Polygalacturonase expression during leaf abscission of normal and transgenic tomato plants

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Abstract. Polygalacturonase (PG, EC 3.2.1.15), an enzyme commonly found in ripening fruit, has also been shown to be associated with abscission. A zone-specific rise in PG activity accompanies the abscission of both leaves and flowers of tomato (Lycopersicon esculentum Mill.) plants. Studies of transgenic plants expressing an antisense RNA for fruit PG indicate that although the enzyme activity in transgenic fruit is < 1% of that in untransformed fruit, the PG activity in the leaf abscission zone increases during separation to a similar value to that in untransformed plants. The timing and rate of leaf abscission in transgenic plants are unaffected by the introduction of the antisense gene. A polyclonal antibody raised against tomato fruit PG does not recognise the leaf abscission protein. Furthermore a complementary DNA (cDNA) clone (pTOM6), which has been demonstrated to code for fruit PG, does not hybridise to mRNA isolated from the abscission-zone region of tomato leaves. These results indicate that the PG protein in abscission zones of tomato is different from that in the fruit, and that the gene coding for this protein may also be different.

Key words: Abscission (leaf) – Leaf (abscession) – Lycopersicon (abscession) – Polygalacturonase – Transgenic tomato (antisense RNA)

Introduction

The phenomenon of abscission occurs at precisely determined positions in a plant and is the result of cell separation mediated via cell-wall dissolution (Sexton and Roberts 1982). It has been proposed that the loss of adhesion between adjacent cells at the site of abscission is regulated by the activity of cell-wall hydrolases. The two principle cell-wall-degrading enzymes which have been studied during the process of abscission are β-1,4-glucanase (cellulase) (EC 3.1.2.4) and polygalacturonase (PG). A rise in cellulase activity has been demonstrated to accompany the abscission of a variety of plant parts including leaves (Horton and Osborne 1967; Goren et al. 1973) and flowers (Hanisch Ten Cate and Bruinsma 1973; Tucker et al. 1984). Although the timing of the increase in cellulase activity correlates closely with the process of abscission (Sexton et al. 1985), the increase is not confined exclusively to the region of cell separation (Tucker et al. 1988).

The relationship between abscission and PG activity has proved more difficult to establish since several conflicting reports have appeared in the literature concerning the activity of the enzyme during this developmental process (Morré 1968; Berger and Reid 1979). Nevertheless, in some abscission systems, such as the leaflets of Sambucus nigra and flowers of tomato, there is a clear association between decline in breakstrength and an increase in PG activity (Tucker et al. 1984; Roberts et al. 1989). Furthermore, the rise in enzyme activity has been shown to be restricted to those cells undergoing cell separation (Roberts et al. 1989). Elevated PG activity is not only found in abscission zones undergoing cell separation but is also associated with the softening of some fruit. In tomatoes, the activity of PG rises dramatically during ripening (Hobson 1964), and is synthesized de novo (Tucker and Griersson 1982; Grierson and Tucker 1983) as a result of the accumulation of PG mRNA (Grierson et al. 1986). The PG activity in fruit has been implicated in the degradation of pectin and consequent changes in texture which accompany tomato ripening (Smith et al. 1990).

The processes of abscission and ripening in tomato have a number of features in common. Cell separation is a key process during both developmental events, and this can be accelerated by application of ethylene. Indeed, there is convincing evidence that ethylene plays an important role in regulating the timing of abscission and ripening in situ (Sexton and Roberts 1982; Grierson and Tucker 1983; Tucker and Grierson 1987). These observations raise the important questions as to whether the...
enzymes which are associated with the two processes are the same, and whether they are coded for by the same gene.

The expression of antisense transgenes has been shown to down-regulate endogenous genes in transgenic plants. For example, Van der Krol et al. (1988) transformed petunia with a chimaeric antisense chalcone-synthase gene and regenerated plants exhibiting an attenuated pattern of floral pigmentation, accompanied by a reduction in steady-state levels of chalcone-synthase mRNA to 1% of that detected in untransformed flower tissue. In addition, tomato plants have been transformed with an antisense PG gene with the resultant down-regulation of the endogenous PG protein in ripe fruit to <1% of normal (Smith et al. 1988; Smith et al. 1990). We have used this transformed tomato material to determine whether the PG that plays a role in fruit ripening is the same enzyme as that associated with tomato leaf abscission, and furthermore, to ascertain whether it is coded for by the same gene.

**Material and methods**

**Plant growth.** Tomato seeds were used from either *Lycopersicon esculentum* Mill. cv. Ailsa Craig or from the progeny of GR98. GR98 is an F1 of GR16, which is a transformed plant that produced fruit with <1% of the PG level of normal fruit (Smith et al. 1990). The seeds were surface-sterilized by washing for 20 min in 50% (v/v) domestic bleach (Domestos; Lever Brothers, Kingston upon Thames, Surrey, UK) rinsed extensively in sterile water and sown in autoclaved Levengton compost (Fisons, Ipswich, UK) in 10-cm pots. Seedlings were grown in computer-controlled greenhouse, maintained at a 20°C day, 15°C night, temperature regime. Supplementary lighting was provided by mercury-vapour lamps to provide a 16-h daylength. Leaf explants were prepared from side-shoots of four-month-old plants which were fruiting. This enabled a comparison to be made between leaf tissue and fruit from the same plants.

**Introduction of construct and transformation.** Production of the construct, introduction of the hybrid gene into the binary transformation vector Bin 19, and its use for the transformation of tomato plants has been described in the paper by Smith et al. (1990).

