Characterization of Two Divergent Endo-β-1,4-Glucanase cDNA Clones Highly Expressed in the Nonclimacteric Strawberry Fruit

Immaculada Llop-Tous, Eva Domínguez-Puigjaner, Xavier Palomer, and Miquel Vendrell*

Departmento de Agrobiologia, Centro de Investigación y Desarrollo, Consejo Superior de Investigaciones Científicas, Jordi Girona, 18–26, 08034 Barcelona, Spain

Two cDNAs clones (Cel1 and Cel2) encoding divergent endo-β-1,4-glucanases (EGases) have been isolated from a cDNA library obtained from ripe strawberry (Fragaria x ananassa Duch) fruit. The analysis of the amino acid sequence suggests that Cel1 and Cel2 EGases have different secondary and tertiary structures and that they differ in the presence of potential N-glycosylation sites. By in vitro translation we show that Cel1 and Cel2 bear a functional signal peptide, the cleavage of which yields mature proteins of 52 and 60 kD, respectively. Phylogenetic analysis revealed that the Cel2 EGase diverged early in evolution from other plant EGases. Northern analysis showed that both EGases are highly expressed in fruit and that they have different temporal patterns of accumulation. The Cel2 EGase was expressed in green fruit, accumulating as the fruit turned from green to white and remaining at an elevated, constant level throughout fruit ripening. In contrast, the Cel1 transcript was not detected in green fruit and only a low level of expression was observed in white fruit. The level of Cel1 mRNA increased gradually during ripening, reaching a maximum in fully ripe fruit. The high levels of Cel1 and Cel2 mRNA in ripe fruit and their overlapping patterns of expression suggest that these EGases play an important role in softening during ripening. In addition, the early expression of Cel2 in green fruit, well before significant softening begins, suggests that the product of this gene may also be involved in processes other than fruit softening, e.g. cell wall expansion.

Fruit softening during ripening is a major factor contributing to postharvest deterioration. Loss of firmness in fruits is mainly due to cell wall disassembly, resulting in a significant increase in polyuronide and hemicellulose solubilization. The mechanisms by which this solubilization occurs are unclear and may differ between species. In tomato, the best-studied fruit, polyuronide solubilization occurs through its depolymerization by hydrolytic enzymes. In this fruit the enzyme PG plays an important role in pectin depolymerization during ripening (Themmen et al., 1982; Brady et al., 1983). However, it has been suggested that other pectolytic enzymes, such as pectate lyase, may also be involved in pectin metabolism accompanying fruit softening (Domínguez-Puigjaner et al., 1997). In contrast to tomato, solubilization of polyuronide in strawberry (Fragaria x ananassa Duch) fruit involves different mechanisms, because no reduction in pectin chain length is observed during softening (Huber, 1984). Huber suggested that increased levels of soluble polyuronide in strawberry fruit are due mainly to the synthesis of a more freely soluble form during ripening and that enzymic hydrolysis of polyuronide is not a likely cause for their solubilization. Accordingly, the activity of the pectolytic PG is found only at very low levels in strawberry fruit (Nogata et al., 1993). However, it has been reported recently that the expression of pectate lyase correlates with the softening of strawberry fruit, suggesting that the action of this enzyme in polyuronide solubilization cannot be excluded (Medina-Escobar et al., 1997).

Although polyuronide solubilization has been generally believed to be the major factor contributing to fruit softening, the expression of a chimeric PG in tomato mutant rin shows that polyuronide degradation and solubilization to near wild-type levels is not sufficient to cause fruit softening (Giovannoni et al., 1989; DellaPenna et al., 1990). This observation suggests that the metabolism of nonpectolytic cell wall polymers such as hemicellulose and cellulose may also play an important role in the decline of fruit firmness during ripening.

Xyloglucans, the predominant hemicellulose in dicotyledonous plants, are thought to play a pivotal role in cell wall architecture, because they can form extensive cross-links between cellulose microfibrils, locking them together (Brett and Waldron, 1996). Enzymes such as xyloglucan endotransglycosylases (Arrowsmith and Silva, 1995), expansins (Rose et al., 1997), and EGases have been proposed as allies cooperating in the modification of the hemicellulose network during fruit ripening. However, the specific contribution of each of these enzymes in fruit softening remains unclear.

