Regulation of Cell-Specific Inositol Metabolism and Transport in Plant Salinity Tolerance

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myo-Inositol and its derivatives are commonly studied with respect to cell signaling and membrane biogenesis, but they also participate in responses to salinity in animals and plants. In this study, we focused on L-*myo*-inositol 1-phosphate synthase (INPS), which commits carbon to de novo synthesis, and *myo*-inositol *O*-methyltransferase (IMT), which uses *myo*-inositol for stress-induced accumulation of a methylinositol, D-ononitol. The *Imt* and *Inps* promoters are transcriptionally controlled. We determined that the transcription rates, transcript levels, and protein abundance are correlated. During normal growth, INPS is present in all cells, but IMT is repressed. After salinity stress, the amount of INPS was enhanced in leaves but repressed in roots. IMT was induced in all cell types. The absence of *myo*-inositol synthesis in roots is compensated by inositol/ononitol transport in the phloem. The mobilization of photosynthate through *myo*-inositol translocation links root metabolism to photosynthesis. Our model integrates the transcriptional control of a specialized metabolic pathway with physiological reactions in different tissues. The tissue-specific differential regulation of INPS, which leads to a gradient of *myo*-inositol synthesis, supports root growth and sodium uptake. By inducing expression of IMT and increasing *myo*-inositol synthesis, metabolic end products accumulate, facilitating sodium sequestration and protecting photosynthesis.

INTRODUCTION

A central focus of our work is to determine genes that underlie mechanisms providing salinity tolerance in higher plants. In addition, we hope to learn how whole plants adapt metabolically. Many researchers have studied tolerance mechanisms in plants. Their findings show the importance of metabolite accumulation, which generally is attributed to osmotic adjustment leading to water retention and/or protection of biochemical pathways. When single enzymes were tested in transgenic plants, the accumulation of mannitol, proline, fructans, trehalose, glycine betaine, or ononitol (Tarczynski et al., 1993; Kavi Kishor et al., 1995; Pilon-Smits et al., 1995; Holmström et al., 1996; Hayashi et al., 1997; Sheveleva et al., 1997) provided marginally higher salinity and cold or drought tolerance. The manipulation of biochemical pathways by the transfer of multiple genes will be an even more appropriate strategy that is expected to provide broader tolerance because salinity tolerance is a multigenic trait (Bohnert et al., 1995; Warne et al., 1995; Bohnert and Jensen, 1996). Salinity tolerance is also a multicellular trait, and the remodeling of complex pathways in transgenic plants will require knowledge of the distribution of such pathways throughout the plant. We have analyzed the *myo*-inositol biosynthetic pathway—leading to methylated inositols—that feeds into a pathway specific to salinity tolerance in a halophyte, the common ice plant (*Mesembryanthemum crystallinum*) (Loewus and Dickinson, 1982; Vernon and Bohnert, 1992).

myo-Inositol is a central component of several biochemical pathways. It is part of at least four cycles in which it is either cycled or shunted to end products with slow turnover. First, inositol phosphates are essential to signaling in almost all organisms; in plants, inositol hexaphosphate provides for phosphate storage (Hübel and Beck, 1996). Second, inositol-containing lipids are components of membranes (Mathews and Van Holde, 1990). Third, galactinol synthesized from UDP-galactose and inositol is the basic substrate for the raffinose series of sugars in plants. These sugars have been implicated in stress tolerance and possibly also in carbohydrate transport (Loewus and Dickinson, 1982). Fourth, inositol may be conjugated to auxins, preventing biological activity and allowing long-distance transport within the plant (Cohen and Bandurski, 1982). In these cases, enzyme cycles exist for the regeneration of inositol without significant net de novo synthesis.

When synthesis is required, it is regulated at the reaction catalyzed by L-*myo*-inositol 1-phosphate synthase (INPS). Synthesis is necessary for at least two pathways leading to

products that are turned over very slowly or not at all. In one of these pathways, D-glucuronate is synthesized directly from inositol and is used in the production of cell wall components, glycoproteins, gums, and mucilage; however, D-glucuronate may also be indirectly synthesized from UDP-glucose. The second pathway is unique and involves monomethylation or dimethylation of inositol. Some of the derivatives of this pathway may not be metabolized further. Inositol itself and these methylated derivatives increase in some animal and plant cell types in association with high external NaCl concentrations and dehydration (Loewus and Dickinson, 1982; Lowell and Nelsen, 1997; Muller et al., 1997).

De novo synthesis of inositol, which has been studied mainly in yeast, proceeds from glucose 6-phosphate through inositol 1-phosphate in two steps catalyzed by INPS and inositol monophosphatase (IMP). Yeast INPS (INO1) is tightly controlled: both inhibition by inositol and activation by choline (Culbertson et al., 1976) lead to efficient partitioning of substrate from the main flux of carbon. The promoter elements, transcription factors, and two-component regulators for feedback inhibition of synthase activity by inositol and enhancement of transcription by choline have been identified in yeast (Bachhawat et al., 1995; Burg et al., 1996). Repression of transcription during periods that do not require inositol and activation during periods of active growth represent fine-tuning of this essential pathway, which also coordinates enzymes that synthesize products downstream of inositol (Lopes et al., 1993).

In some plant species, inositol provides substrate for the production and accumulation of the compatible solutes ononitol and pinitol. This prompted our interest in the regulation of the pathway. Inositol methylation is catalyzed by *myo*-inositol *O*-methyltransferase (IMT), which has been characterized (Vernon and Bohnert, 1992; Rammesmayer et al., 1995) from the ice plant. The product ononitol is epimerized to pinitol, which accumulates to high amounts during salt stress (Adams et al., 1992). Ice plant INPS is regulated to drain substrate efficiently from the primary carbon flux pathway. IMT is also regulated to drain inositol from intracellular pools, most likely by the control of transcription (Ishitani et al., 1996). The role(s) of ononitol and pinitol has not been determined. However, both most likely lower the cytoplasmic osmotic potential and balance sodium accumulation in the plant vacuole. This role is similar to those of inositol and other solutes, which accumulate in renal cells of vertebrates (Burg, 1994).

