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Gene expression of cell-wall degrading enzymes in sapodilla (*Manilkara zapota*) fruit

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ABSTRACT: Sapodilla (*Manilkara zapota*) is a tropical fruit crop grown commercially in Thailand which undergoes a rapid change after harvest. Information on these postharvest changes is rare. In this work, firmness of the fruit flesh in sapodilla cvv. Makok-Yai and Kra-Suay was observed to sharply decrease and was low by day 5 after harvest. The decrease in fruit firmness was hastened after ethylene treatment, and prevented after treatment with 1-methylcyclopropene. Three genes encoding cell wall-degrading enzymes, an endo-β-1,4-glucanase (*MzEG*), a pectate lyase (*MzPL*), and a polygalacturonase (*MzPG*), were isolated. In both cultivars tested, the transcript abundance of the isolated *MzEG* was correlated with fruit growth and not with loss of fruit firmness after harvest. In contrast, the mRNA of the isolated *MzPL* and *MzPG* accumulated during postharvest ripening. Ethylene treatment increased the transcript abundance in both genes. Throughout the treatments the expression of *MzPG* was well correlated with the decrease of fruit firmness, whereas the expression of *MzPL* was not. The PG activity in the fruit flesh was also well correlated with the decrease of flesh firmness. Hence the expression of the isolated *MzPG* was correlated both with PG activity and with firmness. Our data indicated that *MzPG* plays an important role in the rapid softening of sapodilla fruits during ripening.

KEYWORDS: softening, ethylene, pectate lyase, polygalacturonase, endo-β-1,4-glucanase

INTRODUCTION

The tropical fruit sapodilla (*Manilkara zapota*), a member of the Sapotaceae family, is native to Central America[1]. Even though sapodilla is a minor fruit crop in Thailand, this fruit is well known and consumed widely by Thai people. Mature fruit of sapodilla is susceptible to mechanical damage during handling[2]. This may be due to their thin peel. Sapodilla fruit is fully ripe in 4–10 days after harvest, depending on the cultivar[3]. Mamey sapote, another member of the Sapotaceae family, also suffers from a severely limited marketable life due to abrupt softening during post-harvest handling[4]. After harvest, respiration of sapodilla follows a transient increase typical of other climacteric, ethylene-sensitive fruit[1,5]. Exposure of the mature harvested fruit to ethylene hastens ripening[5,6]. Treatment with 1-methylcyclopropene (1-MCP), which blocks the ethylene receptor, has been shown to delay fruit ripening after harvest[7,8].

Fruit softening has been associated with degradation of cell wall components such as pectins, hemicellulose, and cellulose[9,10]. Cell wall modifications during ripening are brought about by many enzymes, including polygalacturonases (PGs), endo-β-1,4-glucanases (namely, EGases and cellulases)[9], and pectate lyases (PLs)[11].

The transcript abundance of genes encoding cell wall-modifying enzymes and the activity of these enzymes in ripening sapodilla fruit has not yet been reported. Here, we studied the expression of genes encoding an EGase, a PL, and a PG in growing fruit as well as in ripening fruit after harvest. Since a relationship was found between the expression of the PG encoding gene, we also determined total PG activity.

MATERIALS AND METHODS

Plant material and treatments

Fruit of *Manilkara zapota* (L.) van Royen, cvv. Kra-Suay and Makok-Yai were obtained from a commercial orchard in Ratchaburi province, Western Thailand. Flowers were tagged at anthesis. Fruit were harvested every two weeks after anthesis, until reaching harvest maturity, i.e., when the peel colour changed from dark green to light green. This occurred by week 36 after anthesis in cv. Kra-Suay, and by week 32 after anthesis in cv. Makok-Yai. Fruit were washed in water to remove the scruff from the skin. Some of the fruit

were air dried, frozen in liquid nitrogen, and stored at −70 °C until use.

Some freshly harvested fruit were selected for uniformity (colour and size). 100 fruits were placed in a sealed plastic container (71 l) and treated with 50 µl/l of ethylene at 25 °C for 20 h (ethylene treatment). 100 other fruits were treated with 1 µl/l of 1-MCP (EthylBloc, Floralife) at 25 °C for 20 h (1-MCP treatment). Release of the gas occurred after adding 1.9 ml of deionized water to 114 mg EthylBloc powder (Floralife) placed in a 10-ml vial. The control group was fruit without any treatment.

After the gas treatments, fruit were placed into corrugated boxes and stored at 25 ± 0.5 °C, with 80–85% relative humidity in the storage room. At daily intervals, 20 fruits were taken from each treatment and assessed for firmness. After five days the measurements were halted because the control fruit were very soft. The equatorial part of the peel and the outer flesh (up a depth of about 5 mm below the peel) was cut into 5 mm cubes, immediately frozen in liquid nitrogen, and stored at −70 °C until further use.

Fruit firmness

Firmness was measured in 20 fruits per treatment, for 1, 3, and 5 days after harvest in the control fruit and for 0, 2, and 4 days after the end of the treatment in the 1-MCP- or ethylene-treated fruit. Firmness was determined at around half way between the pedicel and the remnants of the flower using an Effegi FT-011 penetrometer (Effegi, Alfonsine). A cylindrical plunger, 0.2–1.1 cm in diameter, depending on the ripening stage, was inserted to a depth of 0.5 cm and the force was recorded.

Isolation of cDNA fragments for MzEG, MzPG, and MzPL

The pulp of 'Makok-Yai' sapodilla fruit was used for the isolation of endo-β-1,4-glucanase, pectate lyase, and polygalacturonase genes. Total RNA was extracted from sapodilla pulp, following the method described in Ref. 12, with slight modification as reported in Ref. 5. RNA quantification was measured spectrophotometrically at 240, 260, and 280 nm. The RNA quality was confirmed by gel electrophoresis on 0.8% agarose gel.

In order to obtain specific probes, a degenerate sense primer and antisense primer were designed based on the conserved regions of plant genes. After PCR the reaction products were separated using 1.1% agarose gel electrophoresis. Based on the size of the transcript in other plants, we expected a product of specific size. A single band appeared on agarose gel at this position. It was cut and used for gel extraction (QIAquick, QIAGEN) in order to purify the PCR product. The product was ligated into the pGEM-T easy vector and the ligated plasmid was transformed into *E. coli* DH5-α competent cells. Single white colonies