Manipulation of Strawberry Fruit Softening by Antisense Expression of a Pectate Lyase Gene[1]

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Strawberry (*Fragaria* × *ananassa*, Duch., cv Chandler) is a soft fruit with a short postharvest life, mainly due to a rapid lost of firm texture. To control the strawberry fruit softening, we obtained transgenic plants that incorporate an antisense sequence of a strawberry pectate lyase gene under the control of the 35S promoter. Forty-one independent transgenic lines (Apel lines) were obtained, propagated in the greenhouse for agronomical analysis, and compared with control plants, non-transformed plants, and transgenic lines transformed with the pGUSINT plasmid. Total yield was significantly reduced in 33 of the 41 Apel lines. At the stage of full ripen, no differences in color, size, shape, and weight were observed between Apel and control fruit. However, in most of the Apel lines, ripened fruits were significantly firmer than controls. Six Apel lines were selected for further analysis. In all these lines, the pectate lyase gene expression in ripened fruit was 30% lower than in control, being totally suppressed in three of them. Cell wall material isolated from ripened Apel fruit showed a lower degree of in vitro swelling and a lower amount of ionically bound pectins than control fruit. An analysis of firmness at three different stages of fruit development (green, white, and red) showed that the highest reduction of softening in Apel fruit occurred during the transition from the white to the red stage. The postharvest softening of Apel fruit was also diminished. Our results indicate that pectate lyase gene is an excellent candidate for biotechnological improvement of fruit softening in strawberry.

Temperate fruits can be classified into two categories according to their softening behavior and textural properties (Bourne, 1979). One group includes those fruits that soften greatly during ripening, acquiring a melting texture, whereas the other group comprises fruits that soften moderately and display a crisp fracturable texture. Strawberry (*Fragaria* × *ananassa*, Duch., cv Chandler) is included in the first group, joint to other economically important crops such as tomato (*Lycopersicon esculentum*) and avocado (*Persea americana*). Rapid softening during ripening is one of the main causes of the short postharvest shelf life of these fruits; therefore, any improvement of softening behavior could have a significant commercial importance.

Softening of ripe strawberry fruit occurs mainly by degradation of the middle lammella of cortical parenchyma cells (Perkins-Veazie, 1995). Histological analysis of ripe fruit showed a cell wall thinner than unripe fruit and the lost of intercellular material, the cells being with little contact and separated by considerable intercellular space (Redgwell et al., 1997). The underlying biochemical mechanism of strawberry softening is unclear. The largest changes in the plant cell wall during ripening occur in the pectin component. The percentage of water-soluble pectins increases during ripening but total quantity of polyuronide residues (Woodward, 1972; Knee et al., 1977; Huber, 1984; Redgwell et al., 1997) and polyuronide length (Huber, 1984; Redgwell et al., 1997) are only slightly modified. This last observation is in accordance with the low poligalacturonase activity found in ripe fruit (Abeles and Takeda, 1990; Nogata et al., 1993). Alternative to the role of pectin modification in softening, several authors have focused on the degradation of the cellulose matrix as the primary cause of the lost of fruit firmness. Along this line, Knee et al. (1977) observed that the cell wall became swollen during fruit development and this higher hydration was parallel to change in the neutral sugars of the cell wall fraction as result of a probable degradation of hemicellulose and cellulose. Moreover, endoglucanase activity increased 6 times between the green and red stages of ripening (Abeles and Takeda, 1990). More recently, several endoglucanase genes whose

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expression is correlated with strawberry ripening have been isolated (Harpster et al., 1998; Llop-Tous et al., 1999; Trainotti et al., 1999).

A pectate lyase gene recently isolated from ripe strawberry has been proposed as a new candidate for pectin degradation, contributing to the lost of fruit firmness (Medina-Escobar et al., 1997). The expression of this gene is restricted to ripening fruits and it is inhibited by auxin treatment. Similarly, Domínguez-Puigjaner et al. (1997) isolated a gene with homology to pectate lyase in banana whose expression was induced during ripening of this climateric fruit. Pectate lyases have been extensively studied in pathogenic bacteria, which secreted this enzyme causing depolymerization of pectins in the middle lamella and primary cell wall of higher plants, and consequently the maceration of plant tissues (Henrissat et al., 1995). The degradation of pectins by pectate lyase occurs by a β-elimination reaction in contrast to the hydrolytic mechanism of polygalacturonases. Contrary to the abundant literature about the role of polygalacturonases in fruit ripening, no data on pectate lyase activity in fruit have been reported so far. Thus, the role of these genes in fruit softening remains uncertain. In other plant species, pectate lyase-like genes have been isolated; most of them are related to pollination because they are highly expressed in mature pollen grains or pollen tubes (Rogers et al., 1992; Turcich et al., 1993; Dircks et al., 1996).

In the present work, we present results on transgenic strawberry plants that underexpress the pectate lyase gene by antisense transformation to assess the role of this gene in fruit softening.