EGases (EC 3.2.1.4), commonly referred to as cellulases, are usually assayed by their capacity to degrade the artifical substrate carboxymethylcellulose. Although the natural substrate for plant EGases is unknown, it has been shown (Brummell et al., 1994) that they hydrolyze β-1,4-
linked glucans in vitro, suggesting that xyloglucan is a likely substrate for EGases in vivo. In addition, although plant EGases are unable to degrade crystalline cellulose, they may be able to attack noncrystalline regions of the cellulose microfibrils, modifying the nature of fibril organization (O’Donoghue et al., 1994). EGase activity is associated with several processes that require cell wall weakening, including cell elongation, organ abscission, and fruit softening. Brummell et al. (1994) reported that the softening of fruits such as tomato, avocado, and strawberry accompanies an increase in EGase activity and temporally correlates with a decrease in the average molecular size of xyloglucan.

Ripening-related EGase cDNAs have been characterized from avocado (Tucker et al., 1987; Cass et al., 1990), tomato (Lashbrook et al., 1994), and pepper (Ferrarese et al., 1995; Harpster et al., 1997). However, little effort has been exerted to characterize EGase genes in strawberry fruit, and to date only a partial cDNA clone has been isolated from this fruit (Manning, 1998).

In an effort to understand the essential features of the softening of nonclimacteric strawberry fruit, we have focussed our research on the isolation and characterization of genes encoding EGases that accumulate during ripening. We have found two divergent EGase transcripts expressed in ripe strawberry fruit; each of these shows a different pattern of accumulation during ripening.

**MATERIALS AND METHODS**

**Plant Material**

Our experiments used strawberry (*Fragaria x ananassa* Duch cv Selva) fruits harvested at different stages of ripening, as assessed by the coloration of the fruit surface. We selected the following stages: green (S0), mature-green with a white surface (S1), one-fourth (S2) or three-fourths (S3) surface with red pigmentation, and fully red (S4) fruits. For RNA extraction achenes and the central fibrous core were removed, and the remaining receptacle tissue was immediately frozen with liquid nitrogen and stored at −80°C until needed.

For construction of a cDNA library, we used ripe fruit that had been stored at 4°C for 2 d under a continuous flow of humidified air.

**RNA Preparation and Analysis**

Total RNA was extracted from strawberry fruits or leaves as described earlier by Domínguez-Pujigueran et al. (1997). RNA (20 μg) was fractionated on 1.5% agarose/2.2× formaldehyde denaturing gels and capillary blotted onto a membrane (Hybond N, Amersham) as described by Ausubel et al. (1992). Northern blots were hybridized with random-primed DNA probes synthesized with the Ready-to-Go system (Pharmacia) using full-length Cel1 and Cel2 cDNAs as the templates. Hybridization was carried out at 42°C for 16 h, as described by Amasino (1986). Filters were washed three times for 15 min in 3× SSC and 0.5% (w/v) SDS at 65°C.

**Reverse Transcriptase-PCR**

We used reverse transcriptase-PCR to amplify EGase transcripts from total RNA extracted from ripe strawberries. First-strand cDNA synthesis used Moloney murine leukemia virus reverse transcriptase (Promega), following the manufacturer’s instructions. Approximately 50 ng of the obtained cDNA was used for the PCR amplification with degenerate primers (5′-GGNTAYTAGYGCNGNGAY-AAY-3′ and 5′-CCWACCATRANSACAT-3′), designed on the highly conserved amino acid sequences GYYDAGDN and MSYMVG. The reaction was carried out in a 40-μL volume containing 0.2 μM each primer, 0.2 mM dNTP, and 2.5 mM MgCl₂. The template was denatured at 95°C for 1 min, annealed at 60°C for 2 min, and extended at 72°C for 2 min. We repeated this cycle 35 times and followed it by one additional cycle in which the template was extended for 7 min. Under these conditions the PCR reaction generated two bands. The expected 980-bp band was recovered from the agarose gel, purified, and used as a probe to screen a cDNA library.

