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Simultaneous Transgenic Suppression of LePG and LeExp1 Influences Rheological Properties of Juice and Concentrates from a Processing Tomato Variety

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Processing tomato lines suppressed in the accumulation of ripening-related polygalacturonase or expansin were generated by introduction of transgenes to silence expression of the *LePG* and *LeExp1* genes, respectively. The rheological properties of juice and juice reconstituted from paste produced from lines suppressed in one of these genes, or in both, were compared with azygous controls. When assayed by measuring Bostwick consistency, paste produced from either suppressed LePG or suppressed LeExp1 lines and diluted to 5 °Brix was approximately 18% more viscous than that produced from controls. Simultaneous suppression of LePG and LeExp1 produced a small additional increase in viscosity of 4%. Rheometric flow analysis at 5 or 10 °Brix also showed substantial increases in the consistency index due to suppression of either LePG or LeExp1 alone, and a small additional increase when both genes were suppressed in the same transgenic line. Measurements by laser diffraction and [^{1}H]NMR showed that suppression of LePG or LeExp1 accumulation altered the size distribution of insoluble particles and modified their surface properties. The data are consistent with suppression of LePG increasing serum viscosity, and suppression of either LePG or LeExp1 altering the properties of the insoluble particles and improving some aspect of particle−particle or particle−serum interaction, or both. However, relative to that caused by suppression of either gene alone, the additional increase in viscosity caused by simultaneous suppression of LePG and LeExp1 together was slight.

KEYWORDS: Expansin; *Lycopersicon esculentum*; polygalacturonase; tomato juice; tomato paste; viscosity

INTRODUCTION

The quality of paste produced from processed tomatoes is affected by many factors, including variety, geographical location, growing conditions, ripening stage, postharvest treatment, and processing parameters (*1*). Ideally, paste should be high in viscosity, color, flavor, and nutritive value and low in serum separation. One of the major factors determining commercial value is viscosity, the properties of which are influenced by both the chemical composition and the physical structure of the juice or concentrate. Cell wall components play a major role in rheological properties of juice and diluted paste. The consistency of the serum is primarily due to the amount and size of soluble cell wall pectins (*2*, *3*), and cell wall fragments and ruptured cells are the principal component of the particulate fraction (*4*, *5*). The size and configuration of cell wall fragments in the particulate fraction strongly influence the gross viscosity of the juice and diluted paste (*4*, *6*−*8*). The amount of solubilized pectin, the extent of cell rupturing, and the physical characteristics of the cell wall fragments are affected by the method of processing, particularly the heat treatment to which the processed fruit is exposed and the screen size used for pulping/finishing (*2*, *4*, *6*, *9*, *10*). Thus, the consistency of processed juice and paste is determined predominantly by the components and properties of the fruit cell wall and by how these are modified during processing.

The cell wall is subjected to a prolonged disassembly throughout tomato fruit ripening, resulting in the changes in firmness and texture which make the fruit desirable to eat (*11*). These cell wall changes include increased solubilization and depolymerization of pectins, depolymerization of various cross-linking glycans, loss of pectin side chains, alterations in wall charge and ion content, increased wall swelling and hydration,

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and decreased cell-to-cell adhesion (*11*). The ripening-related enzymes that bring about some of these changes are known. Polygalacturonase (PG) is mainly responsible for pectin solubilization and depolymerization (*12*), pectin methylesterase (PME) increases pectin charge by removing methylester side groups (*13*), and a ripening-related expansin is involved in wall loosening and affects depolymerization of cross-linking glycans (*14*). In tomato, transgenic suppression of *LePG* mRNA and protein accumulation had a substantial effect on the viscosity of juice or paste prepared with a cold break or incompletely effective hot break (*15*, *16*). This was due to reduced depolymerization of soluble pectin in the suppressed LePG line, which increased serum viscosity by up to 2-fold and resulted in increased gross viscosity (*16*). Suppression of LePME activity also caused a significant increase in gross and serum viscosity of juice, presumably since the lack of demethylesterification of pectin protected it from LePG-mediated depolymerization during ripening and subsequent processing (*17*, *18*). However, combining suppression of LePG and LePME into a single transgenic line did not result in increased paste viscosity properties (*19*).

Processed fruit produced from a fresh market tomato variety suppressed in expression of the ripening-related expansin LeExp1 also exhibited increased gross viscosity, due not to altered serum viscosity but to a change in the properties of the particulate fraction (*20*). The aim of the present work was to examine the effects on paste physical characteristics of suppressing LePG and LeExp1 together in a tomato variety optimized for processing, to combine improved serum properties with improved particle properties. Because most commercial processing tomato varieties are grown as hybrids, the inbred parents of an elite hybrid variety were selected as the lines in which to suppress LePG and LeExp1. Two types of constructs containing inverted repeats were employed to bring about gene silencing (see Results), to examine their relative effectiveness. Crossing the silenced *LePG* and *LeExp1* transgenic inbred lines together results in the processing hybrid, which depending upon the segregation of the two transgenes, is of four different genotypes. The rheological properties of concentrates prepared from lines suppressed in both *LePG* and *LeExp1* gene expression were compared with those of concentrates produced from hybrid fruits suppressed in only one of these genes and with azygous controls containing neither transgene.

