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Brief report

Expression of an endopolygalacturonase gene during growth and abscission of peach fruits

LIVIO TRAINOTTI, NICOLETTA RASCIO and GIORGIO CASADORO*

Department of Biology, University of Padua, Via Trieste 75, 35121-Padua, Italy

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*Corresponding author

Polygalacturonase (PG, EC 3.2.1.15) is one of the cell wall hydrolases involved in the cell separation processes which occur during ripening of some fleshy fruits (FISHER and BENNETT 1991), as well as during abscission of leaves and fruits (HUBERMANN and GOREN 1979; RASCIO et al. 1985; TAYLOR et al. 1990; BONGHI et al. 1992; TAYLOR et al. 1993). In particular, endopolygalacturonase of tomato fruit is the most widely known form of this enzyme, having been characterized at molecular and biochemical level (see literature in ZHENG et al. 1992). Endopolygalacturonase activity has also been found in peach during both abscission and development of fruits, while no such activity could be detected during leaf abscission (BONGHI et al. 1992; ZANCHIN et al. 1993).

A few years ago it was observed that polyclonal antibodies raised against a tomato fruit PG (i.e., PG2A) recognized, in soft ripe fruits of peach, a polypeptide with molecular mass similar to that of PG2A. Furthermore, using as a probe a cDNA coding for tomato fruit endopolygalacturonase, the same researchers were able to clone and characterize a 3.5 kb fragment of peach genomic DNA (LEE et al. 1990).

On the basis of sequence analysis it was concluded that, besides an unidentified sequence, it contained about the 3′ half of a gene which showed, in the coding regions, extensive homology with the tomato PG gene. According to the same researchers, such homology could explain the observed cross-reaction between the antibody to tomato fruit PG and a peach polypeptide, which was therefore suggested to be a peach endopolygalacturonase and the product of the partly characterized gene (LEE et al. 1990).

In tomato the gene encoding the fruit endopolygalacturonase seems to be expressed during the fruit ripening, but not during the leaf abscission. In fact, despite a significative rise in PG activity, an antibody to fruit PG did not recognize any leaf abscission protein. Moreover, a cDNA encoding a tomato fruit PG gave no hybridization to mRNA obtained from activated abscission zones of tomato leaves (TAYLOR et al. 1990).

In peach it has recently been shown that a cDNA coding for tomato fruit PG hybridized to mRNA obtained from fruit abscission zones but not from leaf ones where, in any case, no PG activity had been detected (BONGHI et al. 1992).

In peach, cell separation events which show an involvement of endopolygalacturonase, are not restricted to fruit softening and abscission. Recently, it has been found that PG activity can also be detected throughout the fruit growth (ZANCHIN et al. in press).

On the basis of the above findings we considered it of some interest to see whether the endopolygalacturonase activity, observed in the course of different cell separation events in peach, is due to expression of the partly known PG gene (LEE et al. 1990) or, as already observed in tomato (TAYLOR et al. 1990), only some of that activity can be ascribed to expression of that gene.

Materials and methods

Plant material. — Trees of *Prunus persica* (L.) Batsch. cv. Redhaven, grown at a farm near Padova (Italy), were the source of our plant material. Developing fruits were harvested in the field at various stages of development. Young fruits were also collected with their pedicel in order to prepare explants for fruit abscission zones according to BONGHI et al. (1992). Abscission was activated by exposing the explants to ethylene (100 μl/l in air at a flow rate of 0.8 l/min) for 5 days. Tissue samples to be used were frozen in liquid nitrogen and stored at −80°C.

Enzyme extraction and assay. — Endopolygalacturonase was extracted either from fruit mesocarp or from fruit abscission zones. Frozen tissues were ground in a mortar with liquid nitrogen and extracted for 1 h on ice in 4 volumes (w/v) of extraction buffer (100 mM Na acetate pH 4.0, 1 M NaCl, 20 mM β-mercaptoethanol, 5 % PVPP). The homogenate was filtered through four layers of cheesecloth and, after centrifugation (19,000 × g, 30 min), the supernatant was collected and dialyzed overnight against buffer (100 mM Na acetate pH 4.0, 20 mM NaCl). Enzyme activity was measured as described in BONGHI et al. (1992).

Nucleic acid extraction. — RNA was extracted according to the method described in SCHNEIDERBAUER et al. (1991). DNA was obtained as a side product during the first ethanol precipitation and further purified by banding it in CsCl gradient.

PCR amplification of peach genomic DNA. — Figure 5 in the paper by LEE et al. (1990) shows a sequence comparison of the peach endopolygalacturonase and the tomato endopolygalacturonase gene. Based on the published peach sequence, two primers for peach DNA amplification were made (5′ TATTGGTAGCTTGGGAGAAGAC 3′ and 5′ ACATGATTTACAGTCCACAAA 3′, respectively) at the 5′ end of the region named "tomato exon 7" and at the 3′ end of the region "tomato exon 9". The primers comprise a DNA fragment of about 590 bp. Amplification was carried out for 30 cycles and each cycle consisted of: 90 sec at 94°C, 60 sec at 55°C, 90 sec at 72°C. The PCR amplification mixture was size separated on 1.2 % agarose and the expected band was recovered from the gel. After giving it blunt ends with T4 DNA polymerase, the amplified fragment was subcloned in the Sma I site of pUC18 and the recombinant plasmid was named pPG31. Sequencing of the insert by the dideoxy chain termination method (SANGER et al. 1977) confirmed its identity (sequence not shown).

