The expression of a *Vp1*-like gene and seed dormancy in *Mesembryanthemum crystallinum*.

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Seeds of the common ice plant (*Mesembryanthemum crystallinum*) germinate in distinct sub-populations over a time period of more than 4 weeks following imbibition. Distinguishing early (E)- and late (L)-germinating seeds is the expression of a homologue of the transcriptional activator VP1. The deduced amino acid sequence of ice plant VP1 (MVP1) is 39% identical (50% similar) to the sequence of the *Arabidopsis* VP1 homologue, ABI3. The amount of *Mvp1* mRNA, transcribed from a single gene, is different in E and L seeds after water uptake. The levels of the *Mvp1* transcripts are very low in immature and mature seeds and they increased during 6 days of imbibition. This expression profile of *Mvp1* is different from known *Vp1*/ABI3-like genes in other plants. Cycloheximide (at 35 μ M) abolishes the increase of *Mvp1*, and L seeds are turned into E seeds, which develop normally when the inhibitor is applied for a short time during imbibition. E seeds treated for the same time period are developmentally impaired and show no radicle elongation. We suggest that the presence and late disappearance of *Mvp1* in L seeds is responsible for dormancy and after-ripening of late-germinating ice plant seeds.

INTRODUCTION

Dormancy after seed maturation is typically broken when seeds are imbibed, but imbibed seeds or embryos in a number of species remain dormant for extended time periods (Simpson 1990). Dormancy and the capacity for “after-ripening”, the gradual breaking of dormancy by environmental agents, are heritable developmental processes, which include and depend on the synthesis of RNA and specific proteins. The programs are distinct from those controlling seed maturation and germination (Bewley and Black 1994, Goldmark et al., 1992). The mechanisms for maintenance and termination of dormancy are not known in detail, but several approaches have recently brought progress, mainly through the study of genes that are active in dormant seeds. Expression studies have been used to trace the appearance and disappearance of transcripts in dormant seeds or embryos and to follow their fate during after-ripening (Dyer 1993, Goldmark et al., 1992, Johnson et al., 1995, Li and Foley 1995) and biochemical studies have been performed that traced fructose-2,6-bisphosphate in dormant seeds

(Larondelle et al., 1987). Mutants of *Arabidopsis thaliana* with altered seed dormancy have been obtained (Leon-Kloosterziel et al., 1996).

In *Mesembryanthemum crystallinum* (common ice plant) each seed capsule contains approximately 150 seeds. These seeds are not uniform: lightly colored seeds abound in the tip of the capsule followed by increasingly darker pigmented seeds towards the base. This gradient is correlated with dormancy. Seeds with light coloration, which are shed first, germinate immediately after imbibition, i.e., approximately 80% within two days, and sub-populations of pigmented seeds germinate later, with an average 50% germination within 10 to 12 days. A small population of seeds may germinate only after 30 to 40 days (Fukuhara et al., 1999). Since dormancy and uneven germination pose problems during screening of EMS-generated mutant populations, we were interested in studying the basis of the dormancy phenotype.

Dormant seeds of *M. crystallinum* are characterized by increased expression of *Mvp1*, encoding the ice plant homologue of the transcriptional activator VP1. This is different from observations in *A. thaliana*, corn and rice, where *Vp1* transcripts are associated with seed maturation and are low or not detectable in germinating seeds (Giraudat et al., 1992, Hattori et al., 1994, McCarty et al.,

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1991, Parcy et al., 1994). In contrast to the dormant seeds, *Mvp1* transcripts disappear from early-germinating seeds within a few days following imbibition but persists longer in late-germinating seeds (Fukuhara et al., 1999). Here, we characterize the ice plant *Mvp1* transcript. A similar observation has been made about factors distinguishing dormancy types in oats (Jones et al., 1997), indicating that VP1 homologues operate very similar in monocots and dicots. We suggest the existence of a regulatory program that leads to enhanced *Mvp1* transcription and maintains dormancy irrespective of water uptake by late-germinating seeds.

