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A pectate lyase from *Zinnia elegans* is auxin inducible

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Summary

The *Zinnia* mesophyll cell system consists of isolated leaf mesophyll cells in culture that can be induced, by auxin and cytokinin, to reproducibly trans-differentiate into tracheary elements (TE) after 96 h, while in the presence of auxin alone the cells simply elongate. In a search for genes involved in modifications to cell-wall architecture before any overt signs of cell differentiation, a differential hybridization of a 72-h cDNA library with probes from mRNA at time-points of 24 h and 72 h was done revealing a number of transcripts up-regulated between these times. One of these cDNAs shows homology to pectate lyase, a pectin-degrading enzyme. The complete cDNA sequence (*ZePel*) corresponds to a translated protein of 44 kDa with an N-terminal signal peptide of about 2 kDa, and one potential N-glycosylation site. Northern analysis confirms that the strong expression of this gene during TE induction occurs at a very early stage of the process and is due solely to the presence of auxin in the induction medium. *In situ* hybridization studies in young *Zinnia* stems show that *ZePel* expression is associated with vascular bundles and shoot primordia. Recombinant protein made in *Escherichia coli* possesses calcium-dependent pectate lyase activity. Pectate lyase activity is detected in elongating and differentiating *in vitro* cell populations. The role of this enzyme in remodelling the cell wall during cell elongation and differentiation is discussed.

Introduction

The cell wall is continuously modified throughout plant cell growth and differentiation by the secretion of distinct classes of cell-wall polysaccharide and protein, post-insertional modification of polymers within the wall, and regulated hydrolysis of components, to produce functionally adapted cell-wall architectures (Carpita and Gibeaut, 1993; McCann and Roberts, 1991, 1994). We have used a model system, the *Zinnia* mesophyll cell system, to define some of the changes in cell-wall architecture that occur during (i) cell elongation, since it is the cell wall that constrains both the size and shape of the cell, and (ii) cell differentiation, since localized deposition of particular wall polymers is needed to modify the wall for specialized functions (Stacey *et al*., 1995).

When mesophyll cells from the leaves of *Zinnia elegans* cv. Envy (a member of the Compositae) are cultured in a medium containing high levels of auxin, they elongate after 5 days to many times their original length (Roberts and Haigler, 1994; Stacey *et al*., 1995). Alternatively, when cultured in a medium containing a 1:1 ratio of auxin to cytokinin, the mesophyll cells differentiate, without major cell expansion, directly into tracheary elements (TE) by 96 h (Fukuda, 1992, 1996; Fukuda and Komamine, 1980). This model system is unique in plant *in vitro* systems because of the readily inducible synchronous differentiation of up to 60% of the cell population; some other species can be induced to form TE in culture but do so less efficiently and less synchronously (Chasan, 1994). The differentiation process is irreversible after about 48 h and is characterized by a series of cytological changes. Firstly microtubules rearrange in a cortical banding pattern that reflects the position of future secondary thickenings, secondly cellulose is deposited in the incipient thickenings, thirdly lignification occurs, and finally cell death occurs (Fukuda, 1992, 1996).

We have found changes in the repertoire of both protein and polysaccharide cell-wall components, both in the cell wall and secreted into the culture medium, during both cell elongation and cell differentiation (Stacey *et al*., 1995). Many of the uncharacterized proteins in the wall are likely to be enzymes involved in the remodelling and turnover of the polysaccharide framework. In the course of a differential cDNA screen to identify genes whose expression is up-regulated between 24 and 72 h, i.e. early on in our 96-h time–course for TE differentiation, we isolated a cDNA with sequence homology to pectate lyase, a pectin-degrading enzyme.

Pectate lyases (E.C.4.2.2.2) have previously been described as microbial extracellular enzymes that assist pathogenesis by cleaving of polygalacturonate blocks in the plant host cell wall (Collmer and Keen, 1986; Davis *et al*., 1984). Enzymatic cleavage of α-1,4-linked galacturonosyl residues occurs at a pH optimum of 8–11 through a β elimination mechanism, resulting in an unsaturated C-4–

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C-5 bond in the galacturonosyl moiety at the non-reducing end of the polysaccharide produced at the cleavage site (Rombouts and Pilnik, 1980). This mechanism is common to another class of pectin-degrading enzymes, pectin lyases, but pectate lyase is distinguished by its preference for a glycosidic linkage next to a free carboxyl group rather than to an esterified carboxyl group, and by its pH optimum (Pilnik, 1990). All pectate lyases show calcium dependence. In characterizing the *Bacillus subtilis* enzyme (BsPel), Nasser *et al.* (1990) showed that calcium enhanced enzyme activity while EDTA led to enzyme inactivation. Microbial pectate lyases usually exist as multiple, independently regulated isozymes that are 27–80% identical in amino acid sequence, and have subtle differences in substrate specificity (Preston *et al.*, 1992).

The three-dimensional structures of pectate lyase C (PelC) and E (PelE) from *Erwinia chrysanthemi* have been solved and refined to a resolution of 0.22 nm (Yoder *et al.*, 1993 Lietzke *et al.*, 1994, 1996) and, although PelE has only 22% sequence identity with PelC, the core structure of both proteins is very similar. The enzymes fold into an unusual motif of parallel β strands coiled into a large helix. Within the parallel β-helix core, some amino acid side chains form linear stacks and include a novel asparagine ladder. BsPel has a parallel β-helix domain, a loop region and a cleft between that may form the active site (Pickersgill *et al.*, 1994). Calcium binds at the bottom of this cleft and near to it is an arginine residue, conserved across all pectin and pectate lyases, that may be involved in the catalytic reaction. Pectin lyases, however, lack the bound calcium.

Although no pectate lyase activity has been demonstrated in somatic plant cells, cDNA with homology to microbial pectate lyases have been reported in several species, principally in relation to pollen tube growth. Pectate lyase-like genes are expressed in transmitting tissue (McCormick, 1991) together with other hydrolases, β-glucanase, chitinase, proteinase inhibitor, proline-rich proteins and hydroxyproline-rich glycoproteins (Gasser and Robinson-Beers, 1993). Two pollen-expressed genes in tomato, *LAT 56* and *LAT 59*, show homology to *Erwinia* pectate lyases (Wing *et al.*, 1989) and are strikingly similar to the major ragweed pollen allergen (Ambal) (Rafner *et al.*, 1991), to a cDNA clone *LMP131* expressed preferentially in anthers of lily (Kim *et al.*, 1994), and to a tomato cDNA that is predominantly expressed in pistils but also detected at low levels in roots (Budelier *et al.*, 1990). Further homologies were found with the protein sequence of a cedar pollen allergen Cry j I (Sone *et al.*, 1994), pollen-specific maize genes (Turcich *et al.*, 1993), P0149 from alfalfa (Wu *et al.*, 1996) and a genomic clone in tobacco (Rogers *et al.*, 1992).

