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Growth and development of *Mesembryanthemum crystallinum* (Aizoaceae)

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SUMMARY

This review describes the life cycle of *Mesembryantheum crystallinum* L. (the common ice plant, Aizoaceae, Caryophyllales), a halophyte with a developmentally programmed switch from C_3 photosynthesis to Crassulacean acid metabolism (CAM) which is accelerated by salinity and drought. Since there has been controversy regarding the interplay between genes and environmental stimuli during the development of *M. crystallinum*, it is timely to summarize the life cycle for a defined set of conditions. We seek to establish the framework whereby five stages of development can be described in terms of morphology, physiology, and molecular biology. Stages 1 and 2, representing germination and growth of a juvenile form, show a determinate pattern of growth. Although specific genes for salt tolerance can be induced at these stages, stress early in development prevents progression to the

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mature form (stages 3–5) in which the plants advance to mature growth, flowering, and seed development. Growth in stage 3 is indeterminate in the absence of stress, but development and flowering are accelerated by environmental stresses, and CAM is constitutively expressed. Depending on the severity of the stress, plants start to flower (stage 4) and then die from the roots, ultimately with only seed capsules remaining viable, with salt sequestered into large epidermal bladder cells (stage 5). We highlight responses to salinity leading to compartmentation of ions and compatible solutes, turgor maintenance, and CAM. Finally, the molecular genetics of the ice plant are characterized, emphasizing selected genes and their products. We conclude with an analysis of the multiple stages of growth as an ecological adaptation to progressive stress. The initial determinate and inflexible juvenile phase provides a critical mass of plant material which supports the indeterminate, mature phase. Depending on the degree of stress, the mature form is then propelled towards flowering and seedset.

Key words: Mesembryanthemum crystallinum, growth, development, salt stress, CAM induction.

I. INTRODUCTION

Mesembryanthemum crystallinum L., the common ice plant (family Aizoaceae, Caryophyllales), has become synonymous with stress responses modelled at the molecular level (Bohnert *et al.*, 1988, 1994; Cushman & Bohnert, 1996). The interactions between developmental and environmental stimuli have not been systematically charted under controlled-environment conditions, despite many studies which have followed the discovery of Crassulacean acid metabolism (CAM) induction in this plant (Winter, 1978; Winter & von Willert, 1972; Bloom, 1979*a*, *b*; Bloom & Troughton, 1979; Lüttge, 1993). The aim of this paper is to provide a framework for growth and development encompassing responses to salinity, drought and low temperature, as well as CAM. For a defined set of growth conditions, we will review how environmental stimuli can enhance development, depending on the stage in the life cycle.

In terms of ecology and distribution, *M. crystallinum* is native to southern and eastern Africa (Winter, 1978; Winter & von Willert, 1972; Bloom, 1979*a*, *b*; Bloom & Troughton, 1979). It is now introduced into western Australia, around the Mediterranean, along coasts of the western United States, Mexico, and Chile, and the Caribbean (Gutterman, 1980; Sigg, 1981; Winter & Smith, 1996). In its native habitat, the plant germinates and becomes established after a short (winter) rainy season, followed by progressive drought stress coupled with increasing salinity (Winter, 1978; Winter & Troughton, 1978; Bloom, 1979*a*, *b*; Bloom & Troughton, 1979). Such conditions have resulted in the evolution of acclimatory processes which can be defined in terms of anatomical, physiological, biochemical and molecular processes. The seedlings (which are also cold-tolerant), must initially develop a critical mass of plant material, with the formation of up to seven pairs of juvenile leaves. The onset of stress at the end of the rainy season then accelerates the developmental shift from juvenile growth characterized by large leaves of the primary axis, to mature growth, characterized by succulent leaves along developing side shoots. While juvenile plants can respond to salinity, the production of the smaller succulent leaves in mature plants coincides with the

Table 1. *Growth phases of* Mesembryanthemum crystallinum

Phase	Characteristics
Germinating	Only cotyledons present. CAM is not inducible. Compatible solute biosynthesis is inducible in cotyledons by drought, not by salinity stress.
Juvenile	Seven leaf pairs develop along the primary axis. No side shoots, no flowers. C3 photosynthesis. CAM is not inducible by stress. Compatible solute biosynthesis is inducible.
Adult	Side shoots with secondary leaves. No flowers. Primary leaves senesce. CAM becomes gradually inducible.
Flowering	Flowers at the terminus of the primary axis, and in axles of secondary leaves. Epidermal bladder cells become obvious. CAM is always induced.
Seed-forming	Seed capsules are the only viable plant part. No water uptake. Epidermal bladder cells are prominent.

developmental induction of CAM in the entire plant. Under optimal 'garden' conditions, plants can grow to > 20 kg f. wt (Adams *et al.*, 1992), but the magnitude of seedset depends on CAM and on the size the plant had attained in its juvenile growth phase (Winter & Ziegler, 1992). Even though older portions of the plant progressively succumb to salinity, the seed capsules continue to sequester salt and provide fixed carbon for seedset.

At the physiological and biochemical level, work on the plant has provided insights into salinity tolerance and the regulation of CAM. Salinity and drought affect a suite of sugar-alcohol-compatible solutes (inositol, ononitol, pinitol; Paul & Cockburn, 1989; Vernon & Bohnert, 1992*a*; Ishitani *et al.*, 1996) in addition to the activity of ion transporters and specific water-channel proteins associated with roots, stems and leaves. In contrast to many facultative CAM plants in which induction is reversible (Griffiths, 1988; Borland & Griffiths, 1996), the induction of CAM in adult leaves (stage 3, Table 1) of *M. crystallinum* is constitutive, although induction of PEPC (phospho*enol*pyruvate carboxylase) transcripts and protein by salt stress in old leaves of the juvenile stage (stage 2, Table 1) is reversible when salt is removed (Vernon *et al.*, 1988). We distinguish here the inducibility of transcripts for the CAM form of PEPC, which progresses from not inducible in young juvenile leaves (age *c.* 2–4 wk) to readily inducible in old juvenile leaves (age *c.* 6 wk and older), from the induction of the CAM pathway in all its physiological manifestations. Once fully induced, the majority of plant C is derived from net CO_2 uptake at night, mediated by a CAM-specific isoform of PEPC, with malic acid stored in vacuoles. During the subsequent light period, the malic acid is decarboxylated in the cytosol to provide CO_2 for fixation via ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) under low photorespiratory conditions in the chloroplast. Work with *M. crystallinum* has been central to our understanding of the differential regulation of PEPC over the day–night cycle (Winter, 1982), now known to be regulated in part by phosphorylation (Nimmo *et al.*, 1986; Li & Chollet, 1994; Cushman & Bohnert, 1997).

Finally, at the molecular level, changes in gene expression, which are synchronized with, and generate, a developmental pattern, are accelerated by stress. Some genes are controlled transcriptionally by salt stress at all periods of development, such as *Imt1*, encoding a *myo*-inositol O-methyltransferase, the key enzyme leading to the production of compatible solutes, i.e. solutes that will not interfere with biochemical reactions even at high concentration (Vernon & Bohnert, 1992*a*; Ishitani *et al.*, 1996). Although this response might occur during the juvenile phase of growth, the limited capacity to transport sodium into vacuoles (Löw *et al.*, 1996; Tsiantis, Bartholomew & Smith, 1996) and to sequester salt in specialized epidermal bladder cells (EBC) (Lüttge, Fischer & Steudle, 1978; Lüttge, 1993) results in arrested development in juvenile plants which have not attained the capacity to grow side shoots. In contrast, the CAM isoform of PEPC (encoded by gene *Ppc1*) and other CAM-related genes which are not inducible in young juvenile plants (Bohnert *et al.*, 1994), become progressively transcriptionally inducible (Ostrem, Vernon & Bohnert, 1990; Cushman, Michalowski & Bohnert, 1990) in juvenile leaves and are constitutively expressed in mature leaves (Cushman & Bohnert, 1996). The characterization and expression of gene families for the small subunit of RUBISCO (*RbcS*; DeRocher & Bohnert, 1993), of water-channel proteins (*Mip*; Yamada *et al.*, 1995; H. H. Kirch & H. J. Bohnert, unpublished), of CAM genes (Cushman & Bohnert, 1996) transcripts for polyol biosynthesis (Ishitani *et al.*, 1996; D. E. Nelson, G. Rammesmayer & H. J. Bohnert, unpublished and photorespiration (F. Quigley & C. B. Michalowski, unpublished) have been related to developmental stages and magnitude of environmental stress.

In previous studies, plant size and rate of development have been associated with a wide range of experimental variables. In this paper, we seek to establish the pattern of development and gene expression for a defined set of growth conditions used to elicit responses in laboratory experiments, illustrated by new data where appropriate. Our aim is to show how the controversies regarding genes and environments can be resolved by an understanding of which systems are inducible at any given stage of development. We will contend that the plant is, strictly speaking, not 'facultative' in terms of either halophytism or CAM responses. We show that developmental plasticity can be defined throughout the growth cycle by identifying five distinct growth stages, during which the expression of various gene combinations can be altered by environmental conditions. Ultimately, with a small genome of 250–300 Mbp (DeRocher *et al.*, 1990), transformation by *Agrobacterium*-based vectors (H. J. Bohnert & J. C. Cushman, unpublished), together with the already proven utility for transfer of specific genes (e.g. conferring enhanced salinity tolerance in tobacco; Tarczynski, Jensen & Bohnert, 1993; E. Sheveleva, H. J. Bohnert & R. G. Jensen, unpublished), we might identify genes suitable for manipulation into other crop plants (Bohnert & Jensen, 1996*a*, *b*).

