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# Antisense down-regulation of strawberry *endo*-β-(1,4)-glucanase genes does not prevent fruit softening during ripening

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**Abstract**

Strawberry (*Fragaria* × *ananassa* Duch.) fruit softening during ripening is associated with the overlapping presence of two divergent *endo*-β-(1,4)-glucanases (EC 3.2.1.4; EGases), Cel1 and Cel2. Antisense down-regulation of both genes was performed to assess the precise role of these *endo*-β-(1,4)-glucanases on strawberry fruit softening. Constant down-regulation of *cel1* expression throughout ripening was obtained, which was accompanied by reduced Cel1 protein accumulation. However, diminution of the Cel1 protein level together with a reduction of the total EGase activity to 40% of the control level did not affect fruit firmness, thus suggesting that Cel1 protein is not the major determinant of fruit softening during ripening. On the other hand, no significant reduction of Cel2 protein accumulation was found in any of the Cel2 transgenic or Cel1/Cel2 double-transgenic lines obtained. The difficulties encountered to yield a strawberry line with significant reduction of *cel2* expression suggest that this gene might be playing a pivotal role on fruit development prior to ripening, thus accounting for the lack of Cel2 protein down-regulation observed.


*Keywords:* *Endo*-β-(1,4)-glucanase; *Fragaria* × *ananassa*; Fruit softening; Strawberry; Transgenic antisense plants

## 1. Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is a non-climacteric fruit in which ripening is characterized by a set of physico-chemical changes that results in alterations in firmness, coloration, sweetness, sourness and aroma. Changes in strawberry firmness during ripening are controlled by cell turgor pressure and alterations in the composition and structure of cell wall polysaccharides, although it is thought to be mainly produced by alterations in the biochemistry of cell wall. Such changes are attributed to the coordinated action of several enzymes that act on the different cell wall polymers. In strawberry fruit, softening during ripening involves degradation of the middle lamella of cortical parenchyma cell walls [1,2], which results in a significant increase in pectin and hemicellulose solubilisation [3–5]. However, polyuronide solubilisation in strawberry does not come with molecular weight changes suggestive of enzymic modification, while pectolytic activity remains at very low levels during ripening [4,6]. In contrast, the hemicellulosic fraction undergoes a progressive reduction in molecular weight, suggesting the presence of an enzymic hydrolytic activity against xyloglucans, the major component of this fraction in dicotyledonous plants [4].

Plant *endo*-β-(1,4)-glucanases (EGases; EC 3.2.1.4.) are hydrolytic enzymes active against β-(1,4)-glucan links. Although little is known about their real substrate *in vivo*, xyloglucans and non-crystalline regions of cellulose have been proposed as their natural substrates in the cell wall [7]. EGase activity has been associated with several processes involving cell wall weakening, which range from cell wall expansion to fruit ripening and disassembly. In strawberry, high EGase

*Abbreviations:* CMC, carboxymethylcellulose; CMCase, carboxymethylcellulase activity; CTAB, cetyltrimethylammonium bromide; EGase, *endo*-β-(1,4)-glucanase; MS medium, Murashige and Skoog medium

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activity is firstly observed in non-ripened white fruits and further increase occurs during the subsequent ripening process. At the present time, two divergent EGase cDNA clones, *cel1* and *cel2*, have been isolated and characterized from strawberry fruit [8–13]. It has been demonstrated that *cel1* and *cel2* mRNA expression is only partially overlapping and occurs mainly at the cortex parenchyma cells [8–12]. Several reports have implicated Cel1 and Cel2 strawberry EGases in the depolymerisation of xyloglucans [8–12], although a recent report has revealed that Cel1 EGase might also act on cellulose [13].

Other cell wall-modifying enzymes active during strawberry fruit ripening have also been identified, including polygalacturonase [6], expansin [14], pectate lyase [15] and several β-galactosidases [16]. However, the underlying biochemical mechanisms of strawberry fruit softening are still unclear. Antisense technology has been pointed out to be a useful tool in attempts to generate fruits with attenuated softening. The value of this technology is based on its ability of suppressing particular genes involved in fruit softening without altering other desirable components of fruit quality. For instance, reduction of cell wall-modifying enzymes in tomato by transgenic antisense technology altered cracking or viscosity as a result of polygalacturonase and pectin esterase, respectively, down-regulation [17] or, indeed, improved fruit firmness (expansin or β-galactosidase) [18,19]. In strawberry plants, down-regulation of pectate lyase resulted in firmer fruits [20] and Woolley et al. already used antisense technology to assess the role of the Cel1 protein in fruit softening [12]. In the latter study several transgenic plants with reduced *cel1* mRNA levels were obtained, but no significant correlation was found with EGase activity or with fruit firmness [12]. To reconsider the role of Cel1 and Cel2 EGases during fruit development we attempted to down-regulate the corresponding *cel1* and *cel2* genes, both separately and jointly, under the control of a novel fruit specific promoter.