In this paper, we describe the isolation of a cDNA, *ZePel*, with homology to the pectate lyase-like plant sequences and to the microbial enzymes. We detect pectate lyase activity in populations of both elongating and differentiating cells. The mRNA encoding the enzyme is up-regulated *in vitro* during both cell elongation and cell differentiation in response to auxin, but *in situ* hybridization suggests that in *Zinnia* plants it is associated specifically with vascular bundles and shoot primordia rather than with all elongating cells. In addition we demonstrate that the ZePel protein has pectate lyase activity by using bacterially expressed recombinant enzyme. We assayed the recombinant enzyme for calcium dependence, Michaelis–Menten constant and pH optimum.

Results

Isolation and analysis of Zinnia *pectate lyase cDNA*

A cDNA library representing mRNA extracted from cells cultured for 72 h in the TE inductive medium was used to isolate cDNA clones that were induced early in the trans-differentiation process. The library (30 000 recombinant phage) was differentially screened with radiolabelled first-strand cDNA using RNA samples from cells cultured for 24 h or 72 h in inductive medium. One of the isolated cDNA, which was abundantly present under inductive conditions at 72 h but not at 24 h, was selected for further study.

The nucleotide sequence of this cDNA and its predicted amino acid sequence are presented in Figure 1. Without the polyA tail, the cDNA is 1440 nucleotides long and contains an open reading frame (ORF) for a protein of 401 amino acid residues, beginning with the ATG initiation codon at position 28 and ending with a TGA stop codon at position 1230. The calculated molecular mass for this protein is 44 406 Da and the pI is 8.15. Analysis of the predicted amino acid sequence revealed a hydrophobic N-terminal region with characteristics of a signal peptide (von Heijne, 1986) and a potential cleavage site between Ser20 and Ser21 that would leave a protein with a calculated molecular mass of 42 362 Da and a pI of 8.15. The predicted amino acid sequence contains a consensus sequence for N-glycosylation (Asn-X-Ser/Thr) at amino acid residue 38.

Comparison of the cDNA sequence with data in the EMBL database revealed clear homology with other fungal and bacterial pectate lyases (42.2% with *BsPel*, 44.3% with *E. chrysantemi Pel A*), pectin lyases (44.35 with *E. carotova* pectin lyase) as well as with plant allergens (52.3% with *Amb a I*, 53.6% with *Cry j I*), and putative plant pectate lyases (68.7% with tomato *le9612*, 52.1% with tobacco *G10*). The homology was also present at the protein level, as shown in Figure 2. The protein sequence suggests that the protein is likely to be a pectate lyase since the calcium binding site is conserved, Asp179, 203 and 207 in addition to the potential active site residues: RMPRPCR (residues

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GCACGAGACCACCATCACCTCTAAAAAATGGCAACCACAATTCTACCACTAATCCTCTTT     60
                              M  A  T  T  I  L  P  L  I  L  F
ATATCTTCACTAGCCATTGCTTCTTCATCACCAAGTAGAACCCCACATGCCATAGTCAAT    120
I  S  S  L  A  I  A  S  S* S  P  S  R  T  P  H  A  I  V  N
GAAGTCCACAAGAGCATCAATGCTTCCCGTAGAAACTTGGGTTACCTGTCTTGTGGGACA    180
E  V  H  K  S  I  N  A  S  R  R  N  L  G  Y  L  S  C  G  T
GGTAACCCAATTGATGATTGCTGGCGGTGTGACCCTAACTGGGCCAATAACCGCCAGCGT    240
G  N  P  I  D  D  C  W  R  C  D  P  N  W  A  N  N  R  Q  R
CTTGCTGACTGTGCCATTGGGTTTGGAAAGAACGCGATGGGTGGGCGAAATGGAAGGATA    300
L  A  D  C  A  I  G  F  G  K  N  A  M  G  G  R  N  G  R  I
TATGTTGTCACTGATCCCGGGAATGATGATCCAGTGAACCCTGTTCCCGGGACCCTGAGA    360
Y  V  V  T  D  P  G  N  D  D  P  V  N  P  V  P  G  T  L  R
TATGCGGTTATTCAAGATGAACCGTTGTGGATCATCTTTAAACGCGACATGGTAATTCAA    420
Y  A  V  I  Q  D  E  P  L  W  I  I  F  K  R  D  M  V  I  Q
CTACGGCAAGAACTTGTGATGAATTCTCACAAGACCATAGATGGTAGGGGTGTGAATGTG    480
L  R  Q  E  L  V  M  N  S  H  K  T  I  D  G  R  G  V  N  V
CATATTGGTAATGGCCCATGCATTACTATACATTATGCTAGTAATATTATTATACATGGT    540
H  I  G  N  G  P  C  I  T  I  H  Y  A  S  N  I  I  I  H  G
ATTCATATACATGATTGTAAGCAGGCTGGTAATGGTAACATTAGAAACTCACCACATCAT    600
I  H  I  H  D  C  K  Q  A  G  N  G  N  I  R  N  S  P  H  H
AGTGGATGGTGGACACAATCTGATGGTGATGGGATATCCATTTTTGCTAGCAAAGATATA    660
S  G  W  W  T  Q  S  D  G  D  G  I  S  I  F  A  S  K  D  I
TGGATTGATCATAATTCTTTGTCTAATTGTCATGATGGGCTCATTGATGCCATACATGGA    720
W  I  D  H  N  S  L  S  N  C  H  D  G  L  I  D  A  I  H  G
TCTACTGCCATCACTATTTCTAACAATTACATGACTCATCATGATAAAGTTATGTTGTTA    780
S  T  A  I  T  I  S  N  N  Y  M  T  H  H  D  K  V  M  L  L
GGACATAGTGATAGTTATACTCAAGATAAGAACATGCAAGTTACTATTGCATTTAACCAT    840
G  H  S  D  S  Y  T  Q  D  K  N  M  Q  V  T  I  A  F  N  H
TTTGGTGAAGGTCTTGTTCAAAGAATGCCAAGATGTAGACATGGGTATTTCCATGTGGTG    900
F  G  E  G  L  V  Q  R  M  P  R  C  R  H  G  Y  F  H  V  V
AACAATGACTATACACATTGGGAGATGTATGCTATTGGAGGAAGTGCATCTCCTACCATC    960
N  N  D  Y  T  H  W  E  M  Y  A  I  G  G  S  A  S  P  T  I
TATAGCCAAGGCAATAGATTTTTGGCTCCCAATACAAGATTTGACAAGGAGGTGACAAAA   1020
Y  S  Q  G  N  R  F  L  A  P  N  T  R  F  D  K  E  V  T  K
CATGAGAATGCACCCGAAAGTGAATGGAAGAACTGGAATTGGAGATCAGAAGGAGATTTG   1080
H  E  N  A  P  E  S  E  W  K  N  W  N  W  R  S  E  G  D  L
ATGTTAAACGGTGCTTATTTTAGAGAATCAGGTGGACGTGCTGCTTCATCTTTTGCAAGG   1140
M  L  N  G  A  Y  F  R  E  S  G  G  R  A  A  S  S  F  A  R
GCGTCGAGTTTGAGTGGCAGACCATCTACACTTGTGGCGTCAATGACTCGATCAGCCGGA   1200
A  S  S  L  S  G  R  P  S  T  L  V  A  S  M  T  R  S  A  G
GCGCTCGTTTGCCGAAAAGGGTCTCGTTGTTGATTATGGACTAGAGACATATATAAAGTT   1260
A  L  V  C  R  K  G  S  R  C
ATTGCATTTTGTGTTTCTTTCTTGTATTTCTATGTTTTTGTTACTTGCTTTTACTAATAT   1320
GTCATGTCAACCTTTGTGCTTGGTTGGATATGAATCAAGATGAGTCTTTCATATGGACCA   1380
ACAAATTAAATGCAAAAGAGTTGACCAAATGAATATTTATATATTATATTTAATTTCTCT   1440
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Figure 1. cDNA and predicted amino acid sequence of *ZePel*.
The amino acid sequence predicted from the cDNA sequence of *ZePel* is shown in boldface letters. The predicted cleavage site for the mature protein is indicated with an asterisk. The potential N-glycosylation site is underlined. The DNA bases are numbered at the right. The nucleotide and deduced amino acid sequence have been submitted to EMBL, GenBank and DDBJ as accession number Y09541. Residues possibly involved in the active site and the calcium binding are double underlined.

