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RESEARCH PAPER

A cell wall-oriented genomic approach reveals a new and unexpected complexity of the softening in peaches

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Abstract

During ripening, fleshy fruits undergo textural changes that lead to loss of tissue firmness and consequent softening. It is a common idea that this process is the consequence of cell wall dismantling carried out by different and orderly expressed enzymes. For this purpose, by using a single enzyme family approach many enzymes and related genes have been characterized in different fruits. In this work, the softening of the climacteric peach fruits (*Prunus persica* (L.) Batsch.) has been studied by using a genomic approach, and the results obtained are novel and partly unexpected. The genes analysed encode proteins involved in the main metabolic aspects of a primary cell wall: degradation, synthesis, structure. In addition, some genes encoding cell-wall-related proteins with an unknown function have been studied. The gene expression profiles show that the softening actually begins well before the climacteric rise and continues thereafter. Genes whose expression starts before the climacteric rise are mostly down-regulated by ethylene, while genes with a ripening-specific expression are mostly up-regulated by the hormone. A few other genes are apparently insensitive to ethylene. Besides the expected parietal degradation, the softening that results from this study also comprises some repairing of the cell wall performed by enzymes involved in the synthesis of parietal polysaccharides and, especially, by proteins with structural functions. The newly synthesized polysaccharides and the structural proteins would thus help to hold together the fruit cell wall while not preventing the softening.

Key words: Cell wall metabolism, EST, fruit softening, peach, *Prunus persica*.

Introduction

The softening of fleshy fruits is a physiological process that has been thoroughly studied in many laboratories all over the world (see recent reviews by Brownleader *et al*., 1999; Brummel and Harpster, 2001). Such huge interest is due both to a desire for deeper knowledge and to the relevant economic implications of this process. For the latter, it should be pointed out that the extent of the softening of a given fruit is largely responsible for the length of its post-harvest life, for the chances of escaping a successful pathogen attack, not to mention the transportation and storage expenses.

Softening is one of the many physiological events that fall into the more general process of cell separation (Roberts *et al*., 2002). Cell separation occurs whenever the links between cells in a given tissue are loosened as a consequence of the weakening of the cell wall structure. This process involves the expression of different enzymes that irreversibly impair the mechanical properties of the cell wall by acting on specific parietal components.

Early studies of fruit softening mostly used biochemical techniques, sometimes accompanied by morphological analyses, in order to detect possible enzymatic activities particularly involved in cell wall degradation (Dickinson and McCollum, 1964; Hobson, 1964; Knee *et al*., 1977; Pesis *et al*., 1978; Platt-Aloia *et al*., 1980; Crookes and Grierson, 1983). It has been proposed that polygalacturonase activity is particularly important for the softening of tomatoes, while cellulase activity has been proposed to play a comparable role in avocado (see review by Fischer and Bennett, 1991; Abeles *et al*., 1992).

The discovery that many different parietal enzymes can be expressed in fruits, and the use of the very powerful biomolecular techniques, have demonstrated the existence of a more complex situation, where other enzymes are expressed at lower amounts and, in some cases, multiple

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forms of the same enzyme can take part in the softening of a fruit (Rose and Bennett, 1999).

An important purpose of this type of research is that, by using the knowledge thus obtained, it might be possible to interfere with the process in order to curtail the extent of the softening and to prolong the post-harvest life of fleshy fruits. For instance, transgenic tomatoes were obtained with down-regulated polygalacturonase expression (Sheehy *et al*., 1988; Smith *et al*., 1988). Surprisingly, the softening of those tomatoes was comparable to the softening of wild-type fruits. However, compared to the wild-type fruits, the transgenic tomatoes had a longer post-harvest life and presented a superior technological quality when used to make tomato paste (Brummel and Harpster, 2001).

The anti-sense technology was applied in tomato to knock down the expression of other cell-wall-modifying enzymes (Brummel and Harpster, 2001), and interesting results were obtained in the case of expansin (Brummel *et al*., 1999). By contrast, a significant reduction of the softening was obtained in strawberry by down-regulating the expression of a pectate lyase gene (Jiménez-Bermùdez *et al*., 2002).

Identifying the main cell-wall-degrading enzyme/s in a given fruit is preliminary to the above approach. However, by doing so an underestimation might occur of one or more enzymatic activities that, by acting at low amounts on particularly important chemical bonds, might undermine the mechanical properties of cell walls, thus significantly contributing to fruit softening.

It is also important to know the serial expression of the various genes involved in fruit softening, since the activity of a certain enzyme might improve the access of other enzymes to their specific substrates. Therefore, it is necessary to obtain as wide a knowledge as possible of the softening process. At the present time, such information can partly be obtained in the case of tomato by putting together all the separate data obtained by the different laboratories that use tomato as their model fruit.

In this work a genomic approach has been used to study the softening of the climacteric peach (*Prunus persica* (L.) Batsch.) fruits. Peaches are economically relevant, and some varieties have a particularly short post-harvest life; moreover, in these fruits aroma and taste are at their best during the fully ripe stage, that is, when their perishability becomes very high. Present data are limited to a few cell wall enzymes (Pressey and Avants, 1973; Hinton and Pressey, 1974; Downs *et al*., 1992; Glover and Brady, 1994; Bonghi *et al*., 1998). Among them, an endo-polygalacturonase that is highly expressed after the ethylene climacteric rise is regarded as being mainly responsible for the softening of peaches (Lester *et al*., 1994), although there are a few other genes encoding cell wall enzymes that have been reported to be expressed in peach fruits (Glover *et al*., 1996; Hayama *et al*., 2000, 2001).

The more comprehensive approach used in this research has been made possible by the presence in this laboratory of a collection of ESTs representative of preclimacteric peach fruit mRNAs. The pattern of softening that results from the data obtained in this work is novel and complex. By contrast with the commonly held idea of a start of the softening at the onset of the ripening proper, it is shown that softening begins well before the appearance of the ethylene climacteric rise and hence of the ripening proper. It is due to the ordered and sequential expression of many genes encoding proteins involved in the metabolism of the cell wall. Pectins seem to be the parietal components particularly involved in the degradation process. The expression of the various genes shows a differential regulation by ethylene. Finally, the massive cell wall dismantling, as inferred by the expression of the many genes encoding degrading enzymes, appears to be actually counteracted by some synthesis of parietal polysaccharides and, especially, by the expression of genes encoding cell wall proteins with a putative structural function.

Materials and methods

Plant material

Plants of *Prunus persica* (L.) Batsch cv. Redhaven were grown in a field near Padua. Fruits at various stages of development (S1, S2, S3I, S3II, S4I, and S4II; Zanchin *et al*., 1994, corresponding to 40, 65, 85, 95, 115, 120–125 d after full bloom, DAFB, respectively) were collected and used either without or with an ethylene treatment. The hormone treatment was provided by placing whole fruits (attached to a branch for all stages but the S4s) in a sealed chamber and flushing them with ethylene (10 µl l^{-1}) in air at a flow rate of 6 l h^{-1}. Both treated and untreated samples were frozen in liquid nitrogen and stored at –80 °C for subsequent use.