II. STANDARDIZING METHODOLOGY

As the framework for this review we describe a set of growth conditions for standardizing developmental growth stages.

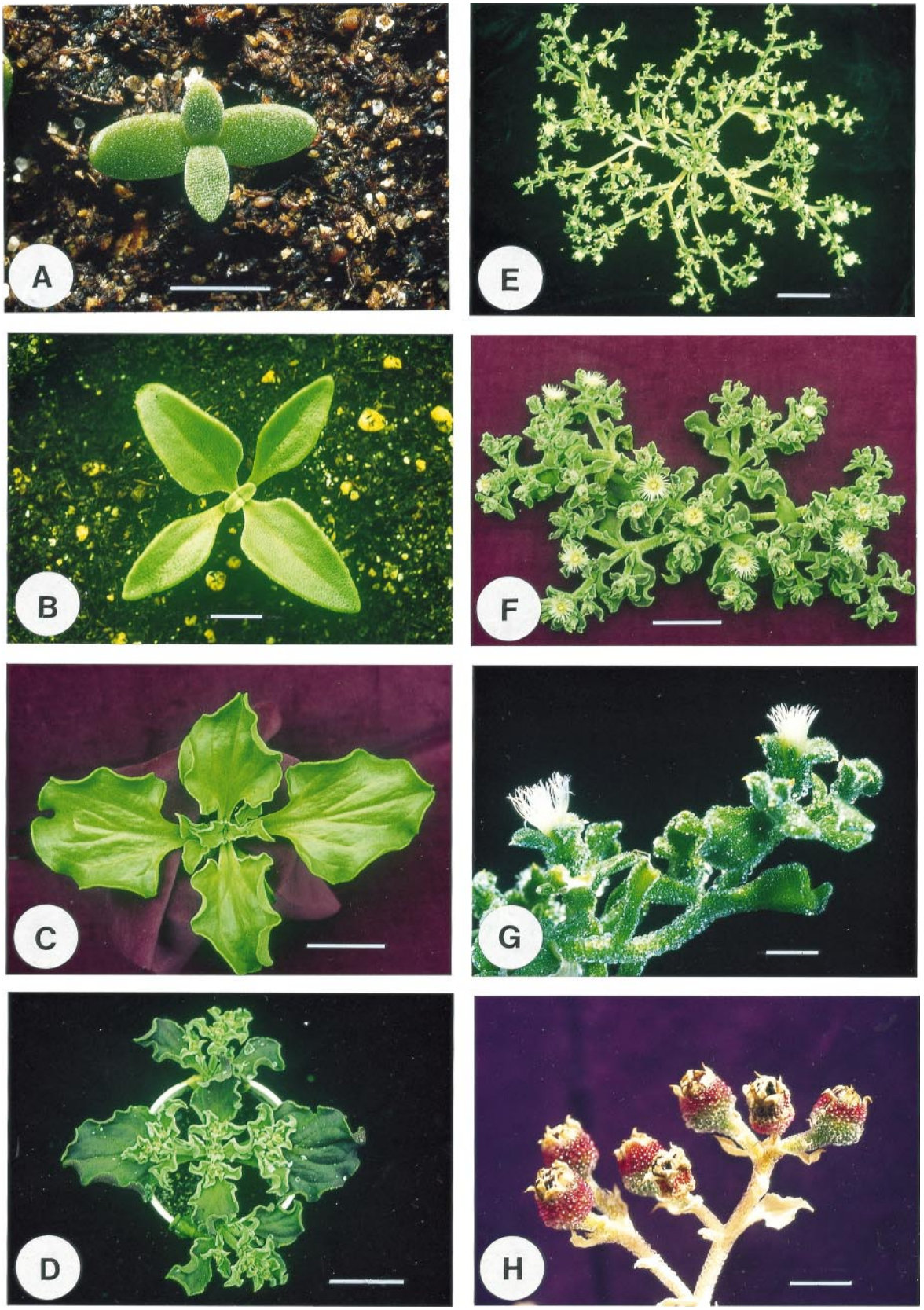

Figure 1. Growth habitus of *Mesembryanthemum crystallinum*. (*a*) Seedling with one primary leaf pair. Bar, 5 mm. (*b*) Juvenile plant (3-wk old). Leaves of first pair are lanceolate. Three pairs of primary leaves (PL) are visible. Bar, 1 cm. (*c*) Transition from juvenile to adult growth. All PL are developed and the first side shoots emerge. Plant is 6-wk old. Bar, 5 cm. (*d*) Adult plant (8-wk old) stressed for two weeks. PL have reached their full fize; further growth is observed with side shoots. Bar, 5 cm. (*e*) Adult stressed plant (12-wk old). Stress was started at age 7 wk. Bar, 5 cm. (*f*) Size depends on the time of start of stress and on ion composition of the medium. The plant was grown in Hoagland's medium containing 4 mM potassium (as nitrate). Plants were stressed at age 5 wk. Plant age is 12 wk. Bar, 5 cm. (*g*) Flowering branch of a 12-wk-old plant. Bar, 1 cm. (*h*) Branch with seed capsules. Only the seed capsules are viable. The epidermal bladder cells (EBC) are pigmented by betalaines. Bar, 1 cm.

1 *Growth of the juvenile form*

Seeds are germinated in vermiculite soaked in half-strength Hoagland's nutrient solution with nitrogen supplied as nitrate only (Hoagland & Arnon, 1938), and seedlings (*c*. 10-d old) are transferred to pots (32-ounce Styrofoam®) containing soil mixture (equal parts by volume of quartz sand, vermiculite and potting compost). Plants are kept well watered in growth chambers (ConViron, ER7 and ER14) or in a growth room illuminated at 300–500 μmol m^{-2} s^{-1}. Light is primarily provided by fluorescent lamps (Sylvania; F96T12/CW/VHO) augmented by incandescent light from 20-W bulbs (four per ER7 chamber). High incandescent light levels lead to betalain production and acceleration of development. Standard conditions are 12 h light/dark at 23/17 °C without humidity control. Plants are watered every second day when less than 4-wk old, or daily as they reach maturity. Initially, the plants receive distilled water (up to week 3) which is gradually replaced by half-strength Hoagland's solution.

For hydroponically grown plants, seedlings are transferred to 4·5-l pots containing half-strength Hoagland's solution. The plants are kept without aeration for 1 wk until vigorous root growth is observed. Later, Hoagland's solution (modified as necessary for experiments) with aeration (25 l h^{-1}) is used. Solutions are exchanged once weekly, the level kept constant by daily additions of water during the week.

2. *Mature growth form*

In order to induce the typical pattern of development, salt stress is initiated after 5 wk of juvenile growth. For plants grown in soil, salt stress is started by watering with half-strength Hoagland's solution plus 500 mM NaCl. Excess solution is left in the trays. Plants are watered three times weekly with the saline nutrient solutions. Once flowering has begun, the plants are no longer watered. Salt stress in hydroponic culture is achieved by replacing the solution with quarter-strength Hoagland's solution plus 400 mM NaCl. Drought conditions are generated by withholding water (compost-grown plants) or by the addition of 15–30 % PEG (mol. wt 3000) in hydroponic nutrient solutions. For treatment with plant growth regulators, ABA and cytokinins (zeatin, 6-benzylaminopurine, 2i) are dissolved in DMSO and added to the hydroponic nutrient solution, with control, NaCl, or plant-growth regulator solutions replaced every second day. For low-temperature conditions, the growth chamber temperature is maintained at 5 °C with conditions otherwise unaltered. Development can be accelerated by modifications in light quality (increasing incandescent lighting), higher light intensity (up to 800 μmol m^{-2} s^{-1}), or increasing the temperature (e.g. to 28 °C) (Thomas, McElwain & Bohnert, 1992*b*).

III. GROWTH AND DEVELOPMENT

The developmental sequence is illustrated in Figure 1, and follows the typical pattern of growth and development for plants maintained under the standard conditions described below.

1. *Stage 1 : Germination of seedlings*

The rate of seed germination depends on the position that individual seeds hold within the capsule, with dormancy lasting from 1 d to more than 30 d. The first seeds to set are larger and will germinate immediately (T. Fukuhara & H. J. Bohnert, unpublished). Staggered germination of seeds, depending on the season and on position in the seed capsule, had previously been reported for a related species, *M. nodiflorum* (Gutterman, 1980). In addition to the standard conditions, seeds will also germinate slowly in 250 mM NaCl. The cotyledons are inconspicuous, and *c*. 4–5 mm long (Fig. 1*a*).

2. *Stage 2 : Juvenile leaves*

Once transplanted, over the next 5–6 wk the juvenile plants grow one leaf pair (primary leaves, PL) per week (Fig. 1*b*, *c*). As long as stress is not imposed early in development, up to seven pairs of PL will be produced which show C_3 photosynthesis and cannot induce CAM (Bohnert *et al*., 1994). When primordia for the seventh leaf pair appear, growth of the primary axis terminates. The primary axis later develops a terminal flower.

