

Research article

## Expression of expansin genes in strawberry varieties with contrasting fruit firmness

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### Abstract

Fruit softening is associated with cell wall disassembly mediated by the action of a complex set of enzymes and proteins. **Expansins, a group of proteins with unknown enzymatic activity, are proposed to be involved in this process.** In order to study the involvement of expansins in strawberry fruit softening we have analyzed the expression level of five expansin mRNAs (*FaEXP1*, *FaEXP2*, *FaEXP4*, *FaEXP5* and *FaEXP6*) in the cultivars “Selva”, “Camarosa” and “Toyonaka”, which differ in fruit firmness during ripening. We have found a correlation between mRNA expression levels and fruit firmness for *FaEXP1*, *FaEXP2* and *FaEXP5*. For these three mRNAs we have observed higher expression levels in the softest cultivar (Toyonaka) than in the other two firmer cultivars (Selva and Camarosa) at the beginning of ripening. This correlation was not found in the case of *FaEXP4* and *FaEXP6*, although both genes displayed a different expression pattern in the three cultivars analyzed. Western-blot analysis revealed that the accumulation of expansin proteins begins earlier in the softest cultivar during ripening.

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### 1. Introduction

Postharvest deterioration of soft fruits is mainly determined by the softening rate, **since fruit softening facilitates pathogen attack, increases postharvest decay and causes quality loss of fresh fruit.** It is believed that **disassembly of the primary cell wall is largely responsible for softening and textural changes during fruit ripening [1,9];** however, the mechanisms involved and the precise role of the enzymes and proteins that modify cell wall structure remains still unclear. Softening has been extensively analyzed in tomato, and experiments performed with transgenic plants suggest that **cell wall changes leading to fruit softening and textural modifications are complex, and involve the coordinated activities of a range of cell wall modifying proteins [4].** Among them, members of the **expansin protein family** that are expressed during fruit ripening seem to be necessary for the softening process. Expansins are proteins present in plant cell walls, and they were first discovered because of their ability to induce cell wall extension in vitro

[16]. **These proteins are encoded by large multigene families and have been found in different tissues of a wide range of plant species.** The expression of expansin mRNA and protein is correlated with growth and/or cell wall disassembly in many plant tissues, such as hypocotyls [16], coleoptiles [7], internodes [5], leaves [14], roots [26], and fruit [3,6,10,12,19]. The relevance of expansins in fruit softening has been demonstrated by using transgenic plants. **The constitutive suppression of *LeEXP1* in tomato rendered firmer fruit while the over-expression of the gene caused higher fruit softening, even at unripe stages [2].** In addition, varieties of peach that differ in firmness show different amount of some expansin mRNAs expressed in the fruit [11]. **A similar correlation between expansin accumulation and the relative firmness of strawberry fruit varieties was found for *FaEXP2* [21].**

In strawberry, seven expansins have been identified so far (*FaEXP1* to *FaEXP7*). Among these, *FaEXP2* and *FaEXP5* are fruit specific and their expression increases along ripening. Instead, *FaEXP3*, 4, 6 and 7 are also expressed in other plant tissues, while the expression of *FaEXP1* has not been characterized yet [6,10,19]. It is well known that different strawberry varieties display a wide range of fruit firmness, although there

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is scarce information about the possible genes or enzymes responsible for this variation. In order to study whether there is a correlation between expansin accumulation and strawberry fruit firmness, we have analyzed the expression of five of these genes during the ripening process in three strawberry cultivars with contrasting softening rates. We have compared *FaEXP2* and *FaEXP5* expression profiles since they are fruit specific and ripening regulated, and *FaEXP4* and *FaEXP6* because they are expressed throughout ripening and are also expressed in other plant tissues [6,10]. We have also included a characterization of *FaEXP1* gene expression in different plant tissues and during fruit ripening.

## 2. Results

### 2.1. Fruit firmness

Strawberry fruit firmness decreased largely from LG to 100% stage in the three cultivars (Fig. 1). Among these, Selva fruits were firmer than fruits in the same stage of Camarosa and Toyonaka cultivars, except in LG stage where firmness of Camarosa and Selva fruits was similar. Fruits from Toyonaka cultivar soften faster than those from Selva and Camarosa cultivars, and the main firmness decrease occurs early at ripening from LG to W stage (Fig. 1). Thereafter, the firmness of these fruits remains low and approximately constant from 25% to 100% stage. In the case of Selva and Camarosa fruits, there is also a large firmness reduction from LG to W stages, but less drastic than in Toyonaka, and then the firmness decreases gradually. In general Selva was the firmest cultivar, Toyonaka was the softest one, and the firmness of Camarosa was intermediate between theirs.