MATERIALS AND METHODS

Generation and Screening of Transgenic Plants. For production of a construct containing an inverted repeat of the *LeExp1* cDNA, a truncated version of the *LeExp1* cDNA was amplified by the polymerase chain reaction (PCR) from a plasmid template using primers that introduced an *Nco*I site at an internal Met at nucleotide 148 (numbering from the translation start ATG) and a *Pst*I site at nucleotide 1008, 216 nucleotides downstream of the translation stop. A second fragment was also amplified, using primers that introduced an *Xba*I site at nucleotide 181 and a *Pst*I site at nucleotide 568. The two PCR-amplified fragments were ligated together at the *Pst*I site, resulting in a construct consisting of a 861 nucleotide region of the *LeExp1* cDNA in the sense orientation, in frame but lacking the first 49 amino acids of the predicted protein, followed by a 387 nucleotide region in the antisense orientation that was complementary to the first part of the sense region. This *Nco*I−*Xba*I ligated fragment was ligated between the cauliflower mosaic virus 35S promoter and the transcription terminator from the *nos* gene of *Agrobacterium tumefaciens* using the *Nco*I and *Xba*I sites, to produce the *35S::LeExp1-IR::nos* construct. For suppression of *LePG* mRNA accumulation, a construct consisting of a truncated sense *LePG* transgene driven by the figwort mosaic virus 34S promoter and attached to an inverted repeat of the *nos* transcription terminator was produced as described previously (*21*). The *35S::LeExp1-IR::nos* and *FMV::LePG::nos-IR* constructs were transferred to binary vector SVS297, which possesses a *nos::nptII* selectable marker gene conferring resistance to kanamycin, and transformed into *A. tumefaciens* strain ABI.

Cotyledons of tomato (*Lycopersicon esculentum* Mill.) were transformed using *A. tumefaciens* harboring one of the two constructs above, essentially as previously described (*22*). The *35S::LeExp1-IR::nos* construct was transformed into tomato inbred cultivar T52, and the *FMV::LePG::nos-IR* construct was transformed into tomato inbred cultivar T53 (proprietary inbred lines of Seminis Vegetable Seeds); these inbreds were the parents from which the processing hybrid variety was generated. Primary transformant seedlings were regenerated and selected by growth on medium containing kanamycin and then grown to maturity in a greenhouse in Oakland, CA. Populations of approximately 50 primary transformants harboring each construct were screened for silencing of the *LePG* or *LeExp1* gene by examining accumulation of mRNA of the appropriate gene in red ripe fruits. RNA was prepared from pericarp of red ripe fruit, separated on denaturing 1.2% agarose gels, and blotted to nylon membranes as previously described (*21*). RNA gel blots were hybridized with labeled probes synthesized from *LeExp1* or *LePG* cDNAs using α-[^{32}P]dCTP, random hexamers, and the Klenow fragment of DNA polymerase I, then washed in 0.1 × SSC, 0.1% sodium dodecyl sulfate (SDS) at 65 °C, and exposed to X-ray film. Gel blots were stripped by washing in boiling 0.1 × SSC and 0.1% SDS and then rehybridized with a probe prepared as above from a plasmid containing a cDNA of wheat ribosomal RNA (*23*).

A primary transformant of each inbred line was selected that showed high-level suppression of *LeExp1* or *LePG* mRNA accumulation and which, by segregation analysis (data not shown), possessed a single locus insertion of the T-DNA. These plants were crossed together, the resulting seeds were collected, and 50 of these were planted out in a greenhouse in Woodland, CA. The developing seedlings were screened by PCR using genomic DNA prepared from young leaves as template and primers specific for the transgenes (one primer complementary to the promoter and the other primer complementary to the transgene coding sequence). Because the two transgenes were segregating independently, four possible genotypes were possible, all in the hybrid processing variety. Plants possessing neither transgene, the *LePG* transgene alone, the *LeExp1* transgene alone, or both transgenes were identified. These plants were grown to maturity in a greenhouse for fruit production.

Immunodetection of LePG and LeExp1 Proteins. Cell wall proteins were prepared from fruit of the various genotypes in the processing hybrid at the mature green (one or two but not all locules liquid), late pink (50−90% light red), and red ripe stages (12 days after breaker) by heating cell wall material in SDS−polyacrylamide gel electrophoresis (PAGE) loading buffer in a boiling water bath and quantifying as previously described (*14*). Cell wall proteins (5 μg) were separated by SDS−PAGE in a 12% polyacrylamide gel and then electroblotted to Immobilon-P membrane (Millipore, Bedford, MA). Protein gel blots were blocked in 5% nonfat dried milk and then reacted with LeExp1 antiserum at a dilution of 1:2500 overnight. Gel blots were washed, reacted with donkey anti-rabbit Ig coupled to horseradish peroxidase (Amersham, Piscataway, NJ) for 1 h, and rewashed, and then detection was carried out with a Western Lightning chemiluminescence kit (Perkin-Elmer, Boston, MA) and exposed to film. For immunodetection of LePG protein, the same gel blots were stripped by incubating in 2% SDS, 62.5 mM Tris-HCl, pH 6.7, and 100 mM 2-mecaptoethanol at 50 °C for 30 min, thoroughly washed, and reblocked as above. Stripped gel blots were reacted with LePG antiserum at a dilution of 1:10 000 for 1 h, then washed, reacted with secondary antibody, and rewashed, and detection was carried out as above.

Preparation of Juice, Paste, and Diluted Paste. Processing hybrid fruits were harvested at the red ripe stage and transported to the laboratory. Approximately 3.5 kg (41−52 fruit) of high-quality fruits without blemishes or pathogen infection were surface wiped and then diced into small pieces, placed into preweighed glass bowls, and weighed. At 30 min after the beginning of chopping, bowls covered with plastic wrap were heated individually in 1300 W output industrial

microwave ovens (model FS-13 EVP, Litton, Memphis, TN) for 6 min at full power and 6 min at half power, a treatment shown previously to completely inactivate endogenous enzyme activities. Bowls were cooled on ice for 10 min, and contents were adjusted to the original weight with distilled water. The diced, cooked fruits were passed twice through a benchtop-scale finisher fitted with a 0.84 mm (0.033 in.) screen to remove seeds and skins. Soluble sugar content in the serum of the resulting juices was determined using an RFM80 0−95% sugar digital refractometer (Bellingham and Stanley, Kent, U.K.). Juice was degassed under vacuum, and 10% of the total was retained as "juice". The remainder of each sample was concentrated by evaporation in a bench-scale, scraped surface evaporator, with vacuum (740 mmHg) applied to reduce the boiling point of the juice to ~20 °C. Aliquots of the sample were removed at approximately 10 and 20 °Brix, and the final sample was removed at 28−32° Brix. At least two preparations of each genotype were carried out on different days, using different harvests of fruit. Samples were diluted to 10.0 or 5.0 °Brix by adding deionized water containing 0.04% NaN_3 (to prevent microbial growth), stored at 4 °C, and mixed at intervals over a period of 24 h, and physical properties were examined as below.