Our results show complex, coordinate regulation of the inositol biosynthetic pathway from glucose 6-phosphate to ononitol in the salt-stressed ice plant, indicating that inositol metabolism provides more than an alteration in the allocation of carbon destined to become a stable osmolyte. The INPS protein is upregulated in leaves and downregulated in the roots, whereas IMT increases dramatically in leaves and marginally in roots. The regulation of enzyme amounts is primarily, possibly strictly, by transcriptional activation of the *Inps* and *Imt* promoters. Regulation of the *Imt* promoter is

mediated by an element near the TATA box. Inositol and ononitol then constitute major phloem carbohydrates that seem to have at least two functions in the root. Inositol is essential for continued root growth, and the amounts of inositol and ononitol are correlated with the concentration of sodium translocated to leaves through the xylem.

We think that *myo*-inositol in the phloem serves to signal photosynthesis capacity to the roots and to sustain membrane biosynthesis dependent on photosynthesis and that it acts as a facilitator of long-distance sodium transport. These results contribute to our emerging awareness of sugars and derivatives as regulatory molecules (Jang and Sheen, 1997; Koch, 1997) and the importance of metabolic flux, as opposed to metabolite accumulation, in signaling between different tissues.

RESULTS

IMT and INPS Protein Analysis

The anti-IMT antibody detected a single protein with an M_r of 41,000 in plant extracts (Figure 1). IMT was not detectable in untreated samples and increased in both seedlings (Figure 1A) and mature leaves (Figure 1B) during the first days of NaCl treatment. Steady state amounts were reached within 3 to 5 days, lagging 1 to 2 days behind mRNA increases (Vernon and Bohnert, 1992). The anti-INPS antibody detected a protein with an M_r of 57,000 in plant extracts, as shown in Figure 2. INPS was present in untreated samples
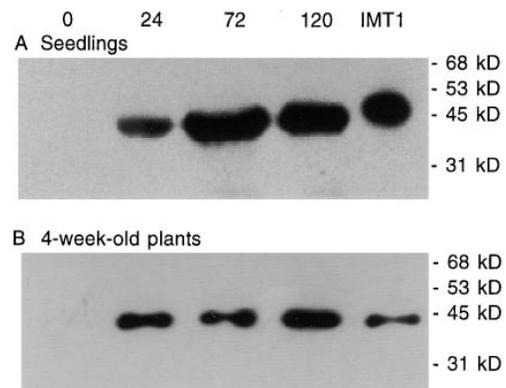

Figure 1. IMT Accumulation after NaCl Treatment.

Total leaf protein (25 μg) was analyzed by SDS-PAGE and immunoblotting with the anti-IMT antibody. Samples were collected at the times (0, 24, 72, and 120 hr) indicated after treatment with 0.5 M NaCl. IMT purified from bacterial extracts was also analyzed. The molecular masses of protein standards are indicated at right.
(A) Accumulation of IMT in seedlings after treatment.
(B) Accumulation of IMT in 4-week-old plants after treatment.

Day 0 Day 2 Night 2 Day 3 INPS-His
A INPS

- 68 kD
- 53 kD
- 45 kD
- 31 kD

8 ng   24   19   36   50

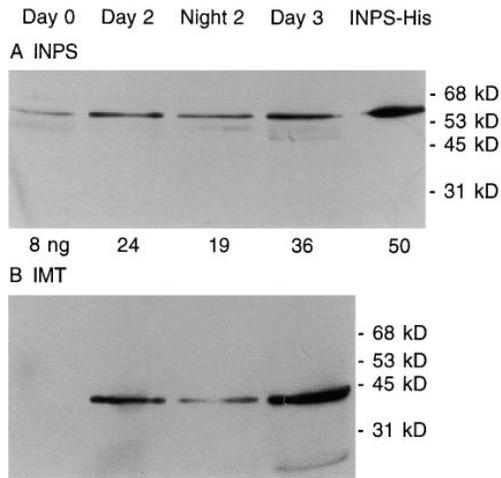B IMT

- 68 kD
- 53 kD
- 45 kD
- 31 kD

Figure 2. Diurnal Fluctuation of IMT and INPS Amounts in Leaves after NaCl Treatment.

Total leaf protein (50 μg) was analyzed by SDS-PAGE and immunoblotting. Samples were collected during the indicated light and dark periods after the initiation of treatment (Day 0). The molecular masses of protein standards are indicated at right.
(A) Fifty nanograms of purified, histidine (His)-tagged INPS was analyzed as the standard. The quantity of INPS present in each sample was determined by digital scanning of the developed film and comparison to the intensity of the signal of the purified protein standard. The quantities are indicated under the figure.
(B) Fluctuation of IMT protein from days 2 to 3.

but increased after NaCl treatment (Figure 2A). This pattern is identical to that observed for mRNA accumulation (Ishitani et al., 1996).

A pronounced diurnal fluctuation of *Inps* and *Imt* mRNAs was observed (Ishitani et al., 1996), with a maximum for both mRNAs shortly after the beginning of the light period and lower levels during the dark period. However, protein amounts did not show as great a fluctuation, although diurnal rhythmicity was evident (Figures 2A and 2B). Availability of the purified INPS recombinant protein allowed us to calculate the actual amount of INPS in each sample. After 3 days of treatment, INPS accumulated to ~0.1% of total protein during the day (Figure 2A) and fluctuated twofold from night to day. IMT levels fluctuated to a greater extent from night to day.

The deduced cDNA sequences of IMT and INPS included N-terminal sequences without any recognizable homology to chloroplast transit peptides, but the localization of INPS activity in chloroplasts has been reported from higher plants (RayChaudhuri et al., 1997). To study whether the two proteins might be located in the cytosol or in chloroplasts, extracts of total cell protein of NaCl-treated plants were compared with protein extracts from chloroplasts (Figure 3). Chloroplasts collected from Percoll gradients were intact as

observed by light microscopy and highly enriched for ribulose-1,5-bisphosphate carboxylase/oxygenase subunit proteins (Figure 3A). Immunological analysis of the samples (Figures 3B and 3C) failed to detect any INPS and IMT in chloroplast protein extracts from leaves of the ice plant (5 weeks old).

