptoethanol; 1 ml/20 ml of culture). Cells were lysed by sonication and extracts were clarified by centrifugation at 10 000 *g* for 20 min. Total protein concentration was determined by the method of Ghosh *et al.* (1988). Supernatants were either used immediately for assays or stored at −70°C with 5% glycerol.

Myo-inositol *O*-methyl transferase assays on *E.coli* extracts were carried out in a 200 μl volume containing 1.0 mg total soluble protein, 50 mM Tris–Cl pH 8.0, 10 mM $MgCl_2$ and 1.0 mM myo-inositol. Assays were pre-incubated at 30°C for 5 min and initiated by the addition of *S*-adenosyl-L-methionine (SAM) to a final concentration of 0.5 mM. SAM stock solution contained unlabeled SAM (Sigma Chemicals St Louis, MO) and ^{14}C-labeled SAM (ICN Biochemicals, Irvine, CA) at a 50:1 ratio. Assays were carried out at 30°C for 60 min and terminated by transfer to ice and chloroform extraction. The aqueous phase was subjected to further processing and HPLC analysis (see below).

Total soluble plant protein was extracted as described by Ostrem *et al.* (1987) using MTEB plus 2 mM leupeptin as extraction buffer. Assays were carried out on crude leaf extracts as with *E.coli* extracts, except that myo-inositol was included at a final concentration of 5 mM and assays were run for 2 h. Assays were terminated with phenol–chloroform (1:1) extraction followed by a chloroform extraction.

HPLC analysis of assay products

Samples were prepared for HPLC by extraction with 2 vol methanol/chloroform/water (12:5:3) followed by addition of 0.4 ml water. A desalting column of AG50WX4 (Bio-Rad, Richmond, CA) and Amberlite IRA-68 (Sigma Chemicals, St Louis, MO) in OH-form was used to desalt extracts and remove charged species (including remaining [^{14}C]SAM). Samples were dried, dissolved in deionized water and filtered through a nylon Acrodisc 13 (Gelman, Ann Arbor, MI). Equal amounts of dissolved carbohydrates from each assay were resolved on a 300 × 7.8 mm HPX87C calcium-form ligand exchange column (Bio-Rad, Richmond, CA) at 85°C with a 0.6 ml/min flow rate using degassed, deionized water as an eluent. Post-column NaOH was added at 0.3 M, 0.6 ml/min, and traces were obtained using a pulsed amperometric detector (Dionex, Sunnyvale, CA) at 35°C and a Spectrophysics SP4290 integrator (Spectrophysics Analytical, San Jose, CA). Fractions were collected at 7.5 s or 0.5 min intervals and scintillation counted.

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References

Ahmad,I., Larher,F. and Stewart,G. (1979) *New Phytol.*, **82**, 671–678.
Bartels,D., Schneider,K., Terstappen,G., Piatowski,P. and Salamini,F. (1990) *Planta*, **181**, 27–34.
Bartels,D., Engelhardt,K., Rancarati,R., Schneider,K., Rotter,M. and Salamini,F. (1991) *EMBO J.*, **10**, 1037–1043.
Bedbrook,J. (1981) *Plant Mol. Biol., Newslett.*, **2**, 24.
Bieleski,R.L. (1982) In Loewus,F.A. and Tanner,W. (eds), *Encyclopedia of Plant Physiology: Plant Carbohydrates I—Intracellular Carbohydrates*. Springer-Verlag, Berlin, Vol. 13A, pp. 158–192.
Binzel,M.L., Hess,F.D., Bressan,R.A. and Hasegawa,P.M. (1988) *Plant Physiol.*, **86**, 607–614.
Bohnert,H.J. *et al.* (1988) *Plant Mol. Biol. Rep.*, **6**, 10–28.
Boyer,J.S. (1982) *Science*, **218**, 443–448.
Briehl,M.M., Flomerfelt,F.A., Wu,X. and Miesfeld,R.L. (1990) *Mol. Endocrinol.*, **4**, 287–294.
Bugos,R.C., Chiang,V.L.C. and Campbell,W.H. (1991) *Plant Mol. Biol.*, **17**, 1203–1215.
Claes,B., Dekeyser,R., Villaroel,R., Van den Buicke,M., Bauw,G., Van Montague,M. and Caplan,C. (1990) *Plant Cell*, **2**, 19–27.
Cohen,A., Plant,A.L., Moses,M.S. and Bray,E.A. (1991) *Plant Physiol.*, **97**, 1367–1374.
Coutinho,C., Bernardes,E., Felix,D. and Panek,A.D. (1988) *J. Biotechnol.*, **7**, 23–32.
Csonka,L.N. and Hanson,A.D. (1991) *Annu. Rev. Microbiol.*, **45**, 569–606.
Cushman,J.C., Meyer,G., Michalowski,C., Schmitt,J.M. and Bohnert,H.J. (1989) *Plant Cell*, **1**, 715–725.
Davis,M.M. (1986) *Handbook Exp. Immunol.*, **76**, 1–13.
De Rocher,E.J., Harkins,K.R., Galbraith,D.W. and Bohnert,H.J. (1990) *Science*, **250**, 99–101.
Dittrich,P. and Brandl,A. (1987) *Phytochemistry*, **26**, 1925–1926.
Dittrich,P. and Korak,A. (1984) *Phytochemistry*, **23**, 65–66.
Dure,L.,III, Crouch,M., Harada,J., Ho,T.-H.D., Mundy,J., Quatrano,R., Thomas,T. and Sung,Z.R. (1989) *Plant Mol. Biol.*, **12**, 475–486.

