

A role for expansins in dehydration and rehydration of the resurrection plant *Craterostigma plantagineum*

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Received 25 November 2003; revised 2 January 2004; accepted 2 January 2004

First published online 20 January 2004

Edited by Ulf-Ingo Flügge

Abstract *Craterostigma plantagineum* is one of the few higher plants capable of surviving desiccation throughout its vegetative tissues. Water loss results in cell shrinkage and a commensurate folding of the cell wall indicating an unusual degree of wall flexibility. We show that wall extensibility undergoes a marked increase during dehydration and rehydration. Similar increases were observed in the activity of expansins in cell walls during these processes suggesting a role for these proteins in increasing wall flexibility. Three α -expansin cDNAs were cloned from dehydrating leaves and transcript levels for one correlated closely with the observed changes in expansin activity during the dehydration and rehydration of leaves.

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Key words: Plant cell wall; Expansin; Drought stress; Resurrection plant

1. Introduction

Plant growth is dependent on water availability and drought stress severely affects crop yields. When confronted with limited water supply, most vascular plants adopt measures to limit water loss and thus avoid, or at least delay, metabolic and cellular damage caused by low water potentials. Such steps include closing of stomata and inhibition of growth in response to abscisic acid (ABA), which has been shown to increase before water limitation becomes potentially damaging [1]. A small number of vascular plants (known as resurrection plants) have evolved an alternative strategy to deal with drought stress. Instead of avoiding water loss they display an extreme level of tolerance to desiccation throughout their vegetative tissues and can rehydrate their tissues and regain metabolic activity within hours when water becomes available [2–4].

The resurrection plant *Craterostigma plantagineum* has served as a model species for molecular investigations of desiccation tolerance [4,5]. During drying, many of the protective mechanisms employed by resurrection plants such as the accumulation of sugars and protective proteins such as late embryogenesis-abundant (LEA) proteins, are also adopted by

non-resurrection plants under drought stress [6,7]. However, resurrection plants also exhibit characteristics not observed in drought-stressed non-resurrection plants. One such feature, seen in *C. plantagineum*, involves extensive cell wall folding during dehydration [3,8], which allows the integrity of plasma membrane to cell wall connections to be maintained as cells shrink due to water loss.

Drought-induced inhibition of leaf growth in non-resurrection plants generally results from decreases in cell wall extensibility [9,10]. In contrast, the ability of some resurrection plants to fold their walls during dehydration indicates an uncommon degree of wall flexibility and is a unique characteristic vital to their survival [8,11].

Cell wall extensibility in plants is determined both by the underlying structure of the wall and the activity of wall-modifying proteins such as expansins [12]. The cell wall loosening properties of expansins are important elements of many developmental and physiological processes in plants. The mechanism of expansin action is thought to involve the disruption of non-covalent bonds between wall polysaccharides [13–15]. Expansins are encoded by large gene families with four sequence-related subgroups now described [16,17]. Most studies have focused on the α -expansins, as they were the first to be characterized and have well-demonstrated wall loosening properties. The role of α -expansins in cell growth through the mediation of pH-dependent cell wall extension is now firmly established and a number of studies have also verified roles in growth [18–20]. In addition, expansins have been shown to be involved in morphogenesis [21,22], germination [23] and fruit softening [24,25].

Expansin expression is also differentially regulated in terms of developmental, hormonal, and environmental factors [26]. For example, in both deep water rice [27] and the flooding-tolerant *Rumex palustris* [28], submergence results in an increase in expansin expression and this correlates with the observed increases in growth. Similarly, maize roots adapted to low water potentials are able to continue growing by increasing the extensibility of their cell walls [29] and this is correlated with an increase in expansin activity and transcript accumulation [30].

Here we report that cell wall extensibility increases markedly in the leaves of *C. plantagineum* during drying and that expansin activity also increases in a similar manner. We further describe the isolation of three expansin cDNAs from *C. plantagineum* leaves and demonstrate that transcript abundance for one of these genes correlates closely with the dehydration and rehydration events that enable desiccation tolerance.

