

Cloning, molecular characterization and expression pattern of a strawberry ripening-specific cDNA with sequence homology to pectate lyase from higher plants

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Abstract

A strawberry fruit cDNA showing sequence similarity to higher-plant pectate lyase genes has been isolated by differential screening of a strawberry fruit cDNA subtractive library. The cDNA contains a 396 amino acids open reading frame corresponding to a 44.8 kDa protein. The transcript is predominantly expressed in ripe fruits and was not detected at high levels in any other plant tissues. The removal of the achenes from unripe green fruits induced the expression of this putative pectate lyase gene. In common with other ripening related genes in strawberry, this induction was partially inhibited by treatment of de-achened fruit with the auxin NAA. Southern blot analysis of genomic DNA indicates that in strawberry there is more than one putative pectate lyase gene. We propose that the ripe fruit expression of this strawberry gene with similarity to pectate lyases could be related to cell wall pectin degradation contributing to strawberry fruit softening.

Introduction

During strawberry ripening softening of the fruit appears mainly due to middle lamella and cell wall degradation, that mostly occurs in the last stages of the ripening process [15]. Increases in both total soluble polyuronides and their associated neutral sugars are indicative of possible alterations in the carbohydrates cross-linking in the cell walls [13, 19]. The loss of pectin stabilization by Ca^{2+} which influences the degree of esterification may also contribute to fruit softening.

Induction of several cell wall hydrolytic enzymes has been demonstrated during strawberry fruit ripening. A clear increase in pectin methylesterase (PME) [4, 5] and cellulase activity in ripe and over-ripe strawberry fruits has been described, which suggests that these enzymes play a role in the fruit softening process

[1, 5]. However, an association between pectate lyase activity, fruit ripening and softening has not yet been reported.

Pectate lyases (EC 4.2.2.2) are pathogen-secreted enzymes that cleave randomly β -1,4-linked galacturonosyl residues of pectins found in the middle lamella and primary cell walls of higher plants resulting in the maceration of plant tissues [7]. Enzymatic cleavage of glycosidic bonds occurs through a β -elimination reaction, which produces an unsaturated C4-C5 bond in the galacturonosyl moiety at the non-reducing end of the polysaccharide and generates 4,5 unsaturated oligogalacturonates [35]. In pathogens, there are multiple and independently regulated pectate lyase isoenzymes which are coded by different genes [2, 7, 14]. Thus, two endo- and exopectate lyase activities have been described, the endoenzymes being much more active in producing maceration [16].

In higher plants, several genes that show similarity with pectate lyases genes from pathogens have been isolated and characterized. These genes are highly expressed mainly in mature pollen grains and in devel-

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opening pollen tubes [27, 31, 34]. Partial degradation of pectin-containing pollen inner walls combined with the action of polygalacturonase could facilitate pollen germination and pollen tube elongation [27, 31, 34]. In addition, another possible role of pectate lyase is to enable the growth of pollen tubes by degrading pectins in the transmitting tissue of the style and then using the degradation products as building blocks for the synthesis of pollen tube walls to allow their continuous extension [27, 31, 34].

At present, only a Japanese cedar pollen protein with pectate lyase activity has been reported [29]. While no biochemical functions have been shown for any plant genes with a pectate lyase sequence similarity, the sequence conservation suggests that they encode this enzyme.

In this paper we report, for the first time in fruits, the isolation and characterization of a strawberry fruit cDNA that shows a high similarity with other putative pectate lyase genes of higher plants. The corresponding gene expression is regulated by auxins and is induced during the strawberry fruit ripening process.

Material and methods

Plant material

The strawberry fruit (*Fragaria* × *ananassa* cv. Chandler, an octaploid cultivar) was harvested at different developmental stages: small-sized green fruits (G1), middle-sized green fruits (G2), full-sized green fruits (G3), white fruits with green achenes (W1), white fruits with red achenes (W2), turning stage fruits (T), and full-ripe red fruits (R).

RNA isolation

Total RNA from a pool of 6-7 strawberry fruits at different ripening stages and from roots, leaves, flowers and stolons was isolated according to Manning [18], and the remaining carbohydrates were removed by passing RNA through a cellulose column [28]. Poly(A)⁺ RNA was obtained using an oligo-dT cellulose column [28].

Double-stranded cDNA generation

Double-stranded cDNA of full-ripe red fruits (R) was obtained from poly(A)⁺ RNA, using a cDNA synthesis System Plus Kit (Amersham) and according to man-

ufacturer's instructions. This double-stranded cDNA was used for subtraction purposes (tracer DNA).

To generate double-stranded cDNA probes for hybridization purposes, double-stranded cDNA from green (G2) and red (R) stages were ligated to an adapter generated by mixing equimolecular amounts of two previously designed oligos with the sequences AATTCGCCATGGATCTAGACC and GGTCTAGATCCATG, thus avoiding unspecific cross-hybridization between the tracer DNA and the probes. After ligation, cDNAs were PCR amplified using the former oligo as a primer. The PCR reaction was carried out under the same conditions as those described below for the pectate lyase cDNA cloning. Afterwards, the PCR products were filtered and retained by a Microcon-100 to eliminate the primers before labelling. Conventional random primer kits were used to label the probes to a specific activity of ca. 10⁸ cpm/μg.

Pectate lyase cDNA cloning

The cloning and isolation of the full-length putative pectate lyase cDNA from strawberry was performed by differential screening of a cDNA subtractive library generated by an MAST-PCR method.

A cDNA subtractive library of the red stage vs green stage was generated by the following procedure: to 2.8 ml (5 mg/ml) of oligo-dT magnetic Dynal beads G2-stage total RNA (2.8 mg) was added. The mixture was allowed to hybridize in ten individual samples, at room temperature, for 10-15 min. Beads were collected using a magnetic support and washed three times in buffer A (10 mM Tris-HCl pH 7.6, 0.15 M LiCl, 1 mM EDTA), and then each sample, containing beads and poly(A)⁺ RNA, was resuspended in 50 μl of cDNA synthesis buffer (50 mM Tris-HCl pH 7.6, 70 mM KCl, 10 mM MgCl₂, 0.25 mM dNTPs, 10 mM DTT, 250 U RNase inhibitor, and 15000 U of M-MLV reverse transcriptase (Gibco-BRL)). The reaction was carried out overnight at 37 °C, in a hybridization oven with continuous rotation. Beads were collected, washed with diethyl pyrocarbonate (DEPC)-treated water, resuspended and boiled for 5 min in new DEPC-treated water, in order to eliminate the poly(A)⁺ RNA used as a template. Yield of the cDNA synthesis was followed in parallel radioactive reactions which resulted in cDNAs between 500 and 3000 bp long (data not shown).