3. *Stage 3 : Adult stems and leaves*

In unstressed plants, mature stems and leaves start to appear as side shoots emerge from the axes of the primary leaves (Fig. 1*d*, *e*) shortly before the termination of growth along the primary axis. Usually, no side shoots develop from the axil of juvenile pair PL1 (or they remain short). Side shoots above PL2, PL3 and PL4 develop most profusely. Side shoots PL5 and PL6 remain shorter, and frequently, above the PL6 internode only two terminal flowers develop, which constitute the PL7 side shoots. In the growth conditions described above, mature shoots have already formed in the axils of PL2, PL3 and PL4, and their development and capacity for induction of CAM is then accelerated by the salt treatment (Fig. 1*d*). The mature growth form is characterized by the growth of side shoots (SS) and secondary leaves (SL) which are morphologically distinct from the PL (Fig. 1*d–f*) and show progressive development of epidermal bladder cells (EBC; see below).

4. *Stage 4 : Flowering*

It is possible to prolong adult growth for many months, but eventually the plants start flowering (Fig. 1*e–g*) because of limiting factors not controlled in growth chambers or soils. However, under 'ideal' growth conditions, plants with 1-m-long side shoots weighing > 20 kg have been observed (Adams *et al*., 1992), whereas unstressed plants in 32-ounce soil containers can start flowering after they reach 1–2 kg after 8 m of seemingly unstressed growth. The onset of flowering is accelerated by any environmental stress, most rapidly by salt stress, and maintained by the cessation of water and nutrients in our defined growth conditions. Comparison of Fig. 1*e* and 1*f*, for example, indicates how stress later, during mature growth, produces a larger plant with fewer flowers (Fig. 1*e*), whereas stress closer to the end of juvenile growth produces a smaller plant with many flowers (Fig. 1*f*). Flowers develop in the axils of secondary leaves and represent the terminal meristems of branches (Fig. 1*g*).

5. *Stage 5 : Seedset*

The development of seed capsules (Fig. 1*h*) occurs some 6 wk after imposition of stress under our defined growth conditions, and is accompanied by a progressive decay of the roots, shoots and leaves, whereas the seed capsules remain photosynthetically viable. This period lasts for several weeks, during which it is possible to measure activity of PEPC, RUBISCO and some photosynthetic oxygen evolution in the seed capsules (data not shown), although the vegetative tissue is dead. The onset of this phase is characterized by the appearance of extremely large epidermal bladder cells (see below).

The pre-eminent characteristics of the five distinguishable developmental stages are summarized in Table 1, together with characters which are inducible at each stage of growth. We now consider in more detail morphological, physiological and molecular attributes which distinguish the juvenile and mature flowering forms, and how the specific effects of salt stress are mediated at each stage of development in terms of underlying biochemical and molecular processes.

IV. EFFECT OF SALT STRESS ON DEVELOPMENTAL PHYSIOLOGY

Many of the plant's reactions to stress can be treated similarly. Our focus has been on salinity stress, because treatment of plants in soil with 500 mM NaCl (400 mM when plants are grown hydroponically) elicits reactions faster than do drought stress or low temperature, but it must be understood that the three stress conditions are not totally identical. For example, the *Imt1* gene is not induced by drought in mature plants, but is induced strongly by low temperature and salinity (Vernon, Ostrem & Bohnert, 1993).

1. *Juvenile and adult leaves*

As indicated, a number of genes can be transcriptionally induced in juvenile leaves by salt stress, such as the induction of genes for inositol biosynthesis and the ice-plant-specific extension of this pathway which leads to the production of pinitol (Paul & Cockburn, 1989; Vernon & Bohnert, 1992*a*; Rammesmayer *et al*., 1995; Ishitani *et al*., 1996; D. E. Nelson, G. Rammesmayer & H. J. Bohnert, unpublished). Similarly, we have recently detected (C. B. Michalowski & F. Quigley, unpublished) transcripts that are induced by salinity stress in juvenile plants encoding components of the 'active methyl cycle' (which provides, among several other functions, methyl groups for pinitol biosynthesis) and transcripts for enzymes that are involved in the scavenging of ROS, reactive oxygen species (Bohnert & Jensen, 1996*b*). These enzymes, superoxide dismutases, ascorbate peroxidases, and glutathione reductase, can be reconciled with a response to salinity stress that is probably ubiquitous in plants. The closure of stomata that accompanies water stress by high salinity elicits a cascade of events leading to increases in ROS (Smirnoff, 1993).

Salt stress of suspension-cultured (non-adapted) cells leads to slow growth, although the cells remain viable, and proline concentrations increase dramatically (Thomas, DeArmond & Bohnert, 1992*a*). Such unadapted cells will, however, show no pinitol increases and, thus, these cells must be considered glycophytic; hence *M. crystallinum* is a halophyte only when organized tissues are present. This view also explains why seedlings and juvenile plants are less successful in coping with stress, and confirms that cellular tolerance and whole-plant tolerance are different. When germinating seedlings are salt-stressed, e.g. by the addition of 200 mM NaCl, development is slow, although these plants can be maintained for months. In these conditions, the bladder cells of the leaves increase in size and serve as sodium-storage organs. Removal of NaCl allows normal development to resume. In standard growth conditions, once side shoots are present, salt deposition leads to increased turgor in these shoots and to the accelerated growth of new cells which can accommodate more NaCl. Incorporation of salt provides a salt gradient and turgor gradient along the axes of the mature plant (Lüttge, 1993). Juvenile leaves and the primary axis seem to have determinate development, thus salt deposition is limited to existing structures and their associated bladder cells, but secondary meristem and side-shoot development become retarded. The shift in gene expression that precedes or accompanies the switch to indeterminate,

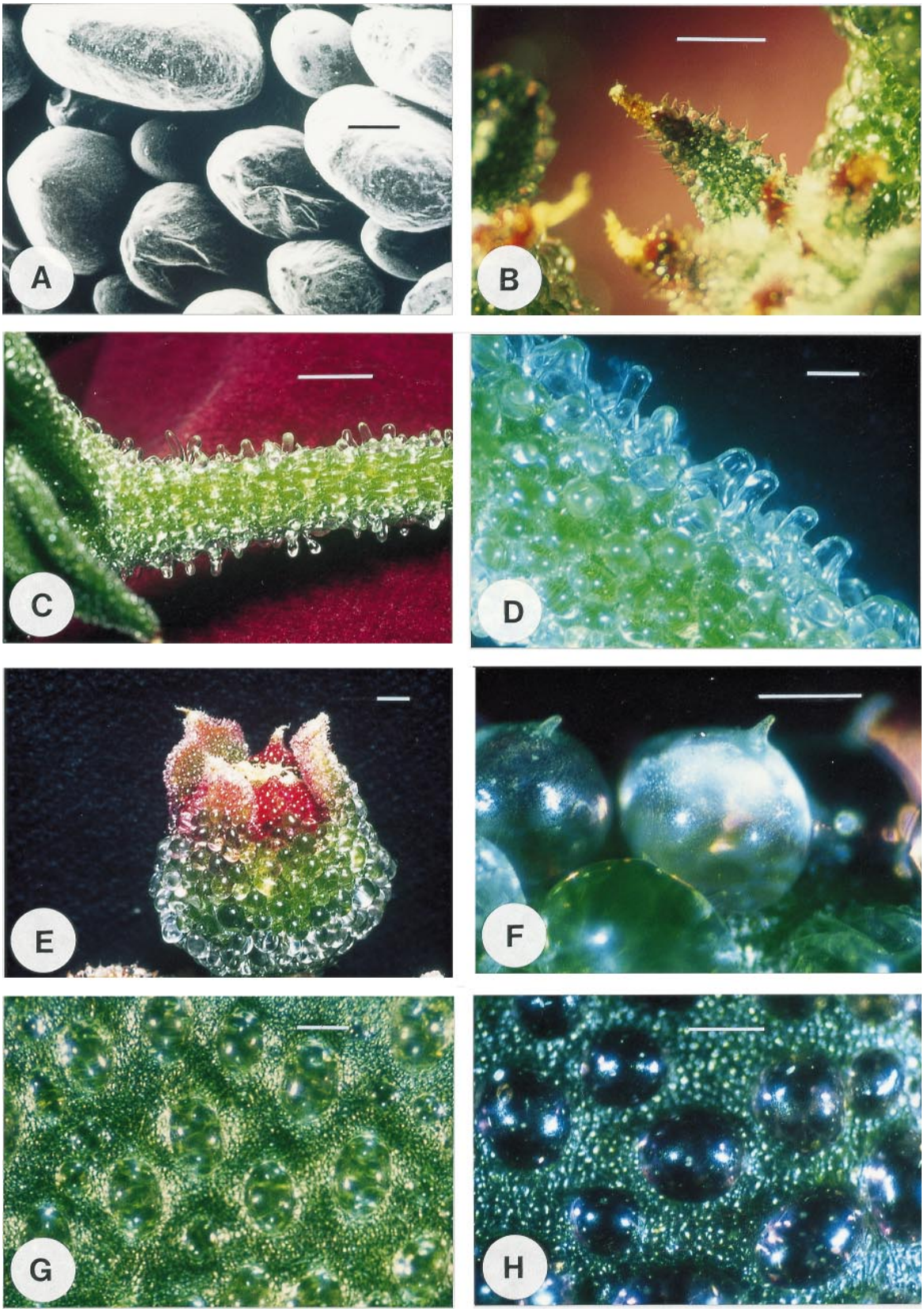

Figure 2. Epidermal bladder cells (EBC). (*a*) EBC by scanning electron microscopy of the surface of a stressed leaf. Bar, 250 μm. (*b*) EBC are modified trichomes. Bar, 1 mm. (*c*) EBC along lateral branch of a stressed plant. Bar, 5 mm. (*d*) Close-up of stem. Bar, 1 mm. (*e*) EBC in seed capsules. Bar, 1 mm. (*f*) Close-up of EBC in seed capsule. Bar, 0·5 mm. (*g*) EBC at the adaxial surface of an unstressed leaf. Bar, 1 mm. (*h*) EBC of the adaxial surface of a stressed leaf. Bar, 1 mm.