Sequence analysis and annotation

A collection of peach ESTs produced in the laboratory from a 3′-end library of fruit mesocarp representative of the S3II (95 DAFB) stage (L Trainotti and G Casadoro, unpublished data) was annotated by database (release of 14 December 2001) similarity searching using the BLASTX software (Altschul *et al*., 1997). By keyword searches of the annotated EST database a first subset of clones corresponding to genes involved in the cell wall metabolism has been selected for the characterization carried out in this work.

The EMBL accession numbers for the DNA sequences of selected contig (Ctg) clones are: Ctg 257, AJ532967; Ctg 251, AJ533510; Ctg 219, AJ533395; Ctg 404, AJ533090; Ctg 124, AJ533611; Ctg 167, AJ533664; Ctg 364, AJ533695; Ctg 874, AJ534047; Ctg 337, AJ533907; Ctg 393, AJ533689; Ctg 6, AJ533390; Ctg 254, AJ533641; Ctg 190, AJ533659; Ctg 285, AJ533938; Ctg 907, AJ532990; Ctg 10, AJ532986; Ctg 125, AJ533670; Ctg 95, AJ533292; Ctg 288, AJ533150; Ctg 152, AJ533682; Ctg 153, AJ533122; Ctg 187, AJ533532; Ctg 188, AJ533253; Ctg 191, AJ533767; Ctg 418, AJ533321; Ctg 444, AJ534072; Ctg 501, AJ534133; Ctg 528, AJ534127; Ctg 612, AJ533776; Ctg 762, AJ533642; Ctg 821, AJ533607; Ctg 839, AJ533259.

Thereafter, this group has been subdivided into three subgroups: (1) genes encoding ‘proteins involved in cell wall weakening’; (2)

Table 1. *List of the 32 cDNAs that have been used in this work*

The subdivision of the cDNAs into four functional groups is based on the results of the BLASTX analyses. The names of the different cDNA clones appear in the first column. In the second column the number of single pass sequences that belong to each contig is indicated. The column 'product' describes the gene product of the corresponding EST and, in parenthesis, the name of the organism from which the sequence with the best BLASTX score was obtained. In the fourth column there are the accession numbers of the protein sequences which gave the best BLASTX score. The fifth column reports the P-FAM numbers of the domains found on the protein sequences corresponding to the accession number of the previous column (NA: not available). The last column shows the scores obtained by the software 'TargetP' with the accessions listed in column four.

	Name	Number of sequences	Product	Accession number	P-FAM	Target-P
Proteins involved in cell wall weakening						
1	Contig 257	9	Pectate lyase [*Arabidopsis thaliana*]	AAB80622	pfam00544	SP: 0.929
2	Contig 251	8	Pectase lyase [*Arabidopsis thaliana*]	AAK25850	pfam00544	SP: 0.978
3	Contig 219	2	Endopolygalacturonase [*Prunus persice*]	AAC64184	pfam00295	SP: 0.982
4	Contig 404	2	Expansin [*Prunus persica*]	BAB19676	pfam01357	SP: 0.965
5	Contig 124	1	Endo-β-1,4-glucanase [*Fragaria ananassa*]	CAB43938	pfam00759	SP: 0.983
6	Contig 167	1	β-galactosidase [*Fragaria ananassa*]	CAC44500	pfam01301	SP: 0.946
7	Contig 364	1	β-1,3-glucanase [*Arabidopsis thaliana*]	NP_180319	pfam00332	SP: 0.825
8	Contig 874	1	Pectin methylesterase [*Arabidopsis thaliana*]	BAB09012	pfam01095	SP: 0.741
9	Contig 337	1	Pectin acetylesterase [*Arabidopsis thaliana*]	NP_198019	pfam03283	SP: 0.874
Proteins involved in cell wall biosynthesis						
10	Contig 393	8	Sucrose synthase [*Psium sativum*]	CAA09910	pfam00862	other: 0.717
11	Contig 006	3	Glucose acyltransferase [*Lycopersicon pennellii*]	AAF64227	pfam00450	other: 0.960
12	Contig 254	3	UDP-glucose transferase [*Arabidopsis thaliana*]	NP_173654	pfam00201	other: 0.449
13	Contig 190	1	α1,4-fucosyltransferase [*Beta vulgaris*]	CAC44377	pfam00852	SP: 0.981
14	Contig 285	1	Glucosyltransferase [*Arabidopsis thaliana*]	NP_181910	pfam00201	other: 0.650
15	Contig 907	1	Cellulose synthase caralytic subunit (RSW1) [A. t.]	NP_194967	pfam03552	other: 0.702
Cell wall proteins with structural function						
16	Contig 010	35	Fibre protein E6 [cotton]	S65062	NA	SP: 0.991
17	Contig 125	9	Proline-rich protein [*Arabidopsis thaliana*]	NP_176440	pfam00234	SP: 0.787
18	Contig 095	4	Allergen extensin-like [*Sambucus nigra*]	AAF16869	pfam01190	SP: 0.865
19	Contig 288	2	LR protein [*Arabidopsis thaliana*]	T07079	smart00366.5	SP: 0.983
20	Contig 152	1	LR protein [*Lycopersicon esculentum*]	T07079	smart00366.5	SP: 0.983
Unknown proteins targeted to the cell wall						
21	Contig 153	10	Snakin-1 [*Solanum tuberosum*]	CAC44032	pfam02704	SP: 0.924
22	Contig 187	2	Unknown protein [*Arabidopsis thaliana*]	NP_192976	PF04043	SP: 0.540
23	Contig 188	1	Unknown protein [*Arabidopsis thaliana*]	NP_192847	pfam03005	SP: 0.575
24	Contig 191	1	Unknown protein [*Arabidopsis thaliana*]	AAF40464	pfam02886	SP: 0.841
25	Contig 418	1	Unknown protein [*Arabidopsis thaliana*]	AAL32685	NA	SP: 0.945
26	Contig 444	1	Unknown protein [*Arabidopsis thaliana*]	NP_187920	NA	SP: 0.954
27	Contig 501	1	Unknown protein [*Arabidopsis thaliana*]	AAK62422	NA	SP: 0.983
28	Contig 528	1	Unknown protein [*Arabidopsis thaliana*]	NP_173069	pfam03348	SP: 0.606
29	Contig 612	1	Unknown protein [*Arabidopsis thaliana*]	NP_200518	NA	SP: 0.709
30	Contig 762	1	Unknown protein [*Arabidopsis thaliana*]	AAFF27920	pfam03141	SP: 0.720
31	Contig 821	1	Unknown protein [*Arabidopsis thaliana*]	NP_191705	pfam00097	SP: 0.815
32	Contig 839	1	Unknown protein [*Arabidopsis thaliana*]	AAF88119	NA	SP: 0.629

genes encoding 'cell wall proteins with structural function'; and (3) genes encoding 'proteins involved in cell wall biosynthesis'. The proteins belonging to the third subgroup are expected to be located in cellular compartments other than the cell wall.