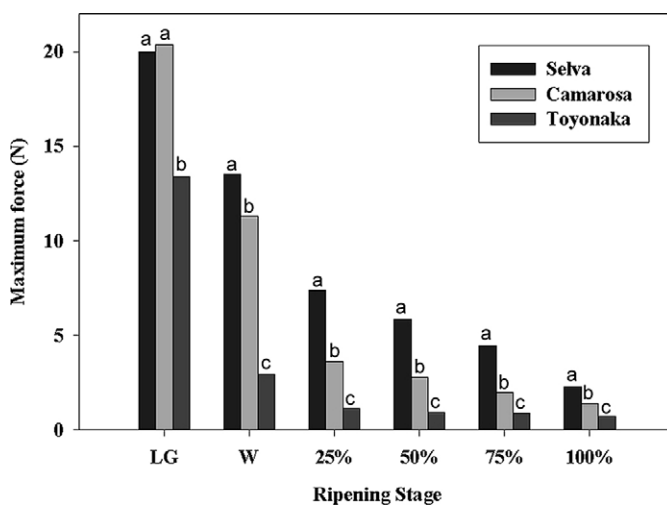


Fig. 1. Changes in fruit firmness of three strawberry cultivars. The maximum force needed to penetrate the fruit was used to compare different ripening stages: large green (LG), white (W), 25% red (25%), 50% red (50%), 75% red (75%) and 100% red (100%). Columns with different letters indicate differences in firmness when the cultivars are compared at the same stage ( $P = 0.05$ ).

### 2.2. Molecular characterization of *FaEXP4* full-length cDNA

The screening of a strawberry cDNA library was performed to obtain the full length cDNA of *FaEXP4* and then to generate a specific probe from its 5' or 3' UTR region. Five positive clones were sequenced and all of them corresponded to the full length cDNA of *FaEXP4*. This cDNA (Gen Bank accession number DQ183068) consists of 1338 bp with an open reading frame of 251 amino acids. The predicted polypeptide has a molecular mass of 26.6 kDa and includes a putative signal peptide of 22 amino acids in its N terminal region as determined by TargetP software, resulting in a mature protein of 24.4 kDa. The program also predicted that this signal peptide directs the protein to the secretory pathway [17].

### 2.3. Expression of *FaEXP1*, *FaEXP2*, *FaEXP4*, *FaEXP5* and *FaEXP6*

All these expansin genes have been found in strawberry fruit, but they show differences in their expression pattern. No data about the expression of *FaEXP1* was found in the bibliography. According to Harrison et al. [10], the expression of *FaEXP4* and *FaEXP6* was not fruit specific. *FaEXP4* mRNA was present throughout fruit development and ripening, but was more strongly expressed during ripening. In the case of *FaEXP6*, a low expression level was found in fruit, and the gene expresses also in roots and at highest levels in stolons. Regarding *FaEXP2*, contradictory results about its tissue specificity have been reported: in Chandler variety, *FaEXP2* mRNA was found only in fruit [6] while in Brighton the message was also detected at low levels in other tissues as leaves, roots and stolons [10]. Finally, *FaEXP5* mRNA has been found only in fruits, and was up-regulated at the onset of ripening [10].

The mRNA abundance of *FaEXP1*, *FaEXP2*, *FaEXP4*, *FaEXP5* and *FaEXP6* was analyzed during strawberry fruit ripening of three cultivars (Selva, Camarosa and Toyonaka) that show differences in fruit firmness during ripening. Specificity of the probes was tested by dot blots in the same hybridization conditions used in the Northern blots, and no cross hybridization was observed (data not shown).

#### 2.3.1. *FaEXP1*

The existence of this gene was reported by Rose et al. [19], who found it in ripe fruit from Chandler cultivar, but no data of its expression was available until now. First, we analyzed the tissue specificity of *FaEXP1* expression in Selva cultivar (Fig. 2). The message was found in all the tissues assayed, including ripe fruit, and the highest expression level corresponded to roots. It was also highly expressed in leaves, sepals and stamens and there was a weaker expression level in ovaries, stems and petioles. The expression profiles of *FaEXP1* during ripening were analyzed in the three strawberry cultivars under study (Fig. 3). The presence of *FaEXP1* was first detected in W stage in the three cultivars (although a very slight signal is also observed in LG), but in the firmest one