RESULTS

Analysis of Transgenic Plants

Forty-one independent transgenic plants were obtained after 20 weeks of selection in 25 mg L^{-1} kanamycin. The presence of the T-DNA in all these plants was confirmed by PCR amplification of both the *nptII* gene and the *35S-antipectate lyase* chimeric gene (results not shown). The absence of bacterial contamination was confirmed by the lack of amplification when using specific primers to amplify the *Agrobacterium tumefaciens VirD1* gene (results not shown).

After being acclimated, transgenic plants were transferred to the greenhouse for runner multiplication and agronomical analysis of the daughter plants. In relation to the vegetative growth, most of the antipectate lyase transgenic plants (Apel lines) displayed growth pattern similar to controls, either non-transformed or transformed with the pGUSINT plasmid. However, 10% of the Apel lines were smaller than control and showed a significant reduction in the leaf area. This modification was maintained after several cycles of runner proliferation.

Twelve plants per transgenic clone were grown in the greenhouse until fruiting and the fruit yield per plant was recorded at the end of the culture. In general, in vitro tissue culture induced a decreased yield during the first growing season after acclimation. In fact, control micropropagated plants showed a 30% reduction in fruit yield when compared with macropropagated controls (22.7 fruits per plant versus 32 in micro- and macropropagated controls, respectively). Five out of the 15 independent β-glucuronidase (GUS) lines (33.3%) showed lower fruit yield than non-transformed controls. In relation to the Apel lines, 13 out of the 41 clones evaluated did not set any fruit, and 20 of them showed fruit yield significantly reduced. Only eight Apel clones, 28.6% of the Apel lines that set fruit, showed an accumulated production similar to that obtained in macro- or micropropagated controls.

Control and transgenic fruits were harvested at the stage of full ripen and their main characteristics were recorded as weight, color, shape, soluble solids, and firmness (Tables I and II). No differences were observed among the controls (micropropagated, macropropagated, and GUSINT transgenic plants) in any of the parameters evaluated, with the exception of soluble solids, where five GUS lines showed higher values than non-transformed plants. Similarly, most transgenic Apel fruits did not show significant differences in weight, color, shape, or soluble solids when compared with controls (Table I). Fruit weight was slightly lower in the Apel lines, although this difference was only statistically significant in Apel 11 and 18. Color and shape were slightly modified and percent of soluble solids was increased in Apel 18, 20, 39, and 45. The anthocyanin content in control and Apel fruits was also similar (mean value of 1.45 $\pm$ 0.09; mean $\pm$ SE). The main differences between Apel and control fruits were observed in firmness (Table II). Fifty percent of the Apel clones analyzed showed a higher external fruit firmness than fruits obtained from control plants. It is noteworthy that most of the Apel lines displayed a statistically significant increment in the internal fruit firmness (obtained after removing the external skin of the fruit), ranging the increment from 149% to a 179% when compared with macropropagated plants. The differences in firmness were not due to a reduction in Apel fruit weight. Plots of fruit size with external and internal firmness are shown in Figure 1. Only in the case of control lines was a low correlation between size and external firmness observed. Thus, the enhanced firmness of Apel lines would not be a secondary effect of a decrease fruit size.

Molecular Characterization and Cell Wall Analysis of Selected Lines

The Apel clone numbers 3, 14, 22, 23, 39, and 48 were selected for further molecular analysis based on

Table I. *Fruit characteristics in controls and transgenic Apel plants*

Three kinds of controls were employed: non-transformed plants conventionally propagated, non-transformed plants micropropagated in vitro, and transgenic plants transformed with the pGUSINT plasmid. Fruits were harvested when fully ripened. Color and shape were measured using standard scales. Data correspond to mean ± SD of a minimum of 40 fruits per clone. Mean separation was performed with the Tukey's HSD test (P = 0.01). Those clones that were significantly different than control macropropagated plants are labeled with an asterisk.