Until this time, protein analyses indicated similar expression patterns for INPS and IMT with respect to inducibility, diurnal rhythmicity, and intracellular location. However, comparison of the two proteins in roots revealed a divergent pattern. Figure 4A shows that the IMT protein was induced in roots, albeit at lower amounts than in leaves. Maximum levels were attained within 1 day after salt stress and were sustained after long-term treatment (120-hr time point not shown). The amount of protein mirrors exactly the appearance and sustained presence of the transcript (Vernon and Bohnert, 1992; Ishitani et al., 1996). Conversely, INPS signals disappeared within 1 day of treatment (Figure 4A) and did not reappear even after long-term (120-hr) treatment. The amount of inositol was measured in roots of both
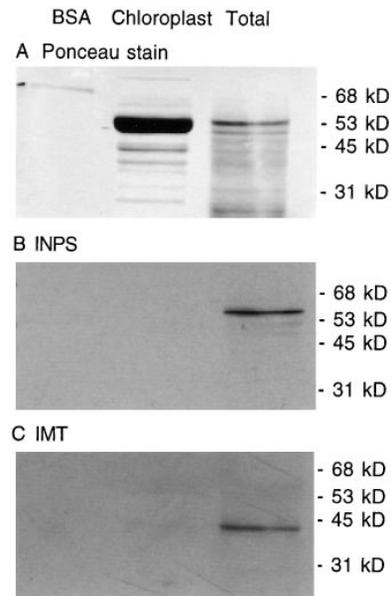
BSA Chloroplast Total
A Ponceau stain

- 68 kD
- 53 kD
- 45 kD
- 31 kD

B INPS

- 68 kD
- 53 kD
- 45 kD
- 31 kD

C IMT

- 68 kD
- 53 kD
- 45 kD
- 31 kD

Figure 3. Localization of IMT and INPS in the Cytoplasm.

Protein (25 μg) extracted from purified chloroplasts and from leaves was analyzed by SDS-PAGE and immunoblotting. Chloroplast samples contained 0.13 μg of protein per μg of chlorophyll. Leaf samples contained 0.28 μg of protein per μg of chlorophyll. The ratio indicates that ~50% of the total cellular protein was located in the chloroplasts. The molecular masses of protein standards are indicated at right.
(A) Ponceau S staining of a nitrocellulose blot after blotting but before detection.
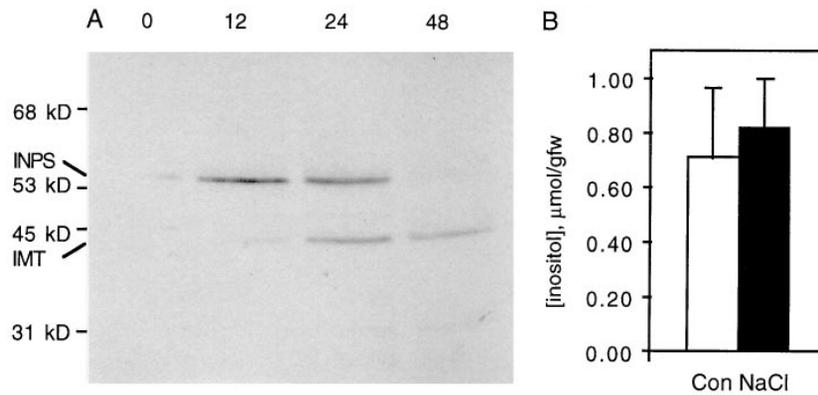**(B)** Detection of INPS.
(C) Detection of IMT.

Figure 4. INPS Decreases and IMT Increases in Roots after NaCl Treatment.

Total root protein (20 μg) was analyzed by SDS-PAGE and immunoblotting.
(A) Detection of INPS (57 kD) and IMT (41 kD). Samples were collected at the hours indicated at the top of the figure after the initiation of treatment. Samples collected after longer periods of time were identical to those reported for the 48-hr time point. Molecular masses of protein standards are indicated at left along with the relative positions of INPS and IMT.
(B) Quantitation of inositol in control (Con; $n = 2 \pm$ SD) and NaCl-treated ($n = 5 \pm$ SD) roots after 72 hr of stress. gfw, grams fresh weight of tissue.

untreated and treated plants and found to remain constant (Figure 4B), indicating that inositol in roots after salt stress might have been mobilized from some unknown storage or that it could have been translocated from the shoot.

Immunocytological Analysis of IMT and INPS

Ice plant leaves show a single epidermal layer with small guard cells on both surfaces, large mesophyll cells, and typical angiosperm vasculature. A comparison of Figures 5A and 5B shows that IMT was induced in mesophyll cells by NaCl treatment. These results are similar to those from the protein gel blot analysis (Figure 1). Protein was present in most or all cells, but different amounts were found in the various cell types. The strongest signals were associated with cells surrounding the vascular tissues. In roots, weak IMT expression was also characteristic of all cell types (results not shown).

In untreated plants, INPS was absent from mesophyll cells (Figure 5C) and located predominantly in companion cells and sieve elements of the phloem and cells surrounding the phloem (Figure 5E). After NaCl treatment, INPS became abundant in mesophyll cells (Figure 5D) but remained approximately constant in the phloem and associated cells (Figure 5F). Thus, the increase in INPS after NaCl treatment (cf. Figure 2A) was primarily in mesophyll cells. In control, nonstressed roots, INPS was localized in the vascular cylinder, and its accumulation was especially strong in cells of the endodermis (Figure 5G). In roots of NaCl-treated plants, the INPS protein disappeared (Figure 5H), indicating that repression in this particular cell type accounted for the decrease observed in the protein gel blot analysis of roots (Figure 4B).

Measurement of Solute Concentrations in Shoots

After treatment with NaCl, sodium and chloride comprised 64% of the total ions found in shoots but only 16% of the total ions in untreated shoots (Figure 6A). Significant increases of inositol and ononitol occurred simultaneously with the accumulation of sodium and chloride (Figure 6B). The data are expressed relative to sucrose, which on an absolute basis did not change significantly with NaCl treatment.