259–264; Figure 1). The BsPel has three aspartic acid residues in the putative active site cleft: Asp184 is a ligand to the putative active site calcium ion, the others being Asp223 and 227. PelE has three Asps also at the active site but PelC has two Asps and a Glu, and the cleft is not so pronounced (Lietzke *et al.*, 1994, 1996; Pickersgill *et al.*, 1994; Yoder *et al.*, 1993). In ZePel, the putative active site is more basic than in pectin lyases due to the contributions of Lys227, Arg262 and Arg264 (as in BsPel). Of these, the Arg264 is absent in pectin lyases. The highly conserved vWiDH region among pectin and pectate lyases is also present in ZePel.

Expression of the pectate lyase mRNA in Zinnia *mesophyll cells* in vitro *and in plant organs*

Mesophyll cells from the leaves of *Zinnia* differentiate directly into TE when cultured in a medium containing a 1:1 ratio of auxin to cytokinin. Alternatively, when cultured in a medium containing high levels of auxin alone, the mesophyll cells elongate after 5 days. To examine whether the expression of the pectate lyase gene is associated with TE differentiation, cell elongation, or both, we analysed the accumulation of the *ZePel* transcript in isolated mesophyll cells cultured under different conditions. RNA gel blot analysis in Figure 3(a)

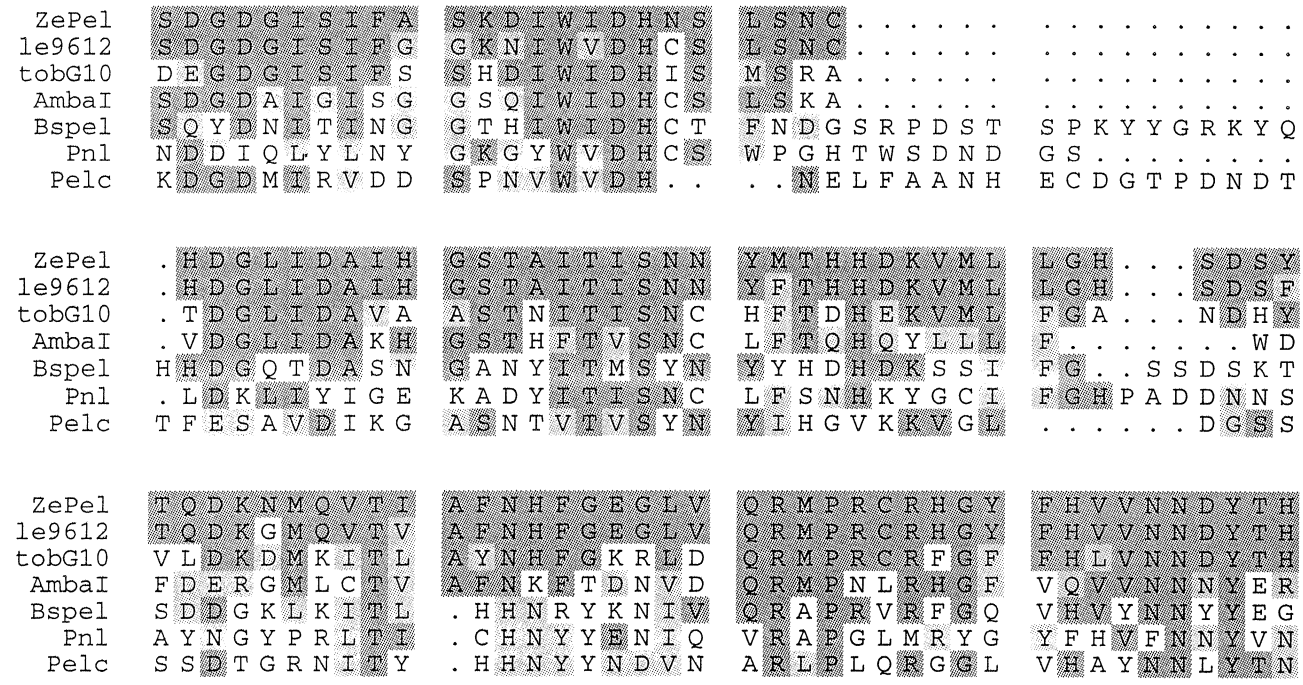

Figure 2. Homology of ZePel to conserved regions of other pectate lyases or pectin-degrading enzymes.
Shown is a multiple alignment of the predicted amino acid sequence of ZePel, from Ser178 to His277, to the predicted amino acid sequences of some homologous sequences in the EMBL database, including tomato style-expressed gene 9612 (le9612), tobacco pollen-specific gene G10 (tobG10), short ragweed allergen (Amb a I), pectate lyase from *Bacillus subtilis* (BsPel), *Erwinia carotova* pectate lyase (Pelc) and pectin lyase (Pnl). The alignment was created using the PILEUP program (Genetics Computer Group) and sequence homology is shown by using the PRETTYBOX program. Dark shading indicates identical residues, and pale shading indicates residues that are conservative substitutions. Gaps introduced to improve the alignment are represented by dots.

shows that mRNA accumulated in inductive cultures (containing 1.0 mg l^{-1} of both auxin and cytokinin), as well as those cultured in the presence of 1.0 mg l^{-1} auxin alone. No mRNA accumulation was found in maintenance medium (1 μg l^{-1} 6-benzylamino-purine (BAP) and 1 μg l^{-1} α-naphthalene acetic acid (NAA)) or in cultures with 1.0 mg l^{-1} BAP only, where neither differentiation nor elongation occurred. A time–course of *ZePel* mRNA accumulation in the cells cultured in the TE induction medium showed a large increase in the accumulation of the corresponding transcript after only 48 h in culture (Figure 3b), at a point when the cells still look indistinguishable from their appearance in the original *Zinnia* leaf, and coincident with the time at which the cells become committed to TE precursor fate (Stacey *et al.*, 1995). As shown in Figure 3(c), NAA alone can induce expression of the *ZePel* gene at the same time or earlier than with the combination of both hormones. The time of appearance of this mRNA is thus much earlier than cell elongation, which does not occur until after 120 h.