MATERIALS AND METHODS

Seeds and plant materials. Lightly colored early-germinating (E) and pigmented late-germinating seeds (L) were separated under a dissecting microscope. Seeds were imbibed on water-saturated paper in a growth chamber (22°C, 12 h light/ dark cycle).

Gene cloning. Total RNA was obtained from 6 day-old (from imbibition) seedlings using the acid guanidinium-thiocyanate-phenol-chloroform procedure (Chomczynski and Sacchi 1987). The RNA was converted into cDNA with SuperScript II reverse transcriptase (Gibco/BRL). cDNA was subjected to PCR (94°C for 1 min followed by 45 cycles of 94°C for 30 s, 45°C for 30 s and 72°C for 1 min). Primers used for the PCR were GCAAGVAAR-MGIATGGC and WAYCTRTASCGCATGTTCCA (I=inosine, M=A+C, R=A+G, S=C+G, V=A+C+G, W=A+T and Y=C+T) corresponding to the conserved motifs (ARKKRMA and WNMRYRF, respectively) of the VP1/ABI3 related gene products (maize (McCarty et al., 1991), *A. thaliana* (Giraudat et al., 1992), rice (Hattori et al., 1994) and *Phaseolus vulgaris* (Bobb et al., 1995)). PCR products were excised from agarose gels and cloned into the pCRII vector (InvitroGene, San Diego). The 3' region of the gene was cloned by 3' RACE using a gene-specific primer and an oligo(dT) primer (Innis et al., 1990). The 5' region of the gene was cloned by inverse-PCR using genomic DNA as a template for PCR (Innis et al., 1990), because all VP1/ABI3 genes reported so far contained no intron within their 5' regions. Inserts of RT-PCR and inverse-PCR clones were sequenced at the University of Arizona DNA sequencing facility. The nucleotide sequence of *Mvp1* has been deposited (DDBJ; AB015183).

DNA and RNA hybridizations. Total genomic DNA from 2-week-old (from imbibition) seedlings of ice plant was purified by the cetyl-trimethylammonium bromide (CTAB) method (Rogers and Bendich 1988) and digested with each of four restriction endonucleases (*Bam*HI, *Eco*RI, *Hind*III or *Xba*I). Five µg of each digested DNA were subjected to electrophoresis in a 1.0% agarose gel in

Tris-acetate buffer (pH 8.0), 1 mM EDTA and 500 ng/ml ethidium bromide. Five µg of each total RNA were fractionated in 1.2% agarose gels in 20 mM MOPS (pH 7.0), 5 mM sodium acetate, 2 mM EDTA, 0.66 M formaldehyde, and 500 ng/ml ethidium bromide. DNA fragments or RNA were transferred to a nylon membrane (Zeta-Probe, BioRad) by capillary transfer. Radiolabeled probes were prepared from the RT-PCR clones with [α -³²P]dCTP and [α -³²P]dATP by random primer labeling (Feinberg and Vogelstein 1983). Hybridizations were carried out in hybridization medium (0.25 M sodium phosphate [pH 7.2], 1 mM EDTA, 7% SDS, 1% BSA, 1% Nonidet P-40 and 0.05 mg/ml denatured salmon sperm DNA) for 16 h at 65 °C. Membranes were washed twice for 30 min each with 20 mM sodium phosphate buffer (pH 7.2) containing 5% SDS and washed twice again for 30 min each with buffer containing 1% SDS at 65°C.

Phylogenetic analysis. Amino acid sequences of Vp1/ABI3-related gene products were aligned by using the ClustalX Ver. 1.6 program (Thompson et al., 1997) and percentage of amino acid identity between the proteins were calculated by the GENEDOC Ver. 2.1 program (Nicholas and Nicholas, 1997).

Inhibitor experiments. The following metabolites and inhibitors were used: ABA (10 and 50 µM), GA3 (100 µM), 2,4-dichlorophenoxyacetic acids (2,4-D, 1 µM), cycloheximide (35 µM), W7 (a calmodulin antagonist, 50 µM), okadaic acid (250 nM), FK506 (an inhibitor of protein phosphatase 2B, 1 µM). The substances dissolved in water were present during imbibition for 12 h to 48 h in different experiments. After rinsing the seeds with water, seeds were placed on moist filter paper for continued germination.