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2. Materials and methods

2.1. Plant material

Detached leaves of 10-week-old *C. plantagineum* plants grown in growth rooms set at 20°C with 16 h day length, were left to dry, under ambient laboratory lighting at 20°C for 0, 3, 8, 24 and 48 h and then rehydrated for a further 3, 5 and 24 h and then stored at –80°C.

2.2. Microscopy

5 mm² sections were cut from freshly harvested, 48 h dehydrated, and 24 h rehydrated leaf tissues, vacuum infiltrated with 4% paraformaldehyde in 0.1 M phosphate buffer and incubated overnight in fixative. Sections were then washed in 0.1 M phosphate buffer and dehydrated through a graded ethanol series before being embedded in LR white resin (Agar Scientific). Semithin 0.5 µm sections were cut with a Leica Ultracut UCT and stained with 0.6% toluidine blue in 0.3% sodium carbonate.

2.3. Wall extensibility

Frozen leaves were thawed and then cut into two halves representing the tip and base of the leaf. These halves were cut longitudinally into strips roughly 2 mm wide and pressed between two tissue paper-covered slides for 5 min under a 300 g weight before being placed between the two clamps of a constant load extensometer as described [31], bathed in 50 mM sodium acetate, pH 4.5 under an imposed load of 22 g. Rates of extension were calculated between 10 and 20 min after the start of the experiment when a steady rate was attained.

2.4. Expansin activity assays

For each time point expansins were extracted from cell walls from 5 g (original fresh weight) of frozen leaf material and precipitated with ammonium sulfate following previously described methods [16]. Precipitates were resuspended in 1 ml of 50 mM sodium acetate, pH 4.5 and desalted on a 5 ml column of Sephadex G25 (Amersham Pharmacia Biotech) in the same buffer. Protein concentrations were calculated using the Coomassie plus protein assay reagent (Pierce). Protein extracts were assayed for expansin activity as described [20]. Briefly, 2 mm wide strips of cellulose/xyloglucan composite were held between the clamps of a custom made extensometer leaving 3 mm of material between the clamps. The composite was bathed in 50 mM sodium acetate, pH 4.5 and allowed to extend for 15 min under an applied force of 5 g. After 15 min the bathing solution was replaced with one containing the desalted extracted proteins. Expansin activity was calculated as the rate of extension of the composite in the 10 min period following protein addition minus the rate of extension in the 10 min period prior to protein addition.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from *Craterostigma* leaf tissue dehydrated for 3 h. RT was carried out with oligo(dT)_{12–18} primers and 200 units of superscript II RNase H[–] reverse transcriptase (Invitrogen) following manufacturer's instructions. PCR was performed with the synthesized cDNA, Taq DNA polymerase (Invitrogen) and consensus α -expansin primers as follows: 5'-GSNCAYGCNACNTTY-TAYGGNG-3' (forward primer) and 5'-YTGCCARTTYTGNCCC-CARTT-3' (reverse primer). PCR cycling parameters were as follows: 92°C for 2 min, 35 cycles of 92°C for 30 s, 50°C for 30 s, 72°C for 1 min, and finally 72°C for 5 min. PCR products were cloned into pCR2.1-TOPO vector (Invitrogen) according to the manufacturer's instructions and sequenced.

Full-length coding sequence for *Cp/Exp1* was obtained by 3' and 5' rapid amplification of cDNA ends (RACE). For 3' RACE, RT was carried out as described above using an adapter oligo(dT) 3' primer (5'-GACCACGCGTATCGATGTCGACTTTTTTTTTTTT-TTIV-3'). PCR was then performed using a gene-specific 5' primer (5'-GTCACAGCCTGTTTCCAAACAC-3'), the adapter primer and Taq DNA polymerase. A further round of amplification was carried out using a nested 5' gene-specific primer (5'-ACTAACGTGGGCG-GTGCT-3') and products cloned into pCR2.1-TOPO and sequenced.

