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# Characterization of cCel3, a member of the pepper endo-β-1,4-glucanase multigene family

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In pepper plants the enzyme endo-β-1,4-glucanase (EGase) is encoded by a multigene family. Here is described the characterization of cCel3, a cDNA which codes for the third EGase known so far in this plant. The known members of this family are present as single copy genes, as demonstrated by a Southern analysis of the genomic DNA. Analysis of the expression of cCel3 demonstrates that the highest levels of the cCel3 mRNA are found in abscission zones of leaves and flowers activated by treatments with the plant hormone ethylene. However, the amounts of the cCel3 transcripts (detectable only by RT-PCR) are always much lower than those of cCel2 so that the latter can be regarded as the "abscission" EGase while cCel3 is likely to perform a role ancillary to that of cCel2.

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In plants many important physiological processes require modifications of the structural properties of the cell walls (FRY 1995; BRETT and WALDRON 1996). These include growth of cells, softening of fleshy fruits, abscission of leaves, flowers and fruits, development of reproductive organs, and others. Due to the biochemical nature of the parietal components, most of the cell wall modifying enzymes are hydrolases which act at the level of the polysaccharidic components.

Though a certain type of enzymatic activity may be remarkable in a given physiological process, it is known that more than one hydrolase can contribute to carry out the process. For instance, the softening of tomato fruits is largely due to the activity of polygalacturonase (TUCKER et al. 1980), but also pectinmethylesterase (HALL et al. 1960), endo-β-1,4-glucanase (DICKINSON and MCCOLLUM 1964), and β-galactosidase (PRESSEY 1983) activities are expressed during the ripening of the tomato berries.

In the last few years such a complex situation was shown to be even more intricate, following the finding that in a plant the same enzymatic activity can be the result of the concomitant expression of divergent members of a polymorphic multigene family (BRUMMELL et al. 1994; LASHBROOK et al. 1994; XU et al. 1996; TRAINOTTI et al. 1997; BRUMMELL et al. 1997).

Recently, three cDNA fragments encoding different endo-β-1,4-glucanases (EGases, also called cellulases; E.C. 3.2.1.4) have been obtained by RT-PCR from pepper RNA, and their cognate genes have been shown to have ethylene-inducible expression (FERRARESE et al. 1995). In particular, the CX1-related gene was expressed at rates detectable by northern analysis during the ripening of fruits (FERRARESE et al. 1995), while CX2 seemed to be especially important for the abscission of leaves (FERRARESE et al. 1995) and flowers (TRAINOTTI et al. 1998). The low expression of the third EGase (CX3) was evidenced only by RT-PCR in the abscission zones of leaves (FERRARESE et al. 1995).

Here we describe the characterization of cCel3, a full length cDNA clone corresponding to the PCR fragment CX3 previously described (FERRARESE et al. 1995). The cCel3 structure, amino acid sequence and genomic organization have been compared with those of cCel1 (HARPSTER et al. 1997; TRAINOTTI and CASADORO, EMBL accession. No.: X97188) and cCel2 (TRAINOTTI et al. 1998), the two EGases of pepper already characterized.

## MATERIALS AND METHODS

### *Plant material*

Plants of *Capsicum annuum* var. *longum* (DC) Sendt. used in this research were grown in pots at the botanic garden of Padua. Tissues were either collected fresh (control) or after placing whole plants in a sealed chamber and flushing them with ethylene (100 µl/l) in air at a flow rate of 6 l/h (treated). In the case of fruits, they were treated with ethylene after harvest. Samples were frozen in liquid nitrogen and stored at −80°C.

### *RNA extraction, gene expression analysis and cDNA library*

Total RNA was extracted as described (LOGEMAN et al. 1987). RNA quantification was checked by means of ethidium bromide staining of agarose gels (not shown). Then equal RNA loading was obtained

by normalizing the non-saturating light intensities of the 28S ribosomal RNAs using the Gel Doc 1000 system (Bio Rad, USA) with the dedicated software (Molecular Analyst). Northern analyses were carried out as described (TRAINOTTI et al. 1998).