We also determined the expression patterns of the pectate lyase gene in different *Zinnia* organs (Figure 4). The mRNA was highly expressed in roots and to a lesser extent in stems, but no significant expression could be seen in fully expanded leaves. We also detected low levels of transcript in flowers and seedlings.

The localization of *ZePel* transcripts *in situ* was investigated by examining cells in sections of stem and root from three-week-old *Zinnia* plants, using the antisense probe. Figure 5 shows that, in stems, *ZePel* mRNA was present in the recent products of cambial divisions on both phloem and xylem sides, and conspicuously localized in the xylem parenchyma cells of young vascular bundles. In roots, the *ZePel* mRNA was located in the outer (most recently formed) part of the xylem and in phloem parenchyma cells. When shoot meristems were examined, the *ZePel* transcript was detected in young leaf primordia.

Southern analysis of ZePel

To see if *ZePel* was part of a family of related genes in *Zinnia*, we hybridized *Xba*I-, *Hin*dIII-or *Xho*I-digested genomic DNA with the *ZePel* cDNA. The DNA gel blot presented in Figure 6 shows that in each digestion only a single band hybridizes under highly stringent conditions (60°C). Although this indicates that the *ZePe*l gene is probably present in the *Zinnia* genome at only one copy per haploid genome, lower stringency blots suggest that

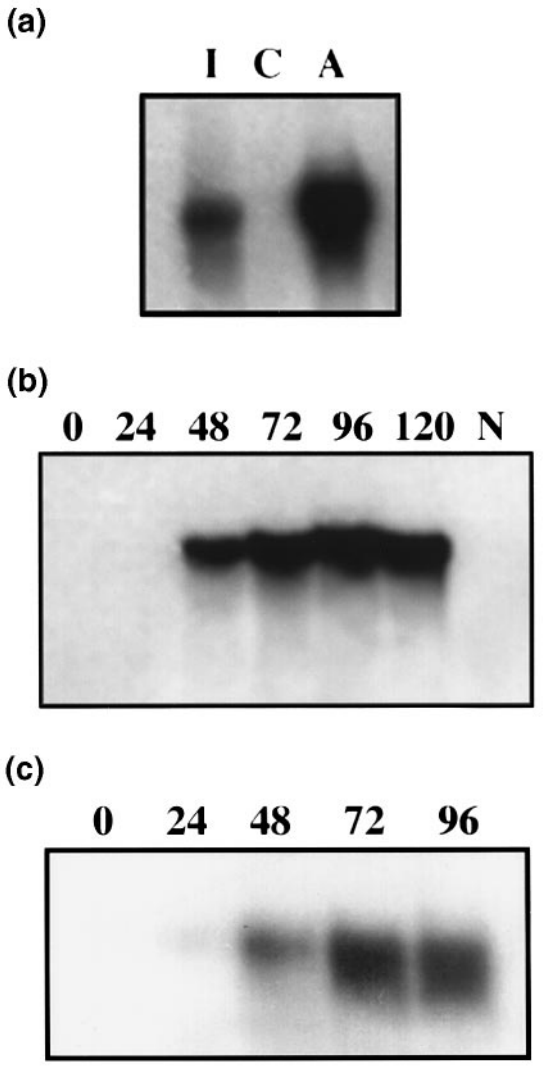

Figure 3. RNA gel-blot hybridization analysis of *ZePel* sequences.
(a) Northern blot analysis of the *ZePel* mRNA extracted from cells cultured 96 h in inductive medium containing 1.0 mgl^{-1} of both auxin and cytokinin (I), medium containing 1.0 mgl^{-1} auxin but no cytokinin (A) and medium containing 1.0 mgl^{-1} cytokinin but no auxin (C).
(b) Time–course of appearance of the *ZePel* mRNA in cells cultured in inductive medium for 0, 24, 48, 72, 96 and 120 h. Lane N represents cells cultured in maintenance medium for 72 h.
(c) Time–course of appearance of the *ZePel* mRNA in cells cultured in medium containing 1.0 mgl^{-1} auxin but not cytokinin for 0, 24, 48, 72 and 96 h.

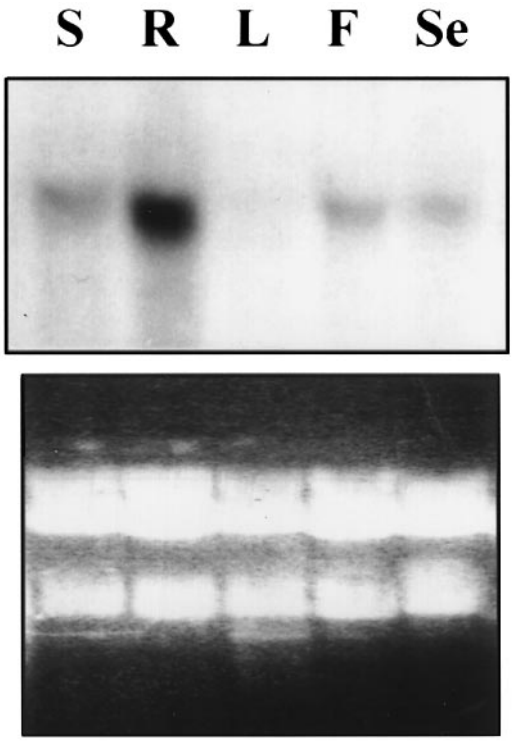

Figure 4. Comparative RNA gel blot analysis of transcripts of *ZePel* in stem (S), roots (R), leaves (L) of 4-week-old *Zinnia* plants, as well as in flowers (F) after the anthesis and in 2-week-old seedling (Se).
An ethidium bromide register is shown at the bottom, demonstrating the equal amount of RNA present in each lane.

there is one other related gene present in *Zinnia* (data not shown).