For 5' RACE, RT was performed with a 3' gene-specific primer (5'-GCCTCCTCTTCTACGGCATGGTACC-3') and a 5' adapter oligonucleotide (5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG-3'). PCR was carried out with the same 3' gene-specific primer and the 5' adapter (5'-AAGCAGTGGTATCAACGCAGAGTGT-3'). In order to improve specificity, a further round of PCR

was performed with a nested 3' gene-specific primer (5'-CTGTAA-GAAACGGGCACAATCCCAGC-3'). Products were cloned and sequenced as before. 3' RACE was also carried out for *Cp/Exp2* and *Cp/Exp3* as described above. The gene-specific primer for *Cp/Exp2* was 5'-CGCCGAGCCCGCCTTCTTGCAG-3' with a nested primer of 5'-ACGAACGTCGGTGGGGTG-3'. For *Cp/Exp3* primers were as follows: 5'-GGCCCAACCCGCGTTTGTACAG-3' and 5'-AG-CAACGTGGGCGGGTTCG-3'.

2.6. Real-time PCR

RT reactions were carried out as described above using 2 µg of DNase-treated RNA and the resulting cDNAs purified using a Qiagen QIAquick PCR purification kit. Real-time PCR was carried out using relative quantitation based on the standard curve method with quantities expressed relative to the *tkt3* (transketolase 3) endogenous control. Primer sequences and the optimized concentrations used were as follows: *Cp/Exp1* forward (100 nM) 5'-GCTCAGTATACAGCTGG-GATTGTG-3', *Cp/Exp1* reverse (200 nM) 5'-TTGAAGTAA-GAGTGTCGTTTATTGTG-3', *Cp/Exp2* forward (300 nM) 5'-GTGCTCTCGGGAACCATAAT-3', *Cp/Exp2* reverse (300 nM) 5'-TTGTAAGTACGCTATCTGCAAGAA-3', *Cp/Exp3* forward (300 nM) 5'-CGCTGAGTACAACGCTGTTC-3', *Cp/Exp3* reverse (50 nM) 5'-GTAATTGGGAGGACAGAAATTTGTG-3', *tkt3* forward (300 nM) 5'-CATCTTGGGTAAAGAACGGAACA-3' and *tkt3* reverse (50 nM) 5'-CAAAACCGATCGTTGTGGTAATC-3'. 25 µl PCR reactions were carried out using the SYBR green PCR master mix (Applied Biosystems) in optical 96-well reaction plates (Applied Biosystems) on an ABI Prism 7000 sequence detection system. Cycling parameters were: 50°C for 2 min, 95°C for 10 min and then 40 cycles of 95°C for 15 s, 60°C for 1 min. All reactions were performed in triplicate or more.

3. Results

3.1. Leaf cell wall extensibility increases during drying

All experiments were carried out on detached leaves of *C. plantagineum* since they are capable of surviving desiccation and resuming physiological activity following rehydration and are hence accepted as representative of whole plant physiology [32]. Examples of cell wall folding are shown in Fig. 1, in which mesophyll and vascular cells in a typical hydrated leaf (Fig. 1A,B) are compared to those following 48 h dehydration (Fig. 1C,D). Water loss results in significant shrinkage of the mesophyll cells accommodated by extensive cell wall folding. Even xylem vessels are affected by dehydration showing longitudinal wall folding between the annular secondary thickenings characteristic of this cell type, leading to a concertina-like appearance (Fig. 1D). Rehydration of leaf tissue following 48 h dehydration resulted in complete recovery and a return to normal cellular morphology within the leaf (not shown).

Wall extensibility was measured in frozen-thawed sections of *C. plantagineum* leaves at different time points during drying and rehydration using a constant load extensometer. Sections were cut longitudinally from either the base of the leaf or from the tip and we found that, in general, for all treatments, the underlying extension rate of the basal sections was three to four times greater than that of the tips under our conditions. Data from base sections are presented in Fig. 2A. Extension measurements become complicated after the tissue shrinks substantially resulting in extremely rapid initial extension as the tissue rearranges. Because of this, extension rates were measured once the tissue reached a steady state (after 10 min). After 48 h drying, tissues have lost almost 85% of their initial fresh weight and are severely shrunken and difficult to handle, as such data are not presented for this time point. Fig. 2A demonstrates that underlying exten-

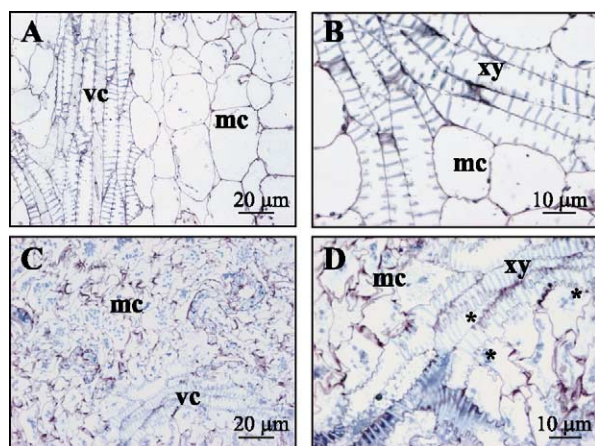


Fig. 1. 0.5 μ m toluidine blue-stained sections of resin-embedded *C. plantagineum* leaf tissue following 0 h dehydration (A, B) and 48 h dehydration (C, D). mc, mesophyll cells; vc, vascular cells; xy, xylem. Areas demonstrating extensive cell wall folding are indicated (*).

sibility increased markedly following 3 h of drying and increased further by 8 h of dehydration. Sections from 24 h dried leaves demonstrated an initial rapid extension before settling to a steady rate of extension lower than those of

freshly harvested leaves. There is also a small significant increase at 5 h of rehydration.

3.2. Expansin activity increases during both dehydration and rehydration

Expansin activity was assessed in cell wall proteins extracted from *Craterostigma* leaf material at various time points throughout dehydration and rehydration. Data, presented in Fig. 2B show that expansin activity increased steadily during the first 8 h of dehydration, reaching a maximum of some three times that of freshly harvested leaves, and subsequently decreased to levels lower than fresh leaves by 48 h without water. During this period of dehydration, the fresh weight of the leaves decreased by 13% after 3 h, 39% after 8 h, 73% after 24 h dehydration and by 48 h without water, leaves weigh only 13% of their original fresh weight. Expansin activ-

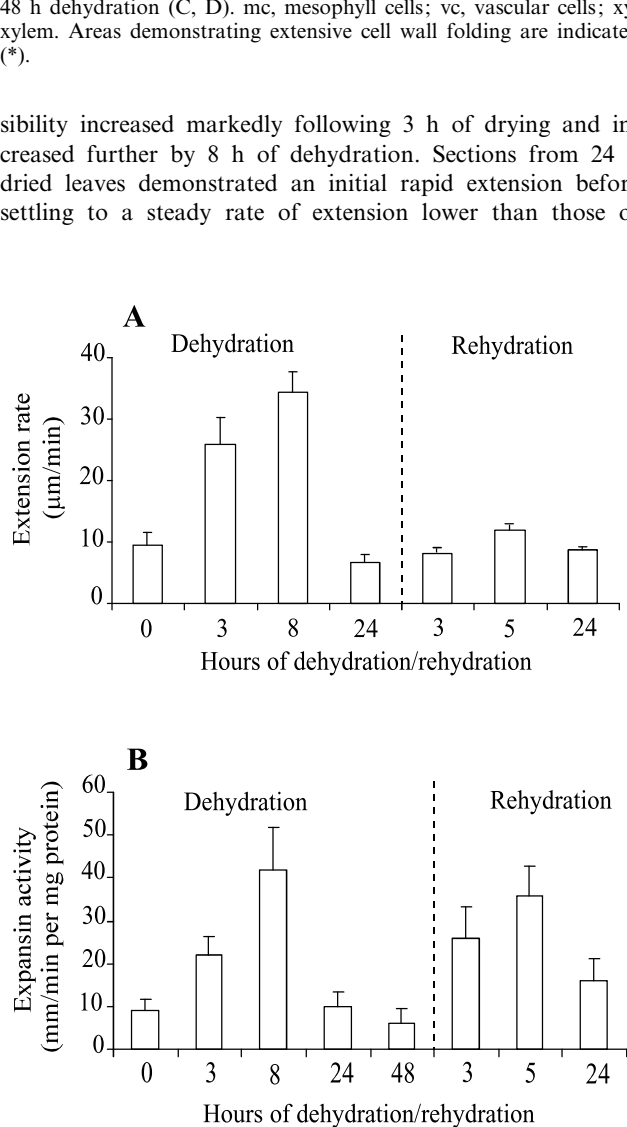


Fig. 2. A: Extension of sections taken from *C. plantagineum* leaves during dehydration and rehydration. Data are averages and standard errors from six measurements. Overall experiments were repeated twice. B: Expansin activity in dehydrating and rehydrating *C. plantagineum* leaves. Data are averages and standard errors from six separate measurements. Overall experiments were repeated three times with similar results.

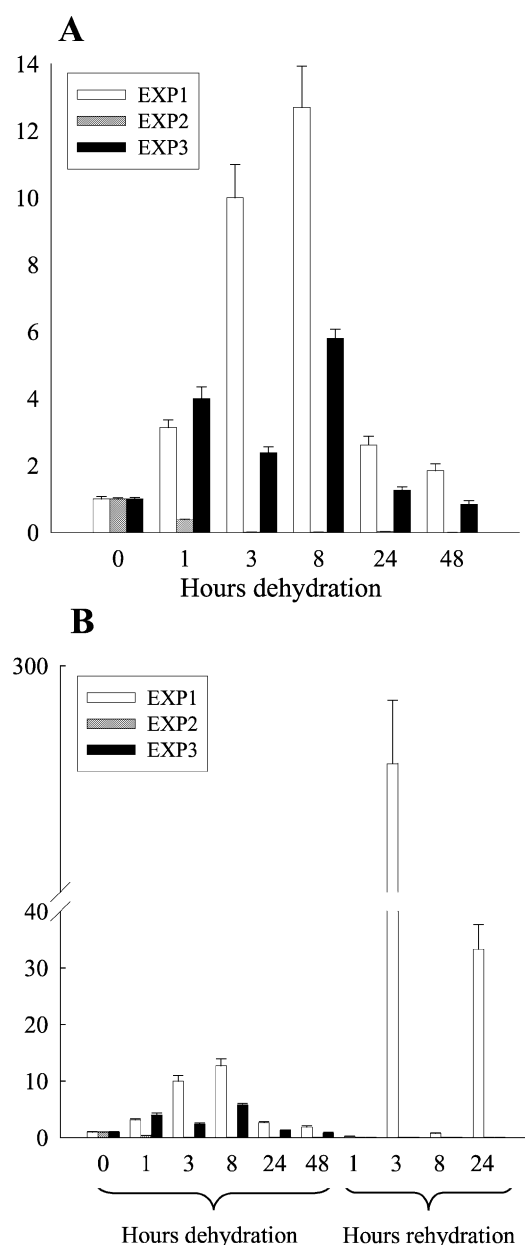


Fig. 3. Expansin transcript abundance at specific stages of dehydration and rehydration, as determined by real-time PCR.

ity increased to about four times that of fresh leaves within 3 h of rehydration (Fig. 2B) and to more than 5-fold following 5 h of rehydration. After 24 h of rehydration activity declined to levels similar to those of fresh leaves.

3.3. Transcript levels of a *C. plantagineum* expansin increase during both dehydration and rehydration

Three distinct expansin cDNAs were amplified by RT-PCR from RNA extracted from *C. plantagineum* leaves dehydrated for 3 h and named *Cp/Exp1*–3 (GenBank accession numbers AY500147, AY500145 and AY500146, respectively). Full-length cDNA sequence was obtained for *Cp/Exp1*, by 5' and 3' RACE, whereas the sequences of *Cp/Exp2* and 3 lack the 5' ends. All three encode predicted proteins with a high degree of homology to previously defined α -expansins. Transcript levels for each of the expansins identified were analyzed during dehydration and rehydration using real-time PCR. Relative quantitation was carried out by constructing standard curves for each primer pair and amounts expressed relative to the *tkt3* gene previously shown to be constitutively expressed throughout dehydration and rehydration [33]. Transcripts for *Cp/Exp1* and, to a lesser extent, *Cp/Exp3* increased relative to *tkt3* during the early stages of dehydration peaking at 8 h (Fig. 3A) and then declined. During rehydration, expression of *Cp/Exp3* remained largely unchanged whilst *Cp/Exp1* increased massively, reaching a peak of approximately 200-fold relative to freshly harvested leaves, following 3 h of rehydration (Fig. 3B). Transcript levels for *Cp/Exp1* had decreased sharply by the subsequent time point of 8 h rehydrating before once again increasing to around 30-fold of control levels after 24 h of rehydration. In contrast, *Cp/Exp2* transcript levels were largely unaffected by either dehydration or rehydration events.

4. Discussion

The ability of cell walls to undergo substantial folding allows cells and tissues of *C. plantagineum* to shrink during desiccation whilst maintaining cellular integrity. This folding is even evident in xylem elements, in which cell walls are typically reinforced to deal with negative pressures associated with water movements in plants. This folding suggests an uncommon degree of wall flexibility. A previous immunolocalization study reported changes in labeling patterns for xyloglucans and esterified pectins during dehydration and rehydration in *Craterostigma wilmsii* (a close relative of *C. plantagineum*) suggestive of changes in wall architecture [34]. Our experiments show that wall extensibility undergoes significant increases during dehydration and rehydration suggesting adaptive changes associated with wall folding during drying. This increase in extensibility is in contrast to the wall rigidification associated with growth inhibition in drought responses of non-resurrection plants [9,10].

Because of their ability to induce wall extension, we examined a potential role for expansins in these processes. Expansin activity increased during the early stages of both dehydration and rehydration in a similar pattern as seen for wall extensibility, suggesting that the activity of this protein may contribute to the observed changes. This suggestion is supported by the increase in transcript levels for *Cp/Exp1* during the early stages of dehydration and throughout rehydration indicating a specific role for this expansin in these processes.

The rise in transcript levels of *Cp/Exp1* was particularly notable during rehydration with levels increasing approximately 200-fold compared to those of fresh leaves just 3 h after the addition of water. Intriguingly, seed imbibition studies with *Arabidopsis* have shown that the transcript levels of an expansin gene increase to a greater extent than those of any other gene on an Affymetrix *Arabidopsis* 22 k microarray chip (Professor Ian Graham, personal communication). Both seed imbibition in *Arabidopsis* and recovery from dehydration in *Craterostigma* leaves, involve plant tissues moving from a desiccated and dormant state to one where they are hydrated and active. Both processes also involve considerable tissue swelling and it may be that expansin action enables cell walls to adapt under these circumstances. In this context it is reported that two expansins from tomato seeds were induced during imbibition, but not apparently on the scale reported here [23,35]. It is notable that expansin activity does not rise by an equivalently high degree during rehydration, as do the levels of transcript for *Cp/Exp1*. One possible explanation for this may be that the adverse effects on metabolism caused by drought stress critically impair translation events during recovery.

Expansins have been shown to be involved in a number of developmental processes where the action of these enzymes permits wall extension, during growth for example, or wall disassembly, such as during fruit ripening. As such, it is not difficult to envisage a role for expansins in the modification of the cell wall in *Craterostigma* during dehydration and rehydration. Not only might this involve increasing wall flexibility during drying, but the plasticizing effects of expansins through their ability to disrupt hydrogen bonds between polymers might be important in the rehydration of the cell wall.

Acknowledgements: This work was funded by CNAP, Department of Biology, University of York. We thank Dr. Peter Scott (University of Sussex) for providing us with *C. plantagineum* plants, Barbara Smith (Technology Facility, University of York) for technical guidance regarding real-time PCR experiments, and Professor Dorothea Bartels (Max-Planck-Institut für Züchtungsforschung) for advice concerning experimental design.

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