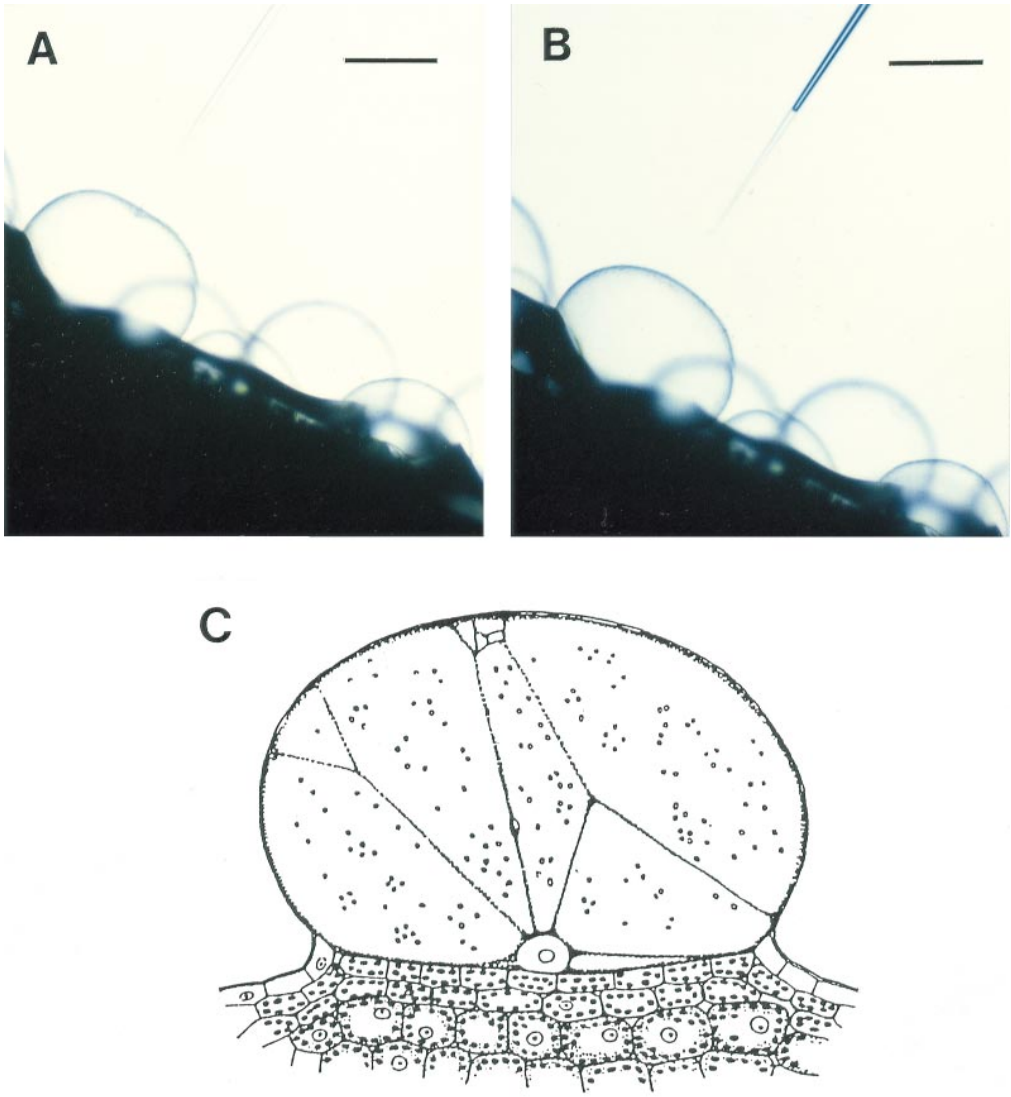

Figure 3. Removal of sap from epidermal bladder cells (EBC). Glass capillaries, with an outer diameter of *c.* 0·5 μm are used to remove sap from the EBC for the determination of ion and metabolite concentrations. (*a*), (*b*) EBC before and after removal of cell sap. The drawing of a bladder cell in panel (*c*) is reproduced from Haberlandt (1904) (see also Kramer, 1979, for ultrastructural analysis).

mature growth allows the increased sodium uptake to drive development towards flowering and seedset.

2. *Bladder cell development*

Epidermal bladder cells (EBC) are present on all tissues, except in the roots, and during all stages of development, although they remain appressed to the leaf or stem surface in young and unstressed plants. These cells comprise such a distinct characteristic of the ice plant that we consider their form and function during development in Figure 2*a–h*. The appearance on the surface of a stressed leaf, when examined by scanning electron microscopy, is shown in Figure 2*a*, although size and position of EBC is dependent on developmental growth stage (Fig. 2*b*, *c*, *e*, *f*). Stress of seedlings, juvenile (primary) leaves (PL), stems and secondary leaves leads to the extrusion of bladder cells. In general, three forms of bladder cell can be distinguished in mature tissues. The first is found along the side shoots (Fig. 2*c*, *d*), characterized as an elongated, 'sausage'-shape due to the limited surface area for development. The second form, found in seed capsules, is round, and includes the largest EBC, exceeding 20 μl of total cell volume (Fig. 2*e*, *f*). The third form, which is found at the tips of leaves and sepals, most clearly indicates that bladder cells are modified trichomes (Fig. 2*b*). This type of EBC is elongated into a hair-like structure in which the base may be enlarged, but all EBC show the tip of the trichome from which they originate at their apex (Fig. 2*f*). Epidermal bladder cells are a store for NaCl (Lüttge *et al.*, 1978; Lüttge, 1993; Adams *et al.*, 1992). The above-ground structures that are present at the beginning of salt stress are used to deposit NaCl into their associated bladder cells, as shown by comparison of the adaxial surfaces of an unstressed (Fig. 2*g*) and a stressed leaf (Fig. 2*h*).

The production of betalaines is usually associated with the stressed state, but pigment production and amount depend strongly on light intensity in addition.

V. IONIC COMPOSITION OF CELLS DURING DEVELOPMENT

The size and large volume of EBC identify them as ideal objects for study of ionic balance, given the ease with which solutes can be extracted using micro-capillaries (Fig. 3). In Table 2 we show the ionic concentration of stressed and unstressed juvenile leaves, in comparison with mature leaves and bladder cells from a stress treatment lasting 5 and 9 wk. Although juvenile leaves were able to increase the salt content and associated inorganic and organic ions after 35 d of stress, it was evident that all ions are accumulated to a lower concentration than by adult leaves stressed for the same period (Table 2). In particular, the juvenile leaves did not accumulate such high concentrations of the compatible solutes proline, ononitol and pinitol, and perhaps are therefore more subject to salt toxicity. At maturity, following the longer-term salt treatment, the bladder cells primarily accumulated inorganic ions, and hence sequestered salt away from those photosynthetic tissues still contributing to seed formation.

In general terms, proline and D-pinitol accumulate in response to stress, and are likely to act as osmoregulatory metabolites (Delauney & Verma, 1993). D-pinitol shows a gradient of accumulation from roots (concentration low), increasing towards the youngest, actively growing portions of the plant. Assuming that these metabolites are located exclusively, or nearly so, in the cytosol, the molar concentration of proline and D-pinitol combined would be high enough to compensate for molar amounts of sodium in the vacuole (P. Adams and H. J. Bohnert, unpublished). Presumably an inducible pathway (Vernon & Bohnert, 1992*a*, *b*; Ishitani *et al.*, 1996; D. E. Nelson, G. Rammesmayer & H. J. Bohnert, unpublished), in conjunction with the osmotic pressure generated by sodium in the vacuole, allows osmotic adjustment in growing tissues and leads to salt tolerance.

There must be other stress-tolerance mechanisms in the ice plant. Among those, we surmise, are mechanisms controlling or perhaps limiting sodium uptake through roots. One such mechanism has been characterized for membranes of roots and shoots represented by the induction of a V-ATPase in vacuolar membranes (Struve *et al.*, 1985; Ratajczak, Richter & Lüttge, 1994; Löw *et al.*, 1996; Tsiantis *et al.*, 1996). This protein complex generates a proton-motive force which is used to drive vacuolar sodium accumulation by secondary active transport, and is upregulated in advance of full CAM activity, while a second proton pump in the tonoplast, a pyrophosphatase, loses activity at this time (Lüttge, 1993; Löw *et al.*, 1996; Tsiantis *et al.*, 1996). However, the V-ATPase is induced specifically in response to salt rather than to an osmotically imposed water deficit (Tsiantis *et al.*, 1996) and therefore there will be other mechanisms which are important to continued water uptake during drought or, indeed, salt stress.