This single-stranded cDNA linked to Dynal beads was used as a 'green' driver cDNA. After boiling, 1 μg

of the 'red stage' double-stranded tracer cDNA was added to the immobilized driver cDNA (14 mg of magnetic beads). Hybridization was carried out in a hybridization oven with continuous rotation, at 65 °C for three days. Hybrid double-stranded cDNAs (corresponding to the green G2 and full-ripe red R stages common cDNAs) were separated from single-stranded (corresponding to the same two common cDNAs) and double-stranded cDNAs (corresponding to red-stage preferentially expressed cDNAs) using a magnetic support. The supernatant was subject to a second round of subtraction. After this second round, no additional significant subtraction was observed. A yield of 46 ng of specific red-stage double-stranded cDNA per μg of input double-stranded total cDNA red-stage was obtained (4.6%). The subtracted cDNA was ligated to an *EcoRI*/*NotI* adapter and size-fractionated with a 200 bp cut using a Sephadex G-50 spin column (Pharmacia). About 5 ng of adapter-ligated specific red-stage double-stranded cDNA was PCR-amplified using a complementary 21-mer (CTGCAGGAATTTCGCGCCGCT) of the lower strand of the adapter as a primer sequence according to the following procedure: an initial extension step at 72 °C for 2 min to extend the 3' recessed ends of the ligated adaptor and thus to generate the primer binding site on the cDNA was carried out before amplification steps. Amplification conditions were: 3 cycles of 95 °C/1 min, 48 °C/2 min (with a ramping time of 1 min), 70 °C/10 min; 22 cycles of 95 °C/1 min, 52 °C/2 min, 70 °C/10 min; and a final extension step of 72 °C/10 min.

The amplified cDNA was digested with *EcoRI* and ligated to λ ZapII using the predigested λ ZapII/*EcoRI*/CIAP cloning kit (Stratagene), according to the manufacturer's conditions, before packaging and plating. A primary titre of 10^7 pfu and 91% of recombinant phage was obtained.

Primary differential screening

The cDNA subtractive library was plated at a density of 2000–2500 pfu/150 mm plate (20 plates). Nylon filters (Amersham, Hybond-N+) were laid onto each plate in duplicate for 2 and 4 min, respectively. Each set of filters was separately prehybridized overnight at 65 °C in 25 ml of prehybridization solution containing 5 $\times$ SSC, 5 $\times$ Denhardt's solution 0.1% SDS and 100 $\mu\text{g}/\text{ml}$ of denatured salmon sperm DNA. The two sets of filters were hybridized, separately, overnight at 65 °C, by adding to the prehybridization solution the same amount of radioactivity (10^8 cpm/ μg) of the

labelled cDNA probes (from the green stage (G2-stage) to one set of filters and from the red stage (R-stage) to the other set). Probes were generated as described above under Double-stranded cDNA generation.

Filters were washed together (twice) at 65 °C for 15 min in 500 ml of 2 $\times$ SSC, 0.1% SDS, and then exposed to X-ray film for three days at –80 °C using an intensifying screen. Positives clones were picked up and resuspended in 1 ml of standard SM buffer.

Secondary and tertiary Southern blot differential screening (PCR-SBDS)

Impure putative differentially expressed clones were subject to a PCR secondary screening as follow: an aliquot of each phage suspension was PCR amplified using T3 y T7 primers and according to the following PCR conditions: one cycle 95 °C/5 min, 50 °C/2 min, 72 °C/2 min; 35 cycles 94 °C/1 min, 50 °C/1 min, 70 °C/2 min; one cycle 94 °C/1 min, 50 °C/1 min, 72 °C/15 min.

A duplicate set of samples, consisting of 10 μl of each PCR amplification mixture, was loaded in the same order and run on the same gel (1.4% agarose). The gel was blotted by alkaline transferred overnight on a Hybond-N+ membrane (Amersham). Each membrane was then prehybridized and hybridized separately as in the primary screening. The phage suspensions that presented positive bands in the Southern differential screening (SBDS) were plated at a low density (about 10–15 pfu/plate). Five lysis plaques were individually isolated and each one was PCR amplified as above. Ten μl of each amplification mixture was loaded and run in a 1.4% agarose gel. Pure phage suspensions with inserts of the same size as the filter-positive signals corresponding to the secondary screenings were selected and subject to an SBDS tertiary screening under identical conditions to those of the secondary screening. Purified positive phages were excised according to the manufacturer's conditions and the BlueScript derivative clones were sequenced.

Northern analysis

The ds-cDNA insert from the putative pectate lyase clone (clone njjs25) was used as a radioactive probe to investigate the differential expression of this gene along the strawberry ripening process by conventional northern analysis [28]. A cDNA corresponding to 18S ribosomal RNA was used as a ribosomal probe to control equal loading of RNA samples. The probes were

labelled to a specific activity of ca. 10^8 cpm/ μ g using a commercial random priming kit (Pharmacia).

20 μ g of total RNA per sample was routinely used for the northern analysis. Filters (Hybond N+, Amersham) were prehybridized at 65 °C for 1 h, in 15 ml of hybridization solution (0.25 M NaH_2PO_4 , 7% SDS, 0.1 mM EDTA). Denatured probes were added to the same hybridization solution and hybridization was carried out at 65 °C for 14–16 h. Filters were washed (twice) for 15 min in 100 ml $0.2\times$ SSC, 0.1% SDS, and then exposed to X-ray film, at –70 °C for 24–48 h.

Auxin treatment

Achenes of two sets of G2 strawberry fruits were carefully removed in the growing plant using the tip of a scalpel blade. One set of de-achened fruits were treated with the synthetic auxin 1-naphthaleneacetic acid (NAA) as a lanoline paste with 1 mM NAA in 1% (v/v) DMSO. The other set of de-achened fruits (control group) were treated with the same paste but without NAA. Both treatments were applied over the whole fruit surface. Fruits were harvested 0, 24, 48, 72 and 96 h after treatment and immediately frozen in liquid nitrogen and stored at –80 °C.

