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Molecular analysis of desiccation tolerance in barley embryos and in the resurrection plant *Craterostigma plantagineum*

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Summary – Two experimental systems, developing barley embryos and the desiccation-tolerant plant *Craterostigma plantagineum,* have been used to isolate cDNA clones specifically expressed in dehydrated tissues. Sequence analysis revealed that most of the isolated cDNA clones show homologies to previously reported genes. Most of the desiccation-induced genes in *Craterostigma* encode polypeptides with substantial homologies to proteins expressed during late embryogenesis in many higher plants. The expression of these genes is induced by abscisic acid treatment in leaves and in undifferentiated callus tissue. Subcellular localization of the corresponding proteins shows that most are cytoplasmic and that 3 dehydration-induced proteins are localized in the chloroplast. In developing barley embryos, some of the cDNA clones are related to genes encoding enzymes involved in sugar metabolism. One of these clones shows high homology to the animal aldose reductases involved in the synthesis of the osmolyte sorbitol. The protein encoded by this clone has been over-expressed in *Escherichia coli* and the purified protein shows aldose reductase activity.

abscisic acid / dehydration / gene expression / Lea-type proteins / water stress

Résumé – Analyse moléculaire de la tolérance à la dessiccation d'embryons d'orge et de la « plante à résurrection » *Craterostigma plantagineum.* *Deux systèmes expérimentaux, à savoir les embryons d'orge et la « plante à résurrection »* Craterostigma plantagineum, *ont été utilisés pour isoler des clones d'ADNc exprimés de manière spécifique dans les tissus déshydratés. L'analyse de séquence de ces clones révèle, dans la plupart des cas, une homologie avec des gènes déjà connus. Chez* Craterostigma plantagineum, *la plupart des gènes induits par la dessiccation codent pour des polypeptides similaires aux protéines exprimées durant l'embryogenèse tardive de plusieurs plantes supérieures. L'expression de ces gènes est induite par des traitements à l'ABA sur des feuilles ou des cals. Les protéines induites par la déshydratation sont pour la plupart localisées dans le cytoplasme mais 3 d'entre elles sont chloroplastiques. Dans les embryons d'orge en développement, certains de ces clones d'ADNc présentent une homologie avec des gènes codant pour des enzymes impliquées dans le métabolisme des glucides. Un de ces clones montre une forte homologie avec le gène de l'aldose réductase animale qui intervient dans la synthèse du sorbitol. La protéine codée par ce clone a été surexprimée chez* E coli. *Après purification, cette protéine possède une activité aldose réductase.*

ABA / déshydratation / expression des gènes / protéines de l'embryogénèse tardive / tolérance à la dessication

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INTRODUCTION

Adaptation to cellular dehydration is one of the most important characteristics that determines the distribution and yield of cultivated plants. Most plant species are able to survive mild water stress but prolonged exposure to stress usually leads to irreversible damage of the cellular structures.

In only a few cases is an organism able to withstand severe dehydration. This ability has been acquired, for example, by baker's yeast *Saccharomyces cerevisiae,* some soil nematodes and mosses like *Tortula ruralis.* In higher plants the ability to survive water loss is normally limited to embryos in developed seeds and to the small group of resurrection plants, which exhibit desiccation tolerance throughout the whole life cycle. Resurrection plants can loose more than 90% of their water, stay in anhydrobiotic state and resume active life a few hours after water becomes available again (Gaff, 1971, 1981; Bartels *et al,* 1990). This survival requires a particular cellular conditioning, which, in the case of embryos, is developmentally regulated and switched on during the embryo maturation; in resurrection plants the conditioning is induced during the dehydration process itself. These 2 systems provide an interesting possibility to study and isolate components that are involved in the development of desiccation tolerance.

We have chosen 2 experimental systems for the molecular analysis of desiccation tolerance: the resurrection plant *Craterostigma plantagineum* and the developing barley embryo. Moreover, the use of *Craterostigma* as a model system offers the advantage that the callus tissue obtained from *Craterostigma* leaves can exhibit desiccation tolerance after treatment with the phytohormone abscisic acid (ABA) (Bartels *et al,* 1991). Thus it is possible to study both undifferentiated and differentiated tissues from the same plant species.