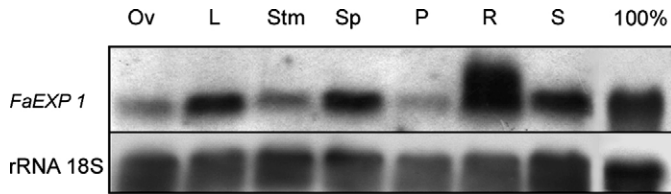


Fig. 2. Northern hybridization analysis of *FaEXP1* in Selva cultivar. Tissues: ovaries (Ov), leaves (L), stems (Stm), sepals (Sep), petioles (P), roots (R), stamens (S) and 100% receptacle (100%). The same membrane was hybridized with a probe for *FaEXP1* (top panel), stripped and then hybridized with a probe for *rRNA 18S* (bottom panel).

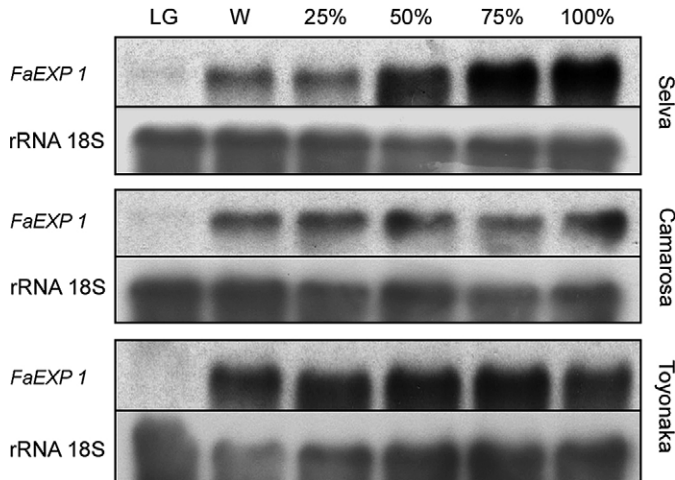


Fig. 3. *FaEXP1* expression throughout strawberry fruit ripening of Selva, Camarosa and Toyonaka cultivars. The stages analyzed are shown on top, the strawberry cultivars on the right and the probe (*FaEXP1* or *rRNA 18S*) on the left of the figure.

(Selva) the expression level was weaker in W and 25% stages than in 50%, 75% and 100%. In the cultivar with intermediate firmness (Camarosa) expression levels were almost the same from W to 100%. On the other hand, in the softest cultivar (Toyonaka) expression levels were higher than in the other two cultivars and almost constant from W to 100%.

### 2.3.2. *FaEXP2*

The analysis of *FaEXP2* revealed different expression profiles in each of the cultivars analyzed (Fig. 4). The transcript was first detected in the W stage in the three cultivars. In Selva cultivar, expression levels in W and 25% stages are low, increase notoriously in 50% and then the expression increases gradually until the end of ripening. The expression level in Camarosa fruits increases from W to 50% stages and then it remains approximately constant. Finally, the expression level in Toyonaka cultivar is high from the W stage and it remains approximately constant through the other stages.

### 2.3.3. *FaEXP4*

The expression profiles of this gene during ripening are shown in Fig. 5. The presence of *FaEXP4* was already detected in LG stage in Selva and Toyonaka cultivars. The expression level in Camarosa and Selva fruits increases in W stage and remains at a constant level in the other stages analyzed. In

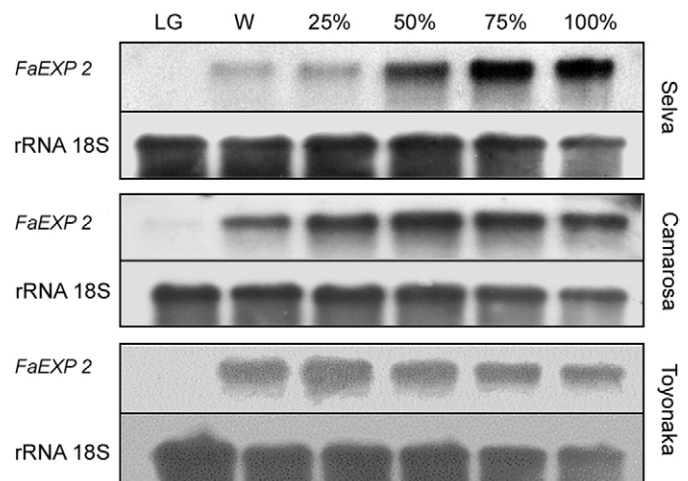


Fig. 4. *FaEXP2* expression throughout strawberry fruit ripening of Selva, Camarosa and Toyonaka cultivars. The stages analyzed are shown on top, the strawberry cultivars on the right and the probe (*FaEXP2* or *rRNA 18S*) on the left of the figure.