VI. WATER TRANSPORT WITHIN THE PLANT

Following long-term stress, the plants tend to die from the roots and basal regions, and we have already discussed the ionic concentrations which contribute to the thermodynamic driving forces for

Table 2. *Ion composition of* Mesembryanthemum crystallinum *at different stages of development and during salt stress*

	Juvenile leaves		Adult leaves		Bladder cells	
ION or metabolite*	5 d control	3 d NaCl	35 d NaCl	61 d NaCl	35 d NaCl	61 d NaCl
Sodium	46	606	687	583	1009	607
Chloride	8	463	767	628	961	701
Potassium	99	47	55	53	42	22
Inorganic cations	9	7	11	5	7	9
Inorganic anions	51	80	59	54	60	54
Organic anions	44	42	6	9	14	29
Soluble sugars	4	3	13	5	0·5	0·7
Proline	0·5	8	11	5	1	1
Pinitol	0	6	9	7	2·2	2·2
Ononitol	0	0	0·2	0·6	0·1	0
Conductivity†	0·21	1·1	1·6	1·2	1·9	1·3

* Amounts are expressed as μmol g^{-1} f. wt, or, for EBC, as concentrations (mM) in EBC sap. It is apparent that EBC are a storage for NaCl, but do not function in accumulating NaCl.

† Conductivity was measured (μS cm^{-2}) using a Radiometer (Copenhagen, Denmark) conductivity meter.

A representative experiment is shown. Four repetitions of this (and modified stress regimes) gave consistently the same results. Plants, in hydroponic culture, were 5-wk-old at the beginning of the experiment (5 d control) and then stressed by the addition of 400 mM NaCl for 3 d, 35 d and 61 d. Bladder cell sap was removed at 35 d and 61 d.

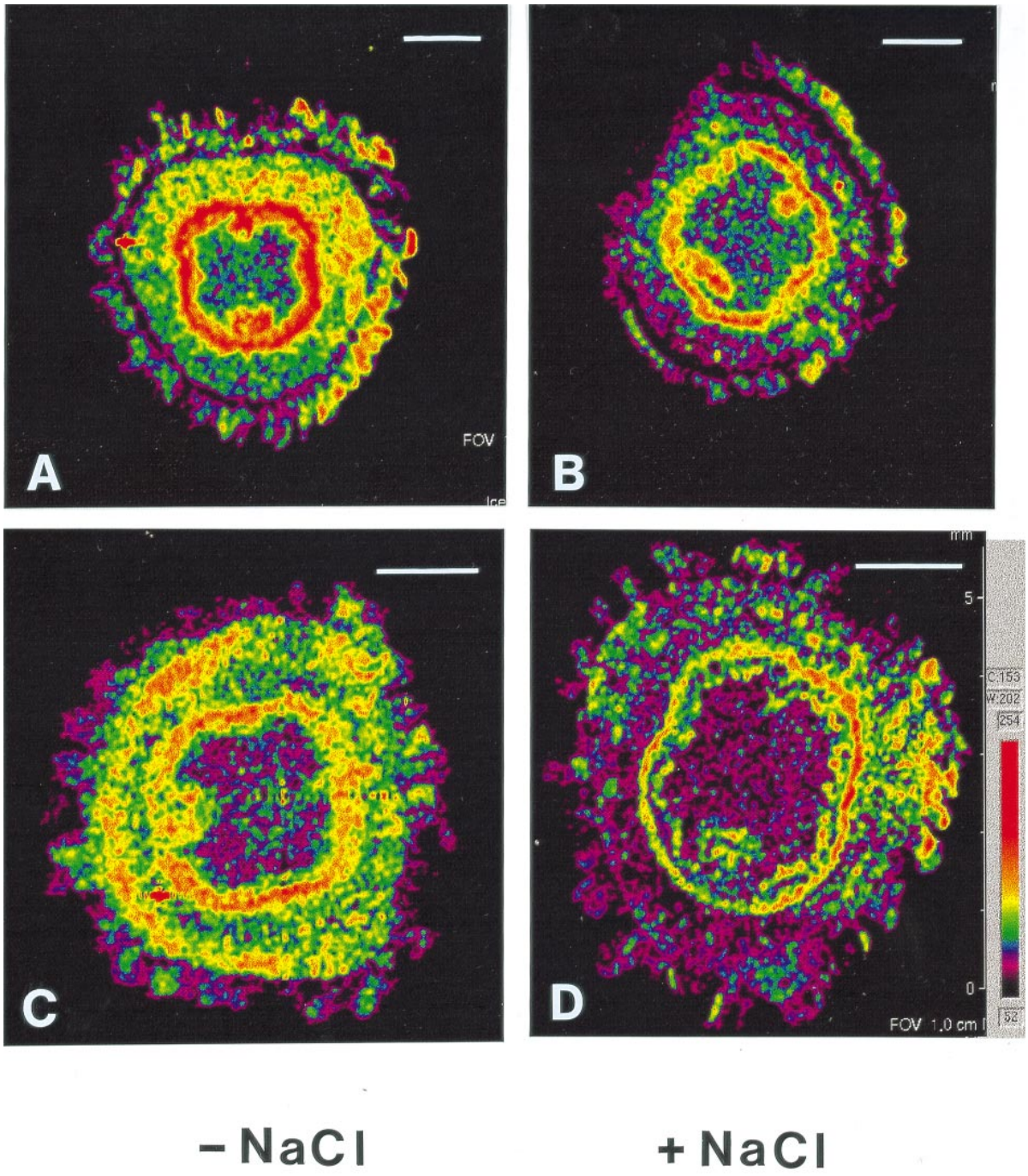

Figure 4. Proton-NMR of stem cross sections of *Mesembryanthemum crystallinum*. (*a*) Side shoot from an unstressed plant, *c*. 1 cm from the meristem. (*b*) Side shoot, similar to (*a*), from a stressed plant (after 7 d of stress by the addition of 500 mM NaCl to the soil). The plants were 8-wk old. (*c*) Side shoot from an unstressed plant, *c*. 10 cm from the meristem. (*d*) Side shoot, similar to (*c*), from a plant stressed by the addition of 500 mM NaCl in soil, as in (*b*). A colour scale, identifying proton mobility, is included in (*d*). Red and yellow represent high proton mobility, blue and black indicate areas with low proton mobility. Images were taken from freshly removed, isolated stem segments which were in contact with water in the NMR tubes.

the transfer of water to apical regions (Table 2). In order to maintain turgor in those remaining cells which are photosynthetically competent, it is important that resources be mobilized and water flow maintained through the tissue despite the stress. We have recently been able to visualize the consequences of salt stress on proton mobility (indicating lower mobility of water) by means of proton NMR-imaging, producing images from stressed and unstressed shoots (Fig. 4). For the unstressed shoot, sections at the tip (Fig. 4*a*) and base (Fig. 4*c*) show that the vascular ring is much brighter than in equivalent sections taken from stressed shoots (Fig. 4*b* and 4*d*, respectively). Indeed, in general terms, proton mobility in the entire unstressed shoot (pith, cortex and EBC) is higher than that in the stressed

state. We interpret these images as indicating the cells to which sodium is preferentially targeted.

A second approach to characterizing the importance of water transport has been the molecular characterization of water-channel proteins, members of a superfamily of genes encoding major intrinsic proteins, MIP (Chrispeels & Agre, 1994). Six water-channel genes and their corresponding proteins have been detected in the ice plant (Yamada *et al*., 1995; H. H. Kirch & J. H. Bohnert, unpublished). For two of these, after injection of their RNA into *Xenopus* oocytes, a water-channel function has been shown (Yamada *et al*., 1995), whereas the remaining MIP have not yet been analysed in the oocyte system. The transport of water is tightly regulated in the ice plant, and the role of water transport through MIP-type water channels can be resolved into a traditional thermodynamic framework for plant–water relations (Steudle, Lüttge & Zimmermann, 1975; Rygol & Zimmermann, 1990; Steudle & Henzler, 1995). However, it is now becoming clear that the members of the ice-plant water-channel protein family are expressed variously in different amounts in different cells, but most highly and predominantly in vascular tissues of all organs (H. H. Kirch & H. J. Bohnert, unpublished).

There seems to be a fundamental difference in MIP regulation that distinguishes the halophytic ice plant from glycophytes. Many glycophytic *Mip* have been detected as water-stress-induced transcripts (Yamaguchi-Shinozaki *et al*., 1992), but in the ice plant, *Mip* transcripts decline following stress. The decline is transient, however, and transcripts recover in a time course that is correlated with the synthesis and accumulation of proline and pinitol (Yamada *et al*., 1995; Ishitani *et al*., 1996). Using MIP-specific (peptide-based) antibodies, we have now shown that *Mip* transcript decline is paralleled by a decline of the corresponding proteins in some, though not all, cells in different tissues, most dramatically in cells of the root tip (H. H. Kirch & H. J. Bohnert, unpublished).

In terms of overall plant water relations, initial loss of turgor following the imposition of salt stress has been suggested as the trigger for the induction of CAM (Winter & Gademann, 1991), while measurements of cell turgor have been made with the pressure probe in EBC and mesophyll cells (Steudle *et al*., 1975; Rygol *et al*., 1986; Rygol, Zimmermann & Balling, 1989; Lüttge, 1993). The data suggest that EBC not only store salt (Lüttge *et al*., 1978) but also act as water reservoirs, accommodating changes in osmotic pressure in the mesophyll cells throughout the day-night cycle (Ruess & Eller, 1981; Rygol *et al*., 1986, 1989; Lüttge, 1993). Such changes might arise indirectly through transpiration driven by environmental conditions, or directly, as a result of malic acid accumulation and decarboxylation associated with CAM activity.

VII. THE SWITCH FROM C_3 TO CRASSULACEAN ACID METABOLISM

The switch from C_3 photosynthesis to CAM has attracted several groups to work with the ice plant. After this switch was first recognized (Winter & von Willert, 1972), the ice plant provided a model to monitor associated changes in enzyme activities (Holtum & Winter, 1982; Winter *et al*., 1982). The results have contributed significantly to understanding the physiology and biochemistry of the CAM pathway (Osmond, 1978; Ting, 1985; Winter & Smith, 1996).