Sequence of the *Imt1* Promoter

When 5′ and 3′ end-specific probes derived from the cloned cDNA were used in DNA gel blot analysis with blots containing DNA cut with a variety of restriction enzymes, single bands were observed in each lane, indicating that the salt-inducible transcript is encoded by a single gene (see also Vernon and Bohnert, 1992). Screening of a genomic library produced three clones. All three lacked a complete 5′ end. Thus, cDNA sequences were used to amplify directly the *Imt* gene from genomic DNA by inverse polymerase chain reaction (IPCR). The sequence of the 3929-nucleotide genomic fragment obtained was identical to the previously characterized cDNA in the overlapping region. Figure 7A presents features of the gene sequence related to transcription and translation.
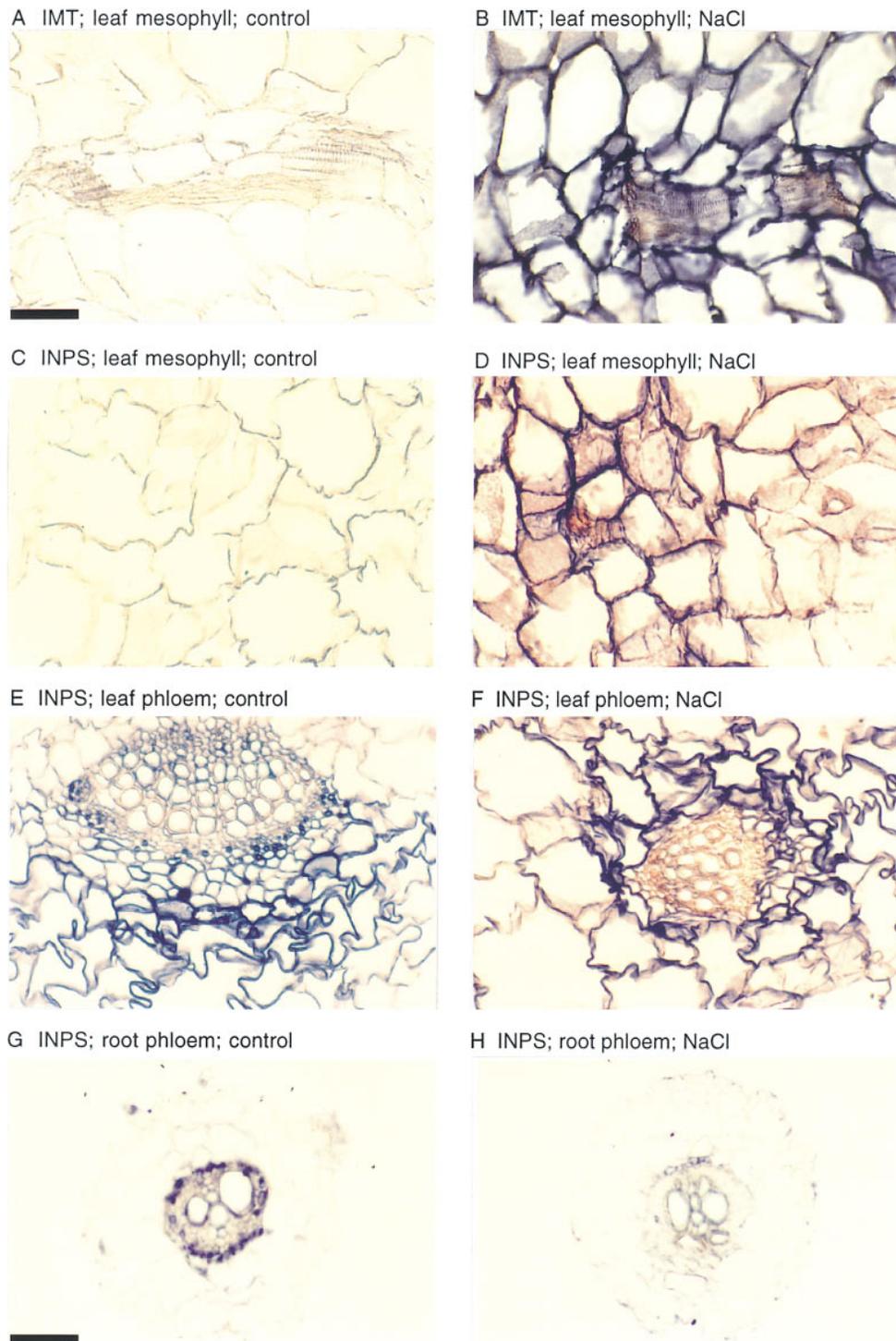
Figure 5. Immunocytological Analysis of INPS and IMT.

Indicated tissues of control plants and plants treated with 0.5 M NaCl were stained with primary antibodies, as indicated, and the goat anti–rabbit IgG–alkaline phosphatase conjugate.

(A) to **(F)** Leaf tissues were magnified equally. Bar in **(A)** = 100 μm for **(A)** to **(F)**.

(G) and **(H)** Root tissues were magnified equally. Bar in **(G)** = 400 μm for **(G)** and **(H)**.

Figure 7B indicates that NaCl-induced transcription (Vernon and Bohnert, 1992) is initiated from a single nucleotide, a cytidine at position 1115. No extension products were obtained with RNA from unstressed leaves. A TATA element conforming to standard composition and distance from the transcription start site (Struhl, 1994) was found 26 nucleotides upstream from this site.

Functional Promoter Analysis by Transient Assays of Reporter Gene Activities

A diurnal cycle of the level of *Imt* RNA has been observed (Ishitani et al., 1996; D.E. Nelson, unpublished data), with maximum accumulation occurring early in the light period of each day. Thus, a specific time course was always followed in the transient assays. Plants were maintained on a 12-hr-day and 12-hr-night cycle. They were irrigated in soil with 500 mM NaCl for 2 hr after the beginning of a light period (day 0). Leaves were detached, and plasmids were introduced the following day (day 1), 2 hr before the end of the light period. Leaves were floated in half-strength Hoagland solution with or without 500 mM NaCl after detachment. Finally, leaves were assayed the morning of day 3, 2 hr after the beginning of the light period, 40 hr after bombardment, and 72 hr after the initial irrigation.
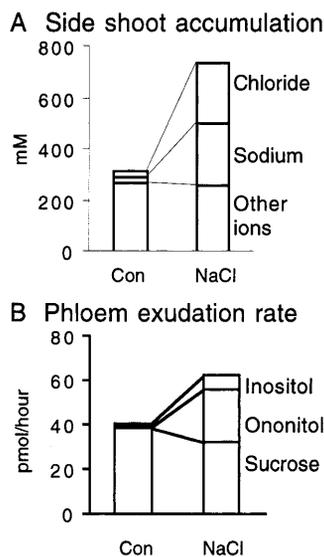