A second subset, consisting of ESTs similar (identity >70% over a minimum of 20 aa) to genes of unknown function (most of them were *Arabidopsis* genes) has been assigned to the cell wall in an indirect way described here. The unknown sequences which gave the best alignment with these ESTs were tested with the TargetP v1.01 software URL: www.cbs.dtu.dk/services/TargetP/ (Emanuelsson *et al*., 2000). Should the unknown protein be predicted to be secreted, the peach EST would also be regarded as corresponding to a secreted protein. In this manner a fourth group of ESTs related to the cell wall and encoding 'proteins with an unknown function' was obtained. As a control, the proteins belonging to the first subset were also tested with the TargerP software and all the proteins expected to be secreted (i.e. those involved in the cell wall degradation and those with a structural function) gave high secretion scores. These two subsets of ESTs (Table 1) represent the cell-wall-related genes used in this study.

RNA extraction, macroarray preparation and hybridization experiments

Total RNA was extracted from fruits as described in Schneiderbauer *et al*. (1991).

To construct the macroarrays, the selected EST clones were picked from freezer stocks and grown overnight in 96 well plates in 200 μl LB plus 50 μg ml^{-1} of kanamycin. Overnight cultures were used directly as templates in PCR amplifications to obtain PCR products for sequence verification and membrane spotting. PCR reactions (50 μl each, run in double: one for sequence verification, the other for macroarray preparation) were assembled as follow: 1×

PCR buffer, 200 µmol l^{-1} dNTP, 10 pmol of each M13 Forward (–20) and M13 Reverse primer, 1 unit *Taq* polymerase, and 2.5 mmol l^{-1} $MgCl_2$. Reaction conditions were the followings: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s. An additional step at 72 °C for 7 min was performed to ensure that the products were extended to their full length. Of each reaction, 5 µl were loaded onto a 1.2% (w/v) agarose gel, along with known concentrations of 100 bp ladder DNA (New England Biolabs), to determine product quality and quantity. All the PCR reactions, visualized by ethidium bromide staining, gave single products of the expected size. Products were present at a minimum of 50 ng μl^{-1}, and did not exceed 100 ng μl^{-1}. A 1/10 volume of spotting solution (bromophenol blue 0.25% w/v, xylene cyanol 0.25% w/v, glycerol 70% w/v) was added to each PCR product to facilitate spotting. Aliquots of each PCR product were spotted in quadruplicate onto a positively charged nylon membrane, Hybond N^+ (Amersham Pharmacia), using a Biomek2000 automated laboratory workstation (Beckman Coulter) with the 96 pins HDRT tool. After spotting, the DNA was denatured by laying the membrane on 3MM paper presoaked with denaturing solution (0.5 mol l^{-1} NaOH, 1.5 mol l^{-1} NaCl) for 5 min. Afterwards, the membranes were neutralized for 5 min on 3MM paper presoaked with neutralizing solution (1 mol l^{-1} Tris pH 7.5, 1.5 mol l^{-1} NaCl), then washed for 10 min with 2× SSC. The DNA was then fixed to the membranes by baking them at 80 °C for 2 h.

For macroarray experiments, ^{32}P-labelled probes consisted of single strand cDNAs obtained by means of retro-transcription of 3 µg of total RNA from each of the six tested developmental stages. Retro-transcription was carried out in the presence of a ^{32}P nucleotide, with an oligo dT_{18} as a primer, 200 units of SuperScript™II RNase H^- Reverse Transcriptase (Life Technologies) for 50 min at 42 °C, followed by an incubation at 70 °C for 15 min. Before applying it to the hybridization solution, the probe was treated with RNase H (2 units, 20 min) followed by heat denaturation (95 °C for 10 min).

For northern analyses equal RNA loading was checked by means of ethidium bromide staining of agarose gels. Total RNA was separated in 6% formaldehyde–1.2% agarose gels and blotted onto Hybond N membranes (Amersham International) using 20× SSC as blotting buffer. DNA probes were ^{32}P-labelled using a random-primed DNA labelling kit (Promega). The membranes were prehybridized (2 h) and hybridized (16–20 h at 60 °C) in 0.5% SDS, 5× Denhardt's solution, 5× SSC, sonicated herring sperm DNA (100 µg ml^{-1}). After hybridization, membranes were washed at 65 °C with solutions containing 1% SDS and decreasing concentration of SSC down to a final wash with 0.5× SSC, and exposed to X-ray films (X-Omat S, Kodak) at –80 °C. Macroarray filters have been exposed to Cyclone Storage Phosphor Screens (Packard) and the image has been acquired with a Cyclone Storage Phosphor System (Packard). The digital image has been analysed with the 'Array Vision' (Imaging Research, Inc) software. The values thus obtained have been normalized by means of an internal standard (i.e. ubiquitin).

Results

Peaches are climacteric fruits whose growth curve is a characteristic double sigmoid where four different stages (i.e. S1, S2, S3, and S4) can be recognized (Zanchin *et al*., 1994). In this laboratory a peach EST collection containing more than 800 unigenes was recently obtained (L Trainotti and G Casadoro, unpublished results). mRNA representative of peach fruits at the preclimacteric stage (i.e. late S3, here reported as S3II) was used to prepare the cDNA library.

An analysis of this collection of annotated ESTs revealed that it contained at least 32 cDNAs encoding proteins related to the cell wall. But for the cDNAs encoding enzymes putatively involved in the biosynthesis of parietal polysaccharides, the relationship to the cell wall was deduced by a computational analysis of homologous sequences that showed the presence of a domain typical for cell wall-secreted proteins (Emanuelsson *et al*., 2000).

On the basis of their putative function, the selected ESTs were divided into four groups (Table 1). As expected, a numerous group comprises cDNAs encoding enzymes involved in the dismantling of the cell wall. Another group is formed by cDNAs encoding enzymes with putative biosynthetic functions. A third group includes cDNAs encoding proteins with a structural function. Finally, a fourth and abundant group is formed by cDNAs encoding proteins with an unknown function, though contig (Ctg) 153 shows significant homology to the potato snakin-1, an anti-pathogen protein (Segura *et al*., 1999).