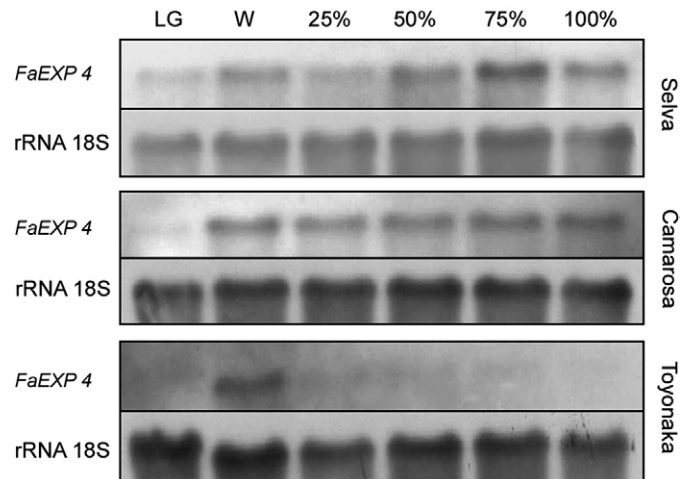


Fig. 5. *FaEXP4* expression throughout strawberry fruit ripening of Selva, Camarosa and Toyonaka cultivars. The stages analyzed are shown on top, the strawberry cultivars on the right and the probe (*FaEXP4* or *rRNA 18S*) on the left of the figure.

Toyonaka cultivar, a maximum expression level was found in W stage, while in the other stages the expression was faint or it was absent.

### 2.3.4. *FaEXP5*

The *FaEXP5* mRNA accumulation was different in the three cultivars analyzed (Fig. 6). The expression in the firmest cultivar (Selva) is very low in W stage, it increases in 25% stage and it reaches a high level at 75% stage. On the other hand, in Toyonaka fruits a high expression level of this gene was detected in W stage, and from 25% to 100% stage the amount of the message remained approximately constant; the expression of this gene was clearly higher than in the other firmer varieties at the beginning of ripening. An intermediate pattern was found in Camarosa fruits, and the expression level remained constant between W and 75% stage and increased at the end of ripening.

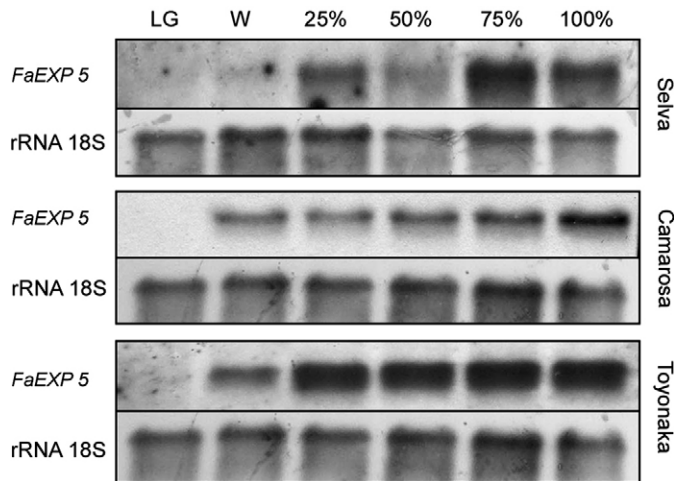


Fig. 6. *FaEXP5* expression throughout strawberry fruit ripening of Selva, Camarosa and Toyonaka cultivars. The stages analyzed are shown on top, the strawberry cultivars on the right and the probe (*FaEXP5* or *rRNA 18S*) on the left of the figure.

### 2.3.5. *FaEXP6*

The comparison of *FaEXP6* mRNA accumulation showed different expression profiles in the three cultivars analyzed (Fig. 7). In Selva, the gene was expressed in all ripening stages, though the expression was particularly higher from stage 25%, despite an apparent decrease in 50% due to a slight difference in the amount of RNA loaded. In the case of Camarosa cultivar, the expression was very low in LG, increased in W stage and then remained approximately constant. Instead, in Toyonaka fruits the expression level of this gene was very low along ripening, and it was almost undetectable at 100% stage.