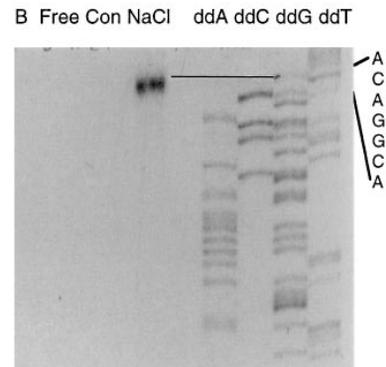| A	
Genbank accession:	U63634
TATA element	1082-1089
Transcription	1115
Translation start	1300-1302
Translation stop	3578-3580
Exon 1	1115-1721
Exon 2	2521-2831
Exon 3	2972-3036
Exon 4	3281-3929

Figure 7. Mapping of the *Imt* Gene Transcription Start Site and Various Features.

(A) GenBank accession number and features relating to transcription and translation of the *Imt1* gene are summarized.

(B) Primer extension products derived from RNA of control (Con) and NaCl-treated leaves were separated side by side with unextended labeled oligonucleotides (Free) and dideoxynucleotide-terminated sequencing ladder products. The unextended labeled oligonucleotides migrated below the portion of the autoradiogram shown. The nucleotide judged to comigrate with the longest extended products is marked by the horizontal bar. The 5′ to 3′ sequence in the region is indicated at right.

Figure 6. Quantitation of Total Ions and Phloem Organic Solutes in Side Shoots after 3 Days of NaCl Treatment (*n* = 6).

(A) Increase of sodium and chloride among total ions after NaCl treatment of side shoots. The concentration is calculated based on total cellular water. Con, control.

(B) Increase of inositol and ononitol exudation in comparison with sucrose in phloem after NaCl treatment.

In control leaves, when either a promoterless or full-length promoter construct was introduced by microprojectile bombardment, no β-glucuronidase (GUS) activity by using X-gluc as substrate was observed, indicating that neither wounding nor oxidative stress that accompanies this treatment induced *Imt* expression. However, in salt-stressed leaves, strong GUS activity was observed when the full-length construct was introduced (data not shown). Also, no activity was observed when the promoterless construct was introduced into NaCl-treated leaves (data not shown). Thus, the induction observed was specific to NaCl and, in addition, was qualitative for the full-length promoter.

To delineate the region regulating salinity responsiveness, seven deletion constructs were tested. When the deletions were introduced to leaves, strong GUS activities similar to those induced by the full-length promoter were observed for all constructs, except for the deletion to position 1100. Deletion to position 1100 resulted in complete elimination of promoter activity with respect to NaCl responsiveness (results

not shown). Therefore, the deletion studies provide preliminary evidence for a salt-inducible domain in this region of the promoter. Additional experiments, including gain-of-function analysis and site-directed mutagenesis, are required to confirm this result.

To confirm the observations made for GUS expression and to quantify the amount of regulation provided by the putative control region, constructs were made for a promoterless luciferase and for the luciferase coding region fused to three deletions, positions 336, 1071, and 1100, of the *Imt* promoter. The 336LUC construct was introduced to both untreated and treated leaves. Luciferase activity was >100 times higher in NaCl-treated leaves than in control leaves (Figures 8A to 8C). Finally, the three deletion constructs and luciferase without any promoter, each introduced independently three times, showed that the salinity responsiveness of the *Imt* promoter is conferred solely by the domain previously identified in the GUS assays (Figure 8D). Deletion of

the promoter to position 1071 resulted in no decrease of responsiveness compared with the deletion to position 336, whereas the deletion to position 1100 was quantitatively identical to the promoterless construct. The location and sequence of the nucleotides in this region are given along with a summary of the deletion end points analyzed and structural features (Figure 9).

DISCUSSION

The ice plant uses several mechanisms through the coordinate expression of sets of genes that enable it to deal with three major and overlapping problems required to be overcome by a salt-tolerant plant (Bohnert and Jensen, 1996). These include (1) the ability to either exclude or take up and compartmentalize NaCl (ion channels, antiporters, symporters,
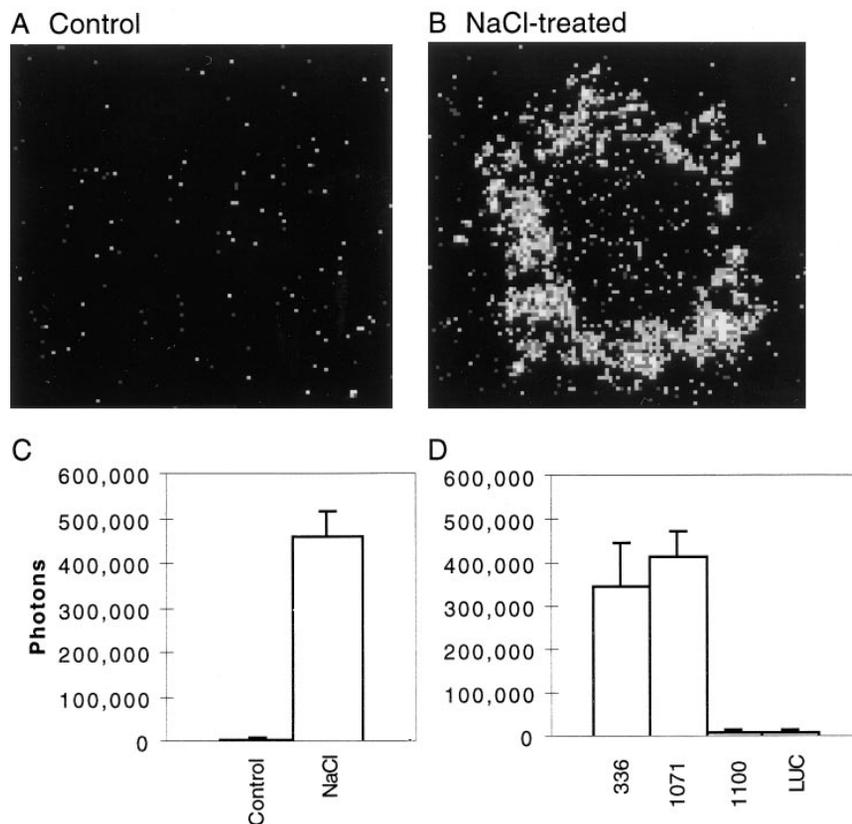

Figure 8. Luciferase Activities of *Imt* Promoter Constructs in Leaves.