Measurement of Viscosity. The rheological properties of diluted pastes were examined in two ways. Bostwick consistency was measured at 5.0 °Brix at room temperature (~20 °C) using a plexiglass apparatus (Fairgate, Cold Spring, NY), recording distance moved by the juice front in 30 s. Rheometric flow behavior was analyzed at 5.0 and 10.0 °Brix using a Haake Rotovisco 20 controlled strain rate rheometer (Haake Buchler Instruments, Saddle Brook, NJ) fitted with an M5/MVII sensor system, and operated at 20 ± 0.1 °C. Samples were equilibrated to temperature before use and then placed in the 2.6 mm gap between the rotating and the stationary cylinders, and shear stress was measured in response to varying shear rate. Shear rate was increased linearly from 4.5 to 450 s^{-1} in 3 min, held at 450 s^{-1} for 1 min, and then decreased linearly from 450 to 4.5 s^{-1} in 3 min. A total of 500 data points was acquired, 250 while increasing and 250 while decreasing shear rate. Flow curves of ascending shear rates were fitted to a power law model using KaleidaGraph software (version 3.09, Synergy Software, Reading, PA).

Particle Size Distribution. The size distribution of particles in juice and in concentrates diluted to 5.0 °Brix was determined using a Coulter LS-230c Laser Diffraction Particle Size Analyzer (Beckman-Coulter, Hialeah, FL), collecting data as 117 size classes on a log scale ranging from 0.04 to 2000 μm, based on the Fraunhofer theory (*24*). A carrying solution of sucrose at 5.0 °Brix was circulated in the analyzer, and then juice or diluted concentrates at 5.0 °Brix were added to obtain an obscuration value of 8−12%. After an equilibration period of 5 min, data were collected for 60 s, using a pump speed of 48% power throughout. The resulting size distribution curves were equalized to the same number of particles. Particle size determinations using light diffraction assumed that particles were spherical and impermeable to light.

NMR Spectroscopy. Spin lattice (T_1) relaxation was measured with a Bruker Advance DRX-500 spectrometer, operating at a proton resonance frequency of 500 MHz, corresponding to a static magnetic field of 11.8 T. Samples were equilibrated first to temperature (20 °C) and then to the magnetic field for 5 min before spectrum acquisition. T_1 measurements were performed using a standard inversion−recovery pulse sequence (180−τ−90−aq). A total of 16 data points was acquired, with a recycle delay between pulse sequences of >5 T_1 to allow the spin system to relax to equilibrium. Data were evaluated using the nonlinear curve-fitting software of the spectrometer.

Statistical Analysis. Variation among genotypes was assessed using one way analysis of variance, followed by Tukey's studentized range test. Statistical analysis was performed using the BMDP/DYNAMIC software package (version 7.0, BMDP Statistical Software Inc., Los Angeles, CA).

RESULTS

Characterization of Transgenic Plants. To generate processing hybrid fruit suppressed in the accumulation of both LePG and LeExp1 proteins, the inbred parents were transformed with constructs to silence *LePG* and *LeExp1* gene expression and these lines were crossed together to produce hybrid fruits of various transgenic genotypes (**Figure 1**). To improve the efficiency of silencing of the target genes, transgene constructs containing inverted repeats were utilized, since these have been shown to increase both the frequency and the extent of posttranscriptional gene silencing (*25*−*27*). To silence *LePG*, a novel strategy involving an inverted repeat of a heterologous terminator sequence was used (*21*). For silencing of *LeExp1*, a construct was assembled consisting of an inverted repeat of the 5′-region of the *LeExp1* coding sequence, with the remainder of the coding sequence and the 3′-untranslated region acting as a spacer. To avoid the possibility that the transgenes themselves would be silenced in the crossed lines, different but equally strong and constitutive promoters were used to control the expression of the *LePG* and *LeExp1* transgenes. These were the figwort mosaic virus 34S (*28*) and the cauliflower mosaic virus 35S promoters (*29*), respectively. Silencing of the expression of the *LePG* gene in one of the inbred parents using an inverted repeat of a heterologous 3′-terminator was highly effective, with most plants showing very low or undetectable amounts of *LePG* mRNA (**Figure 2**). Out of a population of 56 primary transformants, 51 possessed *LePG* mRNA abundances suppressed by 98% or more relative to wild type. In the best lines, *LePG* mRNA accumulation was less than 0.2% of wild type. Suppression of the *LeExp1* gene in the other inbred parent using an inverted repeat of the *LeExp1* cDNA was less effective (**Figure 2**), with only five of 39 primary transformants showing *LeExp1* mRNA abundance reduced by 98% or more. However, a further 15 primary transformants showed either significant reductions in *LeExp1* mRNA accumulation or obvious degradation of the *LeExp1* transcript (e.g., lines 57−19, 57−28, and 57−85), which is typical of posttranscriptional gene silencing. Examination of ribosomal RNA shows that the RNA was not generally degraded, only the silenced transcripts. In both populations of primary transformant plants, a substantial number of individuals exhibited the multiple bands of different

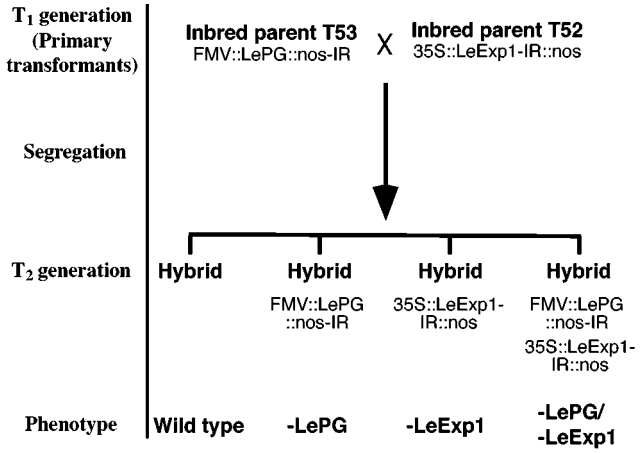

Figure 1. Transformation and crossing strategy to obtain processing hybrid fruit of four transgenic genotypes. The two inbred parents of a hybrid processing line were transformed with different transgene constructs, *FMV::LePG::nos-IR* and *35S::LeExp1-IR::nos*, to suppress accumulation of mRNA and protein of *LePG* and *LeExp1*, respectively. Upon crossing together single locus transformants highly suppressed in *LePG* or *LeExp1* abundance, the resulting population of processing hybrid plants is composed of four genotypes, depending upon the independent segregation of the two transgenes. The presence of the transgenes was determined by PCR.