1.2 µg of DNase 1 (Pharmacia Biotech, Sweden) treated total RNA were used as starting material for the RT-PCR experiments. The first-strand synthesis was carried out with the "SuperScript" kit (Life Technologies, USA) using a 12–18 mers oligo(dT) primer. 2 µl (out of 20) of the first-strand reaction were used for the subsequent PCR amplification. PCR reactions with gene-specific primers (LT51[CATCTTGGGAGATAATCCTC] and LT88[GAGCAGACCATGGATGTTGTG], 10 pM each), 1.5 mM $MgCl_2$ and 1 unit of DynaZyme DNA polymerase (Finnzyme Oy, Finland) were performed in 50 µl volumes using the apparatus "DNA Thermal Cycler" (Perkin Elmer, USA). Denaturation, annealing and extension temperatures were 95°C for 1 min, 60°C for 1 min, and 72°C for 45 s, respectively. This cycle was repeated 25 times. The PCR products (10 µl) were separated by gel electrophoresis, blotted onto Hybond-$N^+$ (Amersham, United Kingdom) membranes and subjected to Southern analysis with a gene specific probe. Hybridization and washing conditions were the same as for northern, but for omission of formamide and temperature raised to 68°C. Filters were then exposed to the electronic screen of the "InstantImager" (Packard, USA) apparatus and band intensities were calculated with the dedicated "Imager" software (Packard). The obtained values were then normalized as percent of the highest one.

A leaf abscission zone cDNA library (TRAINOTTI et al. 1998) was screened using the EGase fragment CX3 (FERRARESE et al. 1995) as probe.

*DNA sequencing and analysis*

DNA sequencing was performed using the T7 DNA sequencing kit (Pharmacia). All the clones were sequenced on both strands using, when necessary, chemically synthesised oligonucleotides to cover the whole length of the clone. Sequence manipulations, analyses, and alignments were performed using the "Lasergene" software package (DNASTAR, USA).

*Genomic DNA extraction and Southern analysis*

DNA was extracted from pepper leaves as described in DELLAPORTA et al. (1983). Aliquots of 10 µg were digested with 100u of the indicated endonucleases for 3 h. The restricted DNA was resolved on an 0.8% agarose gel and transferred to a Hybond $N^+$ (Amersham) membrane using 0.4M NaOH as blotting agent. Hybridisation conditions were the same as for the RT-PCR experiments. The membranes were washed at high stringency (15 min with 0.5 × SSC, 0.1% SDS, 65°C for the final wash), and afterwards they were exposed to x-ray films at −80 °C.

## RESULTS AND DISCUSSION

A cDNA library representing transcripts from ethylene-activated abscission zones of pepper leaves (TRAINOTTI et al. 1998) was screened using as a probe the CX3 cDNA fragment (FERRARESE et al., 1995). A 2352 bp clone was isolated, named cCel3 (*C*apsicum annuum *Cel*lulase *3*) and sequenced on both strands (EMBL accession No.: X97189).

Analysis of the sequence revealed that this clone owes its large size to the fact that it is a chimera. Besides the entire open reading frame (1458 bp) and 5′ and 3′ (188 bp) untranslated regions (UTRs), cCel3 also contains at the 5′ end a fragment of approximately 650 bp unrelated to the cCel3 EGase. In particular, when this fragment is used for a similarity search against the GeneBank database it gives significant homology (BLAST probability score: 4.4e − 38, sequence identity 67%) with an arabidopsis EST (expressed sequence tag, *Arabidopsis thaliana* cDNA clone 110L15T7, Accession No.: T42137). The open reading frame of cCel3 encodes a polypeptide of 53.6 kDa (or 51.4 kDa without the putative signal peptide) whose isoelectric point is 8.46 (8.15 without the putative signal peptide).

To better elucidate the genomic organization of the three pepper EGases isolated by us (i.e., cCel1, cCel2, and cCel3), Southern analyses were carried out by using their entire cDNAs as probes. These analyses (Fig. 1) indicate that all the three EGase genes are present in single copy in the pepper genome. The result obtained with cCel1 confirms data by HARPSTER et al. (1997), but the restriction pattern in the *Eco* R I cut is slightly different, probably because of cultivar variability (same reason may explain the little sequence variation observed at the DNA level between cCel1 and PCEL1).

Multiple alignment (Fig. 2) and analysis of the deduced amino acid sequences of the three pepper EGases revealed a high degree of divergency among them. cCel3 has a percent similarity of 45.2 to cCel1 and 54.4 to cCel2, while cCel1 and cCel2 share a similarity of 49.9. Both cCel3 and cCel1 sequences have putative N-glycosylation sites, while no such site is present in cCel2.