Recombinant ZePel *is a pectate lyase*

The *ZePel* cDNA sequence was mutated to produce initiation codons within convenient *Nco*I sites at the start of the ORF and at the predicted mature N-terminus (Ser20). The two constructs were cloned into the pET3d T7 polymerase-based vector (Studier *et al.*, 1990; Studier, 1991) and their expression induced in BL21 (pLysS) cells with IPTG. No protein expression could be obtained from the construct with its native initiation codon. However, with the predicted signal sequence removed, the construct, upon induction, produced a protein of ⊤ 42 kDa by SDS–PAGE. At this stage no enzyme activity could be detected in protein extracts of this material. *Escherichia coli* expressing the recombinant protein at 28°C and 37°C was fractionated into cytoplasmic, periplasmic and inclusion body extracts and visualized by SDS–PAGE, as shown in Figure 7. Surprisingly at the lower temperature almost all of the recombinant protein was found to be in inclusion bodies. At 37°C, however, a proportion of the protein was found to be soluble (cytoplasmic fraction). This fraction was shown to contain a low level of pectate lyase activity. However, the cytoplasmic protein was also observed to migrate just in front of the inclusion body material upon SDS–PAGE. These gels were electroblotted and the corresponding proteins bands excised and N-terminally sequenced. The inclusion body protein proved to have the expected N-terminus (APSRT), accounting for the polymerase chain reaction (PCR)-induced change of the second codon and removal of the initiating methionine by *E. coli*. In contrast, the

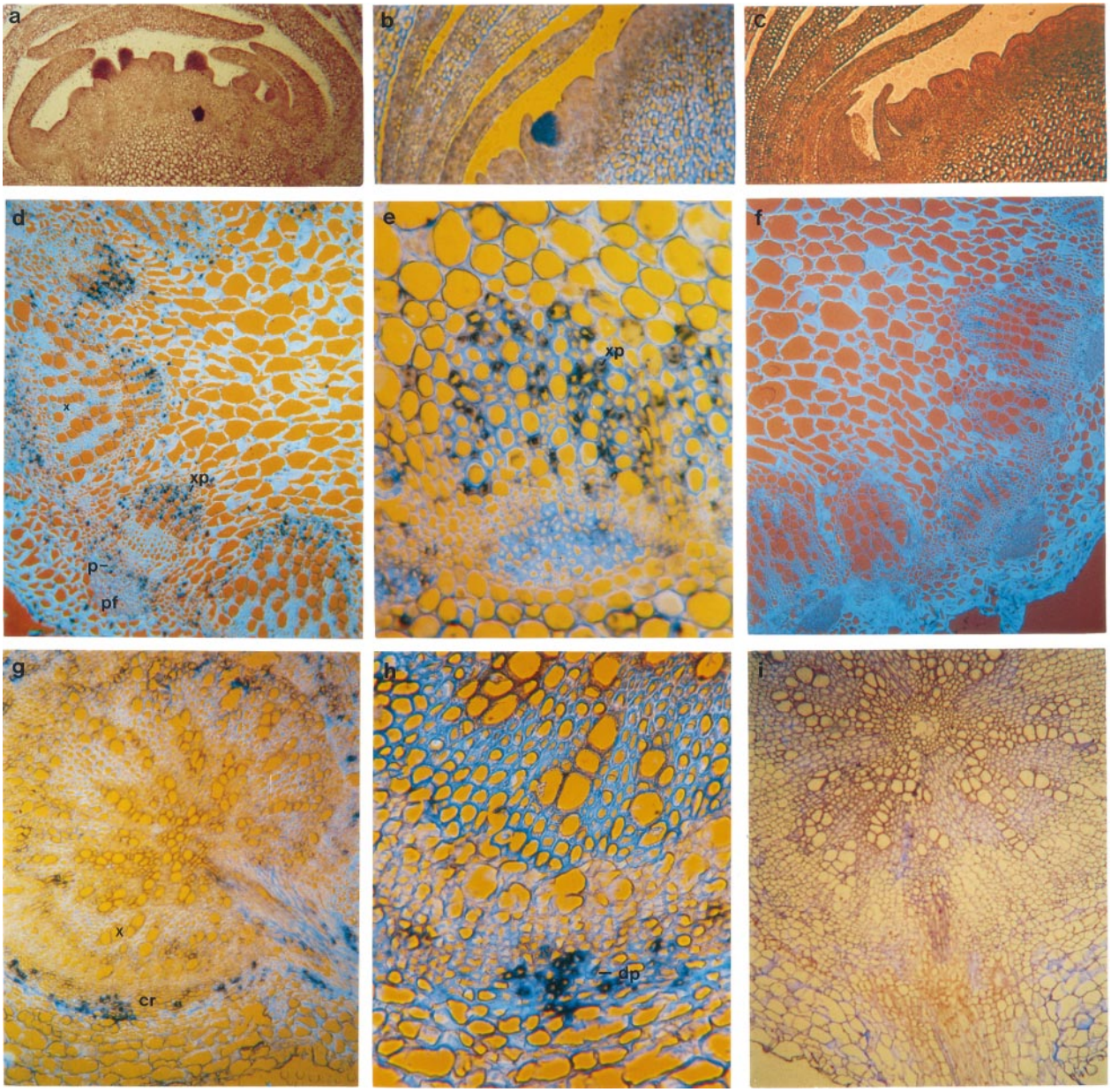

Figure 5. *In situ* hybridization of antisense *ZePel* mRNA on sections of *Zinnia*.
(a, b, c) Vegetative shoot apex sections labelled with the *ZePel* antisense probe (a) (× 50 magnification), or with antisense probe, stained with Calcofluor 0.1%, and visualized under UV light (b) (×75) or with the sense probe (c) (×75). *ZePel* transcript is localized to some young leaf primordia.
(d, e, f) Stem sections labelled with the *ZePel* antisense probe (d) or with the sense probe (f) and stained with Calcofluor 0.1% and visualized under UV light (×50). Higher magnification (×120) of a stem labelled with the antisense probe (e). The transcript is present in the cambial region (d) and in xylem parenchyma cells of young vascular bundles (e).
(g, h, i) Root sections labelled with the antisense probe (g) or with sense probe (i) and stained with Calcofluor 0.1% and visualized under UV light (×50). Higher magnification (×120) of a root labelled with the antisense probe (h). The transcript is present in the outer part of the xylem and in phloem parenchyma cells.
cr, cambium region; dp, differentiating phloem; p, phloem; pf, phloem fibers; x, xylem; xp, xylem parenchyma.

cytoplasmic fraction produced the sequence ASRRN, which is consistent with the loss of an 18-amino acid peptide from the anticipated N-terminus and which correlates with the putative N-glycosylation site. In the plant, glycosylation at this site may protect ZePel from cleavage by proteolytic enzymes.

The recombinant ZePel protein was found to be considerably more soluble in low concentrations of detergent than

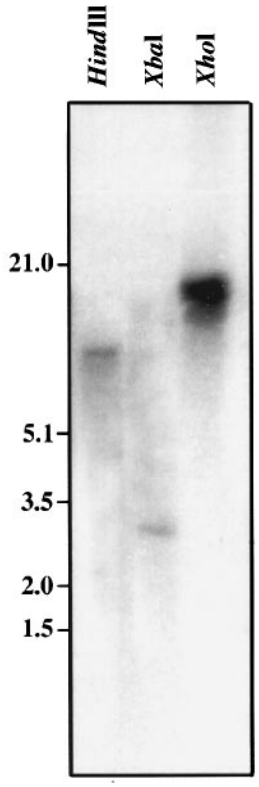

Figure 6. Southern blot hybridization analysis of *ZePel*.
DNA gel blot of *Zinnia* genomic DNA digested with *Hin*dIII, *Xba*I or *Xho*I. The blot was hybridized with the complete cDNA probe. Molecular length markers in kilobases are indicated on the right.

