Overexpression of Polygalacturonase in Transgenic Apple Trees Leads to a Range of Novel Phenotypes Involving Changes in Cell Adhesion

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Polygalacturonases (PGs) cleave runs of unesterified GalUA that form homogalacuronan regions along the backbone of pectin. Homogalacturonan-rich pectin is commonly found in the middle lamella region of the wall where two adjacent cells abut and its integrity is important for cell adhesion. Transgenic apple (Malus domestica Borkh. cv Royal Gala) trees were produced that contained additional copies of a fruit-specific apple PG gene under a constitutive promoter. In contrast to previous studies in transgenic tobacco (Nicotiana tabacum) where PG overexpression had no effect on the plant (K.W. Osteryoung, K. Toenjes, B. Hall, V. Winkler, A.B. Bennett [1990] Plant Cell 2: 1239–1248), PG overexpression in transgenic apple led to a range of novel phenotypes. These phenotypes included silvery colored leaves and premature leaf shedding due to reduced cell adhesion in leaf abscission zones. Mature leaves had malformed and malfunctioning stomata that perturbed water relations and contributed to a brittle leaf phenotype. Chemical and ultrastructural analyses were used to relate the phenotypic changes to pectin changes in the leaf cell walls. The modification of apple trees by a single PG gene has offered a new and unexpected perspective on the role of pectin and cell wall adhesion in leaf morphology and stomatal development.

Polygalacturonases (PGs) are expressed in a wide range of tissues and developmental stages in plants and are encoded by relatively large gene families (e.g. approximately 52 genes in Arabidopsis; The Arabidopsis Genome Initiative, 2000). PGs are associated with fruit ripening, cell separation processes such as leaf and flower abscission, pod and anther dehiscence, pollen grain maturation, pathogen defense, plant-host interactions, and processes of cell expansion, growth, and xylogenesis (for review, see Hadfield and Bennett, 1998; Bergey et al., 1999; Sitrit et al., 1999; Torki et al., 1999; Wang et al., 2000). Endo-PGs cleave runs of unesterified GalUA that form homogalacturonan regions along the backbone of pectin. Homogalacturonan-rich pectin is commonly found in the middle lamella region of the wall where two adjacent cells abut (for example, see Knox et al., 1990; Steele et al., 1997). The best characterized (fungal) endo-PG enzyme requires four to five consecutive runs of unesterified GalUA residues for cleavage; however, little is known about the enzyme activities and specificities of most cloned PGs from plants.

Transgenic plants have been used to study the role of endo-PGs in vivo. In tomato (Lycopersicon esculentum), down-regulation of the fruit-specific PG gene pTOM6 under the control of the constitutive cauliflower mosaic virus 35S promoter showed reduced depolymerization of pectin polymers in fruit (Smith et al., 1990). Overexpression of PG in the ripening-inhibited mutant rin background restored PG activity and pectin degradation in fruit (Giovannoni et al., 1989). In both cases, only the fruit was affected by the transgene expression; therefore, the gene product isolated from tomato fruit appeared to have fruit-specific PG activity. Further experiments where the pTOM6 gene was overexpressed in tobacco (Nicotiana tabacum; Osteryoung et al., 1990) showed that the tomato protein was properly processed and localized in the cell walls of leaves in tobacco. The enzyme showed activity when extracted from transgenic tobacco leaves and tested against tobacco cell wall extracts in vitro. However, no changes in leaf phenotype were observed, nor were there any alterations to the pectins in the tobacco cell walls in vivo.

Apple (Malus domestica Borkh. cv Royal Gala) ripens very differently than tomato and many other fruits in not undergoing cell wall swelling as part of the cell wall modifications during ripening (Redgwell et al., 1997). There is minimal change in viscosity of cell walls, and minimal pectin solubilization or degradation during fruit ripening. This implies that any endo-PG isolated from ripening fruit of apple may have different characteristics to that isolated from ripening tomato fruit. Therefore, results relat-
ing to overexpression of tomato fruit endo-PG may not be the same as overexpression of apple fruit endo-PG.

PG enzyme in ripe apple fruit has been isolated and biochemically characterized as an endo-PG (Wu et al., 1993). The corresponding cDNA (MdPG1, formerly GDPG1; Atkinson, 1994) was isolated from apple cv Golden Delicious and shown to hybridize to an mRNA present in ripe fruit but not in developing fruit or flowers (Atkinson et al., 1998). MdPG1 encoded a protein with 52% amino acid identity to the tomato fruit-specific clone pTOM6. Analysis of the promoter of MdPG1 showed that 532- and 1,460-bp fragments conferred β-glucuronidase expression in ripe tomato fruit, but not in flowers, leaves, or developing fruit (Atkinson et al., 1998).