Based on the sequence characteristics, all the three pepper enzymes belong to the cellulase family E (HENRISSAT et al., 1989), also known as the glycosyl hydrolases family 9 (HENRISSAT 1991). This family can have two different active sites whose sequences

are named signature 1 and signature 2, respectively. It is interesting to note that all three proteins contain the consensus sequence for signature 2 (Fig. 2, dark-shaded box) while, unlike cCel1 and cCel2, the cCel3 protein does not contain the consensus sequence for signature 1 (Fig. 2, light-shaded box).

Here and in previous studies (FERRARESE et al. 1995) it has never been possible to show by northern analysis the expression of the cCel3-related transcripts. In this research, expression analyses (performed first by northern and then by RT-PCR techniques) were carried out in a broader range of tissues (including roots, elongating hypocotyls, auxin treated young green fruits, abscission zones, flowers and fruits) and mRNA expression could only be evidenced in those tissues where ethylene-induced EGase activity is commonly found (Fig. 3). However,

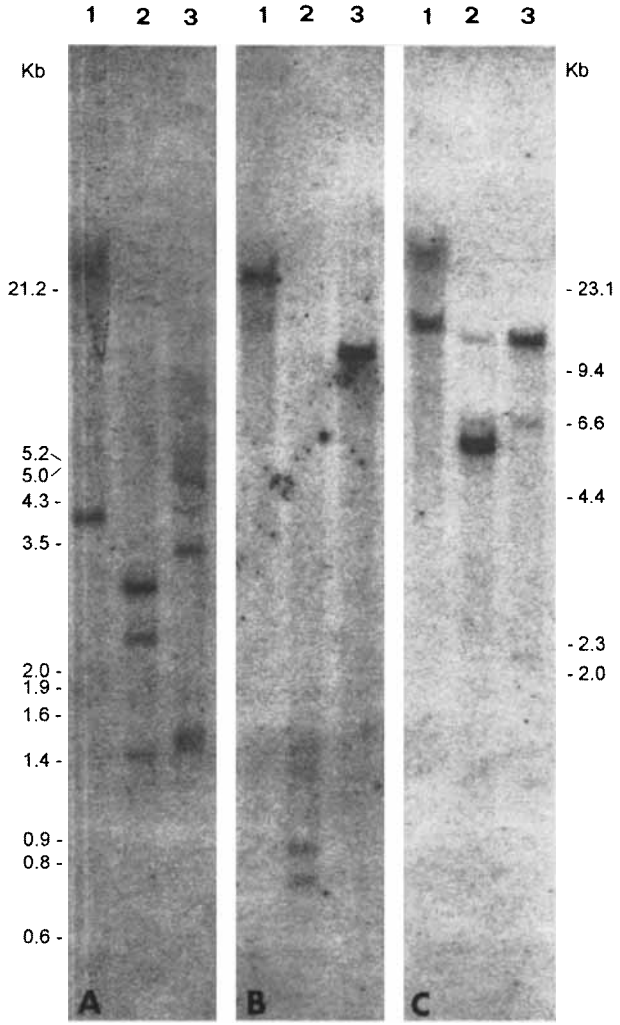


**Fig. 1.** Southern blot analysis of the three pepper EGases: cCel1 (Panel A), cCel2 (Panel B) and cCel3 (Panel C). Genomic DNA (10 µg) was restricted with *Bam* H I (1), *Eco* R I (2) and *Hin*d III (3) endonucleases. DNA molecular size markers are indicated on both sides.

in all the assayed tissues the expression of cCel3 was too low to be detected by northern analysis on total RNA, so we used the more sensitive RT-PCR technique to reveal expression of this elusive EGase in order to try to understand its possible physiological role.

cCel3 is expressed in abscission zones of both flowers and leaves, and the induction of abscission by treatment with exogenous ethylene enhances the mRNA amount (Fig. 3), in accordance with the idea that the expression of its cognate gene can be promoted by ethylene (FERRARESE et al. 1995). A qualitatively similar behaviour had been observed for cCel2 in both types of abscission zones (FERRARESE et al. 1995; TRAINOTTI et al. 1998) while cCel1 seemed less influenced by ethylene in these tissues (FERRARESE et al. 1995; HARPSTER et al. 1997)