Plant	Weight	Color	Shape	Soluble Solid
	g			*°Brix*
Control macropropagated	12.0 ± 4.5	5.5 ± 0.6	5.1 ± 2.1	7.1 ± 2.9
Control micropropagated	12.7 ± 5.4	5.8 ± 0.5	5.2 ± 1.8	8.6 ± 2.5
GUS 1	13.0 ± 6.0	5.7 ± 0.6	5.1 ± 2.1	10.1 ± 1.9*
GUS 2	9.9 ± 4.1	5.1 ± 0.6	4.3 ± 2.2	10.7 ± 3.3*
GUS 3	9.8 ± 4.2	5.6 ± 0.5	3.8 ± 2.1	7.9 ± 2.4
GUS 4	8.2 ± 2.7	5.0 ± 0.5	4.4 ± 2.4	10.5 ± 2.9*
GUS 5	8.4 ± 3.1	5.5 ± 0.5	5.4 ± 1.6	9.5 ± 2.6
GUS 6	12.2 ± 4.7	5.5 ± 0.5	5.3 ± 1.9	9.8 ± 2.7*
GUS 10	12.7 ± 4.6	4.9 ± 0.5	4.4 ± 2.4	9.1 ± 3.1
GUS 11	10.5 ± 4.2	5.8 ± 0.4	5.2 ± 2.1	7.6 ± 2.3
GUS 12	10.6 ± 4.8	5.8 ± 0.4	5.0 ± 2.4	7.3 ± 1.9
GUS 15	11.7 ± 4.5	5.3 ± 0.5	6.7 ± 2.2	9.8 ± 2.1*
Apel 2	11.2 ± 3.3	4.2 ± 0.7*	4.1 ± 3.0	7.3 ± 1.5
Apel 3	9.6 ± 4.2	5.8 ± 0.5	4.7 ± 2.4	7.5 ± 2.8
Apel 11	7.4 ± 3.3*	4.9 ± 0.6	3.7 ± 2.2	9.4 ± 2.1
Apel 14	8.7 ± 3.9	6.0 ± 0.5	4.4 ± 2.3	8.8 ± 2.3
Apel 18	6.1 ± 2.0*	3.0 ± 1.2*	3.0 ± 2.7	10.4 ± 1.9*
Apel 20	8.4 ± 3.1	5.4 ± 0.8	5.2 ± 2.7	10.5 ± 2.7*
Apel 21	12.4 ± 4.7	5.1 ± 1.0	4.1 ± 2.1	5.8 ± 1.7
Apel 22	8.3 ± 3.1	5.3 ± 0.5	3.8 ± 2.1	9.4 ± 2.1
Apel 23	9.8 ± 3.9	6.5 ± 0.9*	3.9 ± 2.3	7.0 ± 1.9
Apel 39	8.4 ± 3.0	5.2 ± 0.3	4.7 ± 2.7	9.8 ± 1.4*
Apel 45	9.6 ± 3.4	5.1 ± 0.6	4.2 ± 1.5	11.0 ± 3.1*
Apel 48	10.2 ± 4.4	5.4 ± 0.5	4.7 ± 2.5	9.2 ± 2.3

their good fruit yield and their higher fruit firmness than control plants. Southern analysis of DNA extracted from these clones is shown in Figure 2. The number of T-DNA insertions ranged from one in the case of Apel 22 to three in the case of Apel 3, 14, and 39. The effect of the antisense transformation on pectate lyase gene expression and protein level in fully ripened fruits is shown in Figure 3. The steady-state levels of pectate lyase mRNA were drastically reduced in all the transgenic lines analyzed (Fig. 3A). The percentage of gene expression in transgenic fruits was always lower than 30% of the control non-transformed fruits. In the Apel lines 22, 23, and 39, the pectate lyase gene expression was totally suppressed (Fig. 3B). The level of pectate lyase suppression was correlated with the internal fruit firmness (r = 0.84, statistically significant at $P < 0.05$). The antisense inhibition of pectate lyase expression was parallel to a reduction at the protein level, as can be observed in the western-blot analysis of Apel 3 (gene expression and protein level partially inhibited) and 23 (both inhibited; Fig. 3C). We tried to measure pectate lyase activity in fruit following different protocols (Collmer et al., 1988; Brooks et al., 1990). However, the results obtained showed a low reproducibility because of strong extract interferences and low sensitivity of assays.

Cell wall material (CWM) was isolated from ripe fruit of control and Apel lines 22 and 39. In vitro swelling of CWM was reduced in the two Apel analyzed when compared with control (Table III). Furthermore, the amount of CDTA-soluble pectins was lower in CWM from Apel fruit (Table III).

Analysis of Fruit Softening during Development

Fruit firmness in the controls and the three transgenic Apel lines that showed 100% pectate lyase inhibition was measured at three different stages of fruit development, (green, white, and full red). In control plants, internal and external firmness decreased along fruit maturation, the values obtained being 508, 185, and 25.6 g mm^{-2} for internal firmness at green, white, and red stages, respectively, and 529, 245, and 29.4 g mm^{-2} for external firmness at the same stages. Transgenic Apel fruits at the green stage showed similar values for internal and external firmness than controls. However, the decrement in internal firmness occurring during fruit maturation from green to white and specially from the white to the red stage was significantly reduced in the Apel clones analyzed (Fig. 4). A similar effect was observed in external firmness of Apel fruits, although the white stage showed similar values than controls.

Table II. *External and internal firmness in controls and transgenic Apel plants*

Fruit were harvested at full ripen and the firmness of intact (external firmness) and peeled fruit (internal firmness) was measured with a penetrometer. Data correspond to mean ± SD of a minimum of 25 fruits per clone. Values in parentheses are the normalized values relative to control macropropagated plants. Mean separation was performed by the Tukey's HSD test (P = 0.01). Those clones that were significantly different than control macropropagated plants are labeled with an asterisk.