The MdPG1 cDNA was overexpressed in apple with the expectation that disruption of cell wall metabolism would occur in ripening fruit. However, we report in this paper a range of novel phenotypes in other plant tissues associated with endo-PG overexpression, providing new information on the involvement of pectin in cell-cell adhesion and stomatal function.

RESULTS

Apple Plants Overexpressing Fruit-Specific PG Have Increased PG mRNA, Protein, and Activity in Mature Leaves

Three independent PG transformants (MdPGS-2, -3, and -4) were identified that exhibited a novel phenotype in tissue culture, characterized by pale green leaves showing necrosis around the edges. The MdPGS transformants were micropropagated to form transgenic lines. DNA gel-blot analysis revealed that MdPGS-2 and -4 each contained a single integrated copy of the PG transgene, whereas MdPGS-3 contained two copies (Fig. 1a).

RNA gel-blot analysis showed that the MdPGS transformants produced easily detectable amounts of the fruit-specific PG mRNA (Fig. 1b) in rapidly expanding leaves. Wild-type apple leaves of equivalent age did not express a homologous message. Antibodies raised against endo-PG from ripening tomato fruit reacted with a polypeptide at 46 kD in MdPGS leaves (Fig. 1c), indicating a correctly translated and processed protein from the PG mRNA. Very low levels of a cross-reacting polypeptide of the same size were observed in control leaves. The increased PG expression and protein in the transformants was reflected in the measured enzyme activity (Table I). In mature leaves of MdPGS-2 and -4, PG activity was almost doubled, and in MdPGS-3, activity increased by approximately 35% above wild-type levels. In wild-type leaves, low enzyme activity was detected (Table I), probably reflecting the very faint immunoreactive band (Fig. 1c).

PG Overexpression Leads to Changes in Leaf Color, Water Relations, and Leaf Abscission

Leaves of PG-overexpressing plants showed a distinct change in leaf color phenotype during maturation compared with wild-type controls. MdPGS-2 and -4 had mature leaves in which the silver color extended throughout the leaf blade (Fig. 2a), whereas in MdPGS-3, the silver color was centered on the leaf veins but did not extend fully into the leaf blade. The
difference in leaf color could be quantified using the L value on a colorimeter (Table II). In all MdPGS transformants, the L value was higher than for wild-type controls. Young leaves did not show a quantifiable change in phenotype compared with controls (data not shown). Microscopic examination of leaves from the MdPGS transformants indicated that the silvery areas of leaves had more airspaces between the epidermis and the palisade cells of the mesophyll, indicating poorer adhesion of cells (Fig. 2c). Staining with ruthenium red showed less pectin on the epidermal cell walls that face palisade cells in all transformants (Fig. 2c). Young transgenic leaves did not show this phenotype.

Because leaves of MdPGS plants appeared more brittle and wilted and abscised more easily, photosynthetic rate and leaf diffusive conductance were compared in silvery and normal leaves (data not shown). Photosynthetic rates were similar in silvery and normal leaves (data not shown). Microscopic examination of leaves from the MdPGS transformants indicated that the silvery areas of leaves had more airspaces between the epidermis and the palisade cells of the mesophyll, indicating poorer adhesion of cells (Fig. 2c). Staining with ruthenium red showed less pectin on the epidermal cell walls that face palisade cells in all transformants (Fig. 2c). Young transgenic leaves did not show this phenotype.

Like wild type, only cell junctions and middle lamellae were labeled. Although mature enough, MdPGS leaves were more enlarged and unevenly shaped than those in wild type (data not shown). Mature MdPGS leaves labeled with JIM7 antibodies in a similar manner to wild type (data not shown). There was little difference in labeling of palisade cells between wild type and MdPGS leaves (data not shown).

Guard cells of both wild-type and transformant stomata showed labeling of highly esterified pectin (JIM7) across the cell wall in a similar manner (Fig. 2h), although stomata were frequently malformed in the transformants. Low-esterified pectin (JIM5) in wild type was also distributed across the whole wall, with areas of increased intensity at the pore edge and near the adjacent epidermal cell (Fig. 2g). In the transformants, JIM5 labeling was strong close to the plasma membrane edge of the guard cell wall and weak to nonexistent near the outer edge.