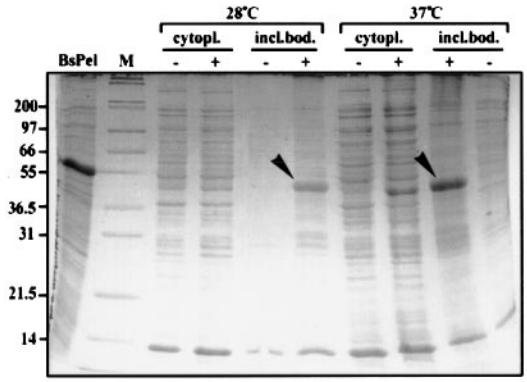

Figure 7. 12% SDS–PAGE of cytoplasmic (C) and inclusion body (IB) fractions of *E. coli* before (–) or after (+) the induction of *ZePel* with IPTG at 28°C and 37°C (molecular weight markers are in kDa).
The *B. subtilis* Pel (BsPel) is shown as marker. A major band of close to the predicted molecular weight for ZePel (44 kDa) is evident in fractions from inclusion bodies upon induction with IPTG at both temperatures (indicated by arrowheads).

other proteins present in the inclusion body material. Extraction of the inclusion bodies with *n*-dodecyl a-D-maltoside produced a solution enriched for the ZePel protein that also proved to be enzymatically active. The

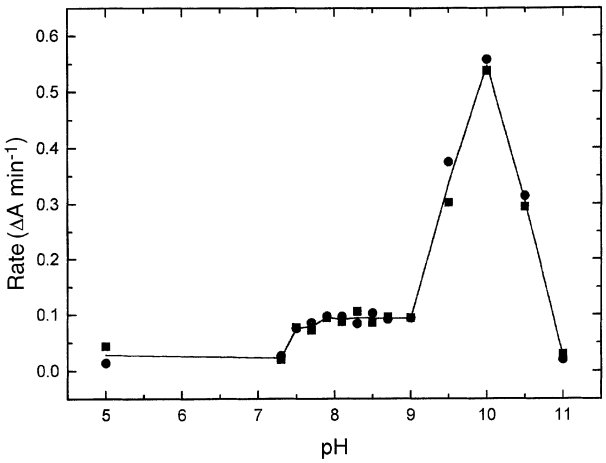

Figure 8. The pH dependence of recombinant *ZePel* enzyme activity, showing maximal activity at pH 10.
The ■ and ● represent the average enzyme activity in duplicate assays performed on different days.

presence of this specific detergent was necessary for the recovery of the activity. The enzyme activity could be eliminated by the addition of 100 μM EDTA, indicating its expected calcium dependence. The enzyme was purified using conventional ion-exchange chromatography in the presence of detergent. The pH optimum of the enzyme was found to be 10, as shown in Figure 8, with a V_{max} of 0.12 ± 0.015 μmol min^{-1} and a K_m of 0.9 ± 0.23 g 100^{-1} ml. The specific activity of the recombinant ZePel protein was 250 U mg^{-1}, similar to the specific activity of 350 U mg^{-1} for cedar pollen allergen (Taniguchi *et al.*, 1995). For comparison, purified recombinant BsPel has a specific activity of 1000 U mg^{-1}.

Pectate lyase activity in Zinnia *cells*

In vitro cultures of *Zinnia* cells were assayed for pectate lyase activity by separating the cells from the culture medium and extracting them with 50 mM Tris, pH 7.5, and 0.1 M NaCl buffer in the presence of 0.1% *n*-dodecyl α-D-maltoside. Extracts from seven-day-old cells cultured in inductive media or in the presence of 1.0 mg l^{-1} auxin alone were then assayed at pH 6.7. A pectate lyase activity of 34.6 mU mg^{-1} was found in the cells cultured in inductive media and of 97.4 mU mg^{-1} in the culture with auxin. A time–course was done for pectate lyase activity at pH 10, where the recombinant enzyme was found to be most active. As shown in Figure 9, the activity could be detected after 4 days when cells were cultured in inductive media or in the presence of 1.0 mg l^{-1} auxin. The specific activity in cells cultured with auxin was higher than that in the inductive medium. No activity could be detected in cells cultured in maintenance medium or in the culture medium of any of the cultures.

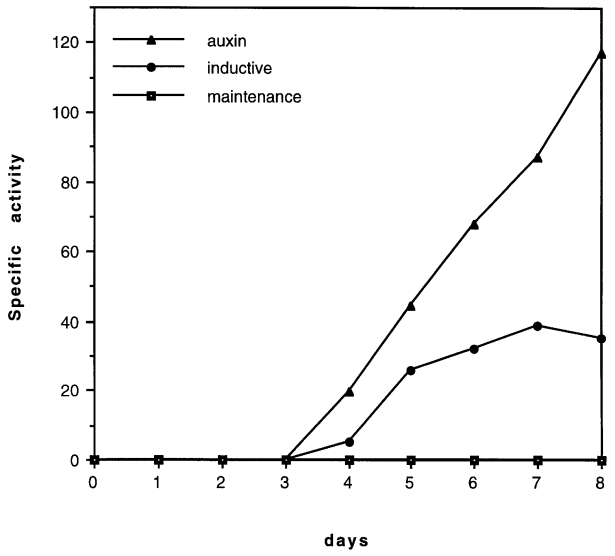

Figure 9. Time–courses of pectate lyase activity at pH 10 in cells sampled from cultures in inductive medium, in medium containing 1.0 mgl^{-1} NAA, or in maintenance medium.
The specific activity was expressed in mU of activity per mg of protein.

Discussion

We have isolated a cDNA clone whose corresponding mRNA is expressed abundantly in cultured cells differentiating into TE. The cDNA sequence has all the conserved features of a pectate lyase. The expression of the protein in *E. coli* produces an active enzyme capable of degrading pectin via the hydrolysis of α-1,4-galacturonosyl residues by β-elimination. Previous attempts to obtain recombinant pectate lyase with enzyme activity in a baculovirus expression system have been unsuccessful (Dircks *et al.*, 1996). However, there is a recent report of pectate lyase enzyme activity in a purified pollen allergen (Cry j I) from Japanese cedar (*Cryptomeria japonica*) (Taniguchi *et al.*, 1995). The optimum temperature and pH for the assay with polygalacturonic acid were 60–70°C and pH 10 (Taniguchi *et al.*, 1995). In this paper, we have demonstrated pectate lyase activity in cell culture and somatic plant cells, at room temperature and within the range of physiological pH.