Both in barley embryos and in *Craterostigma,* the development of desiccation tolerance is correlated with increased concentrations of ABA (Bartels *et al,* 1990) and this hormone is presumably involved in the induction of gene expression during turgor loss.

We have previously reported the isolation of a number of cDNA clones corresponding to transcripts that are induced during desiccation or ABA treatment (Piatkowski *et al,* 1990; Bartels *et al,* 1991, 1992, 1993). In this article we will summarize some regulatory features of corresponding genes in *Craterostigma* and in barley embryos. Furthermore, the structural characteristics of some of the major gene products will be discussed.

RESULTS AND DISCUSSION

The dehydration process in both the resurrection plant and barley embryos is correlated with major changes in the mRNA and protein profiles (Bartels *et al,* 1988, 1990). The major proteins present in the dehydrated state of both organisms are qualitatively, at least in part, similar.

To isolate transcripts relevant to desiccation in both systems, cDNA libraries were constructed using poly A^+ RNA from desiccated *Craterostigma* leaves and developing barley embryos as templates. These cDNA libraries were differentially screened to obtain clones corresponding to transcripts specifically expressed in dehydrated tissues.

Craterostigma plantagineum

A large number of cDNA clones corresponding to transcripts induced during dehydration were isolated. A characteristic feature of these transcripts is their rapid induction during the first hours of water loss (Bartels *et al,* 1990).

The majority of the cDNA clones could be classified into 11 families by crosshybridization. Representative clones of these groups were selected for further analysis, including sequence determination, tissue and cellular localization of the encoded proteins and the expression of the corresponding genes under stress conditions.

Sequence analysis of representative clones revealed that most of them showed significant homologies to previously published genes (table I). Most of the homologies were found to different late embryogenesis abundant (Lea) genes expressed in seeds of higher plants (Galau *et al,* 1986).

Table I. Homologies of isolated *Craterostigma* cDNA clones.

Craterostigma *cDNA clone*	*Encoded protein*	*Homology*[a]	*References*[b]
		Group 2 Lea:	
pcC6–19	dsp 16	rab 16 (rice)	Mundy and Chua, 1988
pcC27–04	dsp 14	dehydrin (barley)	Close *et al,* 1989
pcC86	–	rab 17 (maize)	Vilardell *et al,* 1980
pcC126	–	Lea D11 (cotton)	Baker *et al,* 1988
pcC76	–	radish	Goday *et al,* 1990
		Group 3 Lea:	
pcC3–06	dsp 21	CD8 (carrot) Group 3 Lea (wheat) ABA-inducible gene (barley)	Franz *et al,* 1989 Curry and Walker-Simmons, 1983 Hong *et al,* 1988
pcC163	–	Lea D29 (cotton)	Baker *et al,* 1988
pcC34–62	–	Lea D7 (cotton) pLea 76 (*Brassica*)	Baker *et al,* 1988 Harada *et al,* 1989
pcC26	–	Lea D113 (cotton)	Baker *et al,* 1988
pcC27–45	dsp 15	Lea 14 (cotton)	Galau *et al,* 1993
pcC11–24	dsp 68	LTI 65 (*Arabidopsis*) rd29 (*Arabidopsis*)	Nordin *et al,* 1993 Yamaguchi-Shinozaki and Shinozaki, 1993
pcC13–62	dsp 34	(*Arabidopsis*)	EMBL Data Library (Z17629)
pcC37–31	dsp 22	Elips (pea, barley) cbr (*Dunaliella*)	Grimm *et al,* 1989 Kolanus *et al,* 1987 Lers *et al,* 1991

[a]This list is incomplete due to the rapid accumulation of new sequences; [b]the references for the *Craterostigma* cDNA clones are: Bartels *et al,* 1990; Piatowski *et al,* 1990; and Bartels *et al,* 1993.

One characteristic of the Lea proteins is that they accumulate in mature embryos during the onset of desiccation. They can be induced in premature embryos and in the vegetative tissues of plants by the plant hormone ABA. The occurrence of Lea proteins appears to be ubiquitous among plants and seems to be responsive to a wide range of osmotic stress forms (Skriver and Mundy, 1990; Bray, 1991). Most of the proteins are highly hydrophilic and they have been grouped into several classes according to their primary structure. Group 2 Lea is characterized by a stretch of serines and a lysine-rich repeat sequence. An 11-*mer*-amino-acid sequence motif is found repeated in tandem in group 3 Lea proteins (Dure *et al,* 1989). The high conservation of domains in these proteins suggests that they are functionally significant.