RNA dot blotting and Southern blotting. — Total RNA was applied to Hybond-N membrane (Amersham) by means of a Bio-dot microfiltration apparatus (Bio-Rad). Blotting onto nylon membrane was performed according to the manufacturer's specifications. RNA was fixed by baking the filter in an oven at 80°C for 2 h. RNA blot hybridization was carried out according to Amersham protocol at 42°C with 50 % formamide. Genomic DNA, cut using a number of restriction enzymes, was size fractionated in 0.8 % agarose and blotted onto Hybond-N-membrane (Amersham). Nucleic acids were fixed by baking the filter in an oven at 80°C for 2 h. Southern blot hybridization was carried out, following Amersham protocol, at 60°C. Both in RNA dot blotting and in Southern blotting analyses the probe was the PCR amplified fragment contained in the plasmid pPG31. Probe was made radioactive by the random primer labelling technique (FEINBERG and VOGELSTEIN 1983).

Results and discussion

It is known that growth of peach fruits consists of four main stages (S1, S2, S3, and S4) with unequal growth rates. During the last stage (S4) fruits attain their final size and then start the softening processes. Overall, fruit diameter increase is fast in S1 and S3 and slow in S2 and S4 (ZANCHIN et al. in press).

Our analyses show detectable endopolygalacturonase activity (Fig. 1) throughout the fruit development, with a maximum in S4, followed by a drop when fruit softening has been completed and the fruits are soft ripe. This enzyme, together with other pectin degrading enzymes, is involved both in the weakening of the cell wall matrix and in the hydrolysis of the middle lamella. Therefore, it can be involved in the process of cell enlargement as well as in softening and abscission phenomena. This might explain why endopolygalacturonase activity has been found both during the early stages of fruit growth, when relevant increases in cell diameter occur, and especially in S4 concomitant with the start of an extensive fruit softening.

Dot blot analysis carried out with RNA obtained from mesocarp of fruits collected in the same periods, and using as a probe part of a gene for peach endopolygalacturonase is shown in Fig. 2. Results appear well related to the enzymatic data. Hybridiation can be seen during the early stages of fruit development, with the strongest signal at the level of RNA from Stage S4.

Endopolygalacturonase has also been found to play an important role in the cell separation phenomena which lead to abscission of peach fruits (RASCIO et al. 1985; BONGHI et al. 1992). Upon induction of this phenomenon in explants by means of exogenous ethylene (Fig. 3), enzyme measurements show two moments when activity increases (24 and 96 h, respectively).

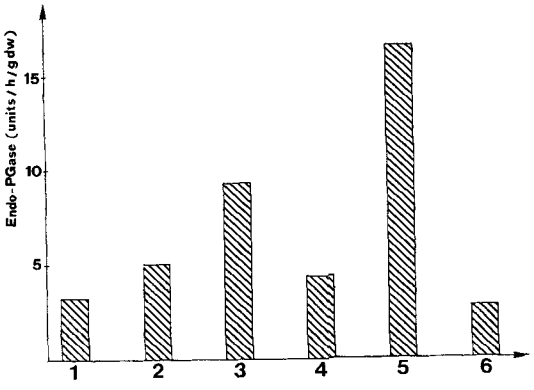

Fig. 1. Time course of endopolygalacturonase activity during fruit development and ripening. 1: 15 days after full bloom (AFB); 2: 40 days AFB (S1): 3: 70 days AFB (S2); 4: 90 days AFB (S3); 5: 110 days AFB (S4); 6: soft ripe fruits.

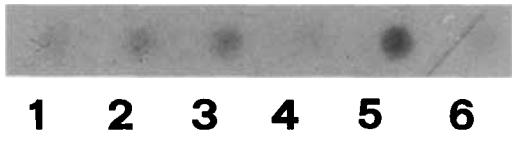

Fig. 2. RNA dot blot analysis of mesocarp from fruits during various stages of development. 1: 15 days after full bloom (AFB); 2: 40 days AFB (S1); 3: 70 days AFB (S2); 4: 90 days AFB (S3); 5: 110 days AFB (S4); 6: soft ripe fruits. 20 μg of total RNA were applied onto a nylon filter which was afterwards hybridized with a radioactive probe consisting of a PCR amplified fragment of peach endopolygalacturonase gene.