The expression of the various ESTs was analysed in fruits at different developmental stages by using the macroarray technique. The occurrence of the ethylene climacteric rise (i.e. S4I) was deduced by following the expression of the peach ACO-1 gene (Tonutti *et al*., 1997; Ruperti *et al*., 2001). Since, on the basis of previous observations (L Trainotti and G Casadoro, unpublished data), the expression of genes involved in the softening of peaches starts before the appearance of the ethylene climacteric rise (i.e. at the S3 stage), analysis of the S3 and S4 stages was expanded by examining tissues obtained by both early (i.e. S3I and S4I) and late (i.e. S3II and S4II) stages.

Figure 1 shows the overall expression of the genes considered in this study. Both in this and in the following figures an arrow indicates the ethylene climacteric rise, as deduced on the basis of ACO-1 gene expression. Six main patterns can be recognized, each with the expression maximum at a different developmental stage. The width of each stripe in the diagram is proportional to the number of genes that show a comparable expression pattern, so this type of analysis indicates that most of the genes considered have maximum expression after the S3II stage, and are therefore relevant for the softening, while only a few of the genes considered appear to be related to the cell wall metabolism that occurs during the growth of fruits. The different behaviour of the various genes in relation to the appearance of the ethylene climacteric peak suggests that they might be differentially regulated by this hormone.

The macroarray analysis yielded more interesting information when the expression of the different genes was evaluated according to their putative function. The expression of genes involved in the weakening of the cell wall is shown in Fig. 2A. But for a pectin-acetylesterase

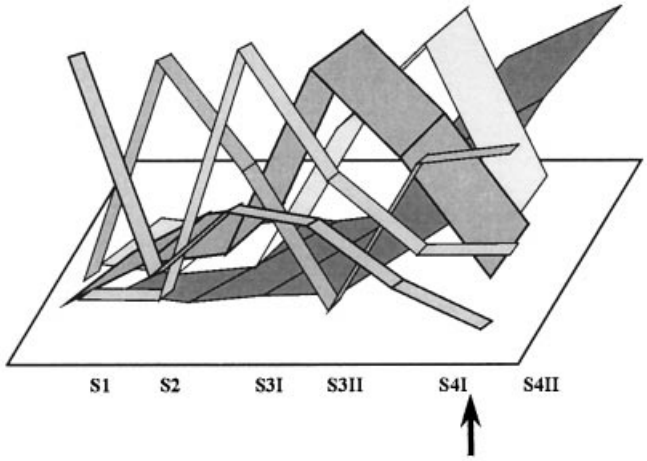

Fig. 1. Expression profiles of the cell-wall-related genes in peach fruits at six different stages that encompass fruit development and ripening (i.e. from S1 to S4II). The figure has been drawn based on the results of the macroarray experiments. The width of each stripe is proportional to the number of genes sharing a similar expression profile. Although most of the genes have maximal expression at the preclimacteric and ripening stages (i.e. from S3II to S4II), genes involved in cell wall remodelling typical of the growing phase can also be found. The arrow indicates the appearance of the ethylene climacteric rise traditionally considered to mark the start of ripening proper.

gene (Ctg 337) whose expression is not detectable, and for an endo-β-1,4-glucanase gene (Ctg 124) with the expression maximum at the S3II stage, all the remaining genes show an expression peak at either the S4I or the S4II stages. In particular, two genes (i.e. the two pectate lyase genes, Ctg 251 and Ctg 257) reach their maximum at the S4I stage and show a significant decrease thereafter, while the remaining five genes (i.e. endopolygalacturonase [Ctg 219], expansin [Ctg 404], β-galactosidase [Ctg 167], pectin methylesterase [Ctg 874], and β-1,3-glucanase [Ctg 364]) continue to increase up to a maximum at the S4II stage.

The expression of genes encoding enzymes putatively involved in the synthesis of parietal polysaccharides is shown in Fig. 2B. In this case, only one gene (i.e. glucose acyltransferase, Ctg 6) reaches the maximum at the S3II stage and significantly decreases thereafter. The sucrose synthase (Ctg 393) encoding gene shows an expression maximum at the S4I stage, while all the remaining genes (i.e. UDP-glucose transferase [Ctg 254], glucosyltransferase [Ctg 285], cellulose synthase catalytic subunit [Ctg 907], and fucosyltransferase [Ctg 190]) have increasing expression during the transition from S4I to S4II.

The expression pattern of the genes encoding proteins with a putative structural function (Fig. 2C) appears of particular interest. Two genes encoding leucine-rich proteins are present, one of them almost undetectable (Ctg 288), the other gene with a low but increasing expression during ripening (Ctg 152). A gene encoding an allergen (extensin-like, Ctg 95) has the highest expression at the S3I stage, decreases in S3II and S4I and increases again at the S4II stage. A proline rich protein encoding gene (Ctg 125) is expressed throughout the fruit life. However, a steep increase in transcript amount is observed in S2 and the amount remains high until stage S4I, while in the subsequent stage S4II the gene expression decreases and becomes comparable to that observed in the young fruitlets (i.e. stage S1). Finally, an EST (Ctg 10) that corresponds to a gene encoding a protein similar to the E6 protein from cotton fibres (John and Crow, 1992) shows an extremely high expression that is clearly ripening-specific. It has to be noted that, in the preclimacteric EST collection, this E6-like gene is by far the most abundant gene among those considered in this study (see Table 1) and makes up about 4% of the mesocarp mRNA at the S3II stage.

As for the genes encoding proteins related to the cell wall but with an unknown function, most of them show an almost undetectable expression throughout fruit growth and ripening (Fig. 2D). The expression of the remaining genes follows three different patterns. Transcripts related to Ctg 188 increase at the S2 stage and remain high till S4I, after which a drop to undetectable levels occurs. The expression of the snakin-1-like gene (Ctg 153) shows a sudden increase at the S3II stage, reaches a maximum at the S4I stage and drops to barely detectable levels thereafter. The remaining three genes (i.e. Ctgs 187, 528 and 821) have increasing expressions during stages S4I and S4II, so they seem to be the only genes that are clearly ripening-related within this group.

A validation of the above described expression patterns was made by northern analyses (Fig. 3). Data are presented for those genes whose expression could be detected by means of this technique. It is noteworthy the fact that these data confirm the result obtained with the macroarray analysis. With regard to the genes involved in cell wall dismantling, it is again the pectin acetylesterase (Ctg 337) which has undetectable expression. The remaining genes of this group show an expression pattern that suggests their relevance for the softening process. All six genes putatively involved in biosynthetic processes have detectable expression. In particular, four of them have maximum expression at the S4II stage, while the expression of the sucrose synthase encoding gene (Ctg 393) reaches its maximum at the S4I stage. Four genes out of the five encoding proteins with structural function have detectable expression. However, it is the only E6-like protein encoding gene (Ctg 10) that clearly shows a significant and ripening-specific expression. As for the 12 genes with unknown function, five of them (Ctgs 191, 444, 501, 612, and 762) have undetectable expression, while of the remaining genes only Ctg 187, 528, 821, and 839 show an increasing expression during ripening that might suggest a possible role in this process.