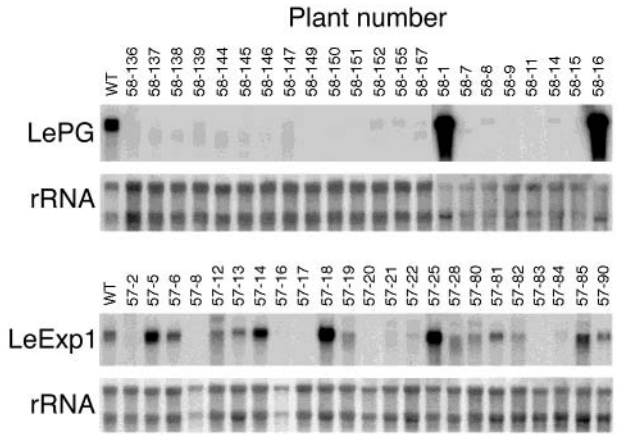

Figure 2. RNA gel blot analysis of primary transformant plants. (Upper panel) Abundance of *LePG mRNA* in red ripe fruit of tomato cv. T53 transformed with an *FMV::LePG::nos-IR* construct. (Lower panel) Abundance of *LeExp1 mRNA* in red ripe fruit of tomato cv. T52 transformed with a *35S::LeExp1-IR::nos* construct. Gel blots of total RNA (5 μg per lane) were hybridized with radiolabeled *LePG* or *LeExp1* probes (only a representative sample of the population is shown in each case). Gel blots were stripped and then rehybridized with a radiolabeled ribosomal RNA probe to assess equal loading of lanes.

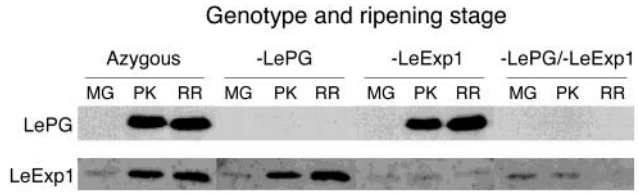

Figure 3. Abundance of immunodetectable LePG and LeExp1 proteins in cell wall extracts of transgenic hybrid fruits. Fruits were harvested at the mature green (MG), pink (PK), or red ripe (RR) stages from four genotypes: azygous (no transgenes present), -LePG (suppressed in accumulation of *LePG* mRNA by an *LePG* transgene), -LeExp1 (suppressed in accumulation of *LeExp1* mRNA by an *LeExp1* transgene), and -LePG/-LeExp1 (suppressed in accumulation of both *LePG* and *LeExp1* mRNAs by both transgenes above). Protein gel blots were reacted with antiserum to LeExp1, and then, the gel blots were stripped and reacted with antiserum to LePG.

sizes that is indicative of the aberrant RNA associated with posttranscriptional gene silencing (*30*).

A member of each T_1 primary transformant population highly suppressed in either *LePG* or *LeExp1* mRNA accumulation and exhibiting segregation of the selectable marker gene consistent with a single locus insertion of the T-DNA was selected. These plants were crossed together, and the resulting seeds were collected. A population of these seeds was planted out, each being a T52/T53 hybrid in which the two transgenes were segregating independently (**Figure 1**). Each plant of this T_2 generation population was examined by PCR for the presence of the *FMV::LePG::nos-IR* and *35S::LeExp1-IR::nos* transgenes (data not shown). The PCR analysis identified groups of plants possessing neither transgene, the *FMV::LePG::nos-IR* trangene alone, the *35S::LeExp1-IR::nos* transgene alone, or both transgenes. Fruits were collected from these four genotypes at the mature green, pink, and red ripe stages, and the accumulation of immunodetectable LePG and LeExp1 cell wall protein was examined (**Figure 3**). In azygous controls, LePG protein was not detectable at the mature green stage but was present at high

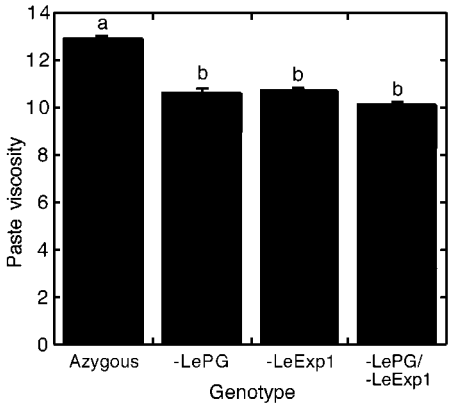

Figure 4. Bostwick consistency of diluted paste. Juice prepared from red ripe fruits of a processing hybrid variety, either an azygous control or suppressed in accumulation of LeExp1, LePG, or both LeExp1 and LePG proteins, was concentrated to 30 °Brix, and the resulting paste was diluted to 5.0 °Brix. Data are means (±SD) of at least three determinations from a representative experiment. Bars not posssessing the same letter are significantly different at $p < 0.01$.

levels at the pink and red ripe stages. In lines possessing the *FMV::LePG::nos-IR* transgene, and consequently very low levels of *LePG* mRNA, immunodetectable LePG protein was also present at very low levels, just detectable in the pink and red ripe stages after prolonged exposure of the blots (data not shown). LeExp1 protein was also not detectable in mature green fruit, although a band of slightly higher molecular weight was present due to other expansins expressed in green fruit cross-reacting with the polyclonal antibody. In azygous controls, LeExp1 protein accumulated at the pink and red ripe stages, but in fruits possessing a *35S::LeExp1-IR::nos* transgene, LeExp1 was present at very low levels. LeExp1 protein was just detectable after prolonged exposure of the blots (data not shown). When LeExp1 protein was suppressed, the cross-reacting band became visible and these nonripening related proteins persisted into ripening but declined in amount as ripening progressed. Suppression of LePG or LeExp1 protein accumulation by the presence of one transgene appeared to be unaffected by the presence of the other transgene, and in the double suppressed LePG/LeExp1 genotype, LePG and LeExp1 were suppressed as effectively as in fruit suppressed in expression of either of these alone.