(A) Digital representation of photon density emanating from a control leaf bombarded with the 336LUC construct.
(B) Digital representation of photon density emanating from a NaCl-treated leaf bombarded with the 336LUC construct.
(C) Count of photons emanating from control and NaCl-treated leaves bombarded with the 336LUC construct ($n = 3 \pm$ SD).
(D) Count of photons emanating from NaCl-treated leaves bombarded with three *Imt* promoter deletion constructs and a promoterless construct ($n = 3 \pm$ SD).
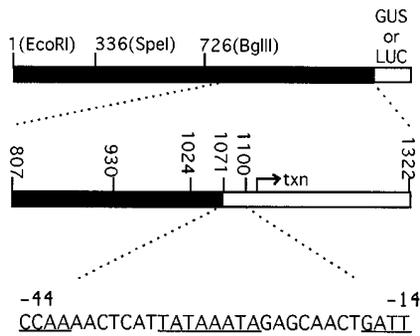
Figure 9. Location and Structure of the Functional Salinity Response Domain.

The bar maps indicate deletions of the full-length constructs that were introduced into leaves. The bar at top indicates deletions made using restriction enzymes. The bar below it indicates deletions made by exonuclease III digestion. Numbers indicate end points of the constructs and correspond to nucleotide positions found under GenBank accession number U63634. The indicated sequence of *Imt* is numbered relative to the transcription start (1071 is −44, and 1100 is −14). The TATA element is underlined. Also underlined are the elements similar to the bipartite element shown to be critical for red/far-red reversible effects mediated through phytochrome in the *L. gibba Lhcb2*1* promoter. txn, transcription start.

and ATPases), (2) the ability to maintain internal water status (enzymes synthesizing osmotica and/or controlling water channel amount and activity), and (3) the ability to prevent direct or indirect damage by NaCl to sensitive cellular structures (enzymes synthesizing compatible solutes and radical oxygen scavenging enzyme systems). Stress-induced changes in tonoplast ATP synthase activity and gene expression have recently been reported for this plant (Löw et al., 1996; Tsiantis et al., 1996). Also, tonoplast Na$^+$/H$^+$ antiport activity increases during stress (Barkla et al., 1995). Amounts of water channel transcripts and of some water channel proteins respond to salinity stress (Yamada et al., 1995; H. Kirch and H.J. Bohnert, unpublished data). For whole plants, salt shock causes wilting for a short period, but full turgor is restored within the period during which (1) the IMT protein increases, (2) the INPS protein is induced in leaves and repressed in roots, and (3) pinitol accumulates.

Although pinitol's function has not yet been proven, we hypothesize that it acts primarily as a cytoplasmic osmoticum but do not exclude its role in oxygen radical scavenging (Smirnoff and Cumbes, 1989) or in the storage of photosynthesis products that are not used under stress conditions (Bohnert and Jensen, 1996). The precursor of pinitol, ononitol, is found in phloem exudates in significant amounts only after stress. Phloem cells and other cells close to the xylem, the major route for long-range sodium transport, will experience higher cytosolic concentrations of sodium and chloride than will cells distant from the vasculature. In the short term,

the presence of IMT and ononitol production in cells close to the vasculature would be more important than would be the abundance of IMT in distant cells.

The following observations together indicate intercellular transport of inositol and its derivatives for a variety of physiological purposes. Gillaspy et al. (1995) demonstrated that IMP, the enzyme catalyzing the dephosphorylation of *myo*-inositol 1-phosphate, is found in the phloem in higher amounts than in other cells. The result also indicates that this cell type is a major site of synthesis. That the phloem has the highest rate of utilization is unlikely, suggesting that the synthesis is for translocation to other sinks. Synthesis of the raffinose series sugars provides another specific example for translocation of inositol to be used in distant tissues and by other biochemical pathways (Bachmann et al., 1994; Bachmann and Keller, 1995). Similar examples are the conjugation of inositol to auxin for long-distance transport from shoot to root (Cohen and Bandurski, 1982) and phytase metabolism (Hübel and Beck, 1996).

Although IMT and INPS are not located in the chloroplast, two important links exist between photosynthetic activity and pinitol accumulation. First, at the biochemical level, synthesis of pinitol proceeds from glucose 6-phosphate, which is most likely derived directly from primary photosynthate. Further, we have argued that the methyl group added to *myo*-inositol may be derived from photorespiration (Bohnert and Jensen, 1996). Methyltetrahydrofolate, produced along with glycolate during photorespiration, enters the active methyl cycle leading to production of *S*-adenosyl methionine, the established cofactor for IMT (Rammesmayer et al., 1995). Second, at the gene expression level, *Imt* and *Inps* mRNA amounts are highest at the beginning of the light period and low by the beginning of the next dark period (Ishitani et al., 1996). The large diurnal regulation of mRNA amounts combined with the more moderate diurnal regulation of protein amounts (Figure 2) imply that INPS and IMT are turned over rapidly during the light period. With the activity of IMT possibly modified by the cytosolic concentrations of monovalent cations (Rammesmayer et al., 1995), relatively constant levels of the enzymes would efficiently use photosynthate.