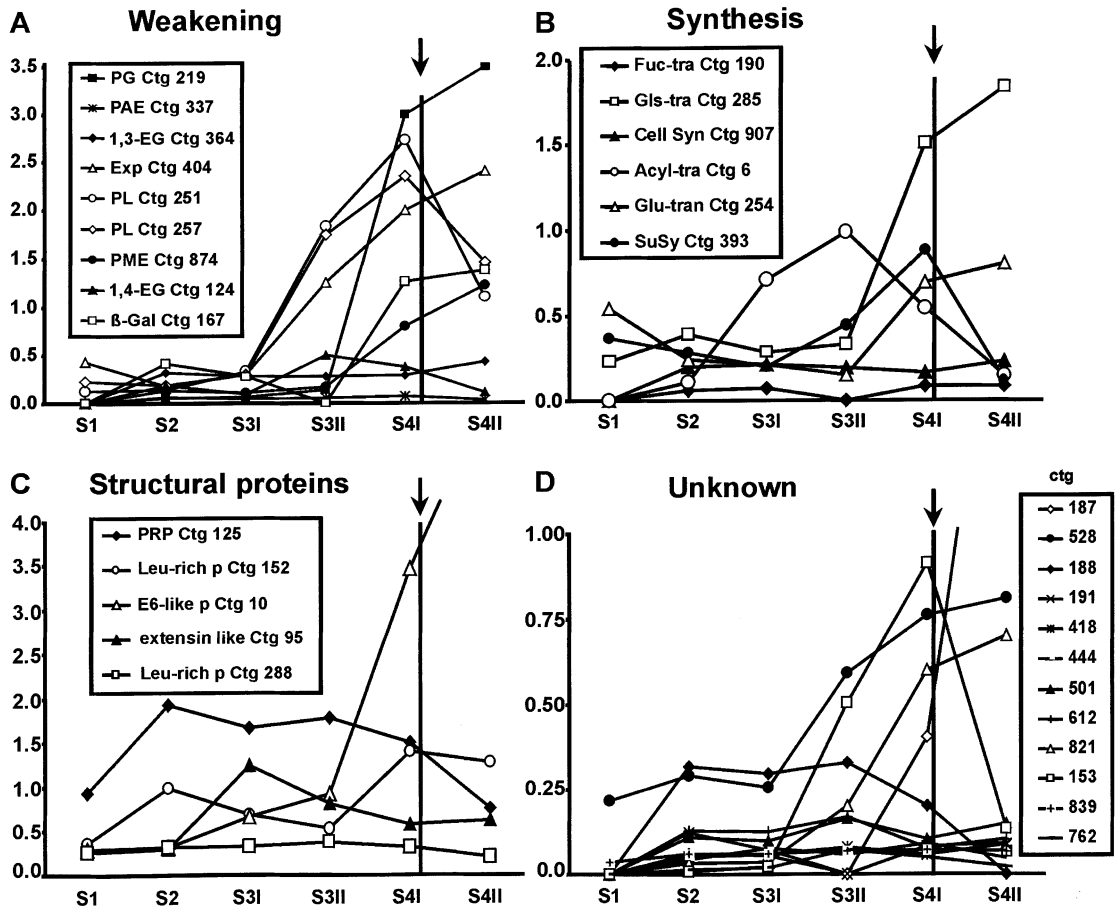

Fig. 2. Macroarray expression data separated by considering the four functional groups that comprise all the proteins encoded by the different cDNA clones: (A) cell wall weakening; (B) cell wall biosynthesis; (C) structural function; (D) unknown. Fruit developmental stages range from S1 to S4II. Values on the ordinate axis are expressed as the ratio of the subtracted volumes of the indicated ESTs and ubiquitin.

Peaches are climacteric fruits and the importance of ethylene for their ripening has long been known. A parallel between the expression pattern of the ACO-1 gene and the actual production of ethylene has been shown in peach fruits (Tonutti *et al*., 1997; Ruperti *et al*, 2001). Therefore, in the present work the appearance of the ethylene climacteric rise has been deduced by monitoring an EST corresponding to the above *ACO-1* gene. In particular, the expression of this gene starts to be detectable in S3II and shows a steep increase thereafter in the subsequent S4I and S4II stages.

The patterns of gene expression in fruits at different stages of development suggest the possibility of different responses to ethylene. In fact, after the high levels of ACO-1 expression observed in S4I, in the subsequent S4II stage some genes show an increased expression while other genes exhibit a decreased expression. Since, on the basis of the overall expression data, the turning point appears to be the passage from preclimacteric (i.e. S3II) to climacteric (i.e. S4I), only these two stages were considered for the analysis of ethylene treatments on the expression of the genes considered in this work. The three paradigmatic patterns shown in Fig. 4 represent the different types of response observed following a treatment with exogenous ethylene.

The genes (i.e. Ctgs 191, 288, 337, 444, 501, 612, and 762), whose expression is undetectable by northern analysis, also have undetectable expression after treatment with ethylene, and this is true for both of the developmental stages considered. As for the other genes, 11 of them appear to be up-regulated by ethylene (Fig. 4A). Of this group, five (i.e. β-galactosidase [Ctg 167], endopolygalacturonase [Ctg 219], β-1,3-glucanase [Ctg 364], expansin [Ctg 404], and pectin methylesterase [Ctg 874]) are involved in cell wall weakening, one gene encodes a protein (i.e. UDP-glucose transferase, Ctg 254) putatively involved in biosynthetic processes, two genes encode proteins (i.e. cotton-E6-like protein [Ctg 10] and leucine-rich protein [Ctg 152]) with structural function, while the remaining three (Ctgs 187, 418, 839) have unknown functions.

Another group of genes appears to be down-regulated by ethylene (Fig. 4B). In the preclimacteric fruits (S3II), the gene expression is not decreased by air as expected, while