Rheological Properties of Juice and Diluted Concentrates. Most of the tomatoes grown for juicing are concentrated to a paste of 30 °Brix or more and are then diluted to the desired soluble solids level, usually 5−10 °Brix. Consequently, we were interested in the viscosity properties of products derived from processed fruits that had been concentrated and then diluted. Gross viscosity as measured by Bostwick consistency showed that pastes diluted from 30 to 5.0 °Brix produced from fruits suppressed in either LePG alone or LeExp1 alone were approximately 18% more viscous than diluted paste from azygous control fruits (**Figure 4**). Diluted paste produced from the fruit genotype suppressed in both LePG and LeExp1 together was more viscous than from fruits suppressed in either of these genes alone but only by an additional 4%. All three transgenic genotypes were significantly different from the azygous control ($p < 0.01$) but not from each other. For this variety, paste diluted to 10.0 °Brix was too thick to be measured using Bostwick consistency. However, rheometric flow properties as measured by shear rate−shear stress analysis could be determined at both 5.0 and 10.0 °Brix. Shear rate−shear stress data were fitted to

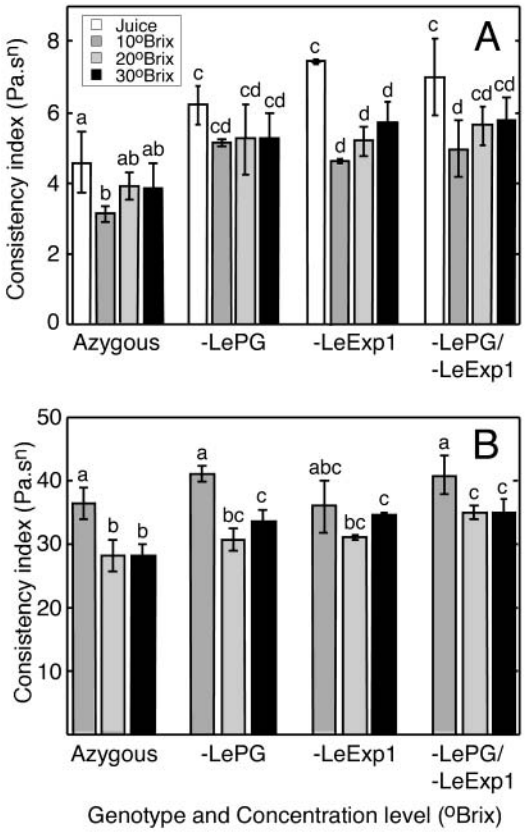

Figure 5. Rheological properties of juice and concentrates diluted to 5 °Brix (**A**) or 10 °Brix (**B**). Shear stress–shear rate data between 4.5 and 300 s^{-1} were fitted to a power law model, from which the consistency index (K) was calculated. Juice was measured at the original °Brix value (azygous, 5.2; -LePG, 4.7; -LeExp1, 4.9; -LePG/-LeExp1, 4.7). Juice was also concentrated to 10, 20, or 30 °Brix as indicated and then diluted to 5.0 (**A**) or 10.0 (**B**) °Brix before measurement. Data are means (±SD) of replicate measurements from two independent paste preparations. Bars not posssessing the same letter are significantly different at $p < 0.05$. Comparisons can be made only between concentration levels within the same genotype or between genotypes at the same concentration level.

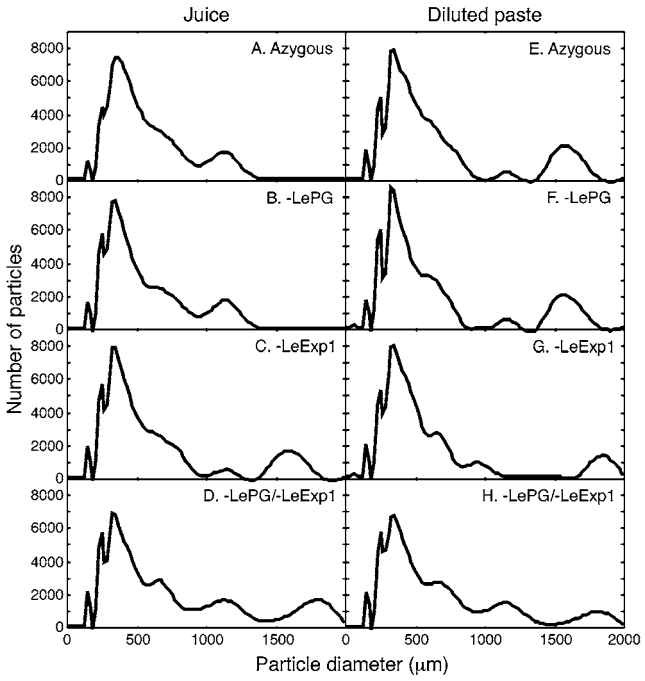

Figure 6. Size distribution of particles in juice (never concentrated) and in 30 °Brix paste diluted to 5.0 °Brix. Juice (**A**–**D**) and diluted paste (**E**–**H**) were produced from fruits of four genotypes, either azygous controls, suppressed in accumulation of LePG protein, LeExp1 protein, or both LePG and LeExp1 protein, as indicated. Measurements were made at 5.0 °Brix in a sucrose carrier solution using laser diffraction. Data are representative profiles (from determinations on three independent preparations).