## 2. Materials and methods

### 2.1. Generation of transgenic strawberry plants

Three different transformation constructs were designed in order to reduce the expression of *cel1* (Cel1 antisense), *cel2* (Cel2 antisense) or *cel1* and *cel2* (Cel1/Cel2 double-antisense) genes. Different cDNA fragments were generated by PCR amplification, adding the corresponding restriction sites to each oligonucleotide. For Cel1 and Cel2 antisense constructs, the corresponding full-length cDNAs were first cloned in antisense orientation between the petunia *FBP7* (floral binding protein 7) promoter and the *Agrobacterium tumefaciens* nopaline synthase (nos) polyadenylation signal in a cloning vector using the *Sal*I and *Xba*I sites. *FBP7* is a fruit-specific promoter with constant expression throughout strawberry fruit development and ripening [21]. The Cel1/Cel2 double-antisense construct was obtained by cloning in antisense orientation and in the same vector a 545 bp fragment from the Cel1 cDNA using the *Sal*I and *Cla*I sites, followed by a 554 bp fragment from the Cel2 cDNA using the *Cla*I and *Xba*I sites. Orientation of the inserts was determined by PCR analysis using oligonucleotides designed to the *FBP7* promoter and nos polyadenylation signal, and was further confirmed by sequencing. The entire cassettes of the different constructs were then cloned into the plant binary transformation vector pBINPLUS [22] using the *Pac*I and *Asc*I sites and transformed separately by electroporation into the kanamycin-sensitive super-virulent *A. tumefaciens* strain AGL0. Strawberry cultivar Calypso was micropropagated *in vitro* on modified MS medium supplemented with benzylaminopurine and indole-3-butyric acid [23]. Cultivar Calypso was used because of its high transformation frequency and fruit yield. Under greenhouse conditions the 'ever-bearing' cultivar Calypso continuously produces fruit, which allows multiple harvests and analyses of the fruits all the year round.

For plant transformation, young leave discs (0.5 $cm^2$) of *in vitro* plantlets were transfected according to Puite and Schaart [24] with *A. tumefaciens* cells harbouring the constructs and regenerated as previously described [21]. Upon regeneration, 20 plantlets of each construct were rooted under selection medium and transferred to soil. Putative transgenic plants were grown to maturity in a greenhouse using standard cultural practices (day length 16 h, 21 °C at day and 18 °C at night). Selected transgenic plants were vegetatively propagated by runner to obtain enough material for subsequent studies.

Fruits were harvested at two different developmental stages, as assessed by the coloration of the fruit surface: fruits with one-fourth surface with red pigmentation (turning) and fully red-ripe fruits. Three to five fruits were collected from each plant for molecular and biochemical analysis. After harvest, receptacle tissue was immediately frozen with liquid nitrogen and stored at −80 °C until needed.

### 2.2. Rapid RNA isolation and analysis

Total RNA was prepared using a modified procedure of that described by Sambrook et al. [25]. Briefly, 1 g frozen tissue was powdered and then blended with 4 ml CTAB buffer pH 8.0 (0.1 M Tris–HCl, 20 mM EDTA, 1.4 M NaCl, 2% (v/v) CTAB, 2% (w/v) polyvinylpyrrolidone, 1% (v/v) 2-mercaptoethanol) together with 4 ml chloroform. After centrifuging at 4000 × *g* for 5 min, the supernatant was washed with chloroform and the RNA precipitated with isopropanol. Precipitated RNA was washed once with 70% (v/v) ethanol and redissolved with the appropriate volume of double-distilled water. Finally, RNA was precipitated with 2 M LiCl and washed again with 70% (v/v) ethanol.

#### 2.2.1. Northern-blot analysis

RNA samples (5 μg) were prepared in 15 mM sodium phosphate buffer pH 6.4, 0.8 M deionized glyoxal and 30% (v/v) dimethylsulfoxide, denatured for 60 min at 50 °C and separated onto 1.4% (w/v) agarose gels in 15 mM sodium phosphate buffer pH 6.4. Fractionated RNA was then capillary-blotted onto Hybond-$N^+$ nylon membranes (Amersham Pharmacia Biotech, The Netherlands) according to manufacturer's instructions and fixed using a UV cross-linker. Probes