The widely accepted view is that, following the inductive stimulus of salt, low temperature, or drought stress (von Willert *et al*., 1985), slight depressions in the photosynthetic rate at midday gradually intensify over successive days to result in complete stomatal closure over the middle part of the day, with peaks in net photosynthesis at the start and latter part of the photoperiod (Winter & Gademann, 1991). In this model, a transient decrease in turgor is thought to trigger the molecular mechanisms underlying CAM induction in *M. crystallinum*, since dark CO_2 uptake develops after the midday depression of photosynthesis (Winter & Gademann, 1991). Following the expression of full CAM activity, *c*. 10 d after the onset of salt stress, leaf turgor increases to a higher level than that before salt treatment. Analysis of carbon isotopic composition of organic material has been used elegantly to infer the dependence on one particular photosynthetic pathway. Although Rubisco generally shows a large discrimination against $^{13}CO_2$, such that leaf material is normally depleted in ^{13}C, the action of PEPC, using bicarbonate as inorganic C source, leads to a much lower discrimination, with organic material relatively enriched in ^{13}C (Farquhar, Ehleringer & Hubick, 1989; Griffiths, 1992). Measurements of carbon isotope composition have been used to show the shift from C_3 to CAM under field conditions (Winter, 1978; Bloom, 1979*a*, *b*; Bloom & Troughton, 1979), and, more recently, that seedset is dependent on CAM activity (Winter & Ziegler, 1992). Most importantly, the molecularly definable induction of CAM in *M. crystallinum* is distinct in that the organic material produced subsequent to the inductive stimulus is constitutively CAM, in contrast with most 'facultative' CAM plants, where the C_3-Rubisco carbon isotopic signal predominates overall (Borland & Griffiths, 1996).

CAM induction cannot be elicited in plants during their juvenile growth phase, whereas individual genes might respond to a stress stimulus in detached leaves (Schmitt, 1990). CAM inducibility coincides with the transition to mature growth, side-shoot emergence and the development of secondary leaves. When unstressed plants enter this phase, CAM develops slowly (Winter & Gademann, 1991), very

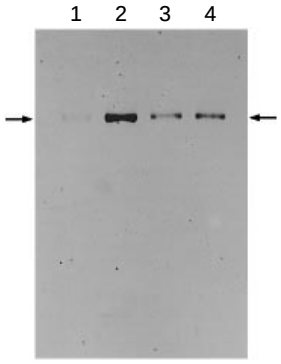

Figure 5. Induction of PEPC in *Mesembryanthemum crystallinum* plants at the juvenile to mature transition. Immunological detection of PEPC after protein blotting of total soluble protein (10 μg per lane). Lane 1, two weak bands are visible which represent the C3- (110 kDa) and CAM-(105 kDa) forms of the enzyme, respectively (arrow). Lane 2, plants stressed by the addition of 450 mM NaCl. Lane 3, plants stressed by the addition of 846 g l^{-1} PEG 3000. Lane 4, plants stressed by withholding water. The plants were of identical age and were stressed for 8 d, NaCl and PEG were added to generate an osmotic pressure equivalent to -20 bar. Salt-stressed plants regained full turgor within 2 d of the addition of NaCl, while PEG-stressed and droughted plants remained without turgor.

similar to the ontogenetic development of CAM in *Peperomia camptotricha* (Ting *et al*., 1996). Stress accelerates CAM induction. Thus, CAM must be viewed as a developmental process whose initiation, speed of transition, and magnitude is ruled by the environment. Persistent low water availability makes CAM constitutive, but the gene for the stress-inducible PEPC, *Ppc1*, maintains its connection to the stress status. Removal of sodium from the soil, for example, leads to a decline in *Ppc1* mRNA, with a half-life of < 6 h and a half-life of the PEPC protein of < 2 d (Vernon *et al*., 1988).

Figure 5 compares the induction of the CAM-specific isoform of PEPC by salt, PEG-treatment and during drought. The polyclonal antibodies against purified PEPC highlight a weak band with an apparent molecular mass of 110 kDa in unstressed plants which represents the C_3-form of the enzyme and an equally weak protein band at slightly faster migration (105 kDa) which represents the CAM-form. The latter increases by at least 50-fold after salt stress (Cushman *et al*., 1989), whereas the increase is less pronounced in response to PEG or by withholding water for 8 d. Both salt and PEG were added to produce an osmotic potential equivalent to -20 bar.

The induction of CAM detected by physiological experiments stimulated interest in the underlying molecular processes (Ostrem *et al*., 1987; Cushman *et al*., 1989). We have used the physiological and biochemical information to isolate genes (see below) for enzymes of the CAM pathway, in order to obtain tools for studying gene expression during pathway switching. It appears that several of the enzymes important for CAM are products of isogenes, presumably duplications of genes which code for anaplerotic, housekeeping functions necessary in C_3, and that these isogenes are specifically induced when the plants enter CAM (Bohnert *et al*., 1992; Cushman & Bohnert, 1996, 1997).

VIII. STRESS AND PLANT-GROWTH REGULATORS

Growth of the ice plant can be influenced experimentally to a large degree by temperature, light quantity and quality, and nutrient supply, representing a variety of conditions that constitute stress (Edwards *et al*., 1989; Schmitt, 1990; Cheng & Edwards, 1991; Lüttge, 1993). Several attempts have been made to ascribe changes in gene expression during environmental stress to alterations in abscisic acid (ABA) and cytokinins. ABA has been proposed to play a significant role in signalling changes in gene expression during water stress (Zeevaart & Creelman, 1988; Skriver & Mundy, 1990), inducing stomatal closure and senescence following translocation from the roots (Horton, 1971; Passioura, 1988; Zhang & Davies, 1989; McAinsh, Brownlee & Hetherington, 1990). Cytokinins have been suggested as anti-senescent and anti-ABA compounds which negatively affect many ABA-induced responses (Sussex, Clutter & Walbot, 1978; Blackman & Davies, 1983). In a series of studies, we have used the rise in PEPC, proline, ononitol/pinitol, and a pathogenesis-related protein, osmotin, as diagnostic tools to compare the effects of NaCl with those of the plant-growth regulators ABA and cytokinin.

Salt treatments cause a rise in endogenous levels of ABA coincident with the increase in levels of proline and PEPC mRNA, although exogenous treatment with ABA did not stimulate these responses in normal growth conditions as outlined above (Thomas *et al*., 1992*b*). Only by increasing temperature and light intensity could responses be stimulated by ABA (Chu *et al*., 1990; Thomas *et al*., 1992*b*; Thomas & Bohnert, 1993). As further evidence against ABA as the singular inducer of *M. crystallinum* gene expression under salt stress, we used an inhibitor of ABA synthesis and showed that PEPC and proline will accumulate under salt stress, while ABA levels are curtailed (Thomas *et al*., 1992*b*). However, the expression of transcripts for a subunit of the vacuolar proton pump (V-ATPase) was enhanced by ABA and did not respond to mannitol-induced drought stress (Tsiantis *et al*., 1996).

Cytokinin levels tend to decrease under salt-stress conditions (Kupier, Schuit & Kupier, 1990; Thomas *et al*., 1992*b*), yet increased exogenous cytokinin can mimic salt-induced responses, greatly increasing PEPCase, proline, ononitol/pinitol, and osmotin (Thomas *et al*., 1992*b*, Thomas & Bohnert, 1993).

Cytokinins are known to play a major role in flower initiation (Bernier *et al.*, 1993; Machackova *et al.*, 1993): thus, increased cytokinin concentration might be used by the ice plant as a signal for the developmental progression from the juvenile to the adult and flowering forms. As an hypothesis, we suggest that ABA signalling might not play a role in the plant's initial adaptation to stress, but that ABA serves as an enhancer of gene expression when the plants experience prolonged stress which propels them into the mature growth phase.

IX. MOLECULAR BIOLOGY

Isolation of nucleic acids or proteins from different tissues followed established procedures; working with the ice plant does not require special precautions. Long-term experience allows an estimation of nucleic acid amounts that can be recovered: *c.* 100–150 μg of RNA from leaf tissue ($\sim$ 50 μg g^{-1} root tissue), and *c.* 25 μg of DNA g^{-1} seedling tissue. In addition, *c.* 1·5 mg of soluble protein can be expected per g leaf tissue. Techniques used to generate cDNA libraries, subtractive cDNA libraries, and genomic libraries, isolation of transcripts and genes, their characterization by DNA sequencing, and the analysis of gene expression have been described (Ostrem *et al.*, 1987; Cushman *et al.*, 1989; Meyer, Schmitt & Bohnert, 1990; Michalowski *et al.*, 1992; Vernon & Bohnert, 1992*a*; DeRocher & Bohnert, 1993; Barkla *et al.*, 1995; Tsiantis *et al.*, 1996; Löw *et al.*, 1996; Ishitani *et al.*, 1996). Organelle, membrane, nucleic acid, and protein isolations pose no problems in unstressed plants. Following salinity stress, however, amounts of nucleases and proteases increase and the addition of nuclease and protease inhibitors becomes essential.

