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Fruit yield and quality of strawberry plants transformed with a fruit specific strawberry *pectate lyase* gene

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ABSTRACT

Two transgenic strawberry lines (Pel 1 and Pel 3) containing the open reading frame of a fruit specific strawberry *pectate lyase* gene (Fa*plC*) under the control of the CaMV35S promoter have been obtained to evaluate the role of this gene on fruit softening. Ripen fruits from both lines showed a significant down-regulation of Fa*plC*, being the percentage of silencing of 84 and 71% on Pel 1 and Pel 3, respectively. The agronomic behaviour of transgenic plants was evaluated during two consecutive years. Fruit set increased in the two transgenic lines when compared with control plants, although Pel 1 showed a significant reduction on fruit weight. Firmness of full ripen fruits from Pel lines was significantly higher than control fruits, while color and soluble solids were not affected. The increase of firmness in Pel lines was maintained when ripe fruits were stored for 3 days at 25 °C. Histological analysis of ripe fruits showed lower intercellular spaces and a higher degree of cell to cell contact area in transgenic fruits when compared with controls. Altogether, these results suggest a direct relationship between pectate lyase gene expression and strawberry fruit softening.

1. Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is one of the most valuable and worldwide cultivated small fruits. This fruit is considered as non-climacteric (Perkins-Veazie, 1995) and its ripening is characterized by a fast softening, acquiring a melting texture in few days. The rapid loss of firm texture constrains the postharvest shelf life of strawberry and also the harvest practices, and frequently, fruits are harvested before being fully mature to accommodate shipping practices, with the consequent reduction in quality (Pritts, 2002). Improvement of textural properties is therefore one of the main objectives of strawberry breeders (Faedi et al., 2002; Graham, 2005; Mercado et al., 2007). The underlying mechanism of strawberry softening still remains unclear. As observed in other fruits, cell wall degradation seems to be the main factor responsible for strawberry softening (Huber, 1984; Perkins-Veazie, 1995). The largest changes that occur in the fruit cell wall are the degradation of the middle lamella of cortical parenchyma cells as well as a dramatic increase in pectin solubilization, although the total quantity and length of pectins is little affected (Woodward, 1972; Huber, 1984; Redgwell et al., 1997). Polygalacturonase (PG) and pectate lyase are among the different enzymes that could be involved in pectin degradation during fruit ripening. These enzymes catalyze the cleavage of unesterified galacturonosyl linkages by hydrolysis (PG) or β-elimination (pectate lyase) mechanisms. PG seems to play a minor role on strawberry ripening, because this activity is low and decreased continuously during fruit development (Nogata et al., 1993). However, a recent study on cultivars differing in fruit firmness showed a positive correlation between PG activity and fruit softening (Lefever et al., 2004). Contrary to the role of PG on strawberry softening, studies performed with transgenic strawberry plants indicate a crucial role of pectate lyase on pectins metabolism and therefore fruit softening (Jiménez-Bermúdez

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et al., 2002; Santiago-Doménech et al., 2008). We have demonstrated that the inhibition of this enzyme by insertion of an antisense sequence of a fruit specific *pectate lyase* gene (*FaplC*) reduces strawberry softening, extends postharvest shelf life and improves quality of processed fruits (Jiménez-Bermúdez et al., 2002; Sesmero et al., 2007; Santiago-Doménech et al., 2008). However, most of transgenic lines containing the antisense sequence showed a significant reduction in fruit yield, due to a reduction of fruit set and fruit weight. Pectate lyase genes in plants were first reported in pollen (Rogers et al., 1992; Turcich et al., 1993; Dircks et al., 1996), suggesting a function for pectate lyase on pollen tube emergence and/or breakdown of the cell wall of transmitting tissue in the style to facilitate its penetration (Marín-Rodríguez et al., 2002). Therefore, the deficient fruit yield of antipel plants could be related to an inhibition of pectate lyase activity in pollen and/or style. In the present work, we have evaluated the effect of transformation with a sense sequence of a fruit specific strawberry *pectate lyase* gene on strawberry fruit yield and softening. We have found that sense transformation do not affect fruit yield although ripen fruits showed a significant inhibition of *pectate lyase* gene expression and, additionally, a reduction of fruit softening.

2. Materials and methods

2.1. Plant material and Agrobacterium-mediated transformation

Leaves of strawberry (*Fragaria* × *ananassa* Duch.) plants, cv. Chandler, micropropagated *in vitro* as described in Barceló et al. (1998) were used as explants for transformation experiments. The *Agrobacterium tumefaciens* LBA4404 strain carrying a binary plasmid with the strawberry *pectate lyase* gene (*FaplC*) in the sense orientation under the control of the 35S promoter was used for transformation. To obtain this construct, a 1.2-kb DNA fragment containing the *FaplC* gene coding sequence was isolated from the clone pNJJS25C (Medina-Escobar et al., 1997) after digestion with EcoRV and purification in a 1% agarose gel. This fragment was subcloned into a pGUSINT-derived plasmid (Vancanneyt et al., 1990) previously digested with SmaI and KpnI to remove the GUS gene. The resulting plasmid, pJPJ4, was checked for the presence of *FaplC* gene in sense orientation by restriction analysis. Leaf disks were inoculated with *A. tumefaciens* containing the pJPJ4 and selected in 25 mg l^{-1} kanamycin, as previously described by Barceló et al. (1998). After 7–8 months of selection, kanamycin resistant plants were acclimated, transferred to the screenhouse and propagated vegetatively by runners to evaluate its agronomical behaviour.