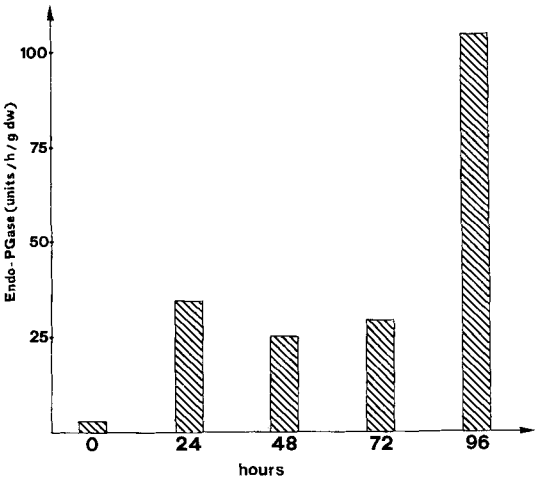

Fig. 3. Time course of endopolygalacturonase activity during abscission of fruit explants induced by treatment with ethylene.

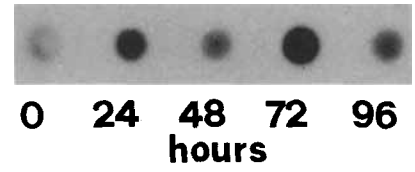

Fig. 4. RNA dot blot analysis of fruit abscission zones showing time course of the phenomenon. 20 μg of total RNA were applied onto a nylon membrane which was afterwards hybridized with a radioactive probe consisting of a PCR amplified fragment of peach endopolygalacturonase gene.

Dot blot analysis with RNA obtained in the course of induced fruit abscission (Fig. 4) again shows a good correlation to enzymatic data. Contrary to the fruit growth, which in the field takes place in over hundred days after full bloom, fruit abscission, experimentally induced and synchronized in explants, occurs in about five days. This might be the reason for the strongest hybridization signal being observed 24 h before the last sampling when the highest enzyme activity is found, soon followed by the fruit shedding.

In tomato, endopolygalacturonase is involved both in fruit ripening and in leaf abscission, even though it seems that two different proteins are active in the two processes (TAYLOR et al. 1990). In peach we cannot make the same comparison because PG does not take part in the abscission of leaves. Instead, it is very important in causing both fruit shedding and their ripening.

The use of a homologous probe, obtained by PCR amplification of a peach endopolygalacturonase gene, has shown that in all the examined situations this gene is transcribed whenever endopolygalacturonase activity is detected. This occurs both when such activity appears more related to cell enlargement phenomena, as in the early stages of fruit growth, and when it is more characteristically related to cell separation phenomena, as in the case of fruit softening or its abscission.

A Southern analysis has been carried out on genomic DNA cut with four different restriction enzymes (Fig. 5). Two of them (Hind III and Eco

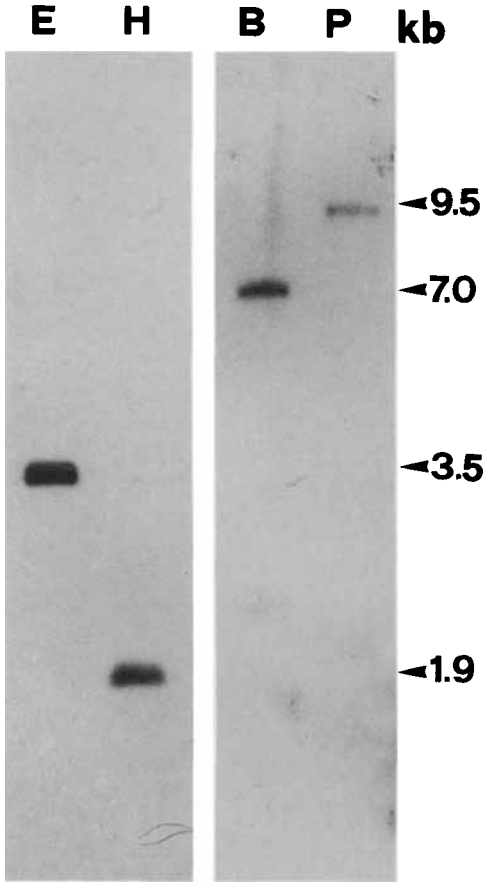

Fig. 5. Southern analysis of peach genomic DNA. 10 μg of DNA were cut with Hind III (H), Eco RI (E), Bgl II (B), Pst I (P) and size separated in a 0.8 % agarose gel. After blotting, DNA was probed with radioactive insert of pPG31 plasmid. Numbers refer to kb size of hybridization bands.

RI) were chosen in order to make a comparison with results by LEE et al. (1990), where the probe was a cDNA coding for tomato PG. The other two (Bgl II and Pst I) were chosen on the basis of a restriction site analysis which showed the presence of one cutting site, outside the 3′ sequence of our probe, but inside the published peach polygalacturonase gene (LEE et al. 1990). This experiment confirms results obtained by LEE et al. (1990) and shows one hybridization band also in the Bgl II and Pst I lanes. Therefore, it clearly appears that our probe can recognize only one polygalacturonase gene in the peach genome.

In conclusion, it seems that, contrary to the finding in tomato (TAYLOR et al. 1990), only the known gene for endopolygalacturonase (LEE et al. 1990) is responsible for such enzymatic activity observed in peach.

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