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# Simultaneous co-suppression of polygalacturonase and pectinesterase in tomato fruit: inheritance and effect on isoform profiles

Howard Simons, Gregory A. Tucker*

*School of Biological Sciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, UK*

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**Abstract**

The simultaneous down regulation of two, or more, genes can be brought about by the transformation of a plant with a single chimeric transgene containing homologous sequences to both target genes. This has been achieved for the two cell wall hydrolases — polygalacturonase and pectinesterase — in tomato fruit. This paper reports the stable inheritance of this co-ordinated gene silencing over two generations. It has also been shown that only two of the three isoforms of pectinesterase in the tomato fruit are silenced by this chimeric construct thus providing some indication of the relative homologies between the gene sequences for these isoforms. © 1999 Elsevier Science Ltd. All rights reserved.

*Keywords:* Tomato (*Lycopersicon esculentum*); Solanaceae; Pectinesterase; Polygalacturonase; Gene silencing

## 1. Introduction

Ripening of tomato fruit is accompanied by major changes in the pectin components of the cell walls. This is exemplified by increased solubility, depolymerisation and de-esterification of the pectin (Tucker & Grierson, 1987; Fischer & Bennett, 1991). Two enzymes, polygalacturonase (PG) and pectinesterase (PE), are thought to be involved in these changes.

The expression of PG is both fruit and ripening specific (Hobson, 1964) and although there are at least three isoforms of this enzyme detectable in the tomato fruit (Mohd Ali & Brady, 1982) these arise from post translational modification of a single gene product. Antisense technology has been used to silence this single PG gene and has resulted in genetically modified fruit with less than 1% of normal PG activity (Smith et al., 1988). The effect of this reduction in PG on wall degradation has been examined and it has been shown that whilst both solubilisation and de-esterification appear unaffected, depolymerisation of the pectin during ripening is markedly inhibited (Smith et al., 1990).

In contrast PE activity is present throughout fruit development and ripening and arises from at least three isoforms (Warrilow, Turner & Jones, 1994) which are likely to be separate gene products. The major isoform in ripe fruit is PE2 and this has been down-regulated by several groups using antisense technology. Ray, Knapp, Grierson, Bird and Schuch (1988) used the 5′ 420 bp *PstI* fragment from a PE2 cDNA to generate their antisense plants. Tucker and Zhang (1996) have shown that whilst PE2 activity was very much lower in these transgenic fruit, levels of the other two isoforms (PE1 and PE3) were both about normal. Tieman, Harriman, Ramamohan and Handa (1992) used an antisense construct that contained the whole of the PE2 cDNA, encoding the mature protein, except for 85 bp at the 3′ end. In a later paper Gaffe, Tieman and Handa (1994) demonstrated that the isoforms they designated as group 2 PE isoforms were

* Corresponding author. Tel.: +44-115-9516126; fax: +44-115-9516122.

*E-mail address:* gregory.tucker@nottingham.ac.uk (G.A. Tucker).

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still present in these PE-2 antisense fruit. The group 2 isoforms were defined as being non-fruit specific, and presumably represented either PE1 and/or PE3.

The two enzymes, PG and PE, have been down-regulated simultaneously in tomato fruit using co-suppression (Seymour, Fray, Hill & Tucker, 1993). These PGPE sense plants were modified by the insertion of a single construct where 244 bp of the PG cDNA, which encodes the N-terminal leader sequence of the mature protein, had been ligated to the entire section of the PE2 cDNA encoding for the mature protein. This had the result of reducing the mRNA, protein and the enzyme activity of both PG and PE2 (Seymour et al., 1993). However, this paper did not examine the effects of this transformation on the other PE isoforms. Furthermore, it has been shown that, in a range of primary transformants, the down-regulation of PG and PE appeared to be co-ordinated (Jones et al., 1996) in that in no instance was a transformant identified in which only one of the two target genes had been silenced. However, it is not known how stable such a co-ordinated silencing may be during subsequent generations.