We have focused here on the genes *Inps* and *Imt*, because the *Imp* gene, encoding *myo*-inositol monophosphatase (GenBank accession number AF037220), was found to be unaffected by salinity stress (D.E. Nelson, unpublished results). Both *Imt* and *Inps* are regulated by salinity and show diurnal rhythmicity in transcript level and protein accumulation. Regulation of gene expression by photoreceptor systems, phytochrome, blue light, and UV-B has been very well documented for plastid-encoded genes and nuclear genes whose products are plastid located (Anderson and Kay, 1997). Examples of diurnal regulation of nonplastid, nonphotosynthetic genes are less common but include nitrate reductase (Deng et al., 1990; Pilgrim et al., 1993) and catalase (Boldt and Scandalios, 1995). The genes of the inositol biosynthesis pathway can probably be added

to this list. Similar to the diurnal rhythmicity of *Inps* and *Imt*, the peaks of nitrate reductase (Pilgrim et al., 1993) and *Cat3* mRNA accumulation associated with photorespiration in Arabidopsis (Zhong et al., 1994) occur at the beginning of the light period. Recently, Degenhardt and Tobin (1996) reported mutations in phytochrome response elements REα and REβ of the *Lhcb2*1* gene of *Lemna gibba*. Mutations that eliminated the red/far-red response of *Lhcb* expression are perfectly conserved in the elements present in the identified functional region (Figure 9), but the function of these sequences remains to be confirmed by further studies, including point mutation analysis.

The lack of developmental control over NaCl induction of IMT accumulation (Figure 1) is consistent with all previous measurements of mRNA levels. The qualitative induction was also observed in GUS activity levels (Figure 8). The lack of any attenuation of promoter strength until position 1100 indicates that the essential elements responsible for salinity induction are located within 10 to 15 nucleotides of the TATA element or possibly that the response is mediated by a yet unidentified protein–protein interaction involving the TATA binding protein and other associated proteins. The presence of sequence elements in this region that are similar to those responsible for phytochrome-mediated regulation of *Lhcb* expression suggests that diurnal rhythmicity may be also controlled here.

The root-specific repression of INPS by salinity was an unexpected result. This repression prevents roots from using phloem-supplied sucrose for inositol and pinitol synthesis. Rather, inositol becomes a major translocated photosynthate (Figures 4 and 6), and inositol synthesis under saline conditions is no longer cell or tissue autonomous. Intracellular compartmentation of separate inositol pools for phosphoinositide synthesis and osmotic function has already been documented (Bersudsky et al., 1994). This situation may be analogous to the existence of intracelluar calcium gradients important for several signal transduction pathways (Bootman and Berridge, 1995). The gradient of INPS in phloem-associated cells reveals a regulatory circuit important far beyond inositol and pinitol synthesis. It seems that in the ice plant, photosynthesis can simultaneously control root growth via inositol supply and osmotic stress protection via ononitol and pinitol availability in the roots.

Based on INPS and IMT transcript increases in leaves under stress, we expected coordinate upregulation of the *Imt* and *Inps* promoters through similar or identical elements. The repression of *Inps* in roots requires revision to a model whereby *Imt* and *Inps* promoter activities are linked sequentially. In the leaves, *Imt* is transcriptionally induced. This induction is proportional to the amount of sodium accumulating in the cells. Increasing activity of the IMT protein leads to a decline in inositol concentration, which induces *Inps* transcription in the short term. Preliminary experiments showed that externally supplied *myo*-inositol reduced INPS protein levels, indicating that feedback inhibition similar to that in yeast exists (D.E. Nelson, unpublished data). *Inps* ac-

tivation could be mediated through an element equivalent to the yeast UASINO element (Lopes et al., 1993), which has, however, not yet been functionally identified in the ice plant *Inps* promoter. Such a model conforms with the immunocytological results. The INPS protein accumulates in mesophyll cells and in particular in phloem companion cells, where inositol is being consumed for the production of ononitol and exported through the phloem at a stress-dependent rate. The decrease in INPS levels in root phloem after long-term stress is likely due to the increased amount of inositol carried by the phloem sap. The low amount of IMT protein detected in roots is consistent with only a transient increase in *Imt1* transcripts in roots. This increase amounted to ∼5% of the increase measured in leaves (Vernon and Bohnert, 1992). Inositol is not consumed by IMT in the root as much as it is in the shoot, because roots do not store NaCl as the shoots do (Adams et al., 1992).

We are continuing to develop this model through analysis of the *Inps* promoter, collection of additional data on ion and organic solute concentrations in the phloem, and determination of the underlying transport mechanism(s). We postulate a sodium-dependent inositol transport mechanism in the roots, which is similar to that in animals, in which Na$^+$/inositol cotransport has been demonstrated as being important for osmotic regulation (Burg et al., 1996). Also, it will be important to know how inositol provision is regulated in glycophytes during salinity stress. Gene probes and antibodies have made possible the analysis of INPS distribution patterns in salt-sensitive species. Salt-dependent inositol synthesis gradients throughout the plant could be a characteristic feature of halophytic plants for which the regulation of sodium uptake is dependent on photosynthetic capacity.

METHODS

Plant Growth Conditions

Mesembryanthemum crystallinum plants were raised in ConViron (Winnipeg, Canada) growth chambers illuminated with 300 μE m^{-2} sec^{-1} from fluorescent and incandescent lights. Standard conditions were 12 hr of light and 12 hr of dark at 23 and 17°C, respectively. For hydroponic roots, seedlings were grown in 4.5-liter containers containing half-strength Hoagland solution with aeration.

Purification of IMT and INPS and Antibody Production

The *Imt* cDNA had been expressed in *Escherichia coli* using the *Imt* start codon (Vernon and Bohnert, 1992). The protein was purified as described in Rammesmayer et al. (1995). For *E. coli* expression of INPS, two primers (5'-GGAATTCCATATGTTTATTGAGAGCTTC-3' and 5'-GGAATTCCTCGAGCTTGTATTCGAGGATC-3') were designed to contain sequences near the translation start and stop sites on the *Inps* cDNA (Ishitani et al., 1996) and the NdeI and XhoI sites for cloning into the vector pET20b (Novagen, Madison, WI). The resulting recombinant protein contains at its C terminus the additional amino

acids LEHHHHHH. The presence of the histidine tag allowed one-step purification by nickel column chromatography. The purified recombinant proteins were used to prepare antibodies in rabbits (HTI Bio-Products, Ramona, CA).

Protein Analysis

Protein from ice plant tissues was extracted either directly or from acetone powders in Laemmli buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 3 μM E-64 (Boehringer Mannheim). Protein concentration was determined using the Bradford reagent (Bio-Rad) on a portion of the extract from which excess SDS was removed by precipitation with 100 mM potassium phosphate buffer, pH 6.8. After SDS-PAGE and electroblotting, we detected protein by using a 1:5000 dilution of the primary antibodies diluted in TBS buffer containing 1% Tween 20 and 1% Carnation instant milk. Goat anti–rabbit antibody conjugated to horseradish peroxidase (Gibco BRL) diluted 1:5000 in the same buffer was used to detect the primary antibodies. Detection of the complex was by chemiluminescence using the ECL reagents of Amersham.