Responses of the ice plant in gene expression to salt stress by the induction of short-term tolerance mechanisms and developmental CAM have been discussed before (Bohnert *et al.*, 1988, 1994; Cushman, 1992; Cushman & Bohnert, 1996). Stress perception and signalling are most likely to be closely related to corresponding mechanisms in the perception of osmotic and ionic disturbances in other organisms (Posas *et al.*, 1996), but a detailed analysis in plants is missing. Signal transduction leads to changes in the transcription of many genes in the ice plant. In general, transcription is induced not only for genes of, for example, CAM enzymes or enzymes essential for osmolyte biosynthesis (Cushman *et al.*, 1989; Vernon & Bohnert, 1992*a*, *b*; D. E. Nelson, G. Rammesmayer & H. J. Bohnert, unpublished), but high or induced transcription has also been observed for genes whose transcripts and encoded proteins decline (*RbcS*; DeRocher & Bohnert, 1993). By contrast, other studies recorded declining transcription but increasing amounts of transcripts and proteins (e.g., for a RNA-binding protein; Breiteneder, Michalowski & Bohnert, 1994), indicating that transcription, transcript stability and protein turnover are affected differentially during the adaptation to water deficit. Highlighting the dependence of the transcriptional status of sets of genes on the developmental phase, transcripts of chloroplast DNA decline at the transition to adult growth without any stress, and stress itself has only a minor effect on chloroplast transcripts (C. B. Michalowski & H. J. Bohnert, unpublished). The mechanism(s) for independent regulation at the different levels of gene expression still remain to be determined. Results from many experiments have indicated that control is exerted at many steps in gene expression: transcript initiation, pre-mRNA maturation, mRNA stability, and translational preference. It is probable that several or all mechanisms contribute and together bring about co-ordinated changes in metabolism and stress tolerance.

Salt stress leads to increases in mRNA amounts, relative to, for example, actin or tubulin transcripts, which remain approximately at the same relative frequency in relation to total RNA in both stressed and unstressed plants. Induction can be observed in less than 1 h for transcripts, and soon after for enzymes that are involved in raising amounts of osmolytes, proline, inositol, ononitol, and pinitol. Judging by the few examples that have been analysed, increases in transcripts and encoded proteins typically reflect increased transcriptional activity.

The prime example of genes induced in all developmental phases at any time in response to salt stress is *Imt1*, encoding a myo-inositol *O*-methyl transferase (Vernon & Bohnert, 1992*a*, *b*), but we have detected other genes in this category (Ostrem *et al.*, 1990; see Cushman & Bohnert, 1997, for a review). In addition to genes for enzymes of the inositol biosynthetic pathway, genes encoding enzymes of the active methyl cycle, and enzymes for scavenging of reactive oxygen species, photorespiration can be considered at the basis of the plant's salt tolerance (Ishitani *et al.*, 1996; Bohnert & Jensen, 1996*a*). Activity increases for enzymes of photorespiration have been documented (Whitehouse, Rogers & Tobin, 1991). Transcripts for water-channel proteins, characterized by transient decline and recovery (Yamada *et al.*, 1995; H. H. Kirch & H. J. Bohnert, unpublished), represent a different, yet, it is most likely, equally important aspect of the plant's salinity-tolerance repertoire.

Many CAM-genes are induced by transcriptional activation during a developmental window at the transition from juvenile to adult growth. Once plants have progressed beyond this stage, these genes are permanently induced, but transcript amounts remain low unless the plants experience stress, which leads to stabilization of the transcripts and to enhanced translation (Cushman *et al.*, 1990; C. B.

Michalowski & H. J. Bohnert, unpublished). Induction of CAM in the juvenile plant is incomplete, presumably because the apparatus that leads to the transcriptional induction of CAM genes is not present, and because the juvenile state of the plant is characterized by a hormonal reaction to stress different from that of older plants (Cushman & Bohnert, 1992; Thomas *et al.*, 1992*b*; Thomas & Bohnert, 1993; Vernon *et al.*, 1993). This behaviour has been most completely studied for the *Ppc1* gene, the CAM-form of PEPC, but it is also found for other CAM-genes; *Gpd1* (glyceraldehyde 1-phosphate dehydrogenase; Ostrem *et al.*, 1990), *Mod1* (Malic enzyme; Cushman, 1993) and *Mdh1* (malate dehydrogenase; Cushman, 1992). Identical behaviour has been shown for the gene encoding PEP-carboxykinase, the enzyme that regulates activity of PEPC (Baur, Dietz & Winter, 1992). The gene encoding PPDK (pyruvate orthophosphate dikinase) shows little induction in response to stress, but increases steadily during development (Fisslthaler *et al.*, 1995). In summary, irrespective of the developmental disposition that leads to CAM (Herppich, Herppich & Von Willert, 1991), stress is an accelerator of the transition necessary to make the complete CAM-syndrome manifest and, as well, is an inducer of the transition from adult to reproductive growth.

The mechanism(s) by which the induction of transcription of CAM-genes is accomplished is not understood. Our preliminary experiments indicate a complex interaction of *cis*-acting DNA sequences of promoters with trans-acting transcription factors (Cushman & Bohnert, 1992). It appears that sequences close to the start of transcription of the *Ppc1* gene are essential for stress induction, but that overall promoter activity depends on sequences remote from the transcription start (Cushman & Bohnert, 1992; Schäffer, Forsthoefel & Cushman, 1995). Among the regulatory elements which govern transcription and induction during stress in the *Ppc1* promoter are elements that have, in other systems, been shown to respond to changes in plant-growth regulator concentrations (Cushman, Meiners & Bohnert, 1993; Thomas & Bohnert, 1993; Thomas, Smigocki & Bohnert, 1995).

x. genetics, ploidy, and mutants

In addition to the analysis of gene expression under stress and the integration of gene expression with physiological behaviour of the ice plant, we have been interested in developing the plant as a halophyte genetic model. The genome size of the ice plant is 250–300 Mbp (Meyer *et al.*, 1990; DeRocher *et al.*, 1990), which are distributed among 2n = 18 chromosomes distinguishable during mitosis (Fig. 6). Surprisingly, the ice plant contains nuclei of different ploidy levels, from 2N to 128N, which led us to label the ice plant a 'multiploid' species (DeRocher *et al.*, 1990), indicating multiple levels of endopolyploidy in nuclei from different tissues. We related this observation to the absolute genome sizes of various species: ploidy levels varied more in plants with small genomes than in plants with large genomes (DeRocher *et al.*, 1990). This hypothesis includes *Arabidopsis thaliana*, with a genome size of *c.* 120–140 Mbp, in which nuclei of higher ploidy levels have also been detected (Galbraith, Harkins & Knapp, 1991; Melaragno, Mehrotra & Coleman, 1993). The ploidy level in the ice plant is cell-specific in that, for example, guard cells and phloem companion cells are 2N (and stress does not change this ploidy level), mesophyll cells range from 4N to 16N, and EBC are 64 and 128N, respectively. Preliminary data indicate that, following stress, ploidy increases in many cells to the next higher level and, in addition, that the methylation status of nuclear DNA changes within the first 2 d after stress (E. J. DeRocher, Y. Burjanov & H. J. Bohnert, unpublished).

We have begun genetic analysis of *M. crystallinum*. The combination of a small genome size, a large foundation of physiological and biochemical knowledge, in-depth analyses of molecular events that accompany reactions to environmental stress, and distinguishable growth forms that change during development and under stress make the plant suited to serve as a genetic model. The ability of the ice plant to survive extreme environmental stresses is not matched by any other genetic model; stress conditions tolerated by the ice plant are lethal to most species and to all crop plants.

After irradiation and EMS treatment of seeds, only preliminary surveys of M2 generations have been conducted. Various mutations in pigmentation, leaf and plant shape have been observed, and some mutants with seemingly altered uptake of monovalent ions have been selected. Figure 7 shows a selection of phenotypes. Frequent were mutants with chlorophyll deficiency (Fig. 7*a*), plants in which juvenile leaves show characteristics intermediate between those of juvenile and mature leaves (Fig. 7*b*), and plants in which internode length and leaf shape of the mature plant habitus are changed (Fig. 7*c*). Most interesting is a phenotype that has been observed several times (Fig. 7*d*). These mutants are characterized by the absence of juvenile leaves, or, in less severe phenotypes, by the presence of very small deformed juvenile leaves, by very slow growth and immediate development of axillary shoots, and tiny mature leaves. Flowers are small yet perfectly shaped and the miniature plants are fertile, but their seeds germinate only after prolonged imbibition (A. Zegeer & H. J. Bohnert, unpublished). These plants, which may be viewed as heterochronic mutants in which the juvenile phase is abolished, seem to be obligate CAM plants.