**cDNA Library Construction and Screening**

We obtained poly(A⁺) mRNA from ripe fruit kept for 2 d at 4°C, using an mRNA-isolation system (PolyATtract, Promega) according to the instructions provided. We recovered double-stranded cDNA from fruit poly(A⁺) RNA using a cDNA synthesis kit (λ-ZAP, Stratagene). The cDNA was ligated to Uni-ZAP XR vector arms and packaged in vitro using a Gigapack-II packaging extract (Stratagene). Using as a probe the cDNA fragment obtained by reverse transcriptase-PCR, we screened approximately 10⁵ clones of the resulting cDNA library. Plaques hybridizing with the probe were purified in a secondary and tertiary screening. Ten plaques were selected and resuspended in 500 μL of buffer (0.1 M NaCl, 8 mM MgSO₄, 50 mM Tris-HCl, pH 7.5), from which 2 μL were used to amplify the insert by PCR with the T3 and T7 primers present in the Uni-ZAP XR vector. The obtained PCR fragment was digested by restriction enzymes and the corresponding restriction map analyzed. Our analysis then used clones with different restriction patterns after the in vivo excision of the corresponding pBluescript.

**Genomic DNA Isolation and Southern Analysis**

We obtained genomic DNA from ripe fruit as described by Prat et al. (1989). DNA extracted from isolated nuclei was purified through a gradient of CsCl containing 1% (w/v) sarcosyl. Total DNA (5 μg) was digested with EcoRI or HindIII and separated by electrophoresis on a 0.8% (w/v) agarose gel. After denaturing, the DNA was transferred to a nylon membrane and hybridized with a random-primed DNA probe prepared from the 3′-untranslated region of Cel1 or Cel2 cDNA. Hybridization and washing conditions were the same as for northern-analysis procedures.
In Vitro Transcription and Translation of Cel1 and Cel2

Plasmid DNA (1 μg) containing Cel1 or Cel2 cDNAs was linearized with restriction enzymes and transcribed using T3 or T7 RNA polymerase (Promega) following the manufacturer’s instructions. The resulting mRNA was purified and in vitro translated in a rabbit reticulocyte lysate (Promega) system, using [35S]Met as an amino acid precursor. To obtain the mature protein from which the signal peptide had been excised, canine pancreatic microsomal membranes (Promega) were added to the translation reaction according to the manufacturer’s protocol.

Protein Analysis

The in vitro translation products were mixed with an equal volume of sample buffer (120 mM Tris-HCl, pH 6.8, 20% [v/v] glycerol, 4% [w/v] SDS, and 10% [v/v] 2-mercaptoethanol). The 35S-labeled protein was fractionated on a one-dimensional 12.5% SDS-PAGE gel, as described by Laemmli (1970).

Gels were stained with Coomassie blue to show nonradioabeled molecular mass markers. After destaining in 30% methanol/7% acetic acid, gels were washed exhaustively with water and incubated afterward with 1 M sodium salicylate, pH 6.0, for 15 min before drying under a vacuum. The dried gels were exposed to preflashed Kodak film.

DNA Sequencing and Sequence Homology Analysis

We used standard dideoxy sequencing (Sanger et al., 1977) to sequence both strands of the Cel1 and Cel2 cDNAs. An automated laser-fluorescent DNA sequencer (Pharmacia) generated the sequence data. The nucleotide sequence data obtained and the amino acid sequence derived were compared with other sequences in the database, using a Genetics Computer Group (Madison, WI) program. The Megalign Program, Clustal method, with a PAM250 residue weight table, generated the phylogenetic tree shown in Figure 2.

RESULTS

Isolation of Strawberry EGase cDNA Clones

EGase transcripts expressed in ripe strawberry fruit were amplified from total RNA by means of reverse transcriptase-PCR using degenerate primers. We obtained a band of the expected molecular mass (980 bp) and used it as a probe to screen a ripe strawberry fruit cDNA library. A large number of hybridizing phage plaques were captured, accounting for the 0.6% of the total clones screened. We selected and purified 10 plaques after two rounds of screening. Restriction endonuclease mapping of the inserts amplified by PCR revealed that the clones could be classified into two groups, from which the longest clones, Cel1 and Cel2, were chosen for sequencing.