**Preparation and treatment of leaf explants.** Explants were prepared from the nodal region of the stem to include 1.5 cm of the stem plus 1.0 cm of the adjacent petiole with the leaf excised. The basal end of the explants were inserted into 1% agar (Oxoid, Basingstoke, Hampshire, UK) in Petri dishes and placed in a desiccator in continuous light at 20°C. For ethylene treatment, the gas was introduced by injection into the desiccator to generate a known concentration. Abscission was determined as described in Roberts et al. (1984).

**Extraction and assay of polygalacturonase.** Excised abscission-zone and non-abscission-zone tissues were homogenized in a mortar and pestle using 3 vols. of 0.1 M sodium citrate, 1 M NaCl at pH 6.0. The homogenate was incubated at 4°C for 3 h and then centrifuged at 12000 g for 20 min. The supernatant was filtered through glass wool and made to 80% saturation with ammonium sulphate. Precipitated protein was pelleted at 12000 g for 20 min, 4°C, resuspended in IM NaCl and dialysed overnight at 4°C against 1 M NaCl. Aliquots equivalent to 0.1 g FW were then assayed for PG activity using the Nelson-Somogyi method as described in Tucker et al. (1980).

**Immunoprecipitation of PG from fruit and abscission zones.** Salt-extractable protein extracts were prepared from both ripe fruit and leaf abscission zones which had been treated with ethylene for 64 h. Ammonium-sulphate-precipitated proteins were dialysed and an equivalent amount of PG activity added to varying amounts of immunoglobulin-G (IgG) antiserum (purified by ammonium-sulphate precipitation) resuspended in phosphate-buffered saline (PBS). This polyclonal serum was raised against purified fruit PG (Tucker et al. 1980). After incubation at 4°C for 18 h, 20 μl of (50 mg·ml⁻¹) protein-A-Sepharose (Sigma, Poole, Dorset, UK) suspended in PBS was added to each mixture. After a further 18 h at 4°C each sample was centrifuged at 11600 g for 2 min and the resulting supernatant assayed for PG activity.

**Extraction of RNA from fruit and leaf abscission zones.** The RNA was extracted from fruit as described in Grierison et al. (1985). RNA was extracted from abscission zones of leaf explants by freezing the tissue in liquid N₂, then grinding to a powder with a mortar and pestle. The frozen powder was transferred to a mortar at room temperature containing homogenizing medium (0.2 M Tris-HCl pH 8.5, 0.2 M sucrose, 0.06 M KCl, 0.03M MgCl₂) (1.75 ml·g⁻¹ tissue), and ground into a frozen slurry. This was then squeezed through Miracloth (Cambridge BioScience, Cambridge, UK) into polypropylene tubes and centrifuged at 12000 g for 20 min at 4°C. The supernatant was decanted into a sterile medical flat with the addition of 0.1 vol. 10% sodium dodecyl sulphate (SDS) plus an equal volume of phenol (dissolved in 50 mM Tris-HCl, pH 7.5). After shaking the solution was centrifuged at 20 000 g for 15 min in a Centaur 2 bench-top centrifuge (MSE; Fisons, Longborough, Leics., UK), the top aqueous layer removed and partitioned against an equal volume of chloroform. The chloroform partitioning step was repeated twice and RNA was then precipitated from the resulting aqueous layer by the addition of 0.1 vol 3 M sodium acetate (pH 6) and 2.5 vol of ethanol at −20°C for 18 h. The RNA was pelleted by centrifugation at 12000 g as previously described then washed twice with 3 M sodium acetate. After a further centrifugation step at 12 000 g the resulting pellet was suspended in 20% 0.5 M potassium acetate and 80% ethanol, and reprecipitated. The pellet was drained, dried in a vacuum desiccator and dissolved in water to an appropriate concentration for its intended use.

**In-situ hybridisation.** Tissue was fixed in freshly prepared 4% paraformaldehyde at 4°C overnight and then dehydrated through an ethanol series. Material was embedded in paraffin wax using Histoclear (Data Diagnostics, Hemel Hempstead, Herts., UK) as a miscible solvent. Thin sections (10–15 μm) were cut using a Cambridge rocking microtome and attached to slides precoated with poly-L-lysine (1 mg·ml⁻¹). Polygalacturonase sense and antisense strand-specific probes were transcribed from the transcription vector pBS-6 (Smith et al. 1988) and labelled using [5,6-³H]UTP (Amerham International, Amersham, Bucks., UK). The percentage incorporation of radiolabel into each of the probes was similar. The ³H–RNA probes were hydrolysed to a modal size of approx. 0.2 kilobases (kb) by incubating in carbonate buffer for 48 min at 60°C (for details see Cox and Goldberg 1988).

Tissue sections were prepared for hybridisation by dewaxing them through Histoclear followed by a graded alcohol/0.85% saline series. Slides containing the hydrated sections were washed in PBS (0.13 M NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄) then incubated in 0.125 mg·ml⁻¹ proteinase K in 50 mM Tris-HCl (pH 7.5), 5 mM EDTA for 10 min, followed by a 2-min wash in 0.2% glycine in PBS. Slides were rinsed in PBS then treated in freshly prepared 4% paraformaldehyde for 10 min. After two further washes in PBS, slides were equilibrated in 0.1 M triethanolamine (pH 8) prior to the addition of acetic anhydride to give a final concentration of 0.5% (v/v). Sections were acetylated for 10 min and then washed in PBS. Slides were then transferred to an ethanol series, dehydrated in fresh 100% ethanol and stored at 4°C for up to 2 h prior to hybridisation.

The ³H–RNA probes, previously made up in 50% deionized formamide, were heated in an 80°C waterbath for 2 min, cooled