Given the complex structure of pectic polysaccharides, their enzymic degradation by pathogens is usually accomplished by the synergistic action of enzymes such as pectin methyl esterase, pectin lyase, endo-and exo-polygalacturonase and pectate lyase. Pectate lyase is particularly effective, and soft-rot symptoms in various plant species can be induced by applying recombinant pectate lyases from *E. coli* (Bartling *et al.*, 1995; Keen and Tamaki, 1986). There is a single pectate lyase secreted from *B. subtilis* (BsPel), which in the absence of pectin methyl esterase activity appears to make it a less aggressive soft-rot pathogen (Nasser *et al.*, 1990). In plants, pectate lyase genes have been described that are expressed primarily in pollen and in the transmitting tissue of plants, and the enzyme may facilitate pollen tube growth. As pectin is abundant in the style, the enzyme may be required to act on this (McCormick, 1991; Ori *et al.*, 1990), or it may be required to protect the open nutrient-rich style from infection by priming the plant's defence mechanisms. The enzymes from *Erwinia* liberate oligosaccharides from purified pectic substances and higher plant cell walls that function as elicitors of active plant defence reactions (Davis *et al.*, 1984; Gardner and Kado, 1976). Only transcripts of the tomato gene (*9612*) have been detected in organs other than flowers, albeit with reduced expression (Budelier *et al.*, 1990). In this paper we report the first detection of a pectate lyase activity in somatic plant cells.

The natural substrates of pectate lyases are not clearly defined. Three *Erwinia* PL isoforms obtained by expression of three pel genes in *E. coli* act synergistically to extend the range of pectin substrates that the bacterium can degrade (Bartling *et al.*, 1995). Incubation of potato tuber tissue with PL3 alone resulted simply in the separation of tuber cells, whereas PL1 and PL2 broke down the wall completely, causing release of cytoplasmic contents. Possibly various isoenzymes degrade pectic subdomains of diverse cell wall types (Bartling *et al.*, 1995). The four pectate lyases secreted by *E. chrysanthemi* have been produced in *E. coli* and the recombinant enzymes release different sizes of oligomers from polygalacturonic acid (Preston *et al.*, 1992). It will be an important task to characterize the substrate specificity for ZePel and other plant pectate lyases now that we are in a position to assay them.

In the *Zinnia* system, *ZePel* expression is up-regulated by auxin prior to either cell elongation or cell differentiation. We have observed that the newly synthesized methyl-esterified pectin deposited in the wall during growth becomes de-esterified when cells reach their mature size (Kim and Carpita, 1992; McCann and Roberts, 1994). This de-esterified and presumably Ca^{2+} cross-linked pectin may be a substrate for pectate lyase in cells that are responding to new growth signals after growth has normally ceased, or where the wall architecture is to become functionally adapted, as in TE formation. Some of the cells isolated from *Zinnia* leaves are spongy mesophyll cells that have a very distinctive shape. During elongation, the characteristic knobbly shape is lost and the cell becomes cylindrical, with the implication that re-modelling of the existing wall architecture must have taken place. During TE formation, there is much evidence that a range of different polysaccharides and proteins becomes deposited in the walls at an early stage (Ingold *et al.*, 1988; Stacey *et al.*, 1995). ZePel may assist in the removal and modification of an existing pectin matrix in order to allow the deposition of newly synthesized wall polymers for a specialized function or to create an architecture that is extensible. A further possibility

is that pectic fragments released by pectate lyase may act as oligosaccharins – polysaccharide fragments that act as cell-signalling molecules to elicit a range of cellular responses (Mohnen and Hahn, 1993).

Cells cultured in the presence of auxin alone have a higher pectate lyase activity than cells cultured in inductive medium. One possibility is that the cell division in the cultures containing auxin results in a higher cell density in a given culture volume. After 8 days of culture in inductive medium, a decrease of activity is detected, which may correlate with a decrease in the percentage of viable cells present after tracheary element differentiation. A second possibility is that the auxin-induced expression may be modulated by other growth factors such as cytokinin. Ye and Varner (1994) showed that the presence of cytokinin in the culture medium changed the expression pattern of an auxin-induced gene.

In the *Zinnia* plant we have shown that *ZePel* expression is correlated with sites of vascular differentiation and with cells that are recent products of meristematic divisions. The localization of the pectate lyase gene expression to new primordia on the flanks of the shoot meristem reveals a transient expression of the gene, as only some primordia are labelled. Auxin induces vascular differentiation as well as being necessary for final maturation of xylem. Jacobs (1952) demonstrated that an auxin flux in a basipetal direction was implicated in xylem differentiation. Sachs (1981) proposed that the pattern of vascular system results from initial local differences in the flow of auxin through cells, which leads to the establishment of preferred channels of auxin transport. These channels become progressively improved pathways of auxin movement and drain the surrounding regions at the same time that their cells are induced to undergo differentiation as vascular elements. The pattern of gene expression, where the formation of new vascular strands is taking place, is consistent with auxin regulation of *ZePel*.

Cell autolysis is a key event in tracheid formation, and this involves disruption of the vacuole to release hydrolytic enzymes. It is possible therefore that *ZePel* is initially targeted to the vacuole rather than to the cell wall. Our assumption that it is a cell-wall protein is founded on the fact that *ZePel* is expressed during cell elongation and at very early stages of cell differentiation when no vacuolar disruption occurs. However, this assumption will only be properly tested by immunolocalization with an antibody raised to the recombinant enzyme. Although expression of *ZePel* is dramatically up-regulated by auxin, it is not a primary auxin response as defined by the appearance of the gene product within minutes of the inductive signal (Abel and Theologis, 1996) and it is not related to the other auxin-regulated genes isolated from the *Zinnia* system (Ye and Varner, 1993, 1994; Demura and Fukuda, 1993), which appear later in the time–course of determination and differentiation. With a pH optimum of 10, and only residual enzymatic activity below pH 7.5, the pectate lyase is likely to be suboptimally active *in vivo*, and this may be a necessary regulatory control for an enzyme that can produce soft-rot symptoms in plants.

Experimental procedures

Plant material and cell culture

Zinnia elegans cv. Envy (Chiltern Seeds, UK) plants were grown from seed in a peat/sand potting compost in a controlled environment room at 20°C with a 16-h day. Mesophyll cells were isolated from the first true leaves of 14-day-old seedlings as described previously (Stacey *et al.*, 1995). Cells were resuspended in culture medium (Fukuda and Komamine, 1980) with 1.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA (inductive medium), 1 μg l^{-1} BAP and 1 μg l^{-1} NAA (maintenance medium) or 1.0 mg l^{-1} BAP or 1.0 mg l^{-1} NAA. Cultures were maintained at 27°C in the dark, shaking at 80 r.p.m., with 3 ml of cell suspension per well in six-well plates (Sterilin, UK) at a density of 10^6 cells ml^{-1}.

cDNA library construction and screening

A *Zinnia* cDNA library was constructed in λZAP (Stratagene, San Diego, CA) using cells cultured in inductive medium for 72 h. The library was screened in duplicate with radiolabelled single-strand cDNA probes from cells cultured in inductive medium for 24 h or 72 h. One of the phages showing differential hybridization with the cDNA probes was selected and converted to the pBluescript SK form by co-infection with R408 helper phage (Stratagene). Hybridization and washes of filters were done at 70°C with the Church and Gilbert (1984) hybridization solution.