Sequence Analysis

Sequence analysis of Cel1 and Cel2 cDNA revealed that they were 1690 and 2503 bp, respectively, in length, including an open reading frame of 1488 and 1683 bp that encoded two proteins homologous to EGases. Comparison of Cel1 and Cel2 sequences revealed that the EGases shared only 46% identity at the amino acid level. When compared with other EGases in the database, the protein encoded by Cel1 showed 78% amino acid identity with the tomato ripening-related Cel2 (Lashbrook et al., 1994). In contrast, the deduced Cel2 protein showed only 49% amino acid identity to the same tomato EGase and similar low values when compared with other plant EGases. Optimal alignment of the two strawberry EGases and tomato Cel2 (Fig. 1) revealed that the Cel2 protein bore several insertions and deletions in its sequence. The longest insertion was found in the C terminus of Cel2 protein, which showed 67 more residues than strawberry Cel1 and 66 more than tomato Cel2. Comparison of this sequence with proteins in the database did not reveal any close homology.

The analysis of the deduced amino acid sequence indicated that both EGases contained a hydrophobic N-terminal sequence characteristic of secreted proteins. As assessed by
the rules of von Heijne (1983), strawberry Cel1 and Cel2 proteins possessed predicted signal sequences of 32 and 24 amino acid residues, respectively. After the signal sequence was processed, Cel1 and Cel2 mature proteins had a calculated molecular mass of 51.5 and 59.6 kD, with basic predicted pI s of 9.4 and 8.6, respectively.

In addition to the different expected molecular masses of Cel1 and Cel2 mature proteins, other interesting differences were deduced from their amino acid sequences. The lack of alignment of some of the Cys residues suggested that the two strawberry EGases had different secondary and tertiary structures. Moreover, they also differed because of the presence of motifs for N-glycosylation, which are frequently found in secreted proteins. Whereas Cel1 protein contained an Asn at position 460 that suits the consensus motif for N-glycosylation (Asn-X-Ser/Thr), the Cel2 EGase did not bear this consensus sequence.

Figure 2 illustrates the evolutionary relationship between plant EGases from which the entire amino acid sequences are available. The phylogenetic tree shows that strawberry Cel2 belongs to a new evolutionary branch that diverged early from other known plant EGases, including strawberry Cel1. Based on this dendogram, the strawberry Cel1 EGase is closely related to the tomato Cel2 expressed during ripening, as well as to other EGases that are involved in cell expansion or abscission processes.

In Vitro Translation of the EGases Encoded by Cel1 and Cel2

Linearized plasmids containing Cel1 and Cel2 cDNA were in vitro transcribed, and the obtained RNA was used to translate the corresponding EGases in a rabbit reticulocyte system. To assess the functionality of the signal peptide, the translation reaction was performed simultaneously in the presence or absence of canine pancreatic microsomal membranes, which caused the cleavage of the signal peptide.

Analysis by one-dimensional SDS-PAGE of the product translated without microsomal membranes indicated that the immature Cel1 mRNA was translated into a 55-kD protein, whereas the Cel2 protein had an apparent molecular mass of 62 kD (Fig. 3, lanes I). The differences in molecular mass are in agreement with the observation that Cel2 cDNA had an open reading frame 195 bp longer than that of Cel1.

The incorporation of microsomal membranes in the translation reaction resulted in the cleavage of the signal peptide and the appearance of the mature protein, which possessed an apparent molecular mass of 52 kD for Cel1 and 60 kD for Cel2 (Fig. 3, lanes M).

Analysis of EGase Gene Expression by Northern Analysis

Northern-blot assay was used to assess the accumulation of Cel1 and Cel2 transcripts in leaves and throughout strawberry fruit development and ripening. Full-length EGase cDNAs were used to obtain the random-primed probes. As shown in Figure 4, we detected no hybridization signal in the leaves, whereas both EGase transcripts were highly expressed in the fruit of the strawberry. However, Cel1 and Cel2 EGases showed distinct temporal patterns of accumulation in the strawberry fruit. Cel2 mRNA was expressed in green fruit and its levels rose as the fruit turned from green to white, remaining at an elevated, constant level until the fruit reached their full-red color. In contrast, expression of the Cel1 gene occurred at a later stage than that of Cel2 and increased as the fruit ripened. The Cel1 transcript was not observed in the green fruit and it was

Figure 2. Phylogenetic analysis of the plant cellulases from which the complete amino acid sequence is available. The phylogenetic tree was obtained as described in the text. Arab., Arabidopsis; Straw, strawberry; Tom, tomato; Avo, avocado; AC, accession number.
EGase and the phylogenetic group to which it belongs. A correlation between the physiological role of a particular process and the phylogenetic tree presented in this paper indicates a poor divergence of several insertions and deletions along the evolutionary branch that diverged early from other plant species such as fruit ripening, cell elongation, or abscission related to EGases that are involved in various physiological processes (Fig. 2). In contrast to previous reports, the phylogenetic tree presented in this paper indicates a poor correlation between the physiological role of a particular EGase and the phylogenetic group to which it belongs.