a power law model (eq 1):

$$\sigma = K\dot{\gamma}^n \quad (1)$$

where σ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (s^{-1}), K is the consistency index (Pa s^n), and n is the flow behavior index (dimensionless). Consistency indices (K) determined in juice and in diluted concentrates at both 5.0 and 10.0 °Brix are shown in **Figure 5**. In the original juices, suppression of LePG, LeExp1, or both LePG and LeExp1 increased viscosity relative to the azygous control (**Figure 5A**). After concentration of juice to 10, 20, or 30 °Brix followed by dilution to 5.0 °Brix, all three transgenic genotypes yielded reconstituted juice more viscous than reconstituted juice from azygous controls. Again, the double suppressed LePG/LeExp1 line was only slightly improved relative to the single suppressed LePG or LeExp1 lines, and all three genotypes were significantly different from the control ($p < 0.05$) but not from each other. Concentration to 30 °Brix followed by dilution to 5.0 °Brix (i.e., the same procedure as in **Figure 4**) gave increases in the consistency index relative to the azygous control of 37, 49, and 50% for the suppressed LePG, LeExp1, and LePG/LeExp1 genotypes, respectively. With all four genotypes, concentration of juice followed by dilution to 5.0 °Brix resulted in reconstituted juice with viscosity lower than that of original juice. Surprisingly, concentration to 10 °Brix followed by dilution to 5.0 °Brix resulted in a greater loss of viscosity than prior concentration to 20 or 30 °Brix. However, when concentrates were examined after dilution to 10.0 °Brix, prior concentration to 20 or 30 °Brix resulted in a greater loss of viscosity than concentration to 10 °Brix alone (**Figure 5B**). The reasons for this discrepancy are not clear but suggest that important and irreversible changes in physical properties occur at around the 10 °Brix concentration level. When analyzed at 10.0 °Brix, the differences between the four genotypes were smaller, but after prior concentration to 30 °Brix, the consistency indices of the three transgenic genotypes were greater than the azygous control by 19, 23, and 25% for the suppressed LePG, suppressed LeExp1, and suppressed LePG/LeExp1 genotypes, respectively. The increased viscosity appears to be associated with changes in the consistency index rather than in the flow behavior index. Values of the flow behavior index n were in the range of 0.33−0.38 in juice and in concentrates diluted to 5 °Brix and in the range of 0.21−0.26 in concentrates diluted to 10 °Brix but were not substantially altered either by final concentration achieved or by genotype (data not shown). Values of n were less than unity, showing the shear thinning behavior of tomato juice.

Particle Size Distribution. The distribution of particle sizes in juice was measured at 5.0 °Brix using juice that had not been concentrated (**Figure 6A**−**D**). All four genotypes showed a distribution of particle sizes with the predominant peak at approximately 350 μm but with different distributions of particle size. Juice prepared from azygous and suppressed LePG genotypes exhibited a similar size distribution profile, with no particles above a diameter of approximately 1300 μm. Juice prepared from the two genotypes suppressed in LeExp1 (either

suppressed in LeExp1 alone or in both LePG and LeExp1 together) contained some larger particles, in the size range of 1500−2000 μm. Relative to the azygous control (**Figure 6A**), juice from all three transgenic genotypes (**Figure 6B**−**D**) also had a greater number of particles smaller than 250 μm. After concentration to 30 °Brix and dilution to 5.0 °Brix (**Figure 6E**−**H**), the size distribution profile of particles was slightly different from juice. With all four genotypes, the profiles showed increased numbers of very small and large particles relative to juice, at the expense of moderate-sized particles. This may be due partly to irreversible aggregation of some particles during concentration and partly to reduced swelling of particles upon rehydration. Suppression of LeExp1 caused the appearance of particles of the largest size (**Figure 6G**,**H**), relative to lines in which LeExp1 was not suppressed (**Figure 6E**,**F**). In the doubly suppressed LePG/LeExp1 genotype, the difference in size distribution of particles between juice and juice reconstituted from paste was slight, and diluted paste of this genotype most closely retained the particle size distribution of original juice. In juice, an increased number of particles with a diameter below 250 μm was correlated with increased viscosity. In diluted paste, there was no obvious correlation between any size class or distribution of particles and viscosity.

Water Proton NMR Relaxometry. The properties of soluble molecules and particulate fragments can affect the interaction of the material with the surrounding solvent, and the motion of molecules can be examined using [^{1}H]NMR relaxometry. T_1 relaxation was found to be double exponential with a fast (of the order of milliseconds) and a slow (of the order of seconds) component. Measurements made at a range of temperatures from 5 to 45 °C using juice from the azygous line showed that the temperature dependence of the two components of T_1 was different (data not shown). For the slow component of the T_1 relaxation constant, increasing temperature resulted in an increase in T_1, showing that an increase in proton mobility or a faster rate of tumbling of water molecules is represented by a greater T_1 value. For the fast component of the T_1 relaxation constant, increasing temperature resulted in a decrease in T_1, showing that an increase in proton mobility or a faster rate of tumbling of water molecules is represented by a smaller T_1 value.

The slow component of the T_1 relaxation constant, which is attributed to the relaxation of bulk water, showed only slight differences with prior concentration or among genotypes (**Figure 7A**). Relative to the azygous control, suppression of LeExp1 did not affect either the value of the T_1 slow component or the trend in T_1 values caused by prior concentration, indicating that LeExp1 does not affect the relaxation behavior of bulk water. However, suppression of LePG, either alone or in combination with suppression of LeExp1, showed a trend of decreasing and then increasing T_1 slow with greater prior concentration and slightly lower T_1 slow values after prior concentration to 10 °Brix. This suggests that suppression of LePG slightly decreases the mobility of bulk water in the serum, possibly due to the increased size of solubilized pectin. The fast component of the T_1 relaxation constant is attributed to the relaxation of protons of water interacting with polymers in the particles or with large molecules in the serum. Within a genotype, the T_1 fast component was not significantly changed by prior concentration, but differences in the values of T_1 fast were observed among genotypes (**Figure 7B**). After prior concentration to 10 or 20 °Brix, all three transgenic genotypes had lower T_1 fast values than the azygous control ($p = 0.002$), with the -LePG genotype having a lower T_1 fast value than the other two transgenic genotypes. After prior concentration to 30 °Brix, only the two transgenic lines suppressed in LePG (-LePG alone and -LePG/-LeExp1) were significantly different from the azygous control ($p < 0.05$).