In this paper the PGPE primary transformant, which demonstrated the greatest down-regulation of PG and PE activity was selected for further study. This plant, known as $T_3$, had been shown to contain a single transgene insertion event (Seymour, personal communication). $S_1$ progeny, homozygous for the transgene, have been subjected to further analysis with respect to PE isoform profiles. In addition to this biochemical analysis of the transgenic fruit this paper also describes an investigation into the inheritance of the double down-regulated PG/PE phenotype by extending this study to an $S_2$ generation.

## 2. Results and discussion

### *2.1. Inheritance assay to select homozygous $S_1$ generation plants*

Seeds were collected from the primary PGPE transformant $T_3$, which had been prepared as, described by Seymour et al. (1993). These were germinated, in the presence of kanamycin, and selected seedlings grown up to provide fruiting plants. These plants represent the $S_1$ population. According to Mendelian genetics 25% of the $S_1$ generation seeds should be homozygous for the transgene, 50% heterozygous and 25% azygous. In order to identify homozygotes, within this $S_1$ population, an inheritance assay, as described in Section 3 and based on the principles of Mendelian genetics was used. Self pollination of the $S_1$ generation homozygotes would yield all homozygous $S_2$ progeny whereas self pollination of heterozygous or azygous $S_1$ plants would yield at least some progeny that are azygous. The construct used for transformation included a kanamycin resistance gene, therefore azygous seedlings would be susceptible to kanamycin whereas homozygous and heterozygous seedlings would demonstrate resistance.

The inheritance assay indicated that, out of the 51 plants screened, 11 (27%) were homozygous for the transgene. Southern blot hybridisation analysis (data not shown) was used to confirm these results.

### *2.2. Enzyme activities in homozygous fruit*

Fruit were picked 5 days after the breaker stage of ripening as characterised by first colour development (B + 5). Total cell wall bound protein was extracted and PE and PG enzyme assays were carried out as described in Section 3. The results from each of the individual $S_1$ plants and corresponding control wild-type fruit are shown in Fig. 1. The PG levels were found to be very low in all transgenic plants compared to wild-types, showing on average 1.5% of wild-type activity. The PE activity was also reduced with levels in transgenic plants showing on average 11% of wild-type activity.

The PGPE primary transformant, $T_3$, which was the parent of the $S_1$ generation, demonstrated PG activity at 43.4% of wild-type and PE activity of 2.8% of wild-type (Seymour et al., 1993). The decrease in PG activity, from parent to $S_1$ progeny, was expected because previous studies had shown that homozygous antisense plants demonstrate a greater degree of silencing than heterozygotes (Fray & Grierson, 1993). Since this has been demonstrated with antisense genes, it may also be true for sense transgenes.

The total PE activity, however, appears to have increased, from 2.8 to 11%. The reason for this is unclear but may be related to seasonal effects or to the relative extraction techniques used in each case. Seymour et al. (1993) used peeled tomato pericarp in their experiments whilst in this paper whole pericarp, including peel, was used.

### *2.3. Analysis of pectinesterase isoforms by heparin affinity chromatography*

Protein was extracted, and then pooled, from the pericarp of three individual $S_1$ generation or Ailsa craig wild-type fruit. The pectinesterase isoforms in each extract were separated by heparin affinity chromatography (Fig. 2). The isoform profile from wild-type fruit clearly shows the presence of three isoforms, termed PE1, PE2 and PE3. In the $S_1$ generation fruit the PE2 isoform was clearly absent as expected, however, it was apparent that a second isoform, PE3, was