DNA extraction

Strawberry genomic DNA was extracted as follows: young leaves were kept in distilled water in the dark at 4 °C for two days. Afterwards, 2 g of leaves were ground under liquid nitrogen into a fine powder and gently resuspended in 25 ml of a warm (65 °C) buffer solution (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 50 mM EDTA, 1% 2-mercaptoethanol, 4% SDS and 6% polyvinylpyrrolidone (PVPP)). The 2-mercaptoethanol, SDS and PVPP were added just before using. The mixture was incubated in a rotatory oven at 65 °C for 1 h with gentle rotation. Then, 8 ml of 3 M potassium acetate, pH 4.8, were added and the resulting mixture was incubated on ice and centrifuged at 10 000 rpm for 10 min. The supernatant mixture was gently filtered through a double layer of Miracloth. Two volumes of cold-freeze ethanol were added and DNA was recovered with a glass microcapillary, washed 2–3 times in fresh cold-freeze ethanol and dried at room temperature. DNA was then resuspended in water.

Southern blot analysis

Genomic DNA (2 μ g) was digested with the restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, and *Hind*III, and fractionated on 0.7% agarose gels and then alkaline transferred to Hybond-N+ membranes. DNA was fixed by UV light using the Stratalinker (Stratagene) and the blot was hybridized using the putative pectate lyase cDNA (njjs25 clone) as a radioactive probe. Hybridization and washing conditions were as in the northern blot experiments.

DNA sequencing and computer analysis of sequences

DNA was sequenced by the dideoxy-chain termination method using an ALF (Pharmacia) automatic sequencer and the Pharmacia DNA Sequencing Kit, following the manufacturer's recommendations. DNA sequence was analyzed for ORFs using the CODON PREFERENCE program from the UWGCG package (version 8.1, August 1995) [8]. Amino acid sequences were analysed and compared with the GenBank (release 94.0, 4/96) and EMBL nucleic acid database (release 46.0, 3/96) and the PIR (release 48.0, 3/96) and SWISPROT (release 33.0, 3/96) databases using TFASTA, BESTFIT, COMPARE, DOTPLOT programs and PRETTY BOX.

Yeast pectate lyase expression vector construction

In order to express in yeast the putative pectate lyase cDNA as a extracellular protein, we used the DLG-3 plasmid (a gift of Dr I.S. Pretorius, University of Stellenbosch, South Africa). This is a multicopy *Escherichia coli*-*Saccharomyces cerevisiae* shuttle plasmid that contains the yeast 2 μ m origin of replication and the URA 3 marker gene. It also carries the glucose-repressible, but strong alcohol dehydrogenase gene (*ADH 2*) promoter and terminator, plus the mating α -factor's secretion signal sequence (MF α 1s) [32].

The strawberry putative pectate lyase cDNA (without its protein leader sequence) was subcloned in frame, into the *Hind*III site of plasmid DLG 3, located at the end of the MF α 1 sequence producing the DLG3-PL derivative plasmid, as follows: to PCR-amplify the pectate lyase cDNA coding sequence (without the leader sequence) two single-stranded primers, containing a *Hind*III restriction site, were synthesized, PL1 (5'-ACGTAAGCTTTTGTGGAAGTAGTAGAGGAG-3') and PL2 (5'-ACGTAAGCTTATATACACCCATTAC

ATATC-3'). The pectate lyase cDNA fragment was amplified with the following profile: (1) initial denaturation 94 °C for 2 min; (2) annealing 55 °C for 30 s, primer extension 72 °C for 2 min, denaturation 94 °C for 30 s; (3) final annealing 55 °C for 2 min, final elongation 72 °C for 5 min. Thirty-five cycles of PCR were performed, after which the amplified DNA fragment was purified and manipulated for subcloning.

Yeast transformation and enzyme assays

Saccharomyces cerevisiae JB811 (Ura 3-52, Leu2, Trp1) cells were transformed with DGL3-PL by the lithium acetate method [10]. Transformants and untransformed JB 811 (control) yeast cells were grown in YPGE medium (1% yeast extract, 2% peptone, 3% glycerol, 2% ethanol) at 30 °C.

For enzymatic assays a 15 ml sample of cell culture was collected at 60 h after inoculation. The cells were harvested by centrifugation at 8000 rpm for 5 min and the supernatant, containing the secreted pectate lyase was concentrated 15-fold by ultrafiltration (collodion bags, Sartorius, Germany). The concentrated supernatant was desalted and dialysed with 10 mM Tris-HCl pH 8.0 by using a Sephadex G-50 gel permeation column (Pharmacia, Sweden), and extracellular pectate lyase activity was determined spectrophotometrically at 235 nm according to Taniguchi *et al.* [29].

Results

Sequence comparison and structural features of the putative pectate lyase protein from strawberry

Differential screening of a subtractive strawberry fruit cDNA library identified more than one hundred ripening-related putative clones (Medina Escobar *et al.*, unpublished data). One of these cDNAs of 1408 bp was sequenced to completion. The 1408 bp strawberry cDNA sequence is presented in Figure 1A. Computer comparisons revealed a high identity of this strawberry sequence with those of higher-plant putative pectate lyase genes previously cloned (identities found were: for *Lycopersicon esculentum*, 74.3% and 68.2%; for *Zea mays*, 60.8% and 52.8%; for *Lilium longiflorum*, 64.3% and 52.86%; for *Nicotiana tabacum*, 61.2% and 56.2% and for Japanese cedar, 58.8% and 48.7% at the nucleotide and the amino acid levels, respectively). However, no significant identities or similarities with EST sequences from *Arabidopsis thaliana*

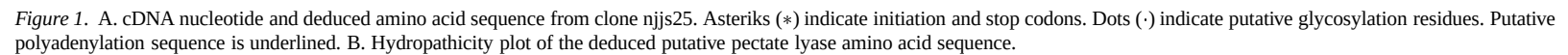
were found. These significant identities suggest that the isolated cDNA corresponds to an mRNA encoding a pectate lyase protein. This conclusion is also strongly supported by a high degree of consensus among the amino acid sequences from cDNAs corresponding to mRNAs potentially encoding higher-plant pectate lyase enzymes and that of the Japanese cedar already shown to have pectate lyase activity (Figure 2).