Ghosh,S., Gepstein,S., Heikkila,J.J. and Dumbroff,E.B. (1988) *Anal. Biochem.*, **169**, 227–233.
Gomez,J., Sanchez-Martinez,D., Steifel,V., Rigau,J., Puigdomenech,P. and Pages,M. (1988) *Nature*, **334**, 262–264.
Gorham,J., Hughes,L.I. and Wyn Jones,R.G. (1981) *Physiol. Plant*, **53**, 27–33.
Gorham,J., Tomar,O. and Wyn Jones,R.G. (1988) *J. Plant Physiol.*, **132**, 678–682.
Gowri,G., Bugos,R., Campbell,W.H., Maxwell,C. and Dixon,R. (1991) *Plant Physiol.*, **97**, 7–14.
Hellebust,J.A. (1976) *Annu. Rev. Plant Physiol.*, **27**, 485–505.
Hoffmann-Ostenhoff,O., Pittner,F. and Koller,F. (1978) In Wells,W.W. and Eisenberg,F. (eds), *Cyclitols and Phospoinositides*. Academic Press, New York, pp. 233–247.
Iraki,N.M., Bressan,R.A., Hasegawa,P.M. and Carpita,N.C. (1989) *Plant Physiol.*, **91**, 39–47.
Ishida,I., Obinata,M. and Deguchi,T. (1987) *J. Biol. Chem.*, **262**, 2895–2899.
Jefferies,R.L. (1981) *Bioscience*, **31**, 42–46.
Laurie,S. and Stewart,G. (1990) *J. Exp. Bot.*, **41**, 1415–1422.
Loewus,F. and Dickinson,D.B. (1982) In Loewus,F.A. and Tanner,W. (eds) *Encyclopedia of Plant Physiology: Plant Carbohydrates I: Intracellular Carbohydrates*. Springer-Verlag, Berlin, Vol. 13A, pp. 193–206.
Loewus,F. and Loewus,M. (1980) In Stumpf,P. and Conn,E. (eds), *The Biochemistry of Plants: Carbohydrate Structure*. Academic Press, New York, Vol. 3, pp. 43–76.
Maniatis,T., Fritsch,E. and Sambrook,J. (1982) *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mason,H. and Mullet,J. (1990) *Plant Cell*, **2**, 569–579.
McCue,K.F. and Hanson,A.D. (1990) *Trends Biotechnol.*, **8**, 358–362.
Mundy,J. and Chua,N.-H. (1988) *EMBO J.*, **7**, 2279–2286.
Nguyen,A. and Lamant,A. (1988) *Phytochemistry*, **27**, 3423–3427.
Ostrem,J.A., Vernon,D.M. and Bohnert,H.J. (1990) *J. Biol. Chem.*, **256**, 3497–3502.
Ostrem,J.A., Olsen,S.W., Schmitt,J.M. and Bohnert,H.J. (1987) *Plant Physiol.*, **84**, 1270–1275.
Paul,M.J. and Cockburn,W. (1989) *J. Exp. Bot.*, **40**, 1093–1098.
Pearson,W.R. and Lipman,D.J. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2444–2448.
Plant,A.L., Cohen,A., Moses,M.S. and Bray,E.A. (1991) *Plant Physiol.*, **97**, 900–906.
Pollard,A. and Wyn Jones,R.G. (1979) *Planta*, **144**, 291–298.
Popp,M. (1984) *Z. Pflanzenphysiol.*, **113**, 411–421.
Sanger,F., Nicklen,S. and Coulsen,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Schmitt,J.M., Michalowski,C.M. and Bohnert,H.J. (1988) *Photosynth. Res.*, **17**, 159–171.
Schobert,B. (1977) *J. Theor. Biol.*, **68**, 17–26.
Singh,N., Nelson,D., Kuhn,D., Hasegawa,P.M. and Bressan,R.A. (1989) *Plant Physiol.*, **90**, 1096–1101.
Skriver,K. and Mundy,J. (1990) *Plant Cell*, **2**, 503–512.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.
Studier,F.W., Rosenberg,A.H., Dunn,J.J. and Dubendorff,J.W. (1987) *Methods Enzymol.*, **185**, 60–89.
Thomas,P.S. (1983) *Methods Enzymol.*, **100**, 255–266.
Ting,I.P. (1985) *Annu. Rev. Plant Physiol.*, **36**, 595–622.
Vernon,D.M., Ostrem,J.A., Schmitt,J.M. and Bohnert,H.J. (1988) *Plant Physiol.*, **86**, 1002–1004.
Vernon,D.M., Ostrem,J.A. and Bohnert,H.J. (1991) *Plant Physiol.*, **s96**, abs. no. 99.
Williams,K.M. (1988) *Comput. Appl. Biosci.*, **4**, 211.
Winter,K. and Gademann,R. (1991) *Plant Physiol.*, **95**, 768–776.
Winter,K. and von Willert,D.J. (1972) *Z. Pflanzenphysiol.*, **67**, 166–170.
Wyn Jones,R.G. and Gorham,J. (1983) In Lange,O.L., Nobel,P.S., Osmond,C.B. and Ziegler,H. (eds), *Encyclopedia of Plant Physiology: Responses to the Environment*. Springer-Verlag, Berlin, Vol. 12C, pp. 35–58.
Yancey,P., Lark,M., Hand,S., Bowlus,R. and Somero,G. (1982) *Science*, **217**, 1214–1222.

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