### 2.4. Expansin protein accumulation

In order to compare expansin abundance along the ripening process, we extracted total proteins from six ripening stages of Toyonaka and Camarosa cultivars and analyzed them by Wes-

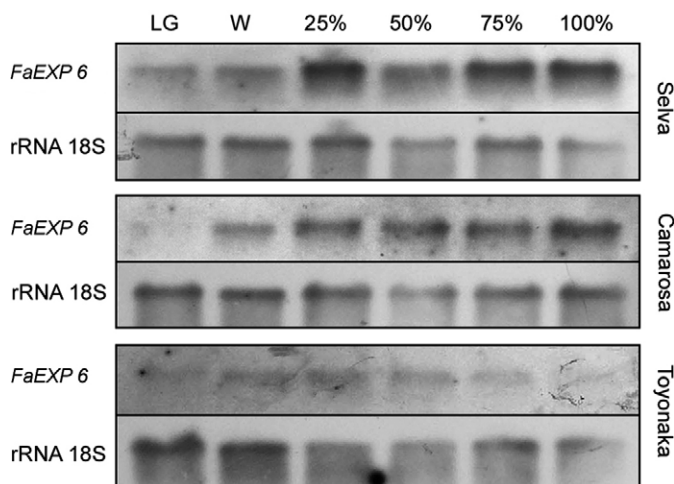


Fig. 7. *FaEXP6* expression throughout strawberry fruit ripening of Selva, Camarosa and Toyonaka cultivars. The stages analyzed are shown on top, the strawberry cultivars on the right and the probe (*FaEXP6* or *rRNA 18S*) on the left of the figure.

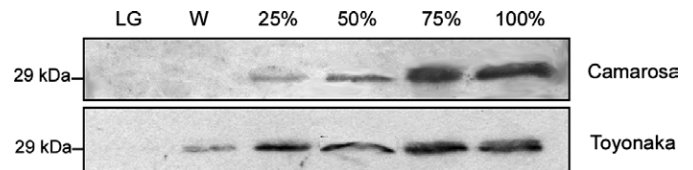


Fig. 8. Western blot analysis of total protein extracts from strawberry fruit. Total proteins (15  $\mu$ g) from Camarosa and Toyonaka strawberry fruits in the stages shown in the figure were separated by SDS PAGE, electroblotted to a membrane and visualized by reaction with antibodies to LeEXP1 from tomato.

tern blot, using an antibody against LeEXP1 from tomato [19]. A difference in the abundance of expansins between both varieties was found (Fig. 8). The antibodies used detected a band of approximately 29 kDa, which is consistent with previous reports [10]. The presence of expansins was first detected in W stage in Toyonaka and in 25% in Camarosa. Moreover, in 25% stage the amount of expansin was nearly twofold higher in the softest cultivar (Toyonaka). The level of expansin remained constant during ripening in Toyonaka, while it increased in Camarosa cultivar.

### 3. Discussion

Fruit softening has been related to the cell wall disassembly due to the action of cell wall enzymes and proteins. Most of the analysis concerning fruit softening by means of transgenic plants have been done in tomato and was reviewed recently by Brummell and Harpster [4]. The suppression of PG or PME expression, two of the enzymes thought to be essential in fruit softening, did not modify tomato fruit softening [15, 23]. No difference in fruit firmness was observed in tomato antisense lines either for XET or  $\beta$ -galactosidase [4]. Strawberry antisense plants with a strong suppression of *FaCell*, which encodes an endo- $\beta$ -1,4-glucanase, did not produce firmer fruits [25]. Instead, strawberry plants with suppression of a pectate lyase gene, presumably involved in pectin disassembly, produced firmer ripe fruits with lower cell wall swelling and pectin solubility [13]. On the other hand, when transgenic tomato plants were generated by suppression or overexpression of the expansin gene *LeEXP1*, this resulted in firmer or softer fruits, respectively [2], indicating a fundamental role for this protein in tomato fruit softening.

Other approach to evaluate the possible role of a particular gene or enzyme in fruit softening is the comparison of cultivars with contrasting firmness. Differences in cell wall metabolism [20] and gene expression [21] in strawberry fruit were studied using this approach. Therefore, in this work we have analyzed the expression of five strawberry expansin cDNAs during ripening of three cultivars that show contrasting softening rates.