It is worth pointing out that, due to the high expression differences of the gene in the various tested tissues, it was impossible to avoid saturating condition for some samples (e.g., LAZT) as PCR cycles could not be reduced too much in order to detect the low expression present in some samples. Anyway, to some extent, the preeminent role of cCel2 over cCel3 during the shedding process can be approximately quantified by comparing the number of positive clones obtained from the screening of the same cDNA library with the two different genes. In particular, the screening of 60,000 plaques with CX2 (ca. 200 bp long) yielded 32 positive clones (0.053%) while only 3 positive clones (0.004%) were obtained by screening 80,000 plaques with the CX3 probe (ca 900 bp long). In our opinion, this result reflects the low expression of cCel3 (less than 10% of cCel2) and suggests that the role of this gene can be ancillary to that of cCel2 during the leaf abscission process.

Also the flower components show expression of the cCel3 gene with the highest level observed in the ovary following treatment with exogenous ethylene (Fig. 3). It is interesting to note that, at the level of the flower corolla, ethylene decreases the expression of the cCel3 mRNA. Such a finding appears particularly interesting since it shows that, besides hormones, also tissue specificity is important to regulate the expression of this gene.

This idea obtains further confirmation by the expression pattern observed in fruits. During the ripening process, both natural and ethylene-induced, it is only the cCel1 gene which has high levels of expression in the fruit tissues, while transcripts of the other two EGase genes remain barely detectable (FERRARESE et al. 1995; HARPSTER et al. 1997). If we consider young developing fruits, treatments with exogenous ethylene cause an increase of cCel1 mRNA (HARPSTER et al. 1997), but a decrease in the case of the cCel3 mRNA (Fig. 3).