Plant	External Firmness	Internal Firmness
	g mm^{-2}	
Control macropropagated	35.7 ± 15.4 (100)	26.8 ± 11.1 (100)
Control micropropagated	33.2 ± 14.2 (93)	24.9 ± 11.9 (93)
GUS 1	21.5 ± 8.6 (60)*	16.7 ± 2.0 (62)*
GUS 2	38.3 ± 11.2 (107)	19.1 ± 5.7 (71)
GUS 3	39.4 ± 13.1 (110)	24.2 ± 13.4 (90)
GUS 4	27.3 ± 13.5 (76)	23.5 ± 10.4 (87)
GUS 5	43.0 ± 9.9 (120)	23.0 ± 7.7 (86)
GUS 6	35.3 ± 12.1 (99)	25.2 ± 12.9 (94)
GUS 10	35.7 ± 10.7 (100)	25.0 ± 10.6 (93)
GUS 11	43.1 ± 10.2 (121)	21.7 ± 8.8 (81)
GUS 12	38.4 ± 13.8 (107)	21.9 ± 9.0 (82)
GUS 15	31.7 ± 13.3 (89)	20.3 ± 7.8 (76)
Apel 2	51.4 ± 11.0 (144)*	45.3 ± 6.0 (169)*
Apel 3	43.5 ± 6.7 (122)	40.0 ± 7.4 (149)*
Apel 11	46.3 ± 11.5 (129)	40.0 ± 8.8 (149)*
Apel 14	52.7 ± 8.9 (147)*	48.1 ± 5.6 (179)*
Apel 18	40.4 ± 9.7 (113)	35.1 ± 7.9 (131)
Apel 20	51.4 ± 8.0 (144)*	45.3 ± 5.2 (169)*
Apel 21	45.9 ± 6.0 (128)	42.2 ± 6.7 (157)*
Apel 22	48.0 ± 5.5 (134)*	45.4 ± 6.3 (169)*
Apel 23	53.4 ± 7.6 (149)*	47.3 ± 8.2 (176)*
Apel 39	47.2 ± 4.5 (132)*	46.9 ± 4.8 (175)*
Apel 45	44.8 ± 4.7 (125)	46.8 ± 9.6 (174)*
Apel 48	44.0 ± 7.5 (123)	43.5 ± 7.9 (162)*

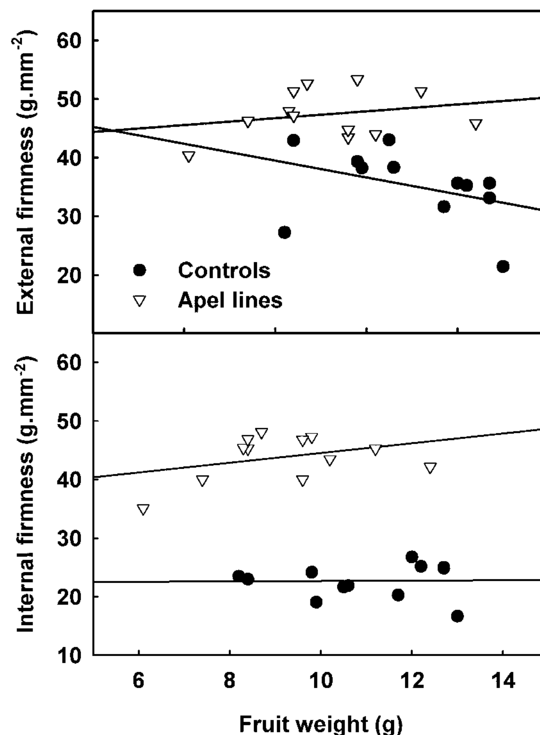

Figure 1. Plots of fruit weight with external and internal firmness in controls (non-transformed and GUS-transformed lines) and Apel transgenic lines.

The electrolyte leakage test was used as an estimation of fruit integrity. At the green and white stages, minor differences were observed between control and Apel lines, with mean values of leaked electrolytes after 1 h of tissue incubation in distilled water of 37.6% and 35.2% for control and Apel lines, respectively, at the green stage, and 44.5% and 47.6% at the white stage. However, at the ripen stage, Apel transgenic lines showed lower percentages of leaked electrolytes than control fruits, 58.7% versus 73.6% for Apel and control fruit, respectively.

Finally, ripen fruit were harvested and maintained for 4 d at 25°C. The percentage of fruits with a semimelted texture after the postharvest period was close to 45% in the case of controls (Fig. 5). This percentage of soft fruit was lower in the three selected Apel lines analyzed.