First, we report the analysis of *FaEXP1* expression and the cloning of the full length for *FaEXP4*. *FaEXP1* gene was found by RT-PCR from tissue fruit of Chandler cultivar [19], though further attempts to clone the full length from a cDNA library were unsuccessful and its expression pattern was not further characterized [6,10]. The gene was re-isolated by RT-

PCR from Selva cultivar and its expression analyzed in different tissues and ripening stages. Data revealed that *FaEXP1* expresses in all the tissues assayed including fruit, but the highest mRNA accumulation was found in roots (Fig. 2). Regarding *FaEXP4*, the full length cDNA obtained encodes for a protein of 251 aa, including a putative signal peptide of 22 aa that drives the protein to cell wall. The protein includes the conserved eight Cys and four Trp residues, characteristics of the  $\alpha$ -expansin family.

To find out if there is a correlation between expansin gene expression and firmness of the different cultivars, we have compared the expression of five expansin genes: *FaEXP1*, *FaEXP2*, *FaEXP4*, *FaEXP5* and *FaEXP6*. The first one was included to characterize its expression, which was unknown until now. Expansins *FaEXP2* and *FaEXP5* were chosen because they are fruit specific and their expression increases during ripening, while the other two expansins (*FaEXP4* and *FaEXP6*) are found not only in fruit but in other tissues also.

The softening of strawberry fruit is progressive, but the main firmness reduction occurs early at ripening between stages LG and 25%. In the case of Selva cultivar, the fruit firmness reduced 63% from LG to the stage 25% while in Toyonaka the corresponding reduction was 91%. Therefore, particular emphasis was put on identifying differences in the expression of expansin genes at early stages in cultivars with contrasting firmness. The analysis of Figs. 4 and 6 revealed a correlation between *FaEXP2* and *FaEXP5* gene expression and fruit firmness at the beginning of ripening. A higher expression of both expansin mRNAs was found in the softer cultivar (Toyonaka) between LG and 25% stages. However, the accumulation of both transcripts increased in the firm cultivars at later stages, equalizing and even surpassing the levels observed in Toyonaka. These results are in agreement with Salentijn et al. [21] who found a *FaEXP2* expression slightly higher in ripe strawberry fruits of a soft cultivar (Gorella) in comparison with that found in firmer cultivars (Holiday and Elsanta).

On the other hand, in Figs. 5 and 7 we show that such a correlation between expansin accumulation and firmness decrease was not found for *FaEXP4* and *FaEXP6* (Figs. 5 and 7). The expression of both genes was higher in the two firmer cultivars (Selva and Camarosa), while a lower level was observed in fruits of the softest variety (Toyonaka).

Regarding *FaEXP1*, the expression of this gene in Toyonaka is higher than in the other two firmer cultivars (Fig. 3) in early ripening stages (W and 25%). Therefore, a correlation between cultivar softness and *FaEXP1* accumulation can be established. It is worth to point out that the expression of this gene is not fruit specific but it also has a high expression level in roots (Fig. 2). The number of expansin genes found in different species has led to several hypotheses about the possible function of so many genes [8]. One possibility is that the numerous expansin genes are expressed in unique patterns according to the characteristic enlargement requirements of each cell type. In this case, the promoters of each gene should specify a unique expression pattern, and there is some experimental evidence supporting this idea [5,19,22]. Another possibility is that they

are redundant, and then the expression of different expansin genes may partially overlap, yet still show differential regulation. We found that the five genes analyzed in this paper are simultaneously expressed from W to 100% stage, so a kind of redundancy can be observed, but only three of them (*FaEXP1*, *FaEXP2* and *FaEXP5*) showed a correlation between firmness and expression at the beginning of ripening, while the other two genes (*FaEXP4* and *FaEXP6*) did not. Moreover, we have found differences in protein abundance between one of the firmer cultivars (Camarosa) and the softer one (Toyonaka) and they were more significant at the beginning of ripening (Fig. 8), which is consistent with the differences in gene expression of the three genes mentioned above. In the softest cultivar, expansin genes involved in the ripening process are up-regulated earlier and this leads to a higher protein accumulation in W and 25% stages, allowing the fruit to soften faster than the other two firmer cultivars (Fig. 1). Altogether, these results suggest that the early accumulation of a set of expansins would contribute to strawberry fruit softening at the beginning of the ripening process, and then could be at least partially responsible for the different softening rate observed in different cultivars. If the early accumulation of expansins weakens the interactions among different components of cell wall [19] or if they act indirectly facilitating the action of cell wall hydrolases at the beginning of strawberry ripening can not be decided with the data obtained in this work, since both interpretations are equally probable. The variation of the activity of cell wall degrading enzymes in these cultivars with contrasting firmness would be useful to develop a hypothesis on the role of expansins in the softening of this particular fruit.