**Table I. PG activity in mature leaves of wild-type apple and MdPGS transformants**

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<thead>
<tr>
<th>Genotype</th>
<th>PG Activity (mmol reducing groups × 100 mg⁻¹ CWM)</th>
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<tr>
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<td>Extraction I</td>
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<td>MdPGS-4</td>
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PG Overexpression Alters Stomatal Functioning and Creates Lesions and Disordered Internal Structure in Mature Apple Leaves

When wild-type and transformant leaves were freshly harvested and placed in the dark for 30 min, only the stomata on mature wild-type leaves closed (Fig. 3a). A similar result was found with abscisic acid (ABA) treatment (data not shown). Closer examination of the stomata revealed a disruption in cell morphology with holes at one or both ends of the stomatal apertures in transformants (Fig. 3, b and c). The holes varied in size but invariably penetrated through the epidermis, and guard cells and neighboring epidermal cells were often separated (Fig. 2, d and e). In young transformant leaves, only a few stomata had holes (Fig. 3d). The pattern of stomatal development looked similar to that in wild-type leaves, although in some cases surface irregularities could be seen adjacent to guard cells.

The lower epidermis of MdPGS leaves was fragile and collapsed frequently before, or during, fixation. When stained with ruthenium red to visualize pectin, staining was reduced compared with wild type (Fig. 2d) and labeling with JIM5 antibodies indicated a reduction in low-esterified pectin (Fig. 2f). Spongy mesophyll cells and cells around the vasculature in MdPGS leaves were more enlarged and unevenly shaped than those in wild type (data not shown). Mature MdPGS leaves labeled with JIM7 antibodies in a similar manner to wild type (data not shown). There was little difference in labeling of palisade cells between wild type and MdPGS leaves (data not shown).

Overexpression of a Fruit-Specific PG Alters Cell Wall Chemistry in Mature Leaves

Overexpression of fruit-specific PG led to significant changes in the chemistry of leaf cell walls of the MdPGS transformants. In most cell wall chemical analyses, results for MdPGS-3 tended to be the same or similar to wild-type values, whereas MdPGS-2 and -4 were significantly different than wild type. This is consistent with the relative endo-PG enzyme activities and mRNA expression levels in these transformants.

On a fresh weight basis, MdPGS leaves had 15% to 22% less cell wall material (CWM) than wild-type leaves, with a tendency for the phenotype to increase as the plants reached senescence (data not shown). Microscopic examination of leaves from the MdPGS transformants indicated that the silvery areas of leaves had more airspaces between the epidermis and the palisade cells of the mesophyll, indicating poorer adhesion of cells (Fig. 2c). Staining with ruthenium red showed less pectin on the epidermal cell walls that face palisade cells in all transformants (Fig. 2c). Young transgenic leaves did not show this phenotype.

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leaves (Table III), and GalUA, the major component of pectin, was reduced by 12% to 26%. However, the molar composition showed an increased proportion of GalUA compared with wild type. Gal and Ara contents of the CWM were markedly reduced in the transformants, but the Xyl content was higher than in wild-type leaves.

To determine which type of pectin had been influenced by overexpression of PG, CWM was extracted sequentially with trans-1,2-diaminocyclohexane-N,N,N’,N’-tetra-acetic acid (CDTA) and Na2CO3. CDTA is a chelator that extracts pectins held in the wall by ionic bonds, whereas the alkaline reagent Na2CO3 extracts covalently linked pectins. There was a difference in partitioning of polymers and in their composition between the subfractions of the CWM of transformants compared with wild type (Table IV). Transformants had a higher yield of CDTA solubles that contained more GalUA on a fresh weight basis than wild type. In comparison, there was a reduction in yield of the Na2CO3 solubles in MdPGS leaves, which also contained less GalUA. Composition analyses of the two pectic fractions showed a very high abundance of GalUA and the neutral monosaccharides Gal, Ara, and Xyl, which are commonly found as side chains of apple-derived pectins (Schols et al., 1995). This and the very low presence of Glc, Man, and Fuc, sugars that are commonly found in non-pectic polysaccharides, indicate that the CDTA and Na2CO3 fractions consist mostly of pectins. CDTA-soluble pectins of transformants had a higher molar ratio of GalUA than the wild type. For Na2CO3 solubles, the molar ratio was lower for MdPGS-2 and 4, but not for MdPGS-3. The Gal, Ara, and Xyl content of the CDTA-soluble pectins was reduced in transformants, whereas for Na2CO3-soluble pectins, Ara and Gal content was unchanged. Xyl was elevated, particularly in MdPGS-2 and -4. These results show that the elevated GalUA levels and reduced levels of Gal and Ara seen in CWM of transformants