DISCUSSION

In the present work, we obtained transgenic plants transformed with an antisense sequence of pectate lyase gene under the control of the double 35S promoter. Ten percent of the Apel lines showed a dwarf phenotype. This effect could probably be induced by the in vitro culture inherent to the transformation process. A similar percentage of dwarf variants was obtained by Nehra et al. (1992) in regeneration experiments of strawberry leaf explants. Fruit yield of most of the Apel lines was significantly reduced. In higher plants, most of the pectate lyase genes have been isolated from mature pollen grains (Rogers et al., 1992; Turcich et al., 1993; Dircks et al., 1996). Pectate lyase activity in pollen must be important for the emergence of the pollen tube from the pollen grain and/or the growth of the pollen tube down the style. Medina-Escobar et al. (1997) did not report pectate lyase gene expression in strawberry flowers, but the presence of a pollen pectate lyase cannot be

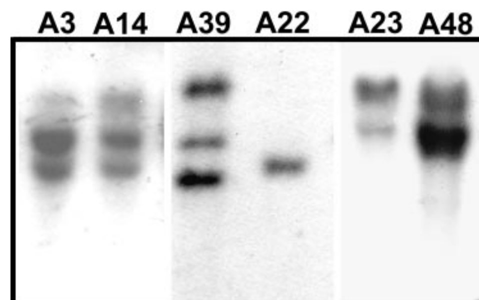

Figure 2. Southern-blot analysis of DNA extracted from selected Apel clones. Membrane was hybridized with the ^{32}P-labeled 35S-antipel-nos chimeric gene (for details, see "Materials and Methods").

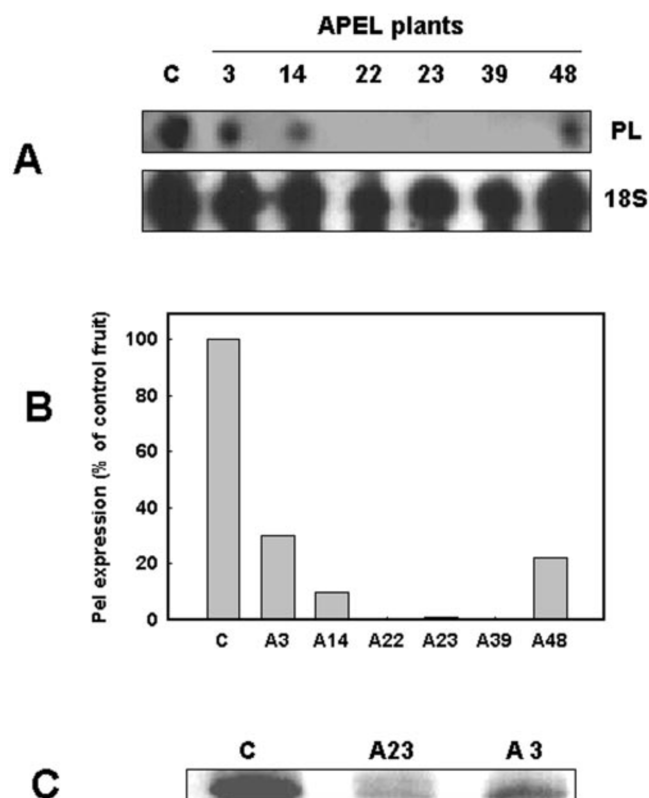

Figure 3. The effect of antisense transformation in pectate lyase gene expression in fruit. A, Northern-blot analysis of RNA isolated from full ripe fruit of selected Apel clones. Membrane was hybridized with the ^{32}P-labeled pectate lyase gene (for details, see "Materials and Methods"). The same blot was hybridized with a ^{32}P-labeled 18S RNA probe as a control. B, Percentage of pectate lyase gene expression in full ripe fruit of selected Apel clones. C, Western blot of ripened fruit-soluble protein extracts prepared from Control and Apel lines 23 and 3, probed with a 1:100 dilution of antiserum raised to a strawberry pectate lyase protein. Each lane contained 100 μg of total fruit protein. In all cases, C represents a control, non-transformed fruit.

completely excluded. Thus, low fruit yield is probably the result of a poor pollination in the antipectate lyase transgenic plants.

At ripening, transgenic Apel fruits showed steady-state levels of the pectate lyase transcripts lower than 30% of those observed in control fruits, the expression being totally inhibited in several clones. In the clones analyzed, the level of pectate lyase inhibition correlated with the internal fruit firmness. In two of them, it has been confirmed that the amount of pectate lyase protein was parallel to the transcript level. Cell wall swelling during softening is common in many fruits, including strawberry (Redgwell et al., 1997). This fact can be related to an increase of pore size due to disassembly of pectin networks induced by pectin-degrading enzymes (Redgwell, 1997; Hadfield and Bennett, 1998). As expected, inhibition of pectate lyase gene resulted in a lower degree of in vitro cell wall swelling and a lower amount of ionically bound pectins, the pectin fraction solubilized by

Table III. *Cell wall swelling and cyclohexane diamine tetraacetic acid (CDTA)-soluble pectins in control and transgenic Apel fruit*

CWM was isolated from fully ripened fruit. For the in vitro swelling, samples were suspended in water in small vials and allowed to settle. The height of the sedimented layer was used as an index of wall swelling. The CDTA-soluble pectins extracted from CWM were expressed as the absorbance at 520 nm mg^{-1} of CWM. Data correspond to mean ± SD of three independent extractions.