An evident difference between Cel1 and Cel2 is the presence of several insertions and deletions along the Cel2 amino acid sequence. The different molecular weight that is deduced from the amino acid sequence was confirmed by in vitro translation of Cel1 and Cel2 mRNA. By using this approach we also showed that both EGases possess a functional peptide signal that was excised to render mature Cel1 and Cel2 proteins with an apparent molecular mass of 52 and 60 kD, respectively. The strawberry EGase Cel2 is larger than most of the known plant EGases, the molecular masses of which range from 49 to 56 kD. The presence of large EGases has also been described in pea, where a 70-kD native protein was found in epicotyls (Byrne et al., 1975), and in tomato, where a native 93-kD EGase was reported recently (Brummell et al., 1997).

Another noticeable difference between Cel1 and Cel2 EGases was deduced from the lack of alignment of the Cys residues, which suggests different secondary and tertiary structures for these two proteins. Moreover, Cel1 and Cel2 also differ in the presence of motifs for N-glycosylation that is often found in proteins directed to the secretory pathway. Whereas one potential site for N-glycosylation is found in the Cel1 mature protein (Asn-X-Ser/Thr), this motif is not present in Cel2 EGase. Although most of the reported plant EGases appear to be glycosylated, the non-glycosylated nature of Cel2 is shared by the EGase BAC1, expressed in bean abscission zones (Tucker and Milligan, 1991). TPP18, also named Cel4, was found in rapidly expanding tissues of tomato (Milligan and Gasser, 1995; Brummell et al., 1997a). Despite the differences discussed above, Cel1 and Cel2 share a basic pI, as deduced from the primary sequence of the mature proteins. The predicted pI of 9.4 for Cel1 and 8.6 for Cel2 is close to the 9.2 and 8.2 deduced for tomato Cel4 and Cel2 (Lashbrook et al., 1994; Milligan and Gasser, 1995) and to the 8.25 expected for the pepper EGase Cel1 expressed in ripening fruit (Harpster et al., 1997). Other plant EGases with basic pIs are the bean

**DISCUSSION**

In this paper we report the characterization of two divergent EGase cDNA clones that have been isolated from strawberry fruit. The EGase encoded by Cel1 cDNA shows a high degree of homology to other plant EGases, especially to tomato EGase Cel2 (Lashbrook et al., 1994) to which it shares 78% identity at the amino acid level. In contrast, Cel2 EGase exhibits a much lower homology with plant EGases in the database, with only 49% identity to tomato Cel2 and 46% to strawberry Cel1. An analysis of the phylogenetic relations among the plant EGases from which the complete amino acid sequence is available shows that the strawberry EGase Cel2 is the only representative of an evolutionary branch that diverged early from other plant EGases. In contrast, the EGase Cel1 is phylogenetically related to EGases that are involved in various physiological events such as fruit ripening, cell elongation, or abscission processes (Fig. 2). In contrast to previous reports, the phylogenetic tree presented in this paper indicates a poor correlation between the physiological role of a particular EGase and the phylogenetic group to which it belongs.

**Southern Analysis**

Southern blots of genomic DNA digested with the restriction enzymes EcoRI and HindIII were hybridized with random-primed probes obtained from either Cel1 or Cel2 3'-untranslated regions (Fig. 5). Comparison of the two Southern blots indicated that Cel1 and Cel2 probes did not cross-react, each probe hybridizing to a different array of fragments. Both probes hybridized preferentially to a single restriction fragment in each lane, although additional faintly hybridizing bands could be seen.

**Figure 4.** Northern analysis of the expression of Cel1 (A) and Cel2 (B) in leaves (lanes L) and fruit at different stages of growth and ripening. Lanes S0, Green fruit; lanes S1, white fruit; lanes S2, fruit with one-fourth red surface; lanes S3, fruit with three-fourths red surface; and S4, fully red fruit. Each lane contained 20 μg of total RNA. Northern blots were hybridized with random-primed probes obtained from full-length cDNAs. Both Northern blots were exposed overnight at −80°C.