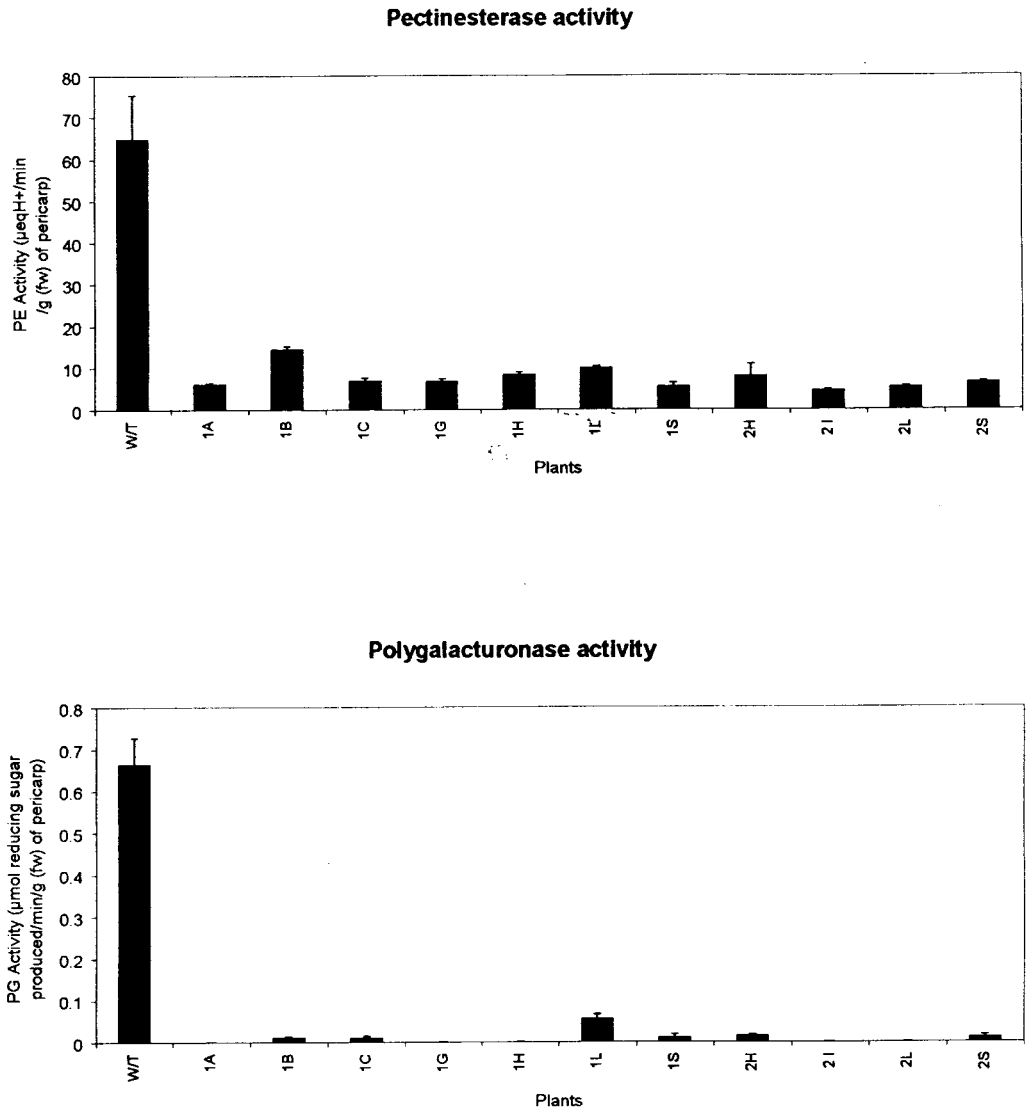


Fig. 1. Total PE and PG activity of the homozygous $S_1$ generation PGPE sense fruit; W/T, Ailsa Craig wild-type activity; error bars show the sd.

also absent compared to the control. It is not possible to comment on any change in the levels of the remaining PE1 isoform in the $S_1$ fruit because the endpoint assay used in these experiments was not quantitative. The $S_2$ generation was also analysed in an identical fashion (data not shown) and these also demonstrated the absence of both PE2 and PE3.