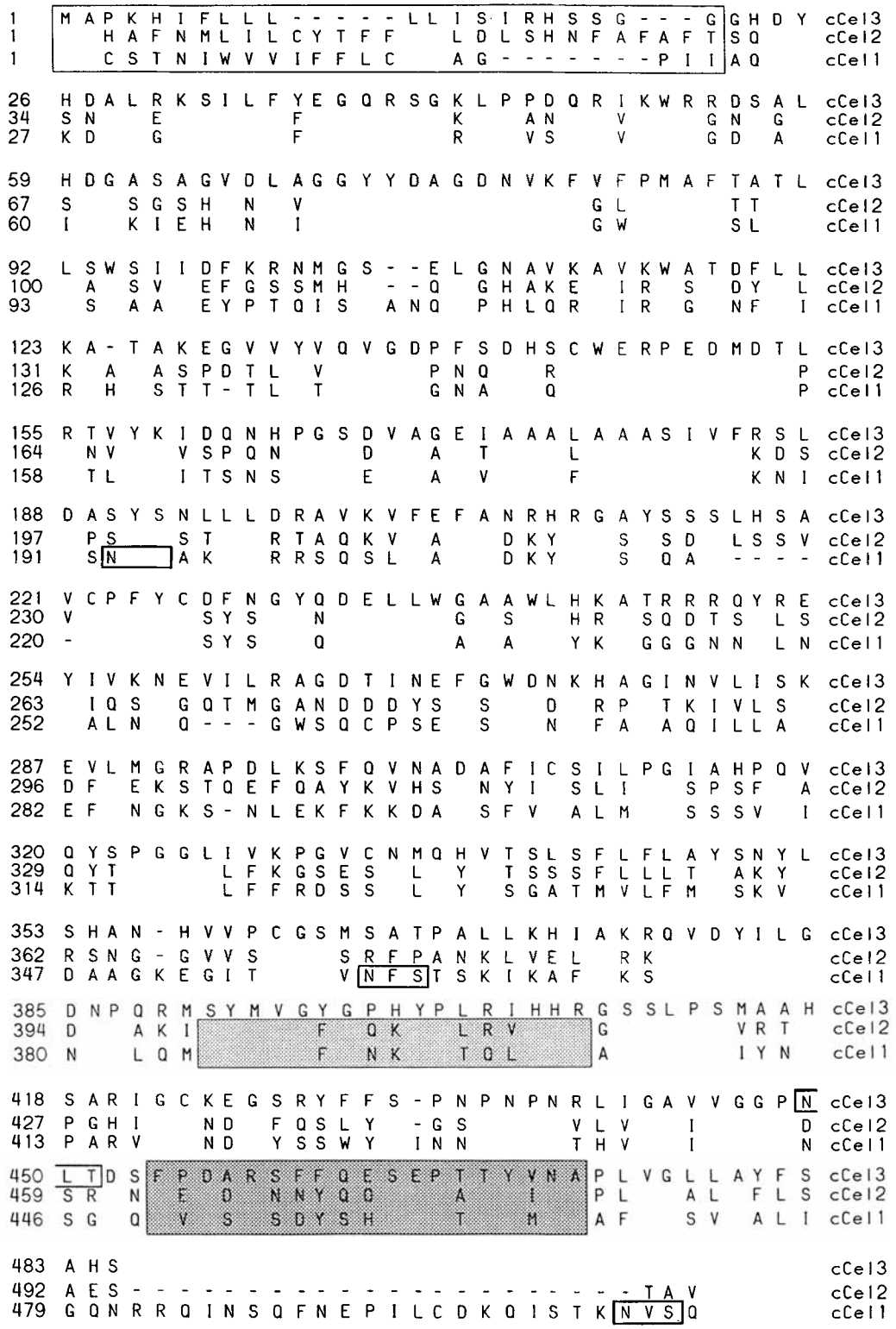


**Fig. 2.** CLUSTAL V alignment of the deduced amino acid sequence of cCel3 with the other two known pepper EGases. Empty spaces under the cCel3 sequence indicate amino acid identity within the three sequences, while dashes within sequences have been introduced by the programme to optimise the alignment. The putative N-glycosylation sites (N-X-S/T) are enclosed in small boxes. The rectangle on the top of the Figure indicates the putative signal peptides as determined by the "Lasergene" software according to VON HEIJNE (1983), while the two at the bottom of the sequences indicate the putative active sites, named Signature 1 (light shaded) and Signature 2 (dark shaded) according to HENRISSAT et al. (1989). Numbers on the left indicate amino acid positions.

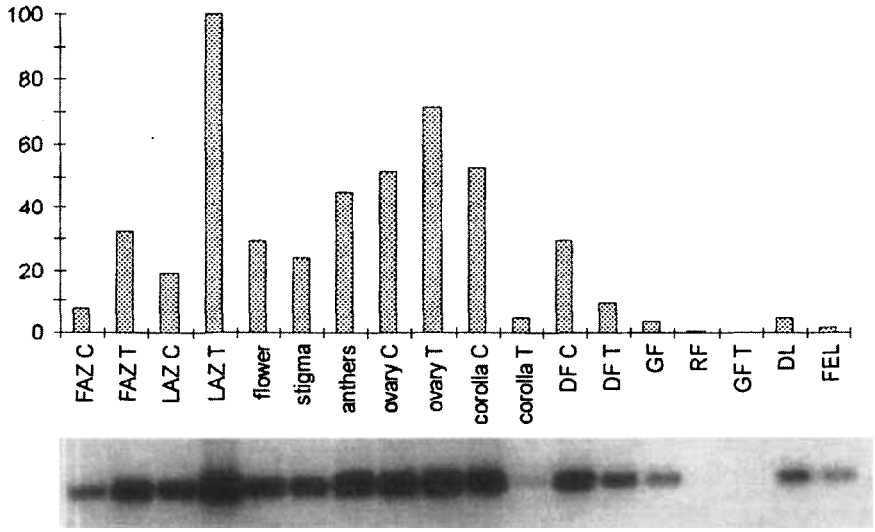


**Fig. 3.** RT-PCR expression analysis of cCel3. Tissue were: FAZ: flower abscission zones; LAZ: leaf abscission zones; DF: developing fruits; GF: mature-green fruits; RF: ripe red fruits; DL: developing leaves; FEL: fully expanded leaves. In some cases the same tissue was treated either with the hormone ethylene (T) or used without any treatment (C).

The above described pattern of expression clearly evidences differences in the behaviour of the three EGases. In our opinion, very important are those relative to the fruit ripening and the abscission of flowers and leaves. In fact, it is clear that cCel1 is the pepper "ripening" EGase (FERRARESE et al. 1995; HARPSTER et al. 1997), while in the leaf and flower shedding process the "abscission" cCel2 (FERRARESE et al. 1995; TRAINOTTI et al. 1998) co-operates with cCel3. It thus appears that, in the tissues examined, cCel3 performs a role ancillary to cCel2 (and to a lesser extent to cCel1) rather than a prominent role on its own.

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