Three putative initiation codons were found in the cDNA pectate lyase sequence (nucleotide positions 88, 115 and 127, respectively) (Figure 1A). By dot-plot comparison (data not shown) between the cDNA pectate lyase sequence and that of other higher-plant pectate lyases sequences, we propose the second ATG (nt 115) as the initiation codon for the strawberry pectate lyase. Furthermore, this second putative initiation codon is the only one with a context that is closely related to the initiation codon consensus sequence from higher-plant ATGGCT [9]. Similarly, three putative initiation codons were found in the *Zea mays* putative pectate lyase gene [31]. The cDNA shows one major open reading frame of 396 amino acids extending from nt 115 to 1302. This ORF size is practically identical to that of G10 *Nicotiana tabacum* putative pectate lyase gene [27]. The predicted molecular mass of the cDNA-deduced pectate lyase protein is 44.8 kDa, with a pI of 7.46. The clone has a 5'-untranslated region of 114 bases and a 3'-untranslated region of 106 bases including the stop codon and 13 residues of the poly(A)⁺ tail. A putative consensus plant polyadenylation site AATATA is also found in this region (nt 1366), which is identical to that of the putative pectate lyase gene of *Zea mays* [31]. In addition, 5'-upstream of this polyadenylation site there is a TAA stop codon (nt 1303) whose context agrees with the rule of the presence of a C immediately upstream and a A immediately downstream of the stop codon utilized in higher-plant genes [9].

Like in the putative tomato pectate lyase (Lat 59) and *N. tabacum* (G10) genes [27, 34], two potential glycosylation sites (amino acids 44 and 76 from the initial methionine) are also found in the deduced strawberry pectate lyase protein (Figure 1A).

As in other higher-plant pectate lyases, the strawberry protein is relatively hydrophilic with a clearly hydrophobic N-terminal region (Figure 1B) [27, 31, 34] and a potential cleavage site between Ser-30 and Arg-31 (Figure 1A) that agrees with the (−3, −1) rule [33] and would give a 30 amino acid transit peptide.

In addition, two highly conserved amino acid motifs present in the Japanese cedar pectate lyase pro-