RNA and DNA gel blot analysis

RNA was purified from different cultured cells and organs as described previously (Shirzadegan *et al.*, 1991). For RNA gel blot analysis, 15 μg of total RNA was electrophoresed on 1% agarose gels containing formaldehyde and blotted onto nylon membranes as described by Sambrook *et al.* (1989). Equal loading of RNA was verified by ethidium bromide staining of the gel before transfer to the membrane. DNA was isolated from leaves as described previously (Rogers and Bendich, 1988). For DNA gel blot analysis, 10 μg of DNA was digested with restriction enzymes, electrophoresed in a 0.7% agarose gel and blotted onto nylon membranes (Rogers and Bendich, 1988). RNA and DNA gel blots were probed with the entire cDNA insert or an *Eco*RI/*Hin*cII-released fragment, which was radiolabelled by random priming using T7 polymerase (Pharmacia, Uppsala, Sweden). Hybridization and filter-washing conditions were done at high stringency (Church and Gilbert, 1984).

DNA sequencing

DNA sequence analysis was performed on both strands with the use of cycle-sequencing dye terminator kits (Abi Prism, Perkin Elmer), according to the manufacturer's instructions. Sequencing reactions were analysed with an ABI 373 A sequencing system. The Genetics Computer Group (GCG, Wisconsin, USA) program

was used for sequence analysis and homology searching of the EMBL databases.

In situ *mRNA hybridization*

A fragment of 0.9 kb *Eco*RI/*Hin*cII fragment of the *ZePel* gene was subcloned into Bluescript KS (+) and SK (+) vectors to provide templates for T7 polymerase to generate sense and antisense RNA. The probes were labelled with digoxigenin-uridine 5′-triphosphate (Boehringer) according to the manufacturer's instructions. The methods for tissue preparation and *in situ* hybridization were as described by Bradley *et al.* (1993).

Expression of recombinant enzyme in E. coli

Two oligonucleotides binding at the N-terminal end of the reading frame of *ZePel* were synthesized to introduce *Nco*I sites either over the initiating ATG (5′-AAACCATGGCAACCACAATTCTACC-3′) or to create a Met-1 and exchange Ser21 to Ala, to remove the putative signal sequence (5′-GCTTCCATGGCACCAAGTAGAAC-CCC-3′). A single terminal oligonucleotide was synthesized to introduce a *Bam*HI site following the stop codon (5′-CTCGGAT-CCATAATCAACAACGAGACCC-3′).

Two independent PCR amplifications of the *ZePel* cDNA (⊤ 100 ng) were performed using the two alternative N-terminal oligonucleotides and the single C-terminal oligonucleotide (500 ng each) with AmpliTaq (Perkin-Elmer, UK) for 25 cycles of 1 min at 94°C to denature, 1 min at 57°C to anneal, and extension for 2 min at 72°C. The PCR product was purified from a 0.8% agarose gel, which was then made blunt ended and phosphorylated using klenow fragment and polynucleotide kinase. This product was ligated upon itself using T4 ligase, which was then cut with the restriction enzymes *Nco*I and *Bam*HI before ligation into a modified form of pBluescript similarly digested and transformed into *E. coli* Sure cells (Stratagene, Cambridge, UK) and plated out on L-agar containing ampicillin (50 μg ml^{-1}), IPTG (1 mM) and X-gal (12.5 mg). The recombinant plasmids carrying the *ZePel* cDNA were sequenced and shown to carry the PCR-induced mutations and otherwise to retain the native sequence. The *ZePel* cDNA were then liberated with *Nco*I and *Bam*HI and gel-purified before ligation into pET3d (Studier *et al.*, 1990) previously digested with *Nco*I and *Bam*HI. These constructs were then transformed into *E. coli* BL21 cells carrying pLysS and plated out on L-agar with ampicillin (50 μg ml^{-1}) and chloramphenicol (30 μg ml^{-1}).

Production of pectate lyase in E. coli

L-broth (500 ml) was inoculated with 1 ml of overnight culture until 0.5 OD 600 nm was reached, and then induced with 0.4 mM IPTG until 1 OD 600 nm. The periplasmic, cytoplasmic and inclusion body fractions were checked for protein expression using 12% SDS–PAGE. The IPTG-inducible protein bands were blotted to Immobilon-P (Millipore, USA) from which the N-terminal protein sequences were determined using automated Edman degradation. For enzyme production the cells were grown in 500 ml L-broth containing chloramphenicol (30 μg ml^{-1}) and ampicillin (50 μg ml^{-1}) with protein induction as carried out above. The cells were French-pressed using a 50 mM Tris–HCl, pH 7.5, buffer and 0.1 M NaCl, 0.1% *n*-dodecyl α-D-maltoside. The culture was centrifuged at 12 000 r.p.m. at 4°C in a Sorvall SS-34 rotor. The clarified supernatant was initially used to determine pectate lyase activity using 50 mM Tris–HCl, pH 8.5, buffer, 0.5% polygalacturonic acid and 1 mM $CaCl_2$ assay conditions (Nasser *et al.*, 1990). The V_{max}, pH optimum and calcium dependence were initially established using the enriched detergent extract before confirmation following purification by ion-exchange chromatography (MonoQ and MonoS; Pharmacia, UK) in the presence of 0.1% *n*-dodecyl α-D-maltoside. The enzyme assays were performed with 50 mM Na acetate within the pH ranges 5–7, with 50 mM Tris HCl within the pH range 7.2–9 and with 50 mM Na_2CO_3 within the pH range 9–11.

Analysis of pectate lyase activity in Zinnia *cells*

Cells, isolated from cultures by filtering, were ground in 50 mM Tris, pH 7.5, and 0.1 M NaCl buffer in the presence of 0.1% *n*-dodecyl α-D-maltoside and centrifuged for 5 min. The supernatant was assayed for enzymatic activity using 50 mM Na_2CO_3, pH 10, and 0.5% polygalacturonic acid by measuring the unsaturated compounds released from polygalacturonic acid at 235 nm during the course of 3 min at room temperature. 5.2 absorbance units per min correspond to the formation of 1 μmol unsaturated uronide per min (Nasser *et al.*, 1990). A unit (U) of Pel activity corresponds to the formation of 1 μmol unsaturated uronide per min. Specific activity was expressed as mU per mg of protein.

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