Figure 2. Phenotype of leaves from MdPGS transformants compared with wild type (WT). a, Silvery leaf phenotype. Scale bar = 10 mm. b, Putative petiole abscission zone showing distribution of low-esterified pectin by labeling with JIM5 antibodies. c, Cross section of upper epidermis and palisade cells of leaves demonstrating reduced cell adhesion in the silvery leaf phenotype (stained with ruthenium red for pectin). d, Cross section of entire leaves, demonstrating the reduced ruthenium red staining of the lower epidermis and holes next to guard cells or stomata. e, Cross section of the lower leaf epidermis showing the hole next to the guard cell in the transformant (stained with toluidine blue). f, Cross section of leaves labeled with JIM5 antibodies, showing reduced labeling of lower epidermal cells in the transformant. g, Cross section of the lower epidermis of leaves labeled with JIM5 antibodies showing altered distribution of label in guard cells of transformant stomata. h, Cross section of the lower epidermis of leaves labeled with JIM7 antibodies showing labeling in the guard cells and neighboring cells. Scale bars in b through h = 10 μm.
could be attributed to ionically bound pectins, whereas elevated Xyl levels in CWM of trans- 
formants were found to be primarily in the cell wall residue after extraction (data not shown).

Strength of cell-to-cell adhesion is to a large part determined by length and DE of pectin 
molecules of the middle lamella (Jarvis, 1984). Because endo-PGs hydrolyze pectins to smaller 
chains, the size of CDTA-soluble pectins was significantly (P<0.05 and P<0.01, respectively) 
shorter and the transformants, we compared the DE of pectin molecules of 
different genotypes. In all MdPGS transformants, the CDTA-soluble pectins 
were found to be primarily in the cell wall 

Table II. Characterization of silver leaf phenotype and abscission 
force

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<th>Genotype</th>
<th>Plant</th>
<th>L Value</th>
<th>Force (N)</th>
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</tr>
<tr>
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<td>2</td>
<td>38.45</td>
<td>2.29</td>
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<tr>
<td></td>
<td>3</td>
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<td>1.67</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
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<td>2.24</td>
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<tr>
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<td>1</td>
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<td>0.74</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>43.15</td>
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<td></td>
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<td></td>
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<td>0.66**</td>
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<td>0.67</td>
</tr>
<tr>
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<td>2</td>
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<td>0.93</td>
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<tr>
<td></td>
<td>Mean</td>
<td>43.87*</td>
<td>0.82**</td>
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DISCUSSION

Constitutive overexpression of a fruit-specific PG in apple plants has led to a range of novel 
phenotypes including disrupted leaf organization, perturbed water relations, malformed and malfunctioning 
stomata, silvery leaves, and changes in leaf abscission. The modification of apple trees by this 
single fruit gene, therefore, has offered a new and unexpected perspective on a number of physiological and 
developmental processes.

The Role of Pectin in Development and Cell Adhesion

Overexpression of PG in apple led to abnormal cell separation within the leaf. Loss of cell adhesion was common to a number of phenotypes. It occurred between upper epidermis and palisade parenchyma, resulting in the silvery color of leaves, between cells of the lower epidermis resulting in stomatal dysfunction, and in the abscission zone of the petioles, resulting in more easily detachable leaves. These phenotypes are consistent with the predicted action of PG. Plant cells are held together in the middle lamella region by pectin forming a gel via Ca^{2+} cross-links (Jarvis, 1984; Knox et al., 1990; Knox, 1992). The strength of adhesion of this gel is largely determined by the length of pectin molecules and their DE. In MdPGS transformants, the CDTA-soluble pectins 
held in the wall by Ca^{2+} cross-links had shorter chains compared with wild type. This results in less 
efficient binding between polymers, a weaker gel, and thus poorer cell adhesion, even though the lower 
DE of the CDTA fraction favors Ca^{2+} cross-linking. The altered monosaccharide content of the side 
chains of the CDTA pectins may also have an impact on cell adhesion.

Analyses of mutants in several plant species have shown alterations to pectin positioning and structure 
that affect cell-to-cell adhesion and normal tissue development. In the Arabidopsis EMB30 mutant, 
pectins (especially low-esterified ones) appear to be misdirected so they accumulate in intercellular 
spaces. Cells of leaves and callus adhere poorly, and are irregular and larger than wild type (Shevell et al., 
2000). Unlike the MdPGS transformants, however, the EMB30 mutant has unaltered neutral sugar com- 
position in the cell wall.