RESULTS AND DISCUSSION

This work started from an observation indicating different classes of seeds in a single seed capsule of the common ice plant (*M. crystallinum*) (Fukuhara et al., 1999). We observed seeds that were lightly colored by betalaines and these seeds germinated early (E seeds), often within the first day following imbibition. Sub-populations show darker betalaine coloration, and these germinated later, with some seeds germinating only 30 to 40 days after they became in contact with water (L seeds). Within single seed capsules, we observed stratification, with higher numbers of lightly colored, early germinating seeds towards the tip of the capsule, while seeds at the base were increasingly more pigmented. The latter invariably germinated late (Fukuhara et al., 1999). We were interested in searching for genes that distinguished the two phenotypes. By RT-PCR, we obtained several transcripts indicative of cell divisions, cell cycle control and water

Fig. 1. Sequence comparisons of VP1-related proteins.

The comparison of amino acid sequences of *Vp1*-related genes includes: MVP1, *Mesembryanthemum crystallinum* (this work: DDBJ/EMBL/GenBank share the accession number, AB015183); AtABI3, *Arabidopsis thaliana* (Giraudat et al., 1992, X68141); OsVp1, *Oryza sativa* (Hattori et al., 1994, D16640); CpVp1, *Craterostigma plantagineum* (Chandler and Bartels 1997, AJ000552). Several regions are underlined: rich in serine and threonine (S&T); regions rich in basic amino acids (B1, B2, B3); NLS – nuclear localization signal.

uptake, such as those encoding *cdc2* and water channel proteins, and studied their expression. While late-germinating seeds took up water and expressed transcripts for water channel proteins, transcripts indicative of cell cycle progression and cell division were expressed much later than in early-germinating seeds (Fukuhara et al., 1999) (and data not shown).

A full-length transcript homologous to the transcriptional activator VP1 was isolated from the ice plant (DDBJ accession: AB015182). The deduced amino acid sequence encoded by *Mvp1* (*Mesembryanthemum Vp1*) was collinear with other VP1 homologues and most similar to that of ABI3 (Fig. 1), the homologue of maize *Vp1* (*viviparous-1*) in *A. thaliana* (Giraudat et al., 1992, McCarty et al., 1989). Identity scores (Table 1) ranged from 39% in comparison with *A. thaliana*, 34% for *Craterostigma plantagineum* (Chandler and Bartels 1997) to 27% in rice

(Hattori et al., 1994). Similarity, allowing for conserved amino acid changes between the seven VP1 sequences available, was approximately 50%. The ice plant *Vp1* encodes a protein of 790 amino acids, longer than other known VP1, due to insertions in several places between conserved domains. Overall, however, the structure of the ice plant protein is similar to other VP1 (Fig. 1), including a large amino terminal acidic domain with several sub-domains, three basic domains and domains rich in hydroxyl amino acids (Fig. 1; labeled B1, B2, B3, and S&T, respectively) (Leung and Giraudat 1998). A putative nuclear localization signal (-RKKR-), found in all VP1, is located in a highly conserved region (Fig. 1; NLS). As in other plants, a single gene encodes *Mvp1* (Fig. 2), documented by Southern hybridizations after digestions of total DNA with four restriction endonucleases.

VP1 serves as a transcriptional activator involved in

Table 1. Sizes and identity of predicted amino acid sequences between VP1/ABI3-like proteins.

	MVP1	PvALF	AtABI3	PtABI3	CpVp1	C-ABI3	ZmVp1	OsVp1	AfVp1
MVP1	790 aa	36%	39%	39%	34%	32%	28%	27%	28%
PvALF		706 aa	40%	48%	32%	32%	26%	25%	25%
AtABI3			720 aa	46%	35%	33%	28%	29%	30%
PtABI3				736 aa	35%	37%	28%	28%	28%
CpVp1					688 aa	32%	30%	29%	31%
C-ABI3						663 aa	27%	26%	27%
ZmVp1							691 aa	64%	58%
OsVp1								728 aa	57%
AfVp1									665 aa