The reason for co-suppression of PE3 in addition to PE2 in these $S_1$ plants is unclear, however, the existence of some sequence homology between the PE2 cDNA fragment used in the PGPE construct and the PE3 gene is a possible explanation. Fray and Grierson (1993) suggest that as little as 150 bp of homology may be sufficient to allow co-suppression. Ray et al. (1988) used only a partial 5′ fragment of the PE2 coding sequence. The cDNA encoding the full mature protein for PE2 was used in the PGPE construct, therefore, it is possible a region of homology between PE3 and PE2 genes may exist at the 3′ end of the PE2 cDNA.

### 2.4. Inheritance of the PGPE down-regulated phenotype

Seeds were collected from the homozygous $S_1$ plants identified previously and used to generate an $S_2$ population. As a control, a new $S_1$ population was vegetatively propagated using cuttings from the original homozygous $S_1$ plants. Fruit from this $S_2$ generation of plants, and the control $S_1$ cuttings, were analysed for total PG and PE activity as shown in Fig. 3. One plant (2H) demonstrated abnormal behaviour compared to all the other lines. Plant 2H showed PG activity that was considerably higher than the 2H control fruit, although the levels seen were still less than wild-

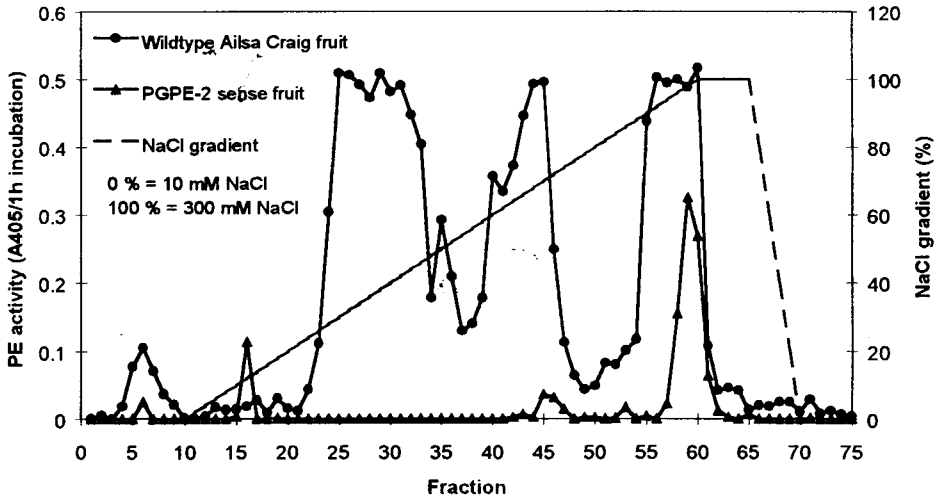


Fig. 2. Heparin affinity chromatography to separate PE isoforms in wild type and PGPE sense fruit.

type. With the exception of plant 2H the rest of the $S_2$ generation demonstrated an average PG activity of 0.14% of wild-type which was similar to the 0.36% activity achieved by the control plants. Fruit from the $S_2$ generation plants demonstrated on average 17% of wild-type PE activity, which was only slightly higher than the 11% of wild-type activity seen in the control plants.

With the exception of plant 2H the PG activity was unchanged during meiosis, however, the PE activity increased by 6%. The size of the cDNA used for co-suppression of PE (1320 bp) was much larger than the cDNA used for co-suppression of PG (244 bp) in the same construct (Seymour et al., 1993). If the increases in activity observed were due to some homology dependent process it is possible that the larger PE part of the transgene would have been more susceptible to modification than the smaller PG fragment.