At least 8 desiccation-induced transcripts from *Craterostigma* can be assigned to different groups of Lea genes (table I). For some of the predicted proteins the homology was found to be only partial and restricted to the conserved domains, whereas in the case of pcC27–45 the encoded protein (dsp 15) was practically identical to the Lea 14 (Galau *et al,* 1993). Moreover, the group 2 Lea protein rab 17 has been shown to be phosphorylated in maize embryos (Vilardell *et al,* 1990). By *in vivo* labelling and immunoprecipitation, it was shown that the homologous protein in *Craterostigma,* dsp 16, is also phosphorylated in desiccating *Craterostigma* leaves. The expression of different Lea-type genes in dehydrated vegetative tissues of *Craterostigma* suggests that the processes occurring in resurrection plants are at least in part similar to those occurring during the maturation of seeds in higher plants.

Homologies have also been found to the non-Lea-like transcripts. Clone pcC11–24 (dsp 68) has partial homology with an *Arabidopsis* cold and desiccation-inducible transcript (Nordin *et al,* 1993; Yamagucchi-Shinozaki and Shinozaki, 1993) (table I). Clone pcC13–62 (dsp 34) has recently been found to be homologous to an unknown *Arabidopsis* transcript (table I). A different class

of genes is represented by the clone pcC37–31 (dsp 22). The predicted structure of this protein contains 3 hydrophobic domains, which are presumably membrane-spanning regions. The dsp 22 is closely related to Elip-proteins (Elip = early light inducible protein), which accumulate in pea and barley during the transition from dark to light, and to a transcript associated with β-carotene biosynthesis in the salt-tolerant algae *D bardawil* (Grimm *et al,* 1989; Kolanus *et al,* 1989; Lers *et al,* 1991).

The subcellular localization was studied for desiccation-related proteins in *Craterostigma* leaves. Immunogold electron microscopy revealed that dsp 68 (pcC11–24) and all Lea-type proteins were localized in the cytoplasm, with one exception, dsp 21 (pcC3–06), which was found in the stroma of chloroplasts. Two other proteins, dsp 22 and dsp 34, were found in chloroplasts associated with thylakoid membranes (Schneider *et al,* 1993). The chloroplastic association together with the homologies found between dsp 22 and Elips suggest a role of this class of desiccation-related proteins in the protection of the photosynthetic membranes.

The cytoplasmic proteins were present in all plant tissues tested (leaves, roots and seeds), whereas the chloroplastic proteins were found only in leaves, with the exception of dsp 21, which was also present in seeds (Bartels *et al,* 1992; Schneider *et al,* 1993).

The expression of the described desiccation-induced genes in *Craterostigma* can also be triggered by ABA, both in leaves and in callus (table II). In callus exogenous ABA treatment is necessary for the induction of these genes and is correlated with the tolerant phenotype. This indicates that ABA is involved in mediating the expression of genes during dehydration.

Both callus and leaves showed an increase in the level of endogenous ABA during desiccation. However, the ABA concentrations in callus never reached the levels found in leaves (Bartels *et al,* 1990), which could be an explanation why drying alone was not sufficient to induce gene expression in callus. Both ABA treatment and dehydration rapidly induced the expression of the water-stress proteins in leaves. However, no protein was found in callus in the cases of dsp 22 and dsp 21 (table II), probably because some chloroplastic components absent in callus are required for translation or stabilization of these proteins. Moreover, dsp 22 protein represents a particular case in which the induction with ABA does not take place in the absence of light (Bartels *et al,* 1992). This result suggests that ABA is important for the induction of the desiccation-related genes, but other environmental factors also modulate their expression.

To analyze ABA inducibility of the dehydration-related genes in *Craterostigma,* promoter analysis was performed with the genomic clone corresponding to pcC27–45. A fragment 5-upstream of the transcription start point (–197 to –542) was shown to contain elements necessary for the ABA inducibility in transient expression assays using the GUS reporter system (Michel *et al,* 1993). Interestingly, an ABA-inducible DNA binding activity located in this region has been found (Nelson *et al,* 1994).