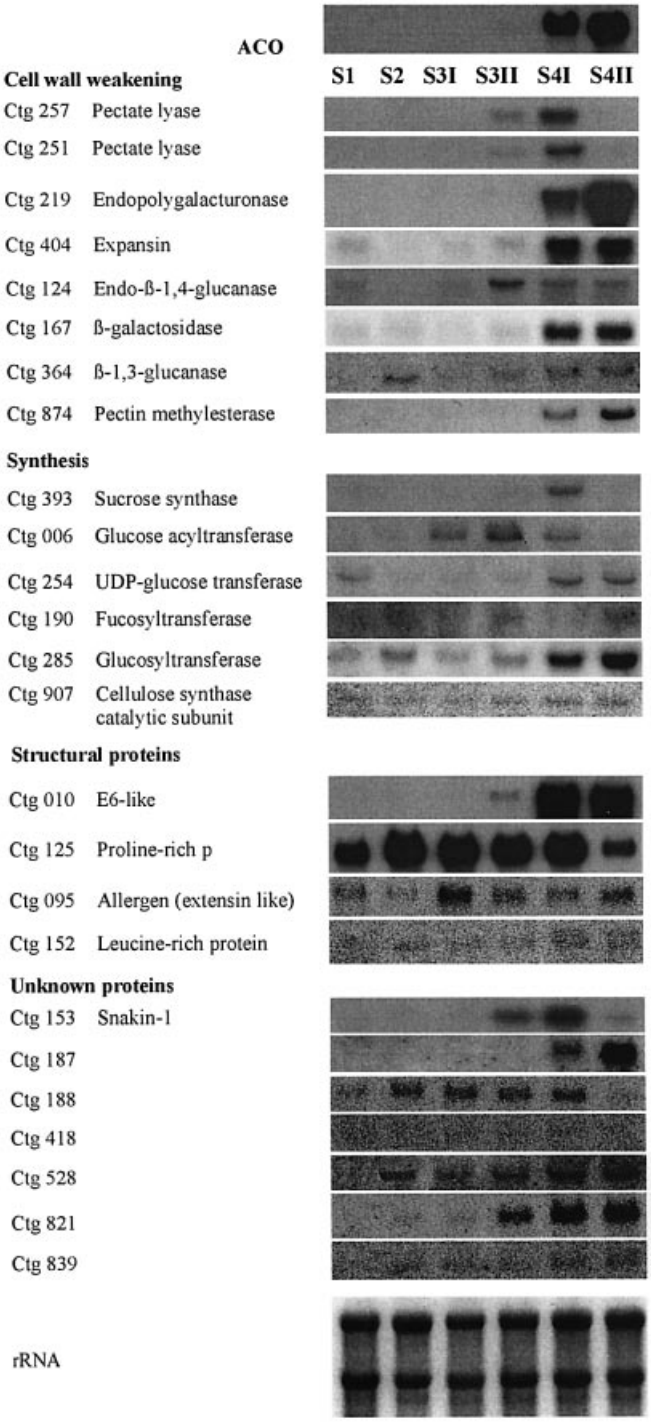

Fig. 3. Northern analysis of the cell-wall-related genes throughout peach fruit development and ripening (i.e. from S1 to S4II). Each lane has been loaded with 10 µg of total RNA. Contigs 337, 288, 191, 444, 501, 612, and 762 did not give any detectable hybridization signal so they have not been included in the figure. The expression profile of the peach ACO-1 gene has been added at the top of the figure as a marker for ethylene climacteric rise. Equal RNA loading is shown by ethidium bromide staining of the ribosomal RNAs.

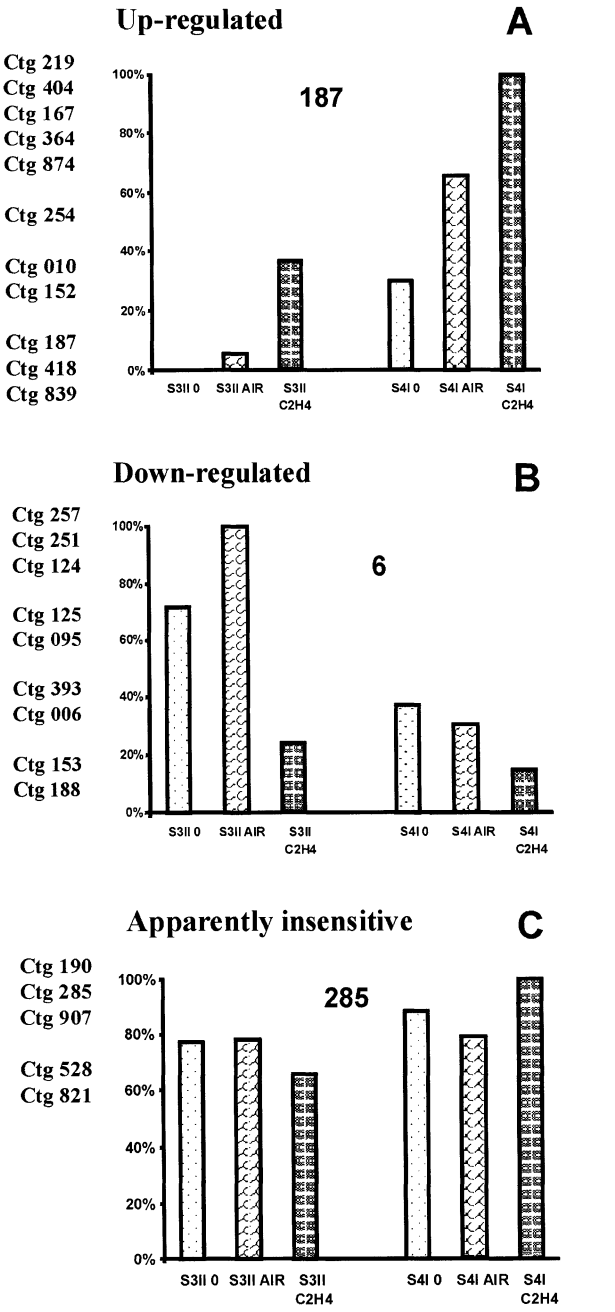

Fig. 4. Treatment of detached peach fruits with exogenous ethylene. The gaseous hormone has been applied for 48 h to peach fruits, at either the S3II or the S4I stages. Although each of the three patterns refers to a specific gene, they are paradigmatic for all the other genes reported close to each pattern. In these experiments Contigs 337, 288, 191, 444, 501, 612, and 762 did not give any detectable hybridization signal. The intensity of the strongest hybridization signal for each experiment has been arbitrarily set to 100% and the other values adjusted accordingly.

it is strongly decreased by the ethylene treatment. By contrast, in S4I fruits, where the endogenous production of ethylene has already started, the gene expression decreases both in air and in ethylene. Among them there are the two pectate lyases and the endo-β-1,4-glucanase encoding genes (Ctgs 257, 251 and 124, respectively), two biosynthesis related genes (i.e. sucrose synthase [Ctg

393] and glucose acyltransferase [Ctg 6]), two genes coding for structural proteins (i.e. proline-rich protein [Ctg 125] and the extensin-like allergen [Ctg 95]), and two other genes with unknown function (i.e. Ctgs 153, 188). Finally, a third and minor group of genes does not show any significant change in expression following the treatment with exogenous ethylene (Fig. 4C). Three of them (i.e. fucosyltransferase, glucosyltransferase and the cellulose synthase catalytic subunit, Ctgs 190, 285 and 907, respectively) belong to the 'synthesis' group, while the remaining two (i.e. Ctgs 528, 821) belong to genes with unknown function.