MVP1 – *Mesembryanthemum crystallinum* (accession No. AB015183); PvALF – *Phaseolus vulgaris* (U28645); AtABI3 – *Arabidopsis thaliana* (X68141); PtABI3 – *Populus trichocarpa* (AJ003165); CpVp1 – *Craterostigma plantagineum* (AJ000552); C-ABI3 – *Daucus carota* (AB005558); ZmVp1 – *Zea mays* (AJ001635); OsVp1 – *Oryza sativa* (D16640); AfVp1 – *Avena fatua* (AJ00140)

Fig. 2. *Mesembryanthemum Mvp1* is encoded by a single-copy gene. Total genomic DNA was digested with *Bam*HI (B), *Eco*RI (E), *Hind*III (H) or *Xba*I (X), followed by size-separation through agarose gel electrophoresis. Hybridization of *Vp1* was carried out using an RT-PCR clone as the probe.

Fig. 3. RNA blot analysis of *Mesembryanthemum Mvp1* in various tissues.

Total RNA was isolated from different tissues of *M. crystallinum* and fractionated by denaturing agarose gel electrophoresis. Lane 1, seeds and seedlings imbibed with water for 6 days; Lane 2, leaves (4.5-week-old); Lane 3, leaf meristems (4.5-week-old); Lane 4, roots (4.5-week-old); Lane 5, leaves (7-week-old); Lane 6, leaf meristems (7-week-old); Lane 7, leaves (2.5-month-old); Lane 8, flower meristems and flower buds; Lane 9, flowers.

seed development and dormancy based on results from maize (McCarty et al., 1989, McCarty et al., 1991), *A. thaliana* (Giraudat et al., 1992, Parcy et al., 1994) and oats (Jones et al., 1997). VP1 serves in this function most likely in all plants, but the gene and protein has been characterized only in a few species. VP1/ABI3 is expressed throughout seed development, persists for a short time in the germinating cotyledon and hypocotyl, but the transcript is not detectable and not inducible (e.g., by ABA or desiccation) during vegetative growth of *A. thaliana* (Parcy et al., 1994). RNA blot hybridizations were car-

ried out with different tissues from the ice plant collected throughout the lifetime of the plant (Fig. 3). A signal for *Mvp1* was obtained only from imbibed seeds and seedlings, which contained germinating (E) and dormant (L) seeds. In developing seed capsules and in dry seeds, *Mvp1* amounts were even lower and signals from RNA hybridizations were barely detectable (Fig. 4A, lanes IS and MS). Because *Mvp1* signals increased for several days after imbibition (Fig. 4A), we hypothesized that early- and late-germinating seed populations might express *Mvp1* differently. A typical germination experiment included

Fig. 4. RNA blot analysis of *Mesembryanthemum Mvp1* in seeds and seedlings.

Total RNA was isolated from seeds and seedlings of *M. crystallinum*, and fractionated by denaturing agarose gel electrophoresis. (A) Lane IS, immature (about one week after flowering) seed capsule; lane MS, mature (dry) seeds; lanes 1–6 seeds and seedlings (combined L and E) imbibed with water for 1, 2, 3, and 6 days; lane 3E, seedlings of E seeds imbibed with water for 3 days. Weak cross-hybridization is observed with rRNA (top). (B) Seeds and seedlings (combined L and E) were imbibed with 35 μ M cycloheximide for 2 days, followed by water for 1 day (lane 1), 2 days (lane 2), 3 days (lane 3) or 6 days (lane 6).

Fig. 5. Germination of E and L seeds.

Seeds of *M. crystallinum* were imbibed in water and germination was measured as the time of appearance of the root tip from the seed coat. Water (left), dry seeds were directly imbibed in water; Cycloheximide (right), dry seeds were imbibed in 35 μ M cycloheximide solution for 2 days and transferred to water.

approximately 150 each of E and L seeds, which had been selected according to coloration and size (Fukuhara et al., 1999). Germination rates of E and L seeds (Fig. 5) confirmed that, indeed, E seeds had germinated to more than 50% between day one and two after imbibition. A 50% germination rate for L seeds, in different experiments, was obtained between day 6 and 10 after imbibition.