Plant	Cell Wall Swelling	CDTA-Soluble Pectins
	%	
Control	100	0.73 ± 0.13
A22	81.9	0.64 ± 0.15
A39	70.9	0.29 ± 0.17

pectin-degrading enzymes such as pectate lyase, indicating a higher integrity of cell wall structure in transgenic ripen fruit. Furthermore, the reduction of leaked electrolytes when fruit pieces were incubated in distilled water is an additional evidence of lower cell wall dissociation in Apel fruit, although decreased membrane deterioration could also contribute to this result. All together, these results indicate that pectate lyase genes play a determinant role in strawberry softening. The expression pattern of pectate lyase gene and the analysis of the Apel fruit firmness at different stages of maturation also support this role. Pectate lyase gene expression is activated at the white stage and showed a maximum level at the full ripe red stage (Medina-Escobar et al.,

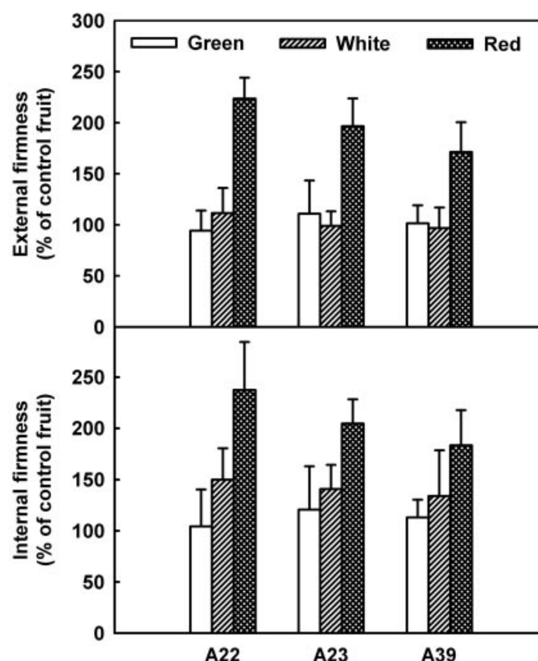

Figure 4. The effect of antisense down-regulation of a pectate lyase gene in the external and internal firmness of fruit at different stages of maturation (green, white, and full red). The values of firmness are expressed as percentage of control, non-transformed fruit. Values are means ± SD of a minimum of 20 fruit per clone.

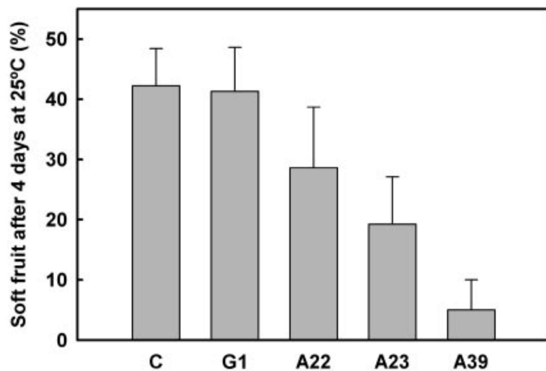

Figure 5. Postharvest softening of controls (C and G1) and Apel transgenic fruit (A22, A23, and A39). Fruit were harvested at ripening and maintained for 4 d at 25°C. The percentage of soft fruit was calculated as the percentage of fruit with internal firmness lower than 15 g mm^{-2}. C corresponds to non-transformed control and G1 corresponds to the GUS1 line. A minimum of 20 fruit per line were analyzed. Bars represent the SE.

1997). In transgenic Apel fruit, the inhibition of pectate lyase only reduces the softening that occurs at this developmental stage, the transition from the white to the red stage.

Fruit softening is a complex process that could involve three sequential steps: loosening of cell wall mediated by expansins, depolymerization of hemicelluloses, and finally polyuronide depolymerization by polygalacturonase or other hydrolytic enzymes (Brummell et al., 1999). Experiments with pectolytic enzymes have de-emphasized the role of pectins in fruit softening. Neither polygalacturonase (Sheehy et al., 1988; Smith et al., 1990) nor pectin methyl esterase (Gaffe et al., 1994) gene expression inhibition reduced softening in tomato. In contrast, underexpression of an expansin gene resulted in a moderate increment in tomato fruit firmness due to a lower depolymerizatin of pectin (Brummell et al., 1999). Polygalacturonase and polymethylgalacturonase apparently play a minor role in strawberry softening because little or no activity has been found in fruit (Barnes and Patchett, 1976; Abeles and Takeda, 1990). On the other hand, pectinmethylesterase activity increases during strawberry ripening (Barnes and Patchett, 1976), but according to Huber (1984), this increment is not sufficient by itself to account for the large changes that occurred in water-soluble polyuronides. Thus, pectin degradation and softening of ripe strawberry must be mediated by a different cell wall hydrolytic enzyme. In this paper, we demonstrate that in a high number of independent transgenic lines, the inhibition of pectate lyase significantly increase strawberry fruit firmness. This result is probably due to a lower pectin solubilization, although a complete analysis of pectin polymer sizes is needed to confirm this hypothesis. However, as observed in tomato (Brummell et al., 1999), it is possible that this enzyme is not the only component that determines the lost of firmness. The slight reduction of the decrement in firmness that occurs during the transition from the green to the white stage in Apel fruits suggests that other cell wall-degrading mechanisms are taking place in unripe fruit. Cellulase (Harpster et al., 1998; Llop-Tous et al., 1999; Trainotti et al., 1999) and expansin (Civello et al., 1999) genes are candidates for cell wall degradation at these stages.