macuinata	..AAFTAPFYS	SRAPL TSA..	..	..	17	
lesculentum	..MS TLFTFSLLL	LAELLVIS..	..	..	20	
njjs25	MHFMTFLL	LLALLVCVSA	SVENGKPVQS	RFVEVVEEER	40	
zmays	MAAAVIRSRRR	AAAAAAAQA	SNN..	..VTSDEERYW	41	
llongiflorum	..MKA AQFFLYSL	..FFASAALS	SAN..	..IAEFDEYW	31	
ntabacum	..MDVYRIRI	SVFLLVLLT	FALLTTAT..	..	26	
jcedar	..	..	..	..	0	
macuinata	..AV	RDP ELVVQEV	QRS LNVS..R	RRLLGYL...	43	
lesculentum	..SI	QDP ELVVQDV	HRS INASLTTR	RRLLGYL...	48	
njjs25	SSFNSSMADR	SNDHWNHVA	DNPEEIASLV	RRLLGYF...	86	
zmays	AERAEVARSR	NLAAYVSDPV	AATNRFNADV	RRLLARYD..G	85	
llongiflorum	QKKSKVAQAK	AKKAYTPHPE	EVTNHFNKAV	HSSEFEGNSTTR	80	
ntabacum	..	..	..	..	40	
jcedar	..	..	..MDSPLCLV	ALLVLISFVTG	17	
macuinata	SCGTGNPIDD	CWRCDPDMAD	NRORLADCAI	GFGKNNAIGGR	DGRIYVVVTD	93
lesculentum	SCGSGNPIDR	LRAAMPQLQK	KSPAELSYCAI	GFGKNNAIGGR	DGRIYVVVTD	98
njjs25	SCCATGNPIDD	CWRCDPQQR	NRKRPAQCAI	GFGKNNAIGGR	DGRIYVVVTD	136
zmays	PCMATNPIDR	CWRCDPQQR	NRKRPAQCAI	GFGKNNAIGGR	DGRIYVVVTD	135
llongiflorum	QCLATNPIDR	CWRCKKNNSA	NRKDLVKVK	GFGKNNAIGGR	DGRIYVVVTD	130
ntabacum	PCRAENPIDK	CWRCDPQQR	NRKDLVKVK	GFGKNNAIGGR	DGRIYVVVTD	90
jcedar	SCFSDNPIDS	CWRGHSNWAQ	NRKDLVKVK	GFGKNNAIGGR	DGRIYVVVTD	66
macuinata	GDDDPVNP KP	CTLRYAVIQD	EPLWIFKRD	VIQLKKEELI	MNSHKTTIDGR	143
lesculentum	GDDDPVNP KP	CTLRYAVIQD	EPLWIFKRD	VIQLKKEELI	MNSHKTTIDGR	148
njjs25	GDDDPVNP KP	CTLRYAVIQD	EPLWIFKRD	VIQLKKEELI	MNSHKTTIDGR	186
zmays	SDDSLTDPKF	CTLRYAVIQD	EPLWIFKRD	VIQLKKEELI	MNSHKTTIDGR	185
llongiflorum	SDDSLTDPKF	CTLRYAVIQD	EPLWIFKRD	VIQLKKEELI	MNSHKTTIDGR	180
ntabacum	SDDSLTDPKF	CTLRYAVIQD	EPLWIFKRD	VIQLKKEELI	MNSHKTTIDGR	140
jcedar	SDDSLTDPKF	CTLRYAVIQD	EPLWIFKRD	VIQLKKEELI	MNSHKTTIDGR	116
macuinata	GASVHIS..GG	PCITITQYVTN	IIHGHIND	CKQGGNXYVR	DSFGHYGWRD	192
lesculentum	GASVHIS..GG	PCITITQYVTN	IIHGHIND	CKQGGNXYVR	DSFGHYGWRD	197
njjs25	GVNVHIA..YG	GCITITQYVTN	IIHGHIND	CKQGGNXYVR	DSFGHYGWRD	235
zmays	GAQVHIM..F	AQITITQYVTN	IIHGHIND	CKQGGNXYVR	DSFGHYGWRD	233
llongiflorum	GANVHIA..GG	AQITITQYVTN	IIHGHIND	CKQGGNXYVR	DSFGHYGWRD	229
ntabacum	GFNVHIQ..NG	AGIKITQYVTN	IIHGHIND	CKQGGNXYVR	DSFGHYGWRD	189
jcedar	GAQVYIGNGG	PCITITQYVTN	IIHGHIND	CKQGGNXYVR	DSFGHYGWRD	166
I						
macuinata	VSDGDGGSIF	GQPPSWDH	CTLENCHDGL	IDAHHGSTAT	TISNNYFRHH	242
lesculentum	VSDGDGGSIF	GQPPSWDH	CTLENCHDGL	IDAHHGSTAT	TISNNYFRHH	246
njjs25	MADGDGGSIF	GS..SHIWDH	CSLNCHDGL	IDAHHGSTAT	TISNNYFRHH	284
zmays	REDGDGGSIF	SS..SNIWDH	VSLARSDGL	IDAHHGSTAT	TISNNYFRHH	282
llongiflorum	REDGDGGSIF	SS..SNIWDH	VSLARSDGL	IDAHHGSTAT	TISNNYFRHH	278
ntabacum	SDGDGGSIF	SS..HDIWDH	ISSRATDGL	IDAHHGSTAT	TISNNYFRHH	238
jcedar	HPQDGDALTE	RTATNIWDH	NSSSNSDGL	IDAHHGSTAT	TISNNYFRHH	216
II						
macuinata	DKVMLLGHSD	ELTSDKSMQV	TVAFNHFGEG	LVQRMPRCRHH	GYFHVNNNDY	292
lesculentum	DKVMLLGHSD	ELTSDKSMQV	TVAFNHFGEG	LVQRMPRCRHH	GYFHVNNNDY	296
njjs25	NEVMLLGHSD	SYTRDKQMQV	TVAFNHFGEG	LVQRMPRCRHH	GYFHVNNNDY	334
zmays	DHVMMLGASD	DSYPQDAVMQV	TVAFNHFGEG	LVQRMPRCRHH	GYFHVNNNDY	332
llongiflorum	DDVMLLGHSD	TYTQDEIMQV	TVAFNHFGEG	LVQRMPRCRHH	GYFHVNNNDY	328
ntabacum	DKVMLLGHSD	HYVLDKSMQV	TVAFNHFGEG	LVQRMPRCRHH	GYFHVNNNDY	288
jcedar	HKVMMLLGHSD	AYSDDKSMQV	TVAFNHFGEG	LVQRMPRCRHH	GYFHVNNNDY	266
macuinata	THWEMYAIGG	SAAPTINSQOG	NRFLAPNDRF	AKEVTKRSD	AQESSEKKK	340
lesculentum	THWEMYAIGG	SAAPTINSQOG	NRFLAPNDRF	AKEVTKRSD	AQESSEKKK	344
njjs25	SADPTINSQOG	SAAPTINSQOG	NRFLAPNDRF	AKEVTKRSD	AQESSEKKK	381
zmays	THWEMYAIGG	NMNPPTINSQOG	NRFLAPNDRF	AKEVTKRSD	AQESSEKKK	380
llongiflorum	THWEMYAIGG	SOHPPTINSQOG	NRFLAPNDRF	AKEVTKRSD	AQESSEKKK	376
ntabacum	THWEMYAIGG	SSGAPTINSQOG	NRFLAPNDRF	AKEVTKRSD	AQESSEKKK	338
jcedar	DPPTAIGG	SSNPPTILS	NSPTAFNESY	KEVVTKRSD	KTSSSC.SN	315
macuinata	NWRSEGDQML	NGAYFFTPSCA	GASSS..HAK	ASSLGPRSSS	LVGTSTTVSAG	388
lesculentum	NWRSEGDQML	NGAYFFTPSCA	GASSS..HAK	ASSLGPRSSS	LVGTSTTVSAG	394
njjs25	NWRSEGDQML	NGAYFFTPSCA	GASSS..HAK	ASSLGPRSSS	LVGTSTTVSAG	396
zmays	VWKESQGDQML	NGAYFFTPSCA	GASSS..HAK	ASSLGPRSSS	LVGTSTTVSAG	428
llongiflorum	TWKESQGDQML	NGAYFFTPSCA	GASSS..HAK	ASSLGPRSSS	LVGTSTTVSAG	424
ntabacum	TWKESQGDQML	NGAYFFTPSCA	GASSS..HAK	ASSLGPRSSS	LVGTSTTVSAG	387
jcedar	VWKESQGDQML	NGAYFFTPSCA	GASSS..HAK	ASSLGPRSSS	LVGTSTTVSAG	364
macuinata	VLSCKKCSR	398				
lesculentum	PVNCCKKCSR	404				
njjs25	..	396				
zmays	PVNCCKKCSR	438				
llongiflorum	AVNCCKKCSR	434				
ntabacum	AVNCCKKCSR	397				
jcedar	VVNCCKKCSR	374				

Figure 2. Comparison of the amino acid sequence of the putative strawberry pectate lyase vs amino acid sequences of putative higher plant pectate lyases using the PrettyBox program. Consensus amino acids are in black. Amino acid homologous substitutions are shaded. I and II, highly conserved amino acid motifs in all higher plant pectate lyase like genes. The accession numbers for the sequences used in the comparison are: X92943 for *Macuinata*, X55193 for *Lycopersicon esculentum*, L20140 for *Zea mays*, Z17328 for *Lilium longiflorum*, P40972 for *Nicotiana tabacum* and D26544 for Japanese cedar.