Confirming experiments performed in the past (Bewley and Black 1994, Simpson 1990), cycloheximide (CHX) incubation of populations of E and L seeds equalized dormancy of the seeds (Fig. 5). L seeds germinated at the same rate as early seeds. In the experiment shown, all seeds had germinated to 50% by day two when the seeds were pre-incubated for one to two days in 35 μ M CHX. Results with this inhibitor of protein synthesis document, first, that L seeds are not impermeable to water, and second, that maintenance of dormancy during imbibition requires protein synthesis.

RNA blot hybridizations of the germination process in L and E seeds in absence or presence of CHX revealed a correlation between germination and the amount of *Mvp1* (Fig. 4). *Vp1* transcripts could not be detected in either immature or mature, desiccated seeds (Fig. 4A, lanes IS and MS). In combined E+L seeds, *Mvp1* appeared during the imbibition period and peaked approximately 6 days after the seeds were placed on water (Fig. 4A, lanes 1, 2, 3 and 6). During longer periods of imbibition, the *Mvp1* signal disappeared gradually (not shown). The increase was due to L seeds alone, because in selected E seeds *Mvp1*

remained a very weak signal during germination (Fig. 4A, lane 3E). In general, we estimated the signal strength of *Mvp1* in E seeds as at least 10-times lower than in late seeds. Incubation of L seeds with CHX did not completely reduce the *Mvp1* signal, but it was, as in E seeds, much reduced compared to untreated L seeds (Fig. 4, compare lanes 6 in panels A and B). This behavior is fundamentally different from that observed for the expression of *Vp1* in other plants. The *Vp1*-related genes from maize (McCarty et al., 1991), *A. thaliana* (Parcy et al., 1994), rice (Hattori et al., 1994) and *P. vulgaris* (Bobb et al., 1995) are expressed predominantly in developing and mature seeds. In these species, *Vp1* is absent or very low once the seeds started to germinate. It has been reported, however, that the *Vp1*-related gene of carrot (*C-ABI3*) is expressed during somatic embryogenesis (Shiota et al., 1998) and *ABI3* gene is expressed during vegetative quiescence processes in *A. thaliana* (Rohde et al., 1999).

Among several chemicals (ABA, GA3, 2,4-D, CHX, W7, okadaic acid, FK506), which were tested for their ability to influence dormancy of either E or L seeds, only CHX was effective in breaking dormancy (Fig. 5). ABA did slow germination of E seeds. Neither GA3 nor 2,4-D had any effects on L or E seeds and inhibitors of phosphorylation chains involved in signal transduction were equally ineffective in breaking dormancy, although various effects on the germination process could be recorded. Okadaic acid, for example, inhibited the formation of root hairs, and the calmodulin antagonist W7 resulted in a shortening of the hypocotyl and primary root (data not included).

Apart from its effect on *Mvp1* expression in L seeds, CHX also influenced early germinating seedlings (Fig. 6). A variable percentage, which depended on the duration of the incubation, of early-germinating seedlings treated with CHX did not develop an elongated radicle. In late-germinating seedlings, this was never observed after a one to two day treatment with CHX (Fig. 6). The most parsimonious explanation is that the E seeds' inability to form a radicle is due to the interruption of protein synthesis by CHX, because E seeds are arrested in a developmental program that follows breaking of dormancy. In contrast, the short treatment of L seeds with CHX breaks dormancy, but does not affect the subsequent developmental programs. Consequently, L seeds develop normal roots. If it is the function of *MVP1* to maintain L seeds dormant, it is a reasonable assumption that a CHX-dependent reduction of *Mvp1* gene expression would break dormancy. The results confirm observations by others that had indicated maintenance of dormancy as an active process, requiring transcription and protein synthesis (Bewley and Black 1994, Goldmark et al., 1992, Johnson et al., 1995).

Fig. 6. Effect of cycloheximide to seedling morphology. E (right) and L (left) seeds were imbibed with 35 μ M cycloheximide (2 days) and then transferred to water (3 days).

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