Discussion

Genomic approaches are being used more and more frequently to obtain a comprehensive understanding of plant physiological processes (Aharoni *et al*., 2000; Aharoni and O'Connell, 2002; Moore *et al*., 2002). Softening is an important metabolic event related to the ripening of fleshy fruits, and its complexity suggests that current knowledge might significantly profit by this type of approach. Therefore, in this work a genomic approach has been used to study the softening of the climacteric peach fruits. The cDNAs used for this study were present in an EST collection recently prepared in the laboratory (L Trainotti and G Casadoro, unpublished results).

An investigation of this collection allowed the selection of 32 cDNAs that are putatively related to the cell wall. These do not include all the possible genes involved in peach softening. However, it is considered that the absence of a few cell-wall-related genes should not weaken the cogency of this study since all the main aspects of cell wall metabolism are represented by the cDNAs used in this work, thus making it possible to obtain a comprehensive picture of the peach softening process.

The result of the macroarray analysis shows that only a limited number of genes has maximum expression during the early stages of fruit growth, while most of the selected genes have expression patterns where the maximum occurs at either the preclimacteric or the ripening stages. This might reflect the fact that the RNA used to prepare the EST library was obtained from peaches at a pre-ripening stage.

The analysis of the expression patterns on the basis of the functional categories adds significant and new information to current knowledge of the peach softening. Six out of the nine genes related to the weakening of the cell walls encode pectin-degrading enzymes. This suggests that pectins are probably the parietal component mostly involved in the dismantling process. The degradation of pectins appears to be finely tuned with different genes being expressed according to highly co-ordinated patterns. Pectate lyases cause massive degradation of pectins (Brett and Waldron, 1996; Marìn-Rodrìguez *et al*., 2002) and they were first studied in fungi that use such enzymes to dismantle the cell walls of the host plants. Pectate lyases were discovered in higher plants many years ago, including strawberry fruits (Medina-Escobar *et al*., 1997). Their role in the softening of strawberries has been demonstrated by the finding that an antisense pectate lyase gene was able to reduce the softening in those fruits significantly (Jiménez-Bermùdez *et al*., 2002). This study's data show that two different pectate lyase genes (Ctgs 251 and 257) start to be expressed at the S3II stage, reach a maximum at the S4I stage, and decline after the ethylene climacteric rise to undetectable levels. By contrast, the other pectin degrading enzymes (i.e. a polygalacturonase, a β-galactosidase and a pectin methylesterase, Ctgs 219, 167 and 874, respectively) show a typical ripening-related pattern with expression that starts at the S4I stage and increases thereafter. So, pectate lyases would appear to have the task to carry out an early and coarse degradation of pectins, thus making them more susceptible to the subsequent attack of other degrading enzymes, among them β-galactosidase and the already known endopolygalacturonase (Lester *et al*., 1994) and pectin methylesterase (Glover and Brady, 1994).

By contrast, only three enzymes (the β-1,4- and β-1,3-glucanases and an expansin, Ctgs 124, 364 and 404, respectively) thought to act on hemicelluloses, have been found and, among them, only the expansin (Ctg 404) is expressed at high level during ripening proper, in agreement with previous data (Hayama *et al*., 2000). Furthermore, a xyloglucan endotransglycosylase (XET) was found to be expressed only during fruit growth (Hayama *et al*., 2001) and an endo-β-1,4-glucanase mRNA was detected at very low levels in S4II fruits (Trainotti *et al*., 1997), thus supporting the idea that pectin degradation prevails over the degradation of hemicelluloses.

Among the genes putatively involved in cell wall biosynthesis, only a glucosyltransferase (Ctg 285) and a UDP-glucose transferase (Ctg 254) show a clearly increased expression during the ripening phases (i.e. S4I and S4II). It appears interesting that a sucrose synthase (SuSy, Ctg 393) and a cellulose synthase catalytic subunit (RSW1, Ctg 907) show a partly overlapping expression during the preclimacteric and early ripening stages. Since it has been suggested that sucrose synthase might be involved in the cellulose biosynthesis by supplying the cellulose synthase with its substrate (Delmer, 1999), the partly overlapping expression of the two genes suggests that some cellulose might also be synthesized during the softening process. On the other hand, some synthesis of polysaccharidic parietal components had already been demonstrated to occur in tomato by feeding mature green berries with radioactive substrates (Huysamer *et al*., 1997*a*, *b*).

New insights into the softening of peaches are supplied by the data relative to the expression of genes encoding cell wall proteins with a putative structural function. A

gene encoding a proline rich protein (Ctg 125) is highly expressed throughout fruit life, though a marked decrease is evident during ripening. Different proline-rich protein encoding genes expressed in fruits have also been described in strawberry and grape. However, while in strawberry (a rosaceous, like peach) the expression profile of those genes did not show a ripening-specific pattern (Aharoni and O'Connell, 2002), in grape Pro/Hyp-rich glycoprotein encoding genes were found to be highly expressed after véraison (Davies and Robinson, 2000). In peaches, a gene encoding an extensin-like allergen (Ctg 95) shows two peaks of expression (S3I and S4II). This suggests that such a structural protein might be required to stabilize the cell walls both during the growth phase that leads to the formation of a 'mature green' fruit and during the late softening phase. Genes encoding extensins have also been shown to be expressed in strawberry fruits, although only one of such genes had a higher expression in ripe fruits compared to the green ones (Aharoni and O'Connell, 2002).

The expression pattern of a gene encoding a protein particularly rich in asparagine residues (i.e. Ctg 10) appears remarkable: the limited expression that starts to be detectable at the S3II stage shows an impressive increase during the subsequent S4I and S4II stages. What might be the function of this protein so highly expressed during the softening of peaches? The deduced protein sequence has a C-terminal domain 55.4% similar to a corresponding domain in the E6 protein from cotton fibres. Moreover, within this domain both proteins share a short (20 amino acids) and highly similar (90%) region found in other cell wall proteins (Demura and Fukuda, 1994) and in asparagine-rich domains of non-classical arabinogalactan proteins (see Fig. 3 in Gaspar *et al*., 2001). The E6 protein has been shown to accumulate in cotton fibres during the late primary cell wall and early secondary cell wall synthesis stages (John and Crow, 1992), so it is also likely that the protein that accumulates during the softening of peaches might fulfil a structural function. To this purpose, it is interesting to note that both proteins are rich in tyrosine residues that are particularly numerous in the C-terminal part of the sequence. Tyrosyl residues of cell wall proteins can undergo oxidative dimerization to yield isodityrosine cross-links (Epstein and Lamport, 1984; McNeil *et al*., 1984; Brady and Fry, 1997), and intramolecular cross-linking by means of isodityrosine has also been identified in *Chlamydomonas* cell walls (Waffenschmidt *et al*., 1993). Further support to the idea that structural cell wall proteins are synthesized during ripening of fleshy fruits can be obtained by the data about the incorporation of radioactive carbon dioxide into parietal components of kiwi fruits induced to ripen by means of ethylene treatments (Redgwell, 1996). In those experiments 'intact fruits showed incorporation of radioactivity into cell wall material during ripening, but over 90% of radioactivity was in cell-wall-associated protein' (Redgwell, 1996). Normally, cell wall enzymes can be easily removed from parietal preparations, so it is probable that some of the radioactivity might have been incorporated into proteins with structural function.