Isolation of Chloroplasts

Five grams of leaf tissue from NaCl-treated plants was ground in 125 mL of buffer (1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM ascorbic acid, 20 mM Hepes, pH 7.5, 0.5 M sorbitol, and 0.25% BSA) by using a Polytron (Brinkmann Instruments, Co., Westbury, NY) at low speed. The extract was filtered through Miracloth (Calbiochem) and centrifuged at $\sim$2000g for 3 min. The pellet was resuspended in 10 mL of grinding buffer and again filtered. The extract was layered on top of a Percoll gradient consisting of 80% Percoll in grinding buffer and 40% Percoll in grinding buffer. After centrifugation (8 min at 4000 rpm; Beckman J-6B; Beckman, Inc., Palo Alto, CA), the layer of intact chloroplasts was collected, diluted 20-fold in grinding buffer, and centrifuged at 4000 rpm for 3 min. The pellet was extracted with 80% acetone and centrifuged. The supernatant was kept for chlorophyll determination (Jagendorf, 1982), and the pellet was resuspended in buffer for SDS-PAGE.

Immunocytology

Leaves and roots of control and NaCl-treated plants were fixed with 2% glutaraldehyde but otherwise were processed as described in Dixon and Klessig (1995). Primary antibody was diluted 1:1000. As the secondary antibody, goat anti–rabbit IgG conjugated to alkaline phosphatase was diluted 1:1000. X-phosphate and nitro blue tetrazolium reagents (Boehringer Mannheim) were used to detect the complex.

Collection of Phloem Exudate and Measurement of Solutes

Side shoots bearing two leaf pairs of 8-week-old plants were excised 4 hr after initiation of the light period. The cut surface was submerged in 2 mL of 10 mM EDTA, pH 8.0, contained in liquid scintillation vials. After a 2-hr period to wash out solutes released directly at the cut surface, the side shoots were placed in fresh media for an additional 4 hr. This method is essentially as described by King and Zeevaart (1974). The buffer was then lyophilized and processed as described by Adams et al. (1992) for determination of sucrose, inositol, and ononitol levels, as described by Adams et al. (1993).

Library Screening, Inverse Polymerase Chain Reaction, and DNA Sequencing

An ice plant genomic library (Meyer et al., 1990) was screened with the *Imt* cDNA. Inverse polymerase chain reaction (IPCR) was used to amplify directly portions of the *Imt* gene. One microgram of genomic DNA was digested with EcoRI (10 units) in a 20-μL volume. After heat inactivation at 65°C for 20 min, the sample was diluted to 450 μL with water. To this, 50 μL of 10 $\times$ ligase buffer and 10 units of T4 DNA ligase were added. Ligations were performed overnight at 16°C. Ligation products were precipitated with 1 mL of ethanol and resuspended in 75 μL of Tris-EDTA. The PCR reaction contained 25 μL of template, 5 μL of 10 $\times$ Taq Extender buffer (Stratagene, La Jolla, CA), 200 μM deoxynucleotidetriphosphates, 1 μM specific primers, 3 units of Taq DNA polymerase, and 3 units of Taq Extender (Stratagene). The reaction cycle was 94°C for 30 sec, 55°C for 30 sec, and 72°C for 3 min for 30 cycles in a DNA thermal cycler (Perkin-Elmer). The primers used were complementary to regions near the translation start site and had the sequences 5'-TCCAAGCTTGAACAA-TTAGCTGGTTTGGC-3' and 5'-TCCAAGCTTGCCATTGGTGTAAGT-AGTC-3'. The primers contain a HindIII restriction site to facilitate cloning. DNA from subcloned fragments was sequenced by standard methods.

Primer Extension

Determination of the transcription start site was done according to Ausubel et al. (1987). An oligonucleotide (5'-GCCATTGGTGTAAGT-AGTC-3') complementary to a sequence near the translation start site (nucleotide position 1302 to 1320) was labeled with γ-^{32}P-ATP and T4 polynucleotide kinase. The labeled primer was annealed to total ice plant RNA (50 μg) in 0.9 M NaCl at 50°C overnight. After precipitation, extension was done with SuperscriptII reverse transcriptase (Gibco BRL). For a size ladder, pDN30 was sequenced with the same oligonucleotide. Primer extensions were performed with RNA from control plants and plants treated with 500 mM NaCl. The extension and sequencing products were analyzed by denaturing PAGE.

Transient Reporter Gene Assays in Leaves

Plasmids were constructed by cloning the *Imt* promoter into a derivative of pGEM3Z (Promega) carrying the β-glucuronidase (*GUS*) reporter gene and the nopaline synthase (*nos*) terminator. This construct carries all three components on a single EcoRI fragment. Double digestion with EcoRI and either SpeI or BglII released fragments with 5' end deletions (335 and 725 nucleotides, respectively). These fragments were subcloned in the SpeI, BamHI, and EcoRI sites of pBluescript KS+ (Stratagene). The construct containing the BglII-EcoRI cassette was further deleted by exonuclease III digestion. For luciferase assays, the *GUS* coding region and *nos* terminator were replaced with a cassette containing a luciferase coding region (Promega) and a pea ribulose-1,5-bisphosphatase carboxylase small subunit terminator region (Hunt, 1988). Plasmids were introduced into control leaves and leaves treated with 500 mM NaCl by microprojectile bombardment (Schaeffer et al., 1995). Leaves were fixed, and GUS activity was detected using 5-bromo-4-chloro-3-

indolyl β-D-glucuronide acid as substrate (Draper et al., 1988). For luciferase assays, 250 μL of substrate solution (1 mM luciferin in 1% Tween 20) was placed in the center of the shot and overlayed with Saran Wrap. After 10 min in the dark, photons were collected for 5 min with the system described by Millar et al. (1992).

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Regulation of Cell-Specific Inositol Metabolism and Transport in Plant Salinity Tolerance

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