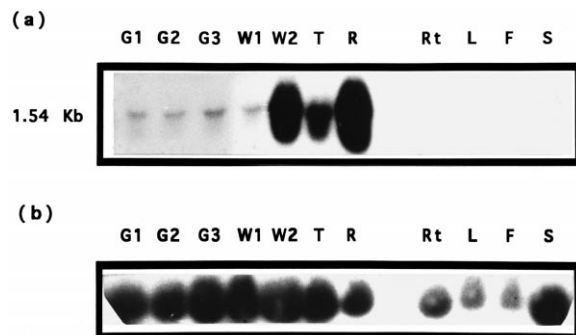


Figure 3. Developmental and spatial expression of putative strawberry pectate lyase gene. Northern analysis of 20 μ g of total RNA isolated from pooled samples of G1, G2, G3, W1, W2, T and R strawberry fruit ripening stages and from roots (Rt), leaves (L), flowers (F) and stolons (St). Size of hybridizing transcript is indicated in kb. a. Northern blot hybridized with the ^{32} P-labelled njjs25 probe. b. The same blot hybridized with a ^{32} P-labelled 18S RNA probe as a control. Hybridization and stringency conditions are described in Materials and methods under Northern analysis.

tein and all potential pectate lyase genes from higher plants and *Erwinia* spp. are also present in the deduced strawberry protein at amino acids residues 262–281 and 317–335 (Figure 2) [6, 27, 29, 31, 34]. These two motifs correspond to the reported substrate-binding region and the specific residues thought to be associated with the binding of Ca^{2+} [31]. These facts strongly suggest that the njjs 25 cDNA corresponds to an strawberry mRNA encoding a putative pectate lyase protein.

Gene expression studies

The spatial and temporal expression patterns of the identified strawberry putative pectate lyase gene have been studied. As shown in Figure 3, the pectate lyase transcript estimated to be 1.54 kb is predominantly expressed in fruits, showing the maximum levels of expression at the full ripe red stage (R). Expression levels in roots, flowers, stolons and leaves were not observed. This expression pattern is consistent with a role for an encoded pectate lyase in the degradation of middle lamella and cell wall pectins that occurs in the strawberry late ripening stages and that leads to fruit softening [19].

In order to ascertain a possible involvement of pectate lyase in the achene metabolism along the ripening process, northern experiments were performed with mRNA isolated from total fruit (achenes plus receptacle), de-achened fruit (receptacle) and removed achenes. As shown in Figure 4, pectate lyase expres-

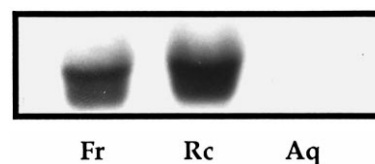


Figure 4. Achenes and receptacle expression levels of the putative strawberry pectate lyase gene. Northern analysis of 20 μ g of total RNA isolated from complete fruit (Fr), receptacle (Rc) and achenes (Aq). Blot was hybridized with ^{32} P-labelled probe corresponding to the putative pectate lyase cDNA (njjs25 clone). The amount of RNA loaded on the gel was controlled by hybridization of the same blot with a ribosomal probe as in Figure 3.

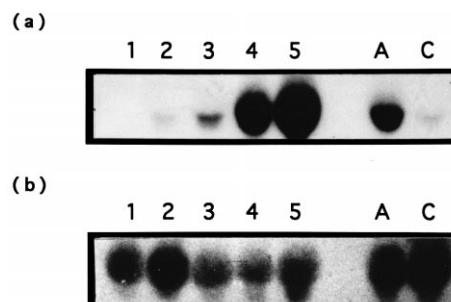


Figure 5. Effects of removing achenes and of auxin-treatment on putative pectate lyase gene expression. Northern analysis of 20 μ g of total RNA isolated from G2-stage strawberry fruit after removing the achenes. 1, 0 h; 2, 24 h; 3, 48 h; 4 72 h; 5 96 h; A, 96 h G2-stage strawberry de-achened fruit treated with NAA; C, G2-stage strawberry fruit with achenes. (a) (b): as described in the legend to Figure 3.

sion was only found in total fruit and receptacle indicating that pectate lyase is not a seed enzyme.

As well as other strawberry fruit genes [20, 25, 26], this putative pectate lyase gene appears to be under the control of auxins. A clear increase in the expression of the pectate lyase gene was found in strawberry G2 de-achened fruits. However, a reduction in the expression was observed in G2 de-achened fruits treated with NAA, and practically no expression was found in G2 normal fruits (with achenes) (Figure 5). The incapacity to revert completely the pectate lyase gene expression by NAA in G2 de-achened fruit can be explained by an incomplete penetration and/or degradation in the receptacle of the NAA.

Southern blot analysis

By using the same probe previously utilized in northern blot studies, we have detected several DNA fragments that hybridized on a Southern blot of genomic strawberry DNA digested with *Bam*HI, *Bgl*III, *Eco*RI

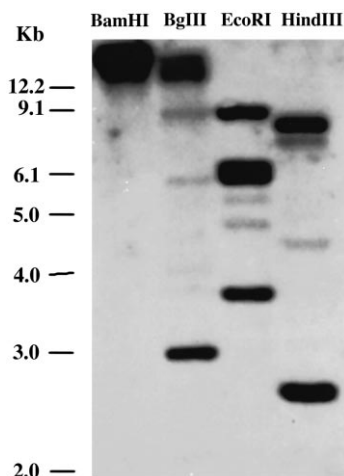


Figure 6. Southern blot of *Fragaria* × *ananassa* cv. Chandler genomic DNA (2 µg) digested with several restriction enzymes and hybridized with the ³²P-labelled strawberry putative pectate lyase probe (njjs25 clone). Hybridization and stringency conditions are described in Materials and methods under Southern blot analysis.

and *HindIII*. Hybridization fragments are all of a large size, ranging between 4 to 6 kb (Figure 6). However, no restriction sites were found in the strawberry cDNA sequence for *BamHI*, *BglIII*, and *EcoRI* and *HindIII*, which suggests that the strawberry putative pectate lyase is probably encoded by a small multigene family. However, the hybridization pattern observed could also be related with the ploidy level of *Fragaria* × *mananassa* cv. Chandler cultivar (octaploid). That issue remains to be further investigated but, nevertheless, at least two different pectate lyase-like genes have been reported in higher plants [27, 31, 34].