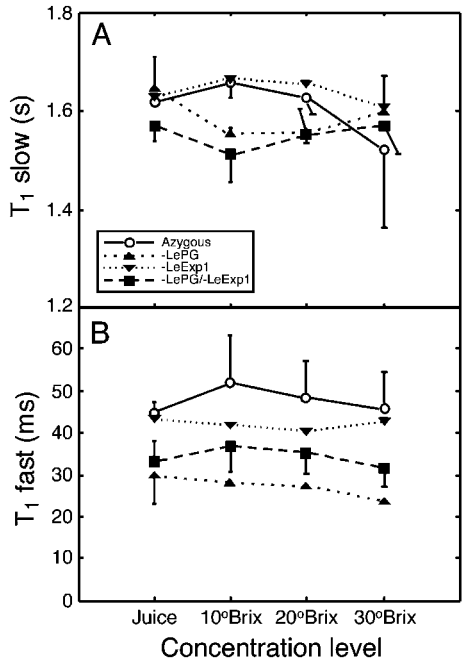

Figure 7. T_1 time constants at 5.0 °Brix determined at 500 MHz by [^{1}H]-NMR. (**A**) Slow component of the T_1 relaxation constant. (**B**) Fast component of the T_1 relaxation constant. Juice and paste were produced from four genotypes of fruit, and measurements were made on juice (never concentrated) and on paste concentrated to 10, 20, or 30 °Brix as indicated and then diluted to 5.0 °Brix. Measurements are means (±SD) of readings from two independent juice or concentrate preparations, except for the -LeExp1 genotype, which is from a single preparation. (**A**) Differences were not significant to $p < 0.05$. (**B**) After prior concentration to 10 or 20 °Brix, all three transgenic genotypes were significantly different from the azygous control ($p = 0.002$).

DISCUSSION

A great increase in the frequency of postranscriptional gene silencing has been seen in populations transformed with transgenes composed of inverted repeats rather than with sense or antisense transgenes (*25*, *27*, *31*). Here, we tried two variants of inverted repeats to examine their relative effectiveness, one consisting of an inverted repeat of the 5′-region of the transgene and the second an inverted repeat of a heterologous termination sequence attached to the transgene. The inverted repeat of the *nos* terminator induced high-level posttranscriptional gene silencing in over 90% of the population. Because the inverted repeat portion of the construct can be made separately and attached to any transgene of known or unknown sequence, such constructs are easy to make (*21*). The inverted repeat of the 5′-region of the transgene was less effective, triggering high-level posttranscriptional gene silencing in only approximately 15% of the population and partial silencing in another 40%. Such a frequency is similar to that expected using sense or antisense constructs (*31*). Recent data suggest that transgene-induced posttranscriptional gene silencing is initiated at the 3′-region of transcripts (*30*), and it is likely that an inverted repeat of the 3′-region of the transgene rather than the 5′-region would have been more effective.

When the inbred parents suppressed in either *LePG* or *LeExp1* mRNA accumulation were crossed together, the resulting

population consisted of four genotypes all in the background of the processing hybrid. Depending on the segregation of the two transgenes, individual plants produced fruits that were either similar to the hybrid wild type or were suppressed in accumulation of LePG, or LeExp1, or both proteins. However, although suppression of the accumulation of both these proteins was very high, it was not complete and traces of activity presumably remained. There is presently no known assay for the activity of ripening-related expansins, which unlike the vegetatively expressed expansins do not appear to cause cell expansion (*11*). Obtaining mutants with disrupted genes would be necessary to achieve complete absence of these gene products.

Tomato juice is a complex mixture composed of swollen insoluble particles (pulp) dispersed in a solution of sugars, organic acids, salts, and pectin (serum). Electrostatic attraction and hydrogen and ionic bonds between charged proteins and pectins in the particles and in the serum appear to keep the insoluble components in suspension (*3*). The natures of both the soluble serum and the insoluble particles influence the consistency of the juice, and both of these are affected by the method of juice preparation. A very high breaking temperature results in juice of the highest viscosity, and this has been attributed to a combination of four factors (*10*): (i) greater inactivation of LePG and consequently reduced degradation of pectin; (ii) increased solubilization of cell wall pectin into the serum; (iii) greater disruption of cell debris resulting in insoluble particles of smaller size; and (iv) more pectin deposited on the surface of particles during drying to paste, which causes a greater content of hydration water in the particles when they are reconstituted to juice. The other main processing factors affecting viscosity are the mechanical stress applied by the finisher blades and the size of the openings in the finisher screen, both of which strongly influence viscosity by affecting the size and shape of the particles (*6*, *8*, *32*, *33*). Particle shape and surface properties may also influence the degree to which cell wall components are solubilized and altered during subsequent high-temperature evaporation. In our experiments, the hot break and the size of the openings in the finisher screen were constant and evaporation was at reduced temperature, so the comparison was between the four genotypes.

Suppression of either LePG or LeExp1 increased the viscosity of reconstituted juice (paste diluted to 5 °Brix) relative to an azygous control, whether measured by Bostwick consistency (essentially shear stress at a fixed shear rate) or using a controlled stress rheometer (which measures shear stress under varying shear rates). Simultaneous suppression of both LePG and LeExp1 gave a small additional increase in viscosity. The viscosity of serum is influenced by the solutes it contains, but the predominant contributor to serum viscosity is the amount and size of soluble pectin. Serum viscosity is rapidly decreased by the presence of active LePG, and it is essential that LePG present in the fruit is inactivated during juice production to prevent depolymerization of pectin, which is of a broad size range including high molecular weight species after a hot break but is of predominantly low molecular weight after a cold break (*2*). A similar retention of high molecular weight pectin (after an incompletely effective hot break) is caused by suppression of LePG activity in transgenic fruit and also results in increased serum viscosity (*16*). However, the molecular weight of the pectin in the insoluble pulp also affects juice consistency (*3*), and the improved viscosity of juice and reconstituted juice from the LePG-suppressed processing hybrid line could be due to higher molecular weight pectin in either the serum or the particles or both.