As reported in the Results, the majority of the cell-wall-related genes with unknown function has almost undetectable expression, so it is difficult to make any hypothesis about their putative role. However, the increasing expression of Ctgs 187, 528, 821, and 839 during the ripening phases suggest the possibility of their involvement in the ripening of peaches. As for the Ctg 153 that shows homology to a potato snakin-1 gene, the possibility exists that, similarly to the potato protein, the protein encoded by Ctg 153 might also be active against plant pathogens. As the cell wall is the first obstacle that a pathogen has to overcome in order to infect a plant cell, it is likely that an anti-pathogen protein is secreted into the cell wall. Therefore, although a direct role in cell wall metabolism is excluded for this protein, such a protective function would confirm its parietal location as detected by the computational analysis.

Peaches are climacteric fruits and ethylene is considered to play a crucial role in the establishment of the ripening syndrome. Analysis of the expression of cell-wall-related genes has shown that if only those genes whose expression was also clearly detectable by northern analysis were considered, about half of them are up-regulated by ethylene. Most of the remaining genes with clearly detectable expression appear to be down-regulated by the hormone, while only a few seem to have an ethylene-independent expression.

Apparently, a similar behavioural pattern is in contrast with the common view that the ripening-related genes are switched on by ethylene in climacteric fruits. However, data obtained with transgenic tomato and melon plants, both with an extremely reduced ability to produce ethylene in fruits, had already demonstrated that both ethylene-dependent and ethylene-independent pathways participate in the establishment of the ripening syndrome (Theologis *et al*., 1993; Hadfield *et al*., 2000). Besides confirming the latter view, these data with the cell-wall-related genes clearly show that the softening of peaches, hence the ripening syndrome, is initiated before the appearance of the ethylene climacteric. Although sound experimental data are necessary to demonstrate it, the hypothesis that the early parietal degradation by both the pectate lyase and the endo-β-1,4-glucanase genes might produce oligosaccharins able to promote the appearance of the subsequent ethylene climacteric rise, as suggested in Dumville and Fry (2000), could be proposed.

In conclusion, although it had been demonstrated in tomato that a limited synthesis of polysaccharides can occur in mature green berries (Huysamer *et al*., 1997*b*), until now the softening of fleshy fruits has mostly been

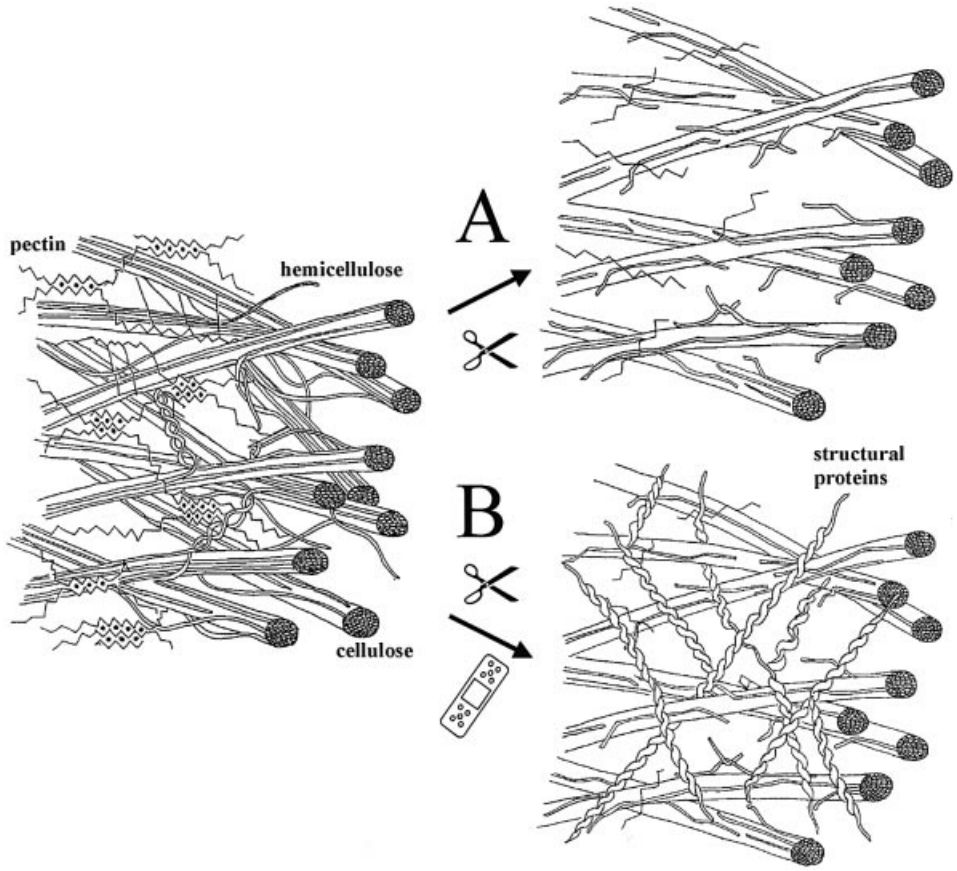

Fig. 5. Proposed model of the cell wall rearrangements that occur during the softening of peach fruits. A simplified model of the cell wall prior to the softening is depicted on the left (redrawn after Carpita and Gibeaut, 1993). In the 'only dismantling hypothesis' the structural properties of the fruit cell walls are badly compromized (pattern A: the degrading enzymes are represented as scissors). In the 'dismantling-repairing hypothesis' that can be drawn from the results of the present work (pattern B), the massive production of the cell-wall-degrading enzymes (scissors) is accompanied by an abundant deposition of newly synthesized cell wall structural proteins (represented as sticky plasters) that would help preventing the rupture of cells while allowing the softening to occur.

regarded as a physiological process where many different degrading enzymes act synergistically to carry out a dismantling of the fruit cell walls (Fig. 5A). The picture of the softening that can be obtained for peaches on the basis of the genomic approach used in this work is quite different from the above idea. Concomitant with the expected massive cell wall dismantling carried out by many different enzymes, a limited synthesis of parietal polysaccharides also occurs and large amounts of structural cell wall proteins are synthesized (Fig. 5B). The deposition of proteins with a structural function into the fruit cell wall would not prevent the accomplishment of the softening. Nevertheless, by acting as molecular sticking plasters, these proteins would partly counteract the massive cell wall dismantling, and thus would help to avoid a possible disruption of the cell walls during the softening process.

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