In conclusion, the reduction of the steady-state levels of pectate lyase mRNA resulted in a high increase in firmness of full ripe fruit and reduced the postharvest softening, without affecting other fruit characteristics such as weight, color, or soluble solids. Thus, this gene is an excellent candidate for biotechnological improvement of strawberry fruit softening. Experiments are in progress to determine the extension of pectin depolymerization in transgenic fruits.

MATERIALS AND METHODS

Plant Material and *Agrobacterium tumefaciens*-Mediated Transformation

Leaves from strawberry (*Fragaria* × *ananassa*, Duch., cv Chandler) plants, micropropagated in vitro in modified Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 2.2 μM kinetin (Barceló et al., 1998), were used as explants for transformation experiments. The *A. tumefaciens* LBA4404 strain carrying a construct with the strawberry pectate lyase gene (*njjs25*) in the antisense orientation (pJLC32a) was used for transformation, following the procedure previously established (Barceló et al., 1998). To obtain such a construct, a 1.2-kb EcorV DNA fragment from plasmid pNJJS25C (Medina-Escobar et al., 1997) was first isolated from a 1% (w/v) agarose gel and subcloned into the calf intestinal alkaline phosphatase-treated *Sma*I site of the plasmid pJC2Ena (Day et al., 1991), containing the 35S-cauliflower mosaic virus promoter with an enhancer, producing the plasmid pJLC30a. This DNA insert contains the njjs25C gene coding sequence, extending from the transcription start point to an EcorV site near the polyadenilation site of the strawberry njjs25C gene. The pJLC30a plasmid was checked for the presence of the pectate lyase gene in the antisense orientation by restriction analysis. Then, the plasmid pJLC30a was cut with *Hin*dIII and *Xba*I and a 2.77-Kb DNA fragment carrying the antisense njjs25 gene flanked by the 35S-cauliflower mosaic virus promoter region and the ocs polyA gene tail was purified from a 1% (w/v) agarose gel and subcloned into the *Hin*dIII and *Xba*I sites of pBINPLUS plant transformation vector (VanEngelen et al., 1995). The resulting plasmid pJLC32a (15.1 kb) was first used to transform *A. tumefaciens* by electroporation.

After 20 to 30 weeks of selection in 25 mg L^{-1} kanamycin, the kanamycin resistant plants were acclimated, transferred to the greenhouse, and grown until fruiting under natural light (200–600 μmol m^{-2} s^{-1}) and photoperiod. These plants were vegetatively propagated by runner to obtain enough copies for further studies.

Phenotypic Analysis of Transgenic Plants

In the first experiment, 41 independent transgenic plants were analyzed. Conventionally propagated plants (macropropagated), micropropagated plants, as well as 15 independent transgenic lines transformed with the 35S-GUSINT plasmid (Vancanneyt et al., 1990), were used as controls. Twelve daughter plants were obtained by runners from each mother plant. Vegetative growth, flowering, and fruit yield were recorded. Fruits were harvested at the stage of full ripeness, when complete fruit surface was red, and the weight, color, shape, soluble solids, and firmness were recorded. Color was measured using the CTIFL (Centre technique interprofessionnel des fruits et legumes, France) code, a standard scale employed by the Strawberry European Network (COST Action 836, European Union). This color code comprises eight categories, increasing the red color from 1 (light orange-red) to 8 (dark wine-red). Color was also assessed by measuring anthocyanin content (Pietrini and Massacci, 1998). Fruit pieces were homogenized in methanol containing 1% (v/v) HCl and maintained at 4°C for 4 h. After centrifugation at 10,000*g*, the absorbances of the supernatant at 530 and 657 nm were recorded, and the anthocyanin content was calculated as $A_{530} - A_{657}$. Fruit shape was measured using the CIREF (Centre Interrejional de Recherche et D'Expérimentation de la Fraise, France) code (Roudeillac, 1987). Percentage of soluble solids was measured using a refractometer Atago N1. Fruit firmness was measured using a penetrometer with 3.1- or 9.6-mm^2 surface needles, and three punctures from opposite sites per fruit. After recording the firmness of intact fruit (external firmness), the surface skin of the fruit was removed and three new punctures were performed to obtain the internal firmness.

Six clones with a yield similar to control plants and the highest values for firmness were selected for molecular analysis and further studies. Thirty-two copies per line were obtained by runner production and grown the next season in the greenhouse until fruiting. In this experiment, fruits were harvested at three different stages: green, mature white, and fully ripened (red), and the weight and internal and external firmness were recorded at each stage. Fruit integrity was estimated by an electrolyte leakage test, based on previous works (Wegener et al., 1996; Tian et al., 2000). Fruit cylinders 1 cm in length were obtained from the central cork of the fruit and incubated for 1 h in distilled water on a gyratory shaker. Then, the cylinders were autoclaved for 20 min to break the tissues and to release all the electrolytes. Conductivity of the solution was measured before and after being autoclaved using a conductance meter (Crison C-525, Crison Instruments, Barcelona, Spain). The leaked electrolytes were expressed as percentage of total electrolytes.