**Figure 5.** Southern analysis of Cel1 (A) and Cel2 (B). Approximately 5 μg of genomic DNA was digested with either EcoRI or HindIII. The Southern blots were hybridized with random-primed probes obtained from Cel1 and Cel2 3'-untranslated regions.
Based on the pattern of expression and hormonal regulation, plant EGases have been classified into two different groups. One group contains EGases whose mRNA levels are stimulated by auxin. These EGases have been found in rapidly expanding tissues such as pea epicotyls (Wu et al., 1996) or poplar stems, roots, and leaves (Nakamura et al., 1995). The second group is formed by EGases whose expression is induced by exogenous ethylene and retarded by exogenous auxin (Tucker et al., 1988; Lashbrook et al., 1994; del Campillo and Bennett, 1996; Koehler et al., 1996). In general, EGases found in abscission zones and ripening fruit fall into this category. However, the extended belief that ripening-related EGases are regulated by ethylene does not take into account nonclimacteric fruits such as strawberry, which has been described to be nonresponsive to exogenous ethylene and insensitive to inhibitors of ethylene synthesis and perception (Given et al., 1988; Abeles and Takeda, 1990). In strawberry it has been proposed that auxin rather than ethylene plays a pivotal role in fruit development and ripening. For instance, it has been demonstrated that the growth of the strawberry receptacle is stimulated by the auxins provided by the achenes and the subsequent decline in the auxin content in achenes as they mature modulates the rate of ripening (Veluthambi and Poovaiah, 1984; Given et al., 1988). Manning (1994) showed that the accelerated strawberry ripening by achene removal involves a set of mRNAs that are qualitatively similar to those expressed in fruit ripened normally. Similarly, the analysis of the expression of an EGase transcript isolated recently from strawberry fruit shows that this gene is negatively regulated by auxin (Manning, 1998). These results, together with the finding that EGase activity increases during strawberry fruit ripening in an ethylene-independent manner (Abeles and Takeda, 1990), suggest that the expression of Cel1 and Cel2 strawberry EGases may be subject to a regulatory control different from the one described for EGases found in climacteric fruits.

The expression of Cel2 mRNA is clearly detected in green fruit and increases substantially thereafter as fruit turn white. This early increase in Cel2 levels is not associated with any apparent change in fruit texture. However, Huber (1984) reported that changes in hemicellulose molecular weight were first detected in fruits at the white stage, indicating that hemicellulose degradation is initiated in green fruit. The expression of the Cel2 transcript in green fruit suggests that the product of this gene may be involved in the early changes in hemicellulose polymer that lead to fruit softening. On the other hand, the early expression of the Cel2 EGase in fruit suggests that the product of this gene may also participate in other processes other than softening, for instance in cell expansion during fruit development. At the onset of ripening the levels of Cel2 mRNA are high and Cel1 starts to accumulate gradually, to reach a maximum in fully ripe fruit. Similar to strawberry, the ripening of tomato is accompanied by an increase in the expression of the EGases Cel1 and Cel2. However, the level of expression of Cel2 was 20-fold higher than that of Cel1, and Cel1 transcript was found to accumulate to a higher level in abscising flowers than in fruit (Lashbrook et al., 1994; del Campillo and Bennett, 1996). In contrast, both strawberry EGases exhibit similar high levels of accumulation in fruit, as deduced from the short exposure time required to obtain a clear signal in the northern blot (Fig. 4). This result suggests that both Cel1 and Cel2 EGases play a significant role in fruit softening. On the other hand, the overlapping expression of Cel1 and Cel2 in fruit suggests that the products of these two genes cooperate in the metabolism of cell wall polymers that occurs during fruit ripening. The fact that these two EGases show distinct biochemical and structural features suggests that they could have different substrate specificity. Experiments to investigate the physiological function of Cel1 and Cel2 EGases are under way.

ACKNOWLEDGMENT

The authors would like to thank Dr. J. Roberts for his suggestions concerning the manuscript.

Received August 13, 1998; accepted December 31, 1998.

The accession numbers for the sequences reported in this article are AF051346 for Cel1 and AF054615 for Cel2.

LITERATURE CITED


Brummell DA, Catala C, Lashbrook CC, Bennett AB (1997b) A membrane-anchored E-type endo-1,4-β-glucanase is localized on Golgi and plasma membranes of higher plants. Proc Natl Acad Sci USA 94: 4794–4799