Suppression of LeExp1 did not increase serum viscosity in a fresh market line but nevertheless increased the gross viscosity of processed fruit, presumably through an effect on the particles (*20*). In the processing hybrid line, a similar increase in the viscosity of juice or reconstituted juice due to suppression of LeExp1 was observed, together with alterations to particle size distribution. Although larger particles contribute to viscosity and the distribution of the population of particles may be important, in juice an increased number of smaller particles has been shown to increase viscosity (*7*, *8*, *32*). This is thought to be because with smaller particles an increased density means a decreased distance between them, and smaller particles have a greater surface area with more opportunity for particle−particle and particle−serum interactions. Suppression of LePG or LeExp1 resulted in small increases in the number of particles below a diameter of 250 μm. Suppression of LeExp1 also resulted in the appearance of some larger particles, above 1400 μm in diameter. The presence of these larger fragments shows that suppression of LeExp1 affects the way the cells and cell walls rupture during processing. Light scattering assumes that particles are spherical, but these larger particles could also be long thin strands. Both particle shape and surface characteristics affect viscosity, with sheetlike or rodlike elongated particles having a greater effect on viscosity than spherical ones (*6*). Suppression of LeExp1 reduced fruit softening during ripening by enhancing cell wall rigidity (*14*), and in fruit suppressed in LePG, cell separation was reduced (*34*). The altered properties of the pericarp could increase the stress or deformation exerted on cells during passage through the finisher screen, consequently affecting cell wall rupturing and resulting in elongated or irregular shaped particles, or with tears creating a rougher surface. Examination of the particles by microscopy is necessary to investigate this possibility.

Relative to the azygous control, all three transgenic genotypes had a lower fast component of the T_1 relaxation time constants obtained by NMR, suggesting that suppression of both LePG and LeExp1 brings about more rapid proton relaxation in the particles or in large soluble molecules, presumably because protons have a greater opportunity to exchange magnetization with nuclei of either water or polymers. This implies an increase in the mobility properties of water associated with the particles, possibly due to changes in the properties of the particles, which could be less hydrated or swollen or more dense. However, this difference is not necessarily achieved in the same manner in the two genotypes, since LePG and LeExp1 may bring about changes in T_1 fast by effects on different cell wall components or on different populations of protons. In whole cell walls prepared from fruits and examined using solid state NMR, the suppressed LePG genotype also exhibited a slower relaxation of proton $T_{1\rho}$, indicating reduced mobility of pectin relative to controls (*35*).

In all four genotypes, when juice evaporated to concentrates of 10, 20, or 30 °Brix was diluted to 5 °Brix, viscosity was lower than in the original juice. This is a well-known phenomenon and was not improved by suppression of LePG or LeExp1. During commercial production of paste, juice is evaporated at elevated temperatures and this is often associated with pectin depolymerization and loss (*36*). Heat can cause the nonenzymatic depolymerization of pectin (*37*), but heat-mediated pectin degradation by hydrolysis or β-elimination is relatively slow and long periods above 75 °C are required (*3*). In the experiments described here, juice was heated during the break, but there was no further heat treatment since these were evaporated at ~20 °C under reduced pressure. Thus, there was

no difference in the amount of heat exposure between the original juice and the juice reconstituted from concentrates, but a decrease in viscosity between juice and reconstituted juice was still evident. This must therefore be attributed partly to a reduction in particle−particle interactions caused by mixing in the evaporator (*3*) and partly to a loss of water from the particles, which is not fully reabsorbed upon rehydration (*38*). Neither of these changes appear to be fully reversible (*3*, *38*).

We have examined the viscosity behavior of juice and reconstituted juice produced from a processing variety of tomato fruit highly suppressed in ripening related LePG and/or LeExp1 protein accumulation. A complementary study examining suppression of the same two enzymes in the fresh market tomato variety Ailsa Craig also found similar changes in the viscosity of processed juice or diluted concentrates, although these differed in magnitude (*39*, *40*). Fresh market varieties produce juice of low viscosity, and the differences between the two studies may be due to a combination of factors including genotype, growing conditions, harvest stage, and the level of suppression achieved. LePG activity seems to predominantly affect serum viscosity, since juice produced from suppressed LePG fruit using a cold break is more viscous than the controls, in which serum pectin is degraded during processing (*15*). Use of an effective microwave hot break removes the difference between the genotypes because LePG is inactivated in controls (*15*). We used a similarly very thorough microwave hot break, but in a commercial setting where the fruit is exposed to greater homogenization before enzyme inactivation and the effectiveness of the break is affected by differential heating in the large volumes of tissue used, the effect of suppression of LePG on viscosity would be expected to be greater. LeExp1 apparently has an effect on the particles rather than the serum. This may be due to altered properties of the cell wall changing the way cells rupture and fragment during the hot break and finishing, resulting in cell wall fragments with irregular or elongated shapes and with jagged edges or tears exposing rough microfibril or pectin matrix surfaces. Such particles may have a different degree of swelling, altered deformability under shear stress, or altered interaction with each other and with soluble pectin in the serum, giving a juice with a higher degree of network structure between the soluble and the insoluble components, and thus improved viscosity. Suppression of both LePG and LeExp1 in the same line resulted in reconstituted juice with a viscosity slightly greater than in lines suppressed in either enzyme alone, and it is possible that this difference would have been greater if the microwave break had been less effective. If the effects of suppression of LePG and LeExp1 are greater in a large-scale commercial situation, lines with reduced amounts of these enzymes will yield increased amounts of a high viscosity reconstituted product. Thus, reducing the activities of ripening-related cell wall proteins such as LePG and LeExp1 may be a route to the selection of improved tomato processing varieties.

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