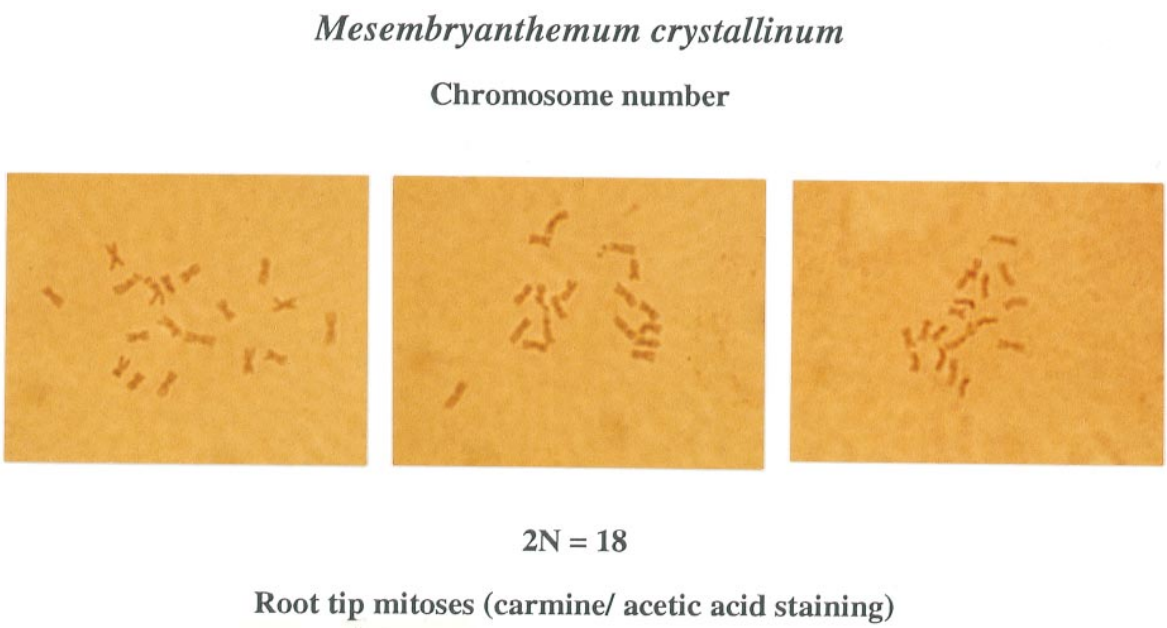

Figure 6. Chromosome number from root squashes of *Mesembryanthemum crystallinum*. The chromosome number in cells of the root tip indicates a set of 9 haploid chromosomes.

Figure 7. Mutations showing altered growth characteristics. (*a*) Chlorophyll-deficient mutant plant. Bar, 2 cm. (*b*) Plant with abnormal juvenile leaves. Bar, 2 cm. (*c*) Plant with lanceolate juvenile and adult leaves. Bar, 2 cm. (*d*) Mutant plant which does not develop juvenile leaves but immediately after germination produces axillary shoots and adult leaves. Bar, 1 cm.

XI. CONCLUSIONS AND FUTURE DIRECTIONS

In *Mesembryanthemum crystallinum*, different physiological, biochemical and molecular markers distinguish the seedlings from the vegetative juvenile and vegetative adult, the flowering, and the seed-producing phase. Each growth phase reacts distinctly differently to environmental stress. We have listed

Table 3. *Features distinguishing the juvenile and adult growth phases* of* Mesembryanthemum crystallinum

Markers of ontogeny	Juvenile growth phase	Adult and flowering growth phases
Leaf shape	Obovate with entire margins; net veined; symmetrical; nearly sessile; only slightly succulent; may be large (> 10 cm^2) declining in size from leaf pair three on	Bilaterally symmetrical; small; more succulent; bearing anthocyanic mucro at apex when young, more pronounced with age; leaves becoming smaller with age
Leaf position	Along primary axis; primary leaves always in pairs; opposite; seven pairs in total	Along secondary axes; oldest secondary leaves in pairs and opposite; becoming alternate later
Growth habit	Orthotropic; determined; primary axis stunted	Plagiotropic; indeterminate; terminal flower at primary meristem
Branching	Equal to number of primary leaves or less	Unlimited side-branch formation; terminal flowers
Betalaines	Absent. Inducible by stress and high light	Strong in flowers and young leaves along secondary and tertiary axes
Flowers	Absent	Present
Bladder cells (EBC)	Present, but empty, appressed	Present, turgid, protruding, salt storage function
Ploidy categories	2–128 N; cell-specific	Increased ploidy in many cells, but not in guard cells
DNA methylation	*CAG-methylation high	*CAG-methylation lower within 2–3 d after stress started†
Pinitol/Ononitol	Absent or low concentration	High and increasing with distance from primary axis

* The reproductive growth phase begins with the emergence of flower primordia, leading to flowering after the plants have been stressed. Different side shoots enter this phase at different times. We distinguish in addition a separate seed-maturation phase which is characterized by viable seed capsules, while the rest of the plant is already desiccated (i.e. no further water supply through roots and stems). This phase can last for several weeks before seeds are dispersed from the septicidal capsules

† DNA-methylation changes have been determined using the differently methylation-sensitive restriction endonucleases, EcoRII and BstNI which recognize the same sequence, and their corresponding methylases (Y. Buryanov and H. J. Bohnert, unpublished). Many nuclei undergo endoreduplication to the next higher ploidy level after plants are stressed.

characteristics that distinguish these growth phases (Table 1) which may be viewed as the reactions to stress of five different plants with different sensitivity to water deficit and different mechanisms to overcome this stress. In general terms, we can describe the changes most effectively by considering the characteristics of the juvenile, as compared with the adult, flowering phase (Table 3). As we have noted before, there are marked changes in leaf shape, position, and growth habit which accompany the change from the determinate juvenile growth form to the development of mature, indeterminate shoots. During this transition, the plant shows a peak in responsiveness to environmental stress, and a suite of molecular processes can now be transcriptionally induced under salinity, drought or low temperature. There are also modifications to the expression of housekeeping genes whose transcripts and proteins assume a new equilibrium (e.g., RUBISCO, FNR, PPDK, chloroplast proteins). The changes in ploidy and DNA methylation (Table 3), which have not been studied in detail, support the notion of a developmentally programmed change in gene expression forming the basis of the plant's tolerance to environmental stress. The existing (always inducible) capacity for production of compatible solutes is enhanced, mature shoots showing greater salt sequestration and development of epidermal bladder cells which store sodium (Table 2). What the ice plant accomplishes through the switch to an indeterminate growth of axillary shoots is the capacity to produce new cells and vacuoles that can serve as repositories for sodium, leading to the generation of turgor, water uptake, and the successful completion of ontogeny.

Most remarkable, however, is the way in which these molecular and biochemical processes provide the capacity for acclimation and flexibility in development that are lacking in glycophytic plants. The native conditions for growth allow germination during a short rainy season, followed by stress (drought and/or salt as water evaporates). Even timing of seed germination is programmed according to seed age and maturity (Guttermann, 1980; T. Fukuhara & H. J. Bohnert, unpublished) to provide a continual spread of germinating seeds for more than 1 m should initial rains fail. The initial amount of water can then be harnessed by up to seven pairs of juvenile leaves, and the plant will continue to proliferate via indeterminate mature branches should favourable conditions persist. Alternatively, once the plants have reached a suitable developmental phase,

the rapid onset of stress can allow a rapid progression towards flowering and seedset which is then fuelled by mobilization of reserves accrued during the juvenile growth phase. The importance of the juvenile phase can be seen in the dwarf mutant (Fig. 7), which from germination grows as if stressed, and having lost the capacity to develop juvenile leaves pays the penalty in terms of maximum size achievable, remaining small but perfectly formed. Detailed analysis of mutants should provide insights into the mechanism of salinity tolerance in the ice plant, and the integration of development and tolerance.

When reactions leading to ionic partitioning, water uptake and osmoprotection are considered, we have outlined some important mechanisms for maintaining osmotic balance and ionic homeostasis, although much work is still needed to understand the detailed interactions. To ensure the capacity for continued carbon assimilation (particularly for flowering and seedset), we think that the switch towards CAM is important, although there must be other biochemical reactions, such as the capacity to synthesize, transport and store malate, or changes in carbohydrate metabolism, which differ between C_3 and CAM plants, or the adjustment of chloroplast metabolism, for which we are only beginning to obtain molecular and biochemical markers.

In addition to these distinct processes, we must also consider the complex interplay between developmental and environmental stimuli: this brings about the adjustment in gene expression leading to the biochemical, physiological and developmental modifications outlined above. Since stress elicits maturation, flowering and seed formation, the contrasting juvenile and mature phases must be regulated by genes which need to become responsive to stress conditions. However, it is evident that various combinations of genes will become activated, and gene products being regulated in terms of transcription and turnover.

Having determined the role of individual genes, they can be transferred into suitable hosts. For instance, having transformed tobacco with the coding region for the ice plant *Imt1*, ononitol accumulation is observed, but the plants are by no means instant halophytes. As has been recently argued, increased salinity tolerance will only be successfully transferred into crop plants once we can identify the requisite suite of genes necessary for specific metabolite synthesis, storage and degradation (Bohnert & Jensen, 1996*b*). While we have not been able reliably to regenerate transformed *Mesembryanthemum* cells, we now need to identify how metabolic complexes are regulated, whether conferring salinity tolerance through maintenance of ion homeostasis, synthesis and accumulation of compatible solutes, or by the induction of CAM. Identifying common promoter and signal perception and transduction pathways for the genes that make up these biochemical pathways is one way to investigate the complex interplay between transcription and the downstream circuits regulating protein synthesis and turnover.

In the ice plant, differential gene expression during each stage of growth, together with contrasting sensitivity depending on juvenile or mature phase, lead to the acceleration of ontogeny. Thus the most important reason for the ice plant's stress tolerance might have been the evolution of a regulatory circuit for cell division and growth under stress to initiate or exploit an indeterminate CAM growth phase. Ultimately, we seek to understand the molecular basis of such a fascinating ecological mechanism, whereby the determinate, juvenile, phase is constrained but must provide a critical mass of C_3 starting-material. The mature life form, by contrast, being indeterminate, allows the plant to exploit favourable conditions for as long as they may occur, thereby optimizing flowering and seedset. Since the plant dies from the root, resources are then transferred progressively towards the last viable unit – the seed capsule.

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