Yeast expression of putative strawberry pectate lyase cDNA

The expression in yeast of the strawberry putative pectate lyase cDNA produced an extracellular biologically active pectate lyase activity. Thus, no pectate lyase activity was detected in the culture medium from untransformed JB 811 cells. However, a clear pectate lyase activity ($0.35 \mu\text{mol min}^{-1} \text{ml}^{-1}$) was observed in the culture medium of JB 811 cells transformed with the recombinant plasmid DGL3-PL 60 h after inoculation. Moreover, at this stage most of the JB 811 transformed cells in these cultures were indeed protoplasts, thus indicating degradation of the yeast cell walls (data not shown). However, practically, no protoplasts were observed in cultures of JB 811 wild-type yeast cells.

This data confirm that the putative strawberry pectate lyase cDNA correspond to a protein with pectate lyase activity. At present the isolation, characterization and purification of this recombinant protein is being performed.

Discussion

In the present paper, we report the isolation and characterization of a strawberry cDNA which contains an ORF amino acid sequence highly homologous to other putative pectate lyase from higher plants. Furthermore, we present evidence of a possible relationship between the expression of this gene and the fruit softening process during strawberry fruit ripening. To our knowledge this is the first time a predominantly expressed fruit pectate lyase cDNA has been isolated from higher plants.

Moreover, the yeast expression studies clearly show that the putative strawberry pectate lyase cDNA corresponds to a protein with pectate lyase activity. At present the isolation, characterization and purification of this recombinant protein is in progress.

During fruit ripening, it is clearly established that cell wall degradation leads to fruit softening [30]. At present, some hydrolytic enzymes, such as polygalacturonase, pectin methyl esterase and cellulose, which catalyse the degradation of pectins or other cell wall polysaccharides, such as cellulose, have been implicated in fruit softening [30]. However, antisense experiments in tomato with cDNAs corresponding to the genes that encode some of these enzymes did not produce a significant reduction in fruit softening [12]. These results seem to indicate that other enzymes not yet characterized could be responsible for the process of fruit softening. In strawberry, an increase in the hydration and disorganization of the cell wall and the enhanced solubility of the middle lamella and wall matrix during ripening accompany fruit softening [19]. Furthermore, a parallel increase in several cell wall hydrolytic enzyme activities with the fruit softening was observed [19]. Moreover, at present the involvement of a pectate lyase enzyme in the degradation of fruit cell walls has not been reported.

In higher plants, a pectate lyase gene is known to be expressed late in pollen development together with genes coding for other pectin degrading enzymes, such as pectin esterase and polygalacturonase [21, 22, 24, 27, 31, 34]. We have not tested the expression of this strawberry gene (njjs25) in mature pollen nor pol-

len tubes. Nevertheless, under our experimental conditions, we have not found any significant expression of this gene in flowers (a sample that includes the pollen tissue). Perhaps the amount of pollen mRNA present in these samples is not sufficient to detect this expression. Accordingly, the possibility that this strawberry gene is expressed in pollen must not be excluded. However, the predominantly fruit-specific expression pattern of the putative strawberry pectate lyase gene, the increasing of expression found during the ripening process (W2 and T stages), and the high expression levels in the R stage indicate a clear relationship between the expression of this gene and fruit ripening. This expression pattern is similar to the expression pattern of other pectin-degrading enzymes in strawberry such as pectin methyl esterase whose activity levels increase during fruit ripening and fruit softening [19]. Also, an increase in cellulase activity during fruit ripening and softening has been described [19]. In contrast, low levels of polygalacturonase were found during the fruit ripening [19]. All of these findings support the hypothesis that the putative pectate lyase reported here plays an important role in the strawberry fruit softening, which is strengthened by the fact that the expression of this strawberry gene is almost entirely restricted to receptacle cells where the fruit softening process occurs.

Furthermore, it has been proposed that strawberry development and ripening processes are under auxin regulation, which stimulates expansion of the receptacle but inhibits ripening [3, 11, 19, 32]. The auxins are synthesized and released by the achenes promoting fruit elongation [3, 11, 19, 23]. Thus, the induction of mRNA populations through strawberry fruit ripening is also produced by removing achenes from unripe fruits [20]. In contrast, the application of the synthetic auxin 1-naphthaleneacetic acid to de-achened receptacle produces a translation pattern similar to that of mature green fruit, suggesting that there exists a relationship between ripening gene expression and the absence of auxin [20]. Other strawberry fruit-expressed genes with no homology with other identified genes are also under auxin control [25, 26]. Recently, a strawberry gene encoding an auxin-binding protein probably involved in auxin perception has been isolated [7]. Similarly, the expression pattern of the strawberry putative pectate lyase gene reported here is negatively regulated by auxin and thus it is putatively induced by the absence of biosynthesis of auxin in the achenes. Thus, the expression of this gene was induced in unripe green fruit by removing the fruit achenes. However, the application of NAA to the de-achened unripe fruits

partially inhibited the expression of the pectate lyase gene, suggesting that, like other strawberry ripening-related genes, the expression of this gene is negatively regulated by the auxins synthesized in the achenes.

In summary, we propose that pectate lyase, in association with other pectin-degrading enzymes, could be responsible for pectin degradation of the middle lamella and cell wall in the strawberry fruit, which results in fruit softening. This proposal is supported by the fact that the gene expression of a pectate lyase like gene occurs predominantly in fruit, with little gene expression in unripe stages nor in other plant tissues. Moreover, like other putative higher-plant pectate lyase genes our putative strawberry pectate lyase protein has an hydrophobic amino terminal region suggesting that this protein may be targeted for secretion to the cell wall [27, 31, 34].

Experiments with antisense RNA are in progress to assess the involvement of this putative pectate lyase gene in the strawberry ripening process.

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References

1. Abeles FB, Takeda F: Cellulase activity and ethylene in ripening strawberry and apple fruits. *Scient Hort* 42: 26–75 (1990).
2. Alfano JR, Ham JH, Collmer A: Use of Tn5tac1 to clone a *pel* gene encoding a highly alkaline asparagine-rich pectate lyase isozyme from an *Erwinia chrysanthemi* EC16 mutant with deletions affecting the major pectate lyase isozymes. *J Bact* 4553–4556 (1995).
3. Archbold DD, Dennis FG: Quantification of free ABA and free and conjugated IAA in strawberry achene and receptacle tissue during fruit development. *J Am Soc Hort Sci* 109: 330–335 (1984).
4. Archer SA: Pectolytic enzymes and degradation of pectin associated with breakdown of sulphited strawberries. *J Sci Food Agric* 30: 692–703 (1979).