Hormonal regulation could play a role in expansin gene expression though scarce information is available. The analysis performed on *FaEXP2* expression revealed no major influence of auxin or ethylene [6]. However, further work is necessary to analyze the possible hormonal regulation of expansin gene family in strawberry fruit.

## 4. Methods

### 4.1. Plant material

Fruits were harvested from field-grown strawberry plants (*Fragaria xananassa* Duch. cv. Selva, Toyonaka and Camarosa) and classified in different stages according to their external coloration degree and size: large green (LG), white (W), 25% red (25%), 50% red (50%), 75% red (75%) and 100% red (100%). The fruits were collected, immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until used. Other vegetative and reproductive tissues analyzed: roots (R), petioles (P), leaves (L), stamens (S), ovaries (Ov), sepals (Sep) and stems (Stm) were obtained from Selva cultivar plants.

### 4.2. Firmness

The firmness of fresh fruits was measured using a texture analyzer (TA.XT2, Stable Micro Systems Texture Technolo-

gies, Scarsdale, NY) fitted with a flat 3 mm probe. Each fruit was penetrated 7 mm at a speed of 0.5 mm s<sup>-1</sup> and the maximum force developed during the test was recorded. Each fruit was measured twice in opposite sides of its equatorial zone and 30 berries at each ripening stage were assayed.

#### 4.3. RNA isolation and RNA gel-blot analysis

Total RNA was extracted from 6 g of pooled samples of frozen fruits (approximately 50) by the hot borate method [24]. Each RNA sample (10 µg) was analyzed by electrophoresis in a 1.2% (w/v) agarose and 1% (v/v) formaldehyde denaturing gel, and then transferred to Hybond-N membranes (Amersham-Pharmacia Biotech UK, Buckinghamshire, UK). Membranes containing total RNA from fruits corresponding to the six ripening stages analyzed (LG, W, 25, 50, 75 and 100%) of each of the three cultivars were prehybridized for 4 h at 42 °C, hybridized with the probes (see below) at 42 °C overnight and then washed for 30 min once at 42 °C and three times at 50 °C with 0.1% w/v SDS and 1 × SSC. The blots were exposed to X-ray films (X-OMAT AR-205, Kodak) at -80 °C for 4 d, and the films were developed according to the manufacturers' recommendation. Blots were stripped of hybridizing probes and hybridized at 42 °C to a probe for strawberry 18S rRNA, and then washed for 30 min with 0.1 % w/v SDS and 1 × SSC once at 42 °C and three times at 55 °C. The films were exposed for 4 h and then developed. The expression of each expansin gene was assayed at least twice in each cultivar analyzed.

#### 4.4. Expansin cDNA cloning and preparation of probes

Expansin cDNAs for *FaEXP1*, *FaEXP4*, *FaEXP5* and *FaEXP6* (GenBank accession numbers AF163812, AF226701, AF226702, AF226703, respectively) were cloned by RT-PCR using specific (*FaEXP4*, *FaEXP5* and *FaEXP6*) or degenerate primers (*FaEXP1*). The RT reaction was performed with 2 µg of total RNA from fruits (small green for *FaEXP1* or 25% for the others). The following primers were used for PCR amplification: *FaEXP4*: sense GGGTTGGGGTGTGGTTCT, antisense GATGCCTCCCC TTCTTCTG; *FaEXP5*: sense TCTCGCCCAGCCCGTCTTC CAGCAT, antisense TGGGTTGCCAATTAGTTCTT; *FaEXP6*: sense TGCGCAAACGACCCGAAC, antisense CAGGCTCACCCCTCACGAT; *FaEXP1*: sense GGAATTCAG CACDGCBYTGTTCAAC, antisense GGCTCGAGYARVACYARGTTGAAG-TA, where B = T, C or G; D = T, G or A; R = A or G; V = A, C or G; Y = C or T. Amplification conditions for *FaEXP4*, *FaEXP5* and *FaEXP6* were: 95 °C for 3 min, 30 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by one cycle at 72 °C for 10 min, and for *FaEXP1*: 94 °C for 3 min, 30 cycles at 94 °C for 1 min, 57 °C for 1 min and 72 °C for 90 s, followed by one cycle at 72 °C for 7 min.