Transgenic plants were evaluated during two consecutive growing seasons, using non-transformed plants as control. To study fruit quality during the first year of analysis, a transgenic line (Apel 39) containing an antisense sequence of the *FaplC* gene which showed a 100% *FaplC* silencing was also used (Jiménez-Bermúdez et al., 2002; Benítez-Burraco et al., 2003). Runners were potted individually in 1 l pots, and after 3 months of culture in the screenhouse, plants were transplanted to 25 l pots, three plants per pot, containing a mixture of peat moss and perlite (3:1). The experiments were carried out in a screenhouse from January to June, under natural temperature and photoperiod. Thirty plants per line and experiment were used.

2.2. Molecular analysis of transgenic plants

Genomic DNA was extracted from young strawberry leaves from plants growing in the screenhouse as described by Mercado et al. (1999). The transgenic nature of plants was confirmed by PCR amplification of both a 220-bp fragment corresponding to the *nptII* gene and a 540-bp fragment corresponding to the chimaeric gene *35S-FaplC*. The primers sequences used for these analyses were: 5′-CGCAGGTTCTCCGGCCGCTTGGGTG-3′ and 5′-AGCAGCCAGTCCTTCCGCTTCAG-3′ for *nptII*; 5′-CTCCGGATTATCTACTGCGTGCTCA-3′ and 5′-GTTCAAGATGCCTCTGCCGACA-3′ for *35S-FaplC*. These last primers amplify 233 bp from the 35S promoter and 307 bp from the *FaplC* gene in sense orientation. For Southern blotting, 5–10 μg of DNA were digested overnight with EcoRI, fractionated in a 0.8% agarose gel and transferred to a Hybond N^+ membrane. The filter was hybridized at 64 °C with a digoxigenin labelled probe obtained by PCR amplification of the *35S-FaplC* gene from pJPJ4 plasmid, using the primers above described. The EcoRI digestion of our plasmid yields a DNA fragment of about 2 kb which hybridizes with the probe.

RNA was isolated from a pool of 5–6 red fruits according to Manning (1991). *FaplC* gene expression was quantified by QRT-PCR analysis, using the iCycler system from Bio-Rad, as previously described by Benítez-Burraco et al. (2003). The reverse transcription reaction contained 1 × RT buffer, 10 mM DTT, 1 mM each dNTP, 0.2 μM specific *FaplC* primers, 1 μg of DNase-treated total RNA and 40 U of Superscript™ RT II. The sequences of *FaplC* primers were 5′-GCGAAAGAGGTGACACATAGA-3′ and 5′-TTCTGGAACTTGTATATTATG-3′. The reaction was heated at 70 °C for 5 min and cooled to room temperature. Afterwards, the reaction was incubated at 42 °C for 5 min, followed by 50 min at 50 °C and 15 min at 70 °C. Transcribed cDNA was subjected to PCR amplification in a reaction mixture containing 30 μl of: 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each specific primers, 3 μl of SYBR Green I (1:15000 diluted), 3 μl of transcribed cDNA, and 2 U of Taq polymerase. The PCR program was: 94 °C for 2 min, followed by 35 cycles of 94 °C 1 min, 55 °C for 30 s and 72 °C for 30 s, and a final step of 72 °C for 5 min. In QRT-PCR analysis, quantification is based on threshold cycle (Ct) values. The Ct is a measurement taken during the exponential phase of amplification when limiting reagents and small differences in starting material have not yet influenced the PCR efficiency. Ct is defined as the cycle at which fluorescence is first detectable above background and it is inversely proportional to the log of the initial copy number. Each reaction was performed in triplicate and the Ct values of each QRT-PCR reactions were normalized in relation to the Ct value corresponding to an interspacer 26S–18S strawberry RNA gene (housekeeping gene). The change of gene expression between the different lines was calculated as previously described (Benítez-Burraco et al., 2003).

2.3. Phenotypic analysis of transgenic plants

Ripe fruits were harvested from March to June and yield estimated as total number of fruits per plant and g of fruits per plant. Fruit quality was evaluated using only normal shape fruits of uniform size and coloration, and weight larger than 5 g. Color was estimated using the CTIFL (Centre Technique Interprofessionel des Fruits et Legumes, France) code. This code comprise eight categories, increasing the red color from 1 (light orange-red) to 8 (dark wine-red). Soluble solids were measured by using a refractometer Atago N1, and firmness by using a hand-penetrometer (Effegi) with 9.62 mm^2 surface needle. Eighty to 250 fruits per line were used each year to evaluate fruit quality parameters, including fruit firmness.

To analyze fruit firmness at different developmental stages, fruits were collected at the green, white 1 (white receptacle with green achenes), white 2 (white receptacle with brown achenes), pink (lower than 25% surface red), ripe (full surface red) and overripe (harvested ripe fruits maintained 3 days at 25 °C in a growth chamber). In this experiment, different needles were used