In the Cnr tomato mutant, pectin structure and cell- 
to-cell adhesion in fruit is altered through a disruption in the deposition of (1→5)-α-L-arabinan that forms the 
branched sidechains of rhamnogalacturonan-I (Orfila et al., 2001). As a consequence, ripe-stage Cnr fruits 
have stronger cell walls throughout the pericarp that do not swell, and there is extensive intercellular space 
in the inner pericarp in comparison with wild type. Orfila et al. (2001) showed that maturation processes
involving middle lamella pectins are altered in Cmr fruit, resulting in the absence or a low level of pectin- /calcium-based cell adhesion.

The Role of PG and Pectin in Stomatal Function

Our study of the stomata in transgenic MdPGS apple plants has confirmed the important role of the cell wall in stomatal functioning (Ziegenspeck, 1938). As a consequence of PG overexpression, guard cells and neighboring epidermal cells are often separated from the lower epidermis of mature transformant leaves. Moreover, holes appeared at each end of the guard cells during leaf growth. These changes reduced the ability of the cells of the stomatal complex to act against each other, which is necessary for open-
ing and closing of stomatal pores. The stomatal response of MdPGS transformants to darkness and ABA therefore was impaired, so that their water requirements were much higher than for wild-type plants. The sites where cell adhesion is lost may be sites where the epidermis is under physical stress.

Stomatal malfunctioning was not evident in young expanding transformant apple leaves, but 75% of the stomata in Arabidopsis leaves develop as secondary stomata from satellite meristemoids during leaf expansion (Geisler et al., 2000). Recent descriptions of guard cell development (Zhao and Sack, 1999) describe thickening of cell walls as important in determining where the division will occur to create the two guard cells. The holes appearing at the end of the guard cells in our MdPGS transformants are adjacent to the position of the cell wall thickening described above.

We are unaware of any other studies that localize or describe specific pectins in stomatal walls. Microscope studies of the stomatal complex showed a high pectin content in wild-type and transformant guard cells relative to other cell types of the leaf, plus a reduction and altered distribution of low-esterified pectins. Recently, Vaughn and Turley (1999) described a pectin sheath surrounding epidermal cells in expanding plant zones such as cotton (Gossypium hirsutum) fibers or hair initials on leaves. The pectin in the sheath had a high proportion of JIM5-reactive pectin, suggesting that pectin with regions of low methyl-ester groups is important in conferring flexibility. The high pectin content of the guard cell wall

<table>
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<th>Table IV. Partitioning and composition of pectic fractions</th>
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<td>Partitioning (on fresh wt basis) and composition (mol %) of pectic fractions from CWM of mature wild-type and MdPGS leaves. Values followed by †, *, or ** within a column are significantly different (P &lt; 0.1, P &lt; 0.05, and P &lt; 0.01, respectively) compared with wild type.</td>
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<tr>
<th>CW Fractions</th>
<th>Pectic Fraction&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% of CWM</th>
<th>GalUA in Pectic Fraction</th>
<th>GalUA</th>
<th>Galactose</th>
<th>Arabinose</th>
<th>Xylose</th>
<th>Composition&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup> CWM extracted from 10 g of leaves set as 100%.<br>
<sup>b</sup> Rha, Fuc, Man, and Glc came to less than 12.2 mol % and showed no significant changes between genotypes. Sum of GalUA and neutral sugars ranged between 416.3 and 466.1 μg mg CWM<sup>−1</sup> with no significant difference between genotypes.

Table III. Yields of CWM and GalUA, and neutral sugar composition of CWM

Yield of CWM, GalUA, and neutral sugar composition of CWM from mature leaves of wild-type apple and MdPGS transformants. Data are presented as amount per fresh wt (yield) or as relative molar ratio of sugars per mg CWM (composition). Values followed by †, *, **, or *** within a column are significantly different (P < 0.1, P < 0.05, P < 0.01, and P < 0.001, respectively) compared with wild type.