To analyze the posharvest softening of fruit, control and Apel fruit were harvested at the full red stage, immersed for a few seconds in an antifungal solution, and maintained for 4 d at 25°C. After this treatment, fruit firmness was measured, and the percentage of soft fruit with a semi-melted texture (internal firmness lower than 15 g mm^{-2}) was recorded. A minimum of 20 fruits per line was employed.

Molecular Analysis of Transgenic Plants

Fully expanded leaves and full ripe red fruits were used for DNA and RNA extraction, respectively. Plant material was collected and immediately frozen in liquid nitrogen and stored at −80°C. Initially, the transgenic nature of the plants surviving in the presence of kanamycin was confirmed by PCR. Two hundred to 500 ng of DNA extracted according to Mercado et al. (1999) was used to amplify the *njjs25* gene in the antisense orientation, including a fragment of the 35S promoter. The possibility of *A. tumefaciens* contamination was checked by the amplification of the *VirD1* bacterial gene in the same DNA samples. The stable integration of the T-DNA in several antisense *njjs25* PCR positive lines was confirmed by Southern analysis. In this case, 5 μg of DNA extracted according to Medina-Escobar et al. (1997) and further purified by electro-elution and phenol extraction followed by concentration with butanol and ethanol precipitation was digested with *Hin*dIII in the presence of 1 mM spermidine, fractionated in a 1.2% (w/v) agarose gel, and then transferred to Hybond N$^+$ membranes. The filter was prehybridized at 65°C and hybridized in hybridization solution (0.25 M phosphate buffer, pH 7.2; 7% [w/v] SDS; and 0.1 mM EDTA). A 1.57-kb fragment (*Xba*I-*Sph*I) containing the double 35S promoter and the nos terminator (pBINPLUS vector) was used as template for radioactive probe in Southern-blot analysis. Double-stranded probes were (μ-32P)-dCTP-labeled by random priming to a specific activity of approximately 108 cpm μg^{-1}. Filters were washed twice for 15 min at 65°C in 100 mL of 2× SSC and 0.1% (w/v) SDS and then exposed to x-ray films for 72 h.

About 20 μg of RNA purified according to Domínguez-Puigjaner et al. (1997) was used for conventional northern analysis. A PCR fragment containing the complete coding region of the strawberry pectate lyase was labeled and used as radioactive probe. Wash and hybridization conditions were as in the Southern analysis. The amount of radioactivity on northern-blot filters was quantified using a Phosphorimager (Bio-Rad, Hercules, CA) system and The Molecular Analyst (Bio-Rad) software.

To extract total fruit protein, 1 g of frozen samples was grinded to a fine power in liquid nitrogen. The powder was extracted with 300 μL of loading buffer (0.35 M Tris-HCl, pH 6.8; 10% [w/v] SDS; 36% [v/v] glycerol; 5% [v/v] β-mercaptoethanol; and 0.012% [w/v] bromphenol blue) by sonication. Samples were centrifuged to precipitate the rough material, and the supernatant was used for western-blot assays. Polyclonal antipectate lyase antibodies were used to determine the levels of pectate lyase protein in sample extracts containing 100 μg of protein, as previously described (Harpster et al., 1998).

CWM was isolated following a method similar to that described by Huber and O'Donoghue (1993). Two grams of ripen fruit samples was homogenized in a volume of phenol:acetic acid:water (2:1:1, w/v). The homogenate was

centrifuged at 4,000*g* for 10 min and the residue was washed successively with water, 85% (v/v) ethanol, and 100% (v/v) ethanol. Afterward, the alcohol-insoluble residue was washed with 5 mL of chloroform:methanol (1:1, v/v) and stirred for 30 min at room temperature. The residue was filtered by glass microfiber filters (Whatman, Maidstone, UK), washed with acetone, and dried in vacuum. In vitro cell wall swelling was estimated as previously described (Redgwell et al., 1997). Chelator-soluble pectins were extracted by incubation of 3 to 5 mg of CWM in 2 mL of 50 mM CDTA for 4 h, and measured with the *m*-hydroxydiphenyl reagent (Blumenkrantz and Asboe-Hansen, 1973). The amount of CDTA-soluble pectins was expressed as the A_{520} per milligram of CWM.

Statistical Analysis

A completely randomized block design was used for characterization of the transgenic plants. In the first experiment, 12 plants per line distributed in four blocks were used. In the second one, 32 plants per clone distributed in four blocks were employed. Data on fruit characteristics were means of 40 to 100 fruits. Three independent extractions of CWM were performed. For the electrolyte leakage test, a minimum of 20 fruits was analyzed. Mean separation was performed by Tukey's HSD test at the 1% level.

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