## 3. Experimental

### 3.1. Preparation of plant material

$S_1$ seeds (80) collected from the $T_3$ PGPE primary transformant (Seymour et al., 1993) were allowed to germinate alongside 20 wild-type Ailsa Craig control seeds. Both the transgenic and the control seeds were treated with 50 μg/ml kanamycin monosulphate and allowed to germinate in the dark. Those transgenic $S_1$ seedlings (51 in total) that appeared to grow better than the controls were selected and glasshouse grown at 22° for 16 h in the light, supplemented as necessary with artificial daylight, and 14° for 8 h in the dark. Wild-type plants were grown in similar conditions as controls.

$S_2$ seeds were collected (20) from fruit of each of the 51 $S_1$ generation plants. These were surface sterilised with 5% (v/v) sodium hypochlorite and rinsed thoroughly and placed on separate filter papers in petri dishes. Control seeds from wild-type plants were treated similarly. The seeds were arranged in groups, with 10 tests and 2 controls per group so that the controls were positioned spatially, in the growth room, near to the experimental seeds they were controlling. The experimental seeds and 1 control per group were treated, ad lib, with a solution containing 50 μg/ml kanamycin monosulphate and 1% (v/v) high nitrogen fertiliser. The other control was treated with a solution containing only 1% (v/v) high nitrogen fertiliser. The seeds were maintained in the dark at room temperature until germination occurred, and when cotyledons formed on any seedling in a group the entire group was then exposed to continuous illumination at a constant temperature of 25°.

The experiment continued until all the wild-type control seedlings, treated with the kanamycin solution, in a group were dead, at which point the number of dead experimental seedlings in that group were recorded. The experimental seedlings were also given a subjective score between 1 and 5 for general vitality compared to the control seeds not treated with kanamycin. A score of 4 indicated that the seedlings were as 'healthy' as the controls and a score of 5 indicated greater vitality than the controls.

A plant was identified as being homozygous for the transgene if none of its progeny seedlings had died by the end of the experiment. Plants were also accepted as homozygotes if one or two seedlings had died, but only if the remaining seedlings had a 'vitality' score of 5. All the other experimental seedlings were designated as coming from non-homozygous plants.