Barley embryos

Barley embryos acquire desiccation tolerance, which can be described as the ability to germinate after a severe dehydration treatment. This property is developmentally regu-

Table II. Expression of desiccation-induced transcripts from *Craterostigma*[a]

cDNA clone	*Untreated leaves*	*Dried leaves*	*ABA-treated leaves*	*Untreated callus*	*Dried callus*	*ABA-treated callus*	*ABA-treated dried callus*
pcC6–19	+	+++	+++	+	+	+++	+++
pcC27–45	–	+++	+++	–	–	++	+++
pcC11–24	–	+++	+++	–	–	++	+++
pcC3–06	–	+++	+++	–	–	+	++
pcC13–62	–	+++	+++	–	–	++	++
pcC37–31	–	+++	++	–	–	–	+

[a]The amount of transcript correlates with the amount of protein in all cases, except for the chloroplastic proteins (pcC3–06, 13–62 and 37–81) in callus, in which the proteins were not detected or a very weak signal was found.

lated and under our conditions embryos have acquired desiccation tolerance 18 d after pollination (dap) (Bartels *et al,* 1988). Several cDNA clones were isolated, which encode mRNAs expressed in 18 dap embryos, but not in younger ones (12 dap).

One of the clones isolated, pG30–44, belongs to the class of Lea transcripts (Velasco and Bartels, unpublished data); the DNA sequence of this cDNA is identical to the published sequences from wheat and barley (Litts *et al,* 1987; Espelund *et al,* 1992). A second clone (pG10–02) shows homology to glucose and ribitol dehydrogenase described in bacteria (Loviny *et al,* 1985; Heilmann *et al,* 1988; Alexander, 1992). Glucose dehydrogenase activity, which correlates with the expression of this protein, has been found in crude extracts of 18 dap embryos.

The clone pG22–69, which is related to the aldo-keto reductase superfamily, has been analyzed in more detail. In animal tissues, the expression of aldose reductases is induced by increasing external osmotic pressure (Bedford *et al,* 1987; Burg, 1988). The enzyme is responsible for the production of sorbitol, a potential osmolyte, during such a stress conditions.

In 18 dap embryos both aldose reductase activity and the pG22–69 product were detected. In order to analyze the enzymatic properties of this protein, the full length cDNA clone was expressed in *Escherichia coli* and purified using metal ion affinity chromatography. The recombinant enzyme showed aldose reductase activity. To further characterize the barley enzyme, site-directed mutagenesis on the cDNA was used to substitute a lysine with a methionine residue. The lysine residue is part of the highly conserved tetrapeptide Ile-Pro-Lys-Ser and has been speculated to be essential for catalytic activity (Bohren *et al,* 1989).

The wild-type enzyme can reduce D,L-glyceraldehyde more efficiently than the mutant one. However, the observed K_m values are higher when compared with animal aldose reductases (Roncarati and Bartels, manuscript in preparation).

To characterize the regulation of this gene, promoter studies were carried out using the GUS-reporter system. The experiments showed that a genomic upstream fragment, spanning position −113 to +76 is sufficient to direct GUS expression in seeds of tobacco transgenic plants (Roncarati and Bartels, manuscript in preparation). The GUS product appears 18–20 dap and increases during seed maturation, thus following the same pattern of expression as the gene in barley embryos. Furthermore, this proximal promoter fragment is about 10-fold more active than the longest (1.4 kb) upstream region tested.

CONCLUSIONS

The analysis of gene expression during dehydration in 2 desiccation-tolerant systems (resurrection plants and developing embryos of higher plants) indicates that the pathways leading to water-stress tolerance are very similar in both systems. Moreover, many of the gene products accumulating during dehydration are homologous in seeds and resurrection plants. This suggests that the genetic information needed for the development of tolerance to cellular dehydration is present in several higher plants. The restriction of the tolerance to the seeds in the majority of plants probably reflects the complexity of vegetative tissues compared with resting embryos. Additional components (*eg,* osmolytes and channel proteins) are probably needed in vegetative tissues to both obtain the tolerance and resume cellular functions upon rehydration.

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