Probes for Northern blot hybridization were prepared by PCR amplification with specific primers for each of the straw-

berry expansins mentioned above and also for *FaEXP2* (GenBank accession number AF159563). The specific primers for preparing *FaEXP5* and *FaEXP6* probes were the same used for cloning them. For *FaEXP1*: sense GCACTGCCG GAAAGCCCTCCATT, antisense TCCGGCTTTGTACT CGGCGATCTTG; *FaEXP2*: sense CTTCTCCTTCTAGC TAGC, antisense GCATGGCCACCAACCCAA and *FaEXP4*: sense CTTGGGACTCTAAACTCC, antisense CAT TACTAGTAGAAAATGG. Amplification conditions for *FaEXP2* were: 95 °C for 3 min, 35 cycles at 95 °C for 1 min, 45 °C for 1 min and 72 °C for 1 min, followed by one cycle at 72 °C for 7 min. For *FaEXP4*: 95 °C for 3 min, 35 cycles at 95 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min, followed by one cycle at 72 °C for 10 min. For *FaEXP5* and *FaEXP6* amplification conditions were the same mentioned above. Gel purified products were used as templates in a random primer labeling reaction.

#### 4.5. Isolation of *FaEXP4* full length cDNA

A full length cDNA for *FaEXP4* was isolated by screening a 75–100% cDNA library of *Fragaria xananassa* constructed in Uni-ZAP XP (Stratagene, La Jolla, CA, USA) from Chandler cultivar [6]. A probe corresponding to the fragment cloned by RT-PCR was used. A total of 5 × 10<sup>5</sup> pfu was plated and plaque lifts were performed. The filters were hybridized as mentioned above. After washing, the filters were prepared for autoradiography and exposed to X-ray films (X-OMAT AR-205, Kodak) at -80 °C for 24 h. Positive plaques were carried through two additional screening rounds for purification, and phagemid DNA was excised. Positive clones were sequenced using T3, T7 and internal primers. A sequencer Perkin-Elmer Applied Biosystems 377 with a 3700 and 3100 capillary column was used (HHMI Biopolymer and W.M. Keck Biotechnology Resource Laboratory, Yale University).

#### 4.6. Sequence analysis

Sequence similarity searches and signal peptide and targeting predictions were performed using the basic local alignment search (BLAST, National Center for Biotechnology Information, Bethesda, MD) and TargetP version 1.1 WorldWideWeb Prediction Server (Center for Biological Sequence Analysis, Technical University of Denmark) [17], respectively.

#### 4.7. Protein extraction and SDS-PAGE analysis

Strawberry fruit tissue (2 g) from a pool of 50 fruits in the six ripening stages analyzed (LG, W, 25%, 50%, 75% and 100%) of Camarosa and Toyonaka cultivars was ground in liquid nitrogen and homogenized in extraction buffer (50 mM Tris, 2% w/v SDS, 2% w/v 2-mercaptoethanol, 1 mM EDTA, 5% w/v sucrose and 1% w/v PVPP, pH 7.0) in the ratio of 1 g of tissue per 3 ml of buffer. Samples were incubated at room temperature for 40 min with agitation, centrifuged 10 min at 10,000 × g and the supernatant precipitated with 1/10 volume

of 100% w/v TCA for 30 min at 4 °C. After centrifugation at 10,000 × g for 5 min, the pellets were washed twice with 80% v/v acetone and dissolved in 0.1 M NaOH/1% SDS. The protein concentration was measured by the modified Lowry method described by Potty [18], using bovine albumin as standard. Equal amounts of total protein (15 µg) were loaded onto a denaturing 1 mm thick 12% w/v polyacrylamide gel and run at 30 mA. Three protein extracts were prepared for each ripening stage.

#### 4.8. Western blotting

Proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane (Hybond ECL, Amersham-Pharmacia) using a gel blotter (Bio-Rad). The transfer was at 4 °C in 25 mM Tris, 192 mM glycine, 0.1% w/v SDS, pH 8.3. The membrane was blocked with 5% w/v non-fat dried milk in Tris buffered saline Tween (TBS-T, 20 mM Tris, 137 mM sodium chloride, 0.1% v/v Tween-20, pH 7.6) for 1 h at room temperature with agitation. To detect expansin proteins the membrane was incubated for 1 h with tomato anti-LeEXP1 antibodies [19] diluted 1:250 in 0.5% w/v non-fat dried milk in TBS-T. The membrane was washed 2 × 15 and 2 × 5 min with TBS-T and incubated for 1 h at room temperature with a 1:2000 dilution of the peroxidase labeled anti-rabbit antibody, and chemiluminescent reagents were used for detection as described by the manufacturer (ECL Western blotting analysis system and detection reagents, Amersham). Western blots were performed three times for both varieties.

#### Acknowledgements

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