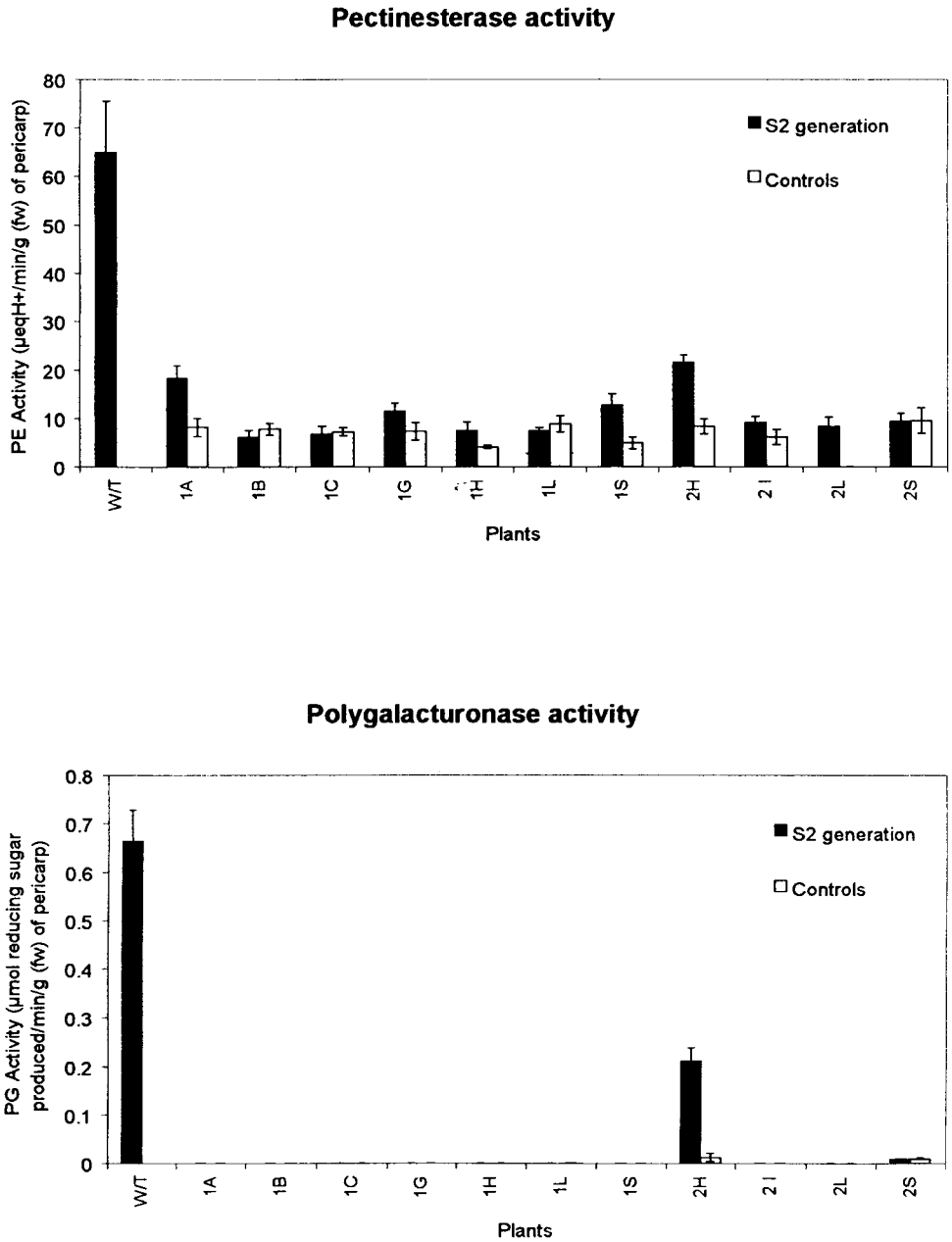


Fig. 3. Total PE and PG activity of the $S_2$ generation PGPE sense fruit. Results are shown for the $S_2$ generation plants and cuttings taken from the homozygous $S_1$ generation plants, which acted as controls. No control was available for plant 2L; W/T, Ailsa Craig wild-type activity; error bars show the sd.

### 3.2. Analysis of enzyme activities

Cell wall bound proteins were extracted from two individual fruit from each plant as described by Tucker, Robertson and Grierson (1980). The extract from each fruit was assayed in triplicate for PG activity according to the method of Tucker et al. (1980). Each extract was also assayed for PE activity using five replicates using a method slightly modified from Cameron, Buslig and Shaw (1992). Assay buffer consisted of 2 mM Tris, 0.15 mM NaCl, 0.002% phenol red, 0.5% citrus pectin (Fluka-Biochemika). The pH was readjusted to 8.5 after preparation; 200 μl of this PE assay buffer was added at time zero to each sample (20 μl) and blank as quickly as possible using a 12 channel multipipette (Anachem) and the plate read immediately on a plate reader (Dynatech MR5000). The plate reader software (RMS) was programmed to carry out a kinetic study taking absorbance readings every 20 s for 20 min at 405 nm. The software measured the best rate of A405/min and determined the activity as μeq $H^+$/min using a pre-programmed standard curve.

The final results were expressed as μeq $H^+$/min/g (fresh weight) of pericarp.

### 3.3. Isoform analysis

Cell wall bound proteins were prepared as described previously. Protein extract was dialysed overnight against 50 mM NaOAc, 10 mM NaCl, and pH 6.0; 2 ml extract was separated on three 5 ml Heparin EconoPac® cartridges (BioRad) used in series. PE was separated in a 5 mM Tris-HCl pH 7.5 mobile phase using a linear NaCl gradient between 10 and 300 mM and a flow rate of 1 ml/min. PE activity was assayed as described above except that instead of a kinetic assay an end point assay was used were the microtitre plate was read after an incubation period of 1 h.

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