5. Barnes MF, Patchett BJ: Cell wall degrading enzymes and the softening of senescence strawberry fruit. *J Food Sci* 41: 1392–1395 (1976).
6. Barras K, Van Gijsegem F, Chatterjee AK: Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. *Annu Rev Phytopath* 32: 201–234 (1994).
7. Collmer A, Keen NT: The role of pectic enzymes in plant pathogenesis. *Annu Rev Phytopath* 24: 383–409 (1986).
8. Devereux J, Haeberli P, Smithies O: A comprehensive set of sequence analysis programs for the Vax. *Nucl Acids Res* 12: 387–395 (1984).
9. Gallie DR: Post-transcriptional regulation of gene expression in plants. *Annu Rev Plant Physiol Plant Mol Biol* 44: 77–105 (1993).
10. Gietz D, Jean A, Woods RA, Schieste RH: Improved method for high efficiency transformation of intact yeast cells. *Nucl Acids Res* 20: 1425 (1992).
11. Given NK, Venis MA, Grierson D: Hormonal regulation of ripening in the strawberry, a non-climateric fruit. *Planta* 174: 402–406 (1988).
12. Gray JE, Picton S, Giovannoni JJ, Grierson D: The use of transgenic and naturally occurring mutants to understand and manipulate tomato fruit ripening. *Plant Cell Environ* 17: 557–571 (1994).
13. Huber DJ: Strawberry fruit softening: the potential roles of polyuronides and hemicelluloses. *J Food Sci* 49: 1310–1315 (1984).
14. Kelemu S, Collmer A: *Erwinia chrysanthemi* EC16 produces a second set of plant-inducible pectate lyase isozymes. *Appl Environ Microbiol* 59: 1756–1761 (1993).
15. Knee M, Sargent JA, Osborne DJ: Cell wall metabolism in developing strawberry fruits. *J Exp Bot* 28: 377–396 (1977).
16. Kobayashi Y, Komae K, Tanabe H, Matsuo R: Approach to maceration mechanism in enzymatic pulping of bast fibers by alkalophilic pectinolytic enzymes produced by *Erwinia* species. *Biotechnol Adv* 6: 29–37 (1988).
17. Lazarus CM, Macdonald H: Characterization of a strawberry gene for auxin-binding protein, and its expression in insect cells. *Plant Mol Biol* 31: 267–277 (1996).
18. Manning K: Isolation of nucleic acids from plants by differential solvent precipitation. *Anal Biochem* 195: 45–50 (1991).
19. Manning K: Soft Fruits. In: Seymour GB, Taylor JE, Tucker GA (eds) *Biochemistry of Fruit Ripening*, pp. 347–373. Chapman and Hall, Cambridge, UK (1993).
20. Manning K: Changes in gene expression during strawberry fruit ripening and their regulation by auxin. *Planta* 194: 62–68 (1994).
21. Mu J-H, Stains JP, Kao T: Characterization of a pollen-expressed gene encoding a putative pectin esterase of *Petunia inflata*. *Plant Mol Biol* 25: 539–544 (1994).
22. Niogret MF, Dubald M, Mandaron P, Mache R: Characterization of pollen polygalacturonase encoded by several cDNA clones in maize. *Plant Mol Biol* 17: 1155–1164 (1991).
23. Nitsch JP: Free auxins and free tryptophan in the strawberry. *Plant Physiol* 30: 33–39 (1955).
24. Pressey R, Reger BJ: Polygalacturonase in pollen from corn and other grasses. *Plant Sci* 59: 57–62 (1989).
25. Reddy ASN, Poovaiah BW: Molecular cloning and sequencing of a cDNA for an auxin-repressed mRNA: correlation between fruit growth and repression of the auxin-regulated gene. *Plant Mol Biol* 14: 127–136 (1990).
26. Reddy ASN, Jena PK, Mukherjee SK, Poovaiah BW: Molecular cloning of cDNAs for auxin-induced mRNAs and developmental expression of the auxin-inducible genes. *Plant Mol Biol* 14: 643–653 (1990).
27. Rogers HJ, Harvey A, Lonsdale DM: Isolation and characterization of a tobacco gene with homology to pectate lyase which is specifically expressed during microsporogenesis. *Plant Mol Biol* 20: 493–502 (1992).
28. Speirs J, Longhurst T: RNA extraction and fractionation. In: Dey PM, Harborne JM (eds) *Methods in Plant Biochemistry*, Vol. 10, pp. 1–33. Academic Press, New York (1993).
29. Taniguchi Y, Ono A, Sawatani M, Nanba M, Khono K, Usui M, Kurimoto M, Matuhasi T: Cry I, a major allergen of Japanese cedar pollen, has pectate lyase enzyme activity. *Allergy* 50: 90–93 (1995).
30. Tucker GA: Introduction. In: Seymour GB, Taylor JE, Tucker GA (eds) *Biochemistry of Fruit Ripening*, pp. 347–373. Chapman and Hall, Cambridge, UK (1993).
31. Turcich MP, Hamilton DA, Mascarenhas JP: Isolation and characterization of pollen-specific maize genes with sequence homology to ragweed allergens and pectate lyases. *Plant Mol Biol* 23: 1061–1065 (1993).
32. van Rensburg P, van Zyl WH, Pretorius IS: Expression of the *Butyrivibrio fibrisolvens* endo- β -1,4-glucanase gene together with *Erwinia* pectate lyase and polygalacturonase genes in *Saccharomyces cerevisiae*. *Curr Genet* 27: 17–22 (1994).
33. Von Heijne G: A new method for predicting signal sequence cleavage sites. *Nucl Acids Res* 14: 4683–4690 (1986).
34. Wing RA, Yamaguchi J, Larabell SK, Ursin VM, MacCormick S: Molecular and genetic characterization of two pollen-expressed genes that have sequence similarity to pectate lyases of the plant pathogen *Erwinia*. *Plant Mol Biol* 14: 17–28 (1989).
35. Yoder MD, Keen NT, Jurnak F: New domain motif: the structure of pectate lyase C, a secreted plant virulence factor. *Science* 260: 1503–1507 (1993).