<table>
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<tr>
<th>Genotypes</th>
<th>Yield</th>
<th>Composition&lt;sup&gt;a&lt;/sup&gt;</th>
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<th>Galactose</th>
<th>Arabinose</th>
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<td>MdPGS-2</td>
<td>0.618</td>
<td>0.135</td>
<td>47.5†</td>
<td>6.8**</td>
<td>13.6**</td>
<td>27.4</td>
<td></td>
</tr>
<tr>
<td>MdPGS-3</td>
<td>0.624</td>
<td>0.114</td>
<td>45.4†</td>
<td>6.8**</td>
<td>12.5**</td>
<td>27.6</td>
<td></td>
</tr>
<tr>
<td>MdPGS-4</td>
<td>0.674</td>
<td>0.131</td>
<td>40.8</td>
<td>7.3***</td>
<td>14.3*</td>
<td>31.6†</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Rha, Fuc, Man, and Glc came to less than 9.3 mol % and showed no significant changes between genotypes. Sum of GalUA and neutral sugars ranged between 408.9 and 908.9, 823.3, and 794.8 for MdPGS-2, -3, and -4, respectively, all significantly greater than control (P < 0.05). Sum of GalUA and neutral sugars for Na<sub>2</sub>CO<sub>3</sub>-soluble fractions was between 587.7 and 626.5 μg mg<sup>−1</sup> with no significant difference between genotypes (P < 0.05).
Overexpression of Polygalacturonase in Apple

PG Overexpression and the Silvery Leaf Phenotype

The silvery leaf phenotype of MdPGS transformatant leaves resembles the “silverleaf” phenotype that occurs in apple in response to infection by the systemic fungus Chondrostereum purpureum. The silverleaf symptoms develop in the leaves as they mature and are believed to be due to airspaces caused by partial separation of the epidermal cells from the palisade cells (Tetley, 1932; Spiers et al., 1987) as was seen in the MdPGS transformants. Endo-PG produced by the fungus is responsible for the development of the pathogenic symptoms (Miyairi et al., 1977). A comparison of infected apple leaves with healthy leaves showed altered pectin partitioning, a high content of methyl-esterified pectin, and some low-\( M \) pectin occurring in infected leaves (Miyairi et al., 1997). In the silvery leaves of MdPGS transformants, we also found altered pectin structure and partitioning.

In MdPGS transformants, young leaves were not silvery, did not detach easily, and stomata were still functioning. Changes in cell wall chemistry were already present but not as marked as in mature leaves. These results suggest that endo-PG can interrupt cell wall metabolism only at a particular stage in leaf development. It is possible that PG has no substrate available in developing leaves. Pectin is synthesized and incorporated into the cell wall in a highly methoxylated form (Zhang and Staehelin, 1992), and only during development are the methyl-groups removed by pectin-methyl-esterase, thereby creating a substrate for PG (Kim and Carpita, 1992). PG is also immobilized in specific sections in the cell wall (Steele et al., 1997). If the cell wall structure loosens during maturation, PG may then be able to come in contact with potential substrates. Pectins have been reported as mediators of wall porosity in soybeans (Glycine max), and a mild treatment with PG apparently enlarged the channels (Baron-Epel et al., 1988). Another possibility is that a continuous modification of pectin structure in the transformant leaves (because of the use of a constitutive promoter) results in altered cell-cell association that is “revealed” only as cell expansion continues. Interestingly, fungal endo-PG applied to mature apple leaves has no effect (silvering does not occur and the polysaccharides remain large), whereas if young expanding leaves are treated with the fungal enzyme, symptoms develop and the polysaccharides are degraded (Miyairi et al., 1997).

The Role of PG in Leaf Abscission

Our results also provide the first direct evidence that expression of a single nonabscission-related PG gene can have a significant role in cell-to-cell adherence in the abscission region, leading to a loss of structural strength.

Endo-PGs have previously been identified and characterized in the abscission zone of several plant species including tomato (Taylor et al., 1990; Kalaitzis et al., 1997; Hong et al., 2000) and Brassica napus (Petersen et al., 1996). Taylor et al. (1993) correlated increases in PG activity only with the onset of abscission. Experiments with transgenics contributed to the theory that PG action was not one of the prime determinants of abscission in leaves. Antisense down-regulation of the tomato fruit-specific PG gene pTOM6 in tomato did not appear to affect leaf abscission (Taylor et al., 1990). Constitutive overexpression of the same gene in tobacco did not result in an increase in leaf abscission although PG activity was elevated (Osteryoung et al., 1990), suggesting that substrate-enzyme interactions were not appropriate for fruit-specific PGs to act in the leaf abscission zone.

In wild-type apple, in regions where the abscission zone develops, low-esterified pectin was only
present in cell junctions and middle lamellae. In the MdPGS transormants, levels of low-esterified pectins were increased and distributed almost across the whole cell wall. This should improve cell adhesion (Jarvis, 1984). However, if modifications to the pectins of these cell walls occurred in a similar manner to those in the leaves, then the reduction in cell adherence during detachment could be due to the decrease in size of the CDTA-soluble pectins, which are more abundant in the transformants. With a DE < 35% in all genotypes, they would still be recognized by JIM5. Earlier examinations of the abscission zone in various organs have shown that during the separation phase, pectins are solubilized and the middle lamella swells (Sexton and Roberts, 1982). This fits well with our microscopic studies with localization of the low-esterified pectin in the abscission zone of the petiole.

CONCLUSIONS

 Constitutive overexpression of a fruit-specific PG in apple plants led to profound effects on leaf morphology, plant water relations, stomatal structure and function, and leaf attachment. This has not been the case in other transgenic plant studies. In tobacco, constitutive expression of a tomato fruit PG had little or no effect on tobacco plant morphology or development, although the enzyme was appropriately processed and showed activity in vitro (Osteryoung et al., 1990). Is the difference between tomato fruit PG and apple fruit PG one of mobility in the cell wall, of substrate availability, or of substrate preference? We recently have transformed Arabidopsis with the same apple gene and find a similar set of phenotypic changes in Arabidopsis leaves as observed in transgenic apple trees. Hence, it may be that apple fruit PG is less specific in the choice of its substrates than tomato fruit PG. Our results in apple suggest that it would be useful to carry out a fuller examination of the action of specific PGs on pectins, and the changes in pectins during leaf development.

MATERIALS AND METHODS

Plant Transformation and Growth

The apple (Malus domestica Borkh. cv Royal Gala) PG cDNA clone MdPG1, formerly GDPG1 (Atkinson, 1994), was used as the starting point for vector construction. Initially, the NptI site present in pSPortI was removed by blunting, then the PG cDNA insert was excised with BamHI and Smal, blunted, and cloned into the Smal-site of pART7 (Gleave, 1992). This construct was digested with NptI and cloned into the binary pART27. The binary was electropropated into Agrobacterium tumefaciens strain LBA4404.

Transgenic apple shoots were produced using the method of Yao et al. (1995). Independent transgenic shoots were micropropagated from axillary buds to form transgenic lines. Transgenic lines were maintained in a containment greenhouse under identical conditions (ambient light and temperature) to wild-type plants, except MdPGS transformants were given supplementary watering. Plants were 3 years old at the time of experimentation.

DNA and RNA Gel-Blot Analyses

Because previous transformations of apple cv “Royal Gala” with the empty binary vector pART27 (Yao et al., 1995) showed that all transformants were similar to wild type, we used wild-type plants as controls for these and all further analyses.

Apple genomic DNA was extracted from young, expanding leaves as described in Dellaporta et al. (1983). DNA gel-blot analysis, apple leaf RNA extractions, and RNA gel-blot analysis were performed as described by Schröder et al. (1998).

PG Extraction, Activity Assays, and Western Blotting

PG enzyme was extracted using the method of Bergey et al. (1999). Aliquots of enzyme (600 μL) were incubated with 1% (w/v) poly-GalUA, pH 4.5 (100 μL) and 0.3 M Na-acetate, pH 4.5 (150 μL). Duplicate samples boiled in water for 5 min before addition of the substrate served as blanks (assay modified after Pressey, 1986). The mixtures were incubated overnight at 30°C, and PG activity analyzed by measuring reducing end groups using 4-hydroxybenzoic acid hydrazide with GalUA as standard (Lever, 1972). After subtraction of the corresponding blank, PG activity was expressed in mmol reducing end groups generated per 100 mg CWM. Protein concentration was estimated using the protein assay (Bio-Rad Laboratories, Hercules, CA).

For immunoblotting, proteins were separated on 1.5 mm 10% (w/v) SDS-Tris-Tricine gels (Schägger and von Jagow, 1987) using a Mini-Protean II electrophoresis system (Bio-Rad). Proteins were electroblotted onto Immobilon-P transfer membranes (Millipore, Bedford, MA) using a Trans-Blot SD Semi-Dry transfer cell (Bio-Rad) following the manufacturer’s instructions (Millipore). Blots were blocked overnight at 4°C with TBS-T20 (10 mM Tris base, 150 mM NaCl, and 0.1% [v/v] Tween 20) containing 5% (w/v) nonfat dried milk powder and incubated with tomato (Lycopersicon esculentum) endo-PG antibody (1:1,000 [w/v] dilution) in blocking solution for 1 h at ambient temperature. After washing in TBS-T20, the blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgE (1:1,000 [w/v], Sigma, St. Louis) in blocking solution. After washing in TBS-T20 (3 × 15 min), the bound antibodies were visualized using 1-Step nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Pierce Chemical, Rockford, IL).

Measurement of Force for Leaf Detachment

Petioles of mature, fully expanded leaves were wrapped three times with string using slipknots. The string was firmly attached with glue to the petiole, leaving at least 1 cm to the leaf abscission zone. The other end of the string was attached to a hook attachment on a Materials Testing
Machine (model MN 44, Instron, Canton, MA) with a 100 N load cell. The petiole was positioned at right angles to the direction in which the breakforce was applied. Tensile force was applied at a constant 10 mm min⁻¹. The maximum force required for leaf removal was recorded for at least five leaves from three plants of each transgenic line and the wild-type control. The experiment was carried out in spring, summer, and early autumn.

Color Readings and Photosynthesis, Leaf-Diffusive Conductance, ETR, and Photon Efficiency

The silvery leaf phenotype was measured on mature leaves on the upper surface using a Chromameter (Minolta, Osaka) and the CIELAB model for hue (h) and lightness (L) (ASTM, 1966) and the standard illuminant D₆₅ (emits the equivalent of average daylight including UV region with correlated color temperature of 6504K). C (chromaticity), L, and h values were measured on seven leaves of each tree of each transgenic line. All leaf gas-exchange measurements were made with a climate-controlled minicuvette system (CMS 400, Walz, Effeltrich, Germany) as described by Buwalda et al. (1991). The dewpoint temperature of air entering the cuvette was set at 5.2°C and the air temperature in the cuvette was set at 24°C. The reported photosynthetic rate and leaf diffusive conductance were the values recorded at a photosynthetically active radiation of 900 μmol m⁻² s⁻¹. Maximum ETR and photon efficiency of electron transport were measured using a MINIPAM (Walz, Effeltrich, Germany) according to the method of Laing et al. (2000).

Microscopy

Light microscopy observations were carried out on a Vanox AHT 3 microscope (Olympus, Tokyo) fitted with a 35-mm film camera and a CoolSnap digital camera (Roper Scientific, Tucson, AZ). Lamina tissue from young and mature leaves and petiole tissue from mature leaves were excised and fixed in either 2% (v/v) formaldehyde with 0.1% (v/v) glutaraldehyde in 0.1 M phosphate buffer, or 2% (v/v) formaldehyde with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer. Tissue was subjected to light vacuum to infiltrate airspaces, washed in buffer, dehydrated with an alcohol series, and embedded in LR White resin (Hallett et al., 1992). The embedded material was sectioned at a thickness of 0.5 or 1 μm and stained with 0.05% (w/v) toluidine blue in borate buffer (pH 4.4) for gross morphological examination and 0.02% (w/v) ruthenium red for general localization of pectic material. Antibodies JIM5 and JIM7 were used to localize low-esterified pectin and high-esterified pectin, respectively (Sutherland et al., 1999). Direct observation of intact unfixed leaves of wild-type and transformed leaves was carried out after exposure to light, up to 45 min in the dark and exposure for 30 min to 3 μM ABA brushed over the epidermis.

For low-temperature scanning electron microscopy, leaf tissue material was mounted on copper specimen stubs and rapidly frozen in liquid nitrogen and either processed and observed immediately or stored under liquid nitrogen. Material was processed and transferred for observation on a cryostage in a Philips PSEM 505 scanning electron microscope (Philips, Eindhoven, The Netherlands) using an EMScope SP2000 sputter cryo-system (Falloon et al., 1989).

Cell Wall Analyses

Tissue from mature leaves (10 g, midribs excised) was ground in liquid N₂ and homogenized by polytronizing in 40 mL of methanol:chloroform:water:formic acid (16:5:1:1 [v/v]). Homogenates were left at −20°C overnight and centrifuged (6,000g, 10 min). Pellets were resuspended by polytronizing using 50 mL of methanol:chloroform:water (16:5:1 [v/v]) and centrifuged. Extraction of the twice more washed pellet with dimethyl sulfoxide to give CWM, and sequential extraction of CWM with CDTA and Na₂CO₃ to give CDTA solubles, Na₂CO₃ solubles, and residue was carried out after Redgwell et al. (1988). Fractions were dialyzed extensively against water for 7 d using dialysis membranes with a molecular mass cutoff of 3.5 kD (Spectra/Por, Spectrum) to minimize loss of pectic fragments possibly created by the action of PG, and then freeze dried. The composition of neutral monosaccharides was analyzed by gas liquid chromatography according to Albersheim et al. (1967). GalUA content was quantified (Blumenkrantz and Asboe-Hansen, 1973; Ahmed and Labavitch, 1977) using n-GalUA as a standard. The DE was measured after Wood and Siddiqui (1971).

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