

## Expression of a heterologous expansin in transgenic tomato plants

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**Abstract.** Expansins are cell wall proteins thought to play an important role in growth and other events involving cell wall modifications. Whereas the expression patterns of many isoforms have been characterised, the nature of their activity is still poorly understood. Large amounts of active expansins are necessary to undertake biochemical studies and identify their substrates. We report here the successful expression of a recombinant expansin [*CsExp1*, isolated from cucumber (*Cucumis sativus* L.) hypocotyls] in transgenic tomato (*Lycopersicon esculentum* Mill.) plants, under the control of a constitutive promoter. In some transformants, *CsExp1* transcript and protein accumulated to high levels, and expansin activity extractable from the cell walls was increased up to about 20-fold the activity measured in wild-type plants. These results confirm the identity of the *CsExp1* coding sequence, and will enable large quantities of active expansin to be obtained for further studies.

**Key words:** Cell wall – *Cucumis* (expansin) – Expansin – Growth (expansin) – *Lycopersicon* (transgenic) – Transgenic tomato

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### Introduction

Expansins were first identified for their ability to induce the extension of isolated cell walls under tensile stress (McQueen-Mason et al. 1992). They are good candidates for “wall loosening factors” allowing stress relaxation in the cell walls during cell expansion. Besides their involvement in growth, expansins seem to play a role in other physiological events involving wall modifications (for recent reviews, see Shieh and Cosgrove

1998; McQueen-Mason and Rochange 1999). For example, it has been proposed that they could play an active role in cell wall disassembly during fruit ripening (Brummell et al. 1999).

Expansins comprise large multigene families, which might reflect a need for finely regulated expression, and/or differential biochemical properties such as substrate specificity or activity. Despite the rapid development of expansin research over the last few years, the biochemical basis of their action in cell walls is still poorly understood. It does not appear to consist of a simple hydrolytic activity (McQueen-Mason et al. 1993) and might involve an alteration of non-covalent links within, or between, wall polymer networks. To investigate the interaction of expansins with these networks, Whitney et al. (2000) have recently developed the following approach: composite materials made of cellulose and various hemicelluloses are produced in *Acetobacter xylinus* cultures, and their mechanical properties analysed following treatment with expansins. Such studies should help to identify the substrates of expansins within the cell walls, and to characterise better their activity. However, substantial amounts of active protein are needed to undertake such studies. The purification of expansins from growing plant tissues is time-consuming, and yields small quantities of protein even in favourable systems such as cucumber hypocotyls (McQueen-Mason 1995).

We describe here the successful expression of a heterologous putative expansin coding sequence in transgenic tomato plants under the control of a constitutive promoter. Our results both confirm the identity of this coding sequence and report the development of an efficient expression system for recombinant expansins.

### Materials and methods

#### *Plant transformation*

The *CsExp1* expansin coding sequence from cucumber (formerly CuEx S1, Shcherban et al. 1995) was inserted between the

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Cauliflower Mosaic Virus 35S promoter and nos 3' polyadenylation signal. This construct was introduced into pDE1001 binary vector (Denecke et al. 1992), then mobilised into *Agrobacterium tumefaciens* strain LBA 4404 pAL4404 (Hoekema et al. 1983). Transformation of tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) was performed as described by Fillati et al. (1987). Primary (T0) transformants were allowed to self-pollinate and T1 seeds collected. The presence of the transgene was detected by polymerase chain reaction in the T1 transformants analysed as described below.

#### Analysis of RNA gel blots

Total RNA was extracted from leaves following the method of Verwoerd et al. (1989). A 12- $\mu$ g sample of RNA from each plant was run on formaldehyde denaturing gels and blotted onto a nylon membrane (Magnacharge; MSI, Westborough, Mass., USA). A DNA probe corresponding to *CsExp1* was labelled with alkaline phosphatase (Alk Phos Direct, Amersham, UK). Hybridisation, stringency washes and detection were performed according to the manufacturer's instructions, using CDP-Star as a substrate for alkaline phosphatase.

#### Extraction of cell wall protein

Growing leaves were harvested from wild-type and transgenic tomato plants, frozen at  $-80^{\circ}\text{C}$ , and homogenised in a blender in cold buffer: 25 mM Hepes, 5 mM DTT, 2 mM EDTA, 3 mM sodium metabisulfite, 1% (w:v) polyvinylpyrrolidone (molecular weight 40 000) and 0.1% Triton X-100 (pH 7.0). Wall material was collected on a nylon screen (47  $\mu\text{m}$  mesh), extracted once more in homogenisation buffer, and rinsed three times with cold distilled water. Proteins were solubilised from wall fragments for 90 min on ice in 1 M NaCl, 25 mM Hepes, 5 mM DTT, and 2 mM EDTA (pH 7.0). After removal of cell wall fragments by filtration, proteins were precipitated by addition of 390  $\text{g l}^{-1}$   $(\text{NH}_4)_2\text{SO}_4$  then resuspended in 50 mM sodium acetate, (pH 4.5). Protein concentration was determined by the method of Bradford (1976) and adjusted to 100  $\mu\text{g ml}^{-1}$  for all samples.

#### Analysis of protein immunoblots

Cell wall proteins (10  $\mu\text{g}$  for each sample) prepared as described above were separated by SDS-PAGE and electro-transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell, Germany). After blocking overnight at  $4^{\circ}\text{C}$  with 5% BSA, the membrane was incubated with *CsExp1* rabbit antiserum (raised against recombinant protein expressed in insect cells) at a 1:5000 dilution, washed, reacted with a goat anti-rabbit antibody coupled with horseradish peroxidase, and re-washed. Bound antibodies were detected using luminol as a substrate (ECL, Amersham, UK).

#### Extensometer assays

A pellicle of cellulose/xyloglucan composite (Whitney et al. 1995) was cut into strips (0.2 cm  $\times$  1 cm). These were pressed under 300 g weight for 5 min, then clamped in the extensometer (distance between clamps = 5 mm). Extension under a constant load of 0.2 N was recorded using a direct-voltage displacement transducer (described by Cosgrove 1989). Extension was first recorded for 20–30 min in 50 mM sodium acetate (pH 4.5), then the buffer was replaced by 100  $\mu\text{l}$  of protein extract. Expansin activity was calculated as the difference between extension rates before and after addition of the extract.

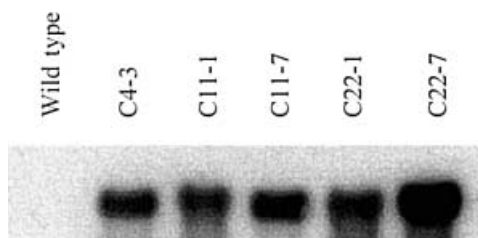
## Results and discussion

### Accumulation of *CsExp1* transcript and protein in transformants

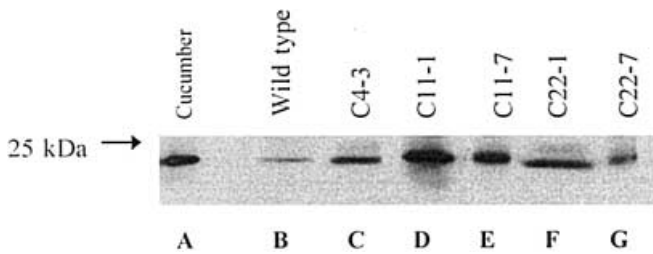
Expansins were first identified in the growing region of etiolated cucumber hypocotyls (McQueen-Mason et al. 1992), and two isoforms have been well characterised in this system. Following partial protein sequencing and screening of a cDNA library, two closely related putative expansin coding sequences (*CsExp1* and *CsExp2*) were isolated (Shcherban et al. 1995). Attempts to express recombinant expansins in bacteria (*Escherichia coli*) or yeast (*Pichia pastoris*) have so far proved unsuccessful, as the expressed proteins were inactive (data not shown). As an alternative approach, we expressed *CsExp1* in transgenic tomato plants, under the control of the CaMV 35S promoter.

Nineteen independent transgenic lines were obtained by *Agrobacterium tumefaciens* mediated transformation. Gel blot analysis was performed on RNA from mature leaves of all primary transformants (not shown), and some T1 transgenics, using a probe corresponding to *CsExp1* (Fig. 1). No signal was detected with RNA from a wild-type plant, indicating that the *CsExp1* probe does not cross-hybridise with tomato endogenous expansin transcripts. The T1 transformants analysed here show high levels of accumulation of *CsExp1* mRNA, demonstrating effective transcription of the transgene.

Protein immunoblot analysis confirmed the accumulation of *CsExp1* protein in the transformants (Fig. 2). Polyclonal antibodies raised against partially purified *CsExp1* recognised a band at the expected molecular weight in cucumber crude wall protein extracts (Fig. 2, lane A). Lanes C to G show expansin presence in crude protein extracts from cell wall material from transgenic plants, at a higher level as compared to wild-type plants (lane B). The faint band observed in the wild-type samples probably corresponds to the endogenous tomato expansins. A comparison between Fig. 1 and Fig. 2 reveals that the correlation between transcript and protein levels does not always agree, suggesting differences in translation rates, or protein stability between the different lines.



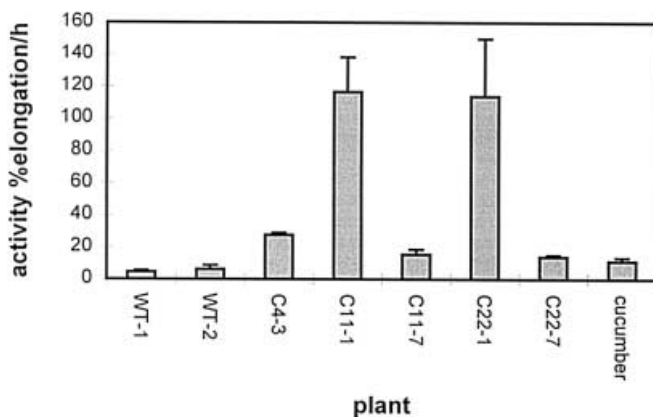
**Fig. 1.** Accumulation of *CsExp1* steady-state mRNA in transgenic tomato plants. Total RNA was extracted from leaves of primary transformants, and from a wild-type control plant. After gel separation and blotting, the RNA was hybridised with a DNA probe corresponding to the full-length *CsExp1* coding sequence



**Fig. 2.** Protein immunoblot analysis. Cell wall proteins (10 µg/sample) were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. The presence of CsExp1 protein was detected with a polyclonal antibody raised against recombinant CsExp1 expressed in insect cells. *A*, 4-day-old dark-grown cucumber hypocotyls. *B–G*, growing leaves of wild-type and T1 transformants

### Expansin activity

Expansin activity in crude cell wall protein extracts was measured in the following manner: a strip of cellulose/xyloglucan composite produced in *Acetobacter xylinus* cultures (described by Whitney et al. 1995) was stretched under a constant load and its elongation was recorded over time. After the addition of an extract containing expansins, a marked increase in elongation rate can be observed and used as a measure of expansin activity. This method was adapted from the reconstitution assay described by McQueen-Mason et al. (1992), which used cucumber hypocotyl wall specimens as a substrate. The cellulose/xyloglucan composite we used here extends rapidly in response to expansins and provides higher sensitivity and reproducibility than plant-derived substrates (Whitney et al. 2000). Preliminary experiments using cucumber hypocotyl reconstitution assays confirmed the results presented here (data not shown). Expansin activity was measured in protein extracts from mature leaves of T1 plants (Fig. 3). Extracts from mature leaves of wild-type plants showed a basal level



**Fig. 3.** Expansin activity in cell wall protein extracts. Crude cell wall protein extracts were prepared from growing leaves of transgenic and wild-type tomato plants. Expansin activity in these extracts was assayed using an extensometer, by measuring the increase in extension rate of a cellulose/xyloglucan composite after addition of the extract. The activity was calculated as the difference in extension rates (% increase in length per hour) before and after addition of the extract. Data are means and SE of six replicate measurements

of expansin activity, resulting from the expression of endogenous expansin genes. Expansin activity has previously been identified in growing tomato leaves (Keller and Cosgrove 1995). In all transgenic plants analysed, expansin specific activity was significantly higher than in the wild type, with a maximum of about 20-fold. In some lines activity was also much higher than that extracted from the apex of etiolated cucumber hypocotyls (Fig. 3). In these experiments, protein extracts are from mature leaves, each of which is several grams in weight. In contrast, only the apical 2–3 cm of an etiolated hypocotyl contains expansin activity (McQueen-Mason 1995) and very large numbers of plants need to be grown to yield worthwhile quantities of this protein.

This article may represent the first report on the successful production of active recombinant expansin. Our results show that: (i) The *CsExp1* cDNA encodes an active expansin. We previously failed to obtain expansin activity from transgenic bacteria and yeast and suggest that the success obtained in transgenic plants could be attributed to better folding and processing of the protein. In a recent report, Brummell et al. (1999) described the overexpression of an expansin isoform in tomato fruit. Although this had a profound effect on fruit softening, the authors did not measure expansin activity. (ii) The cDNA contains all the information for secretion of the protein to the cell wall, which is consistent with the finding of a putative hydrophobic signal peptide at the N-terminus of the sequence (Shcherban et al. 1995). This enables us to study the effect of increased expansin levels in vivo. (iii) Transgenic plants seem to be quite tolerant to high levels of expansin activity in their cell walls, which makes them a good expression system for recombinant expansins. In addition, they are easy to grow to obtain large amounts of material. Purification of recombinant expansins from transgenic plant material is complicated by the presence of endogenous expansins in the cell walls (Keller and Cosgrove 1995) which are difficult to separate from recombinant forms. However, it may be possible to overcome this by expressing recombinant expansins containing an epitope tag (such as an oligo-histidine tag) to aid in purification. Extracts from the transformants described in this report have already been used for studies of expansin action on composite polymer networks (Whitney et al. 2000). Site-directed mutagenesis studies can also be undertaken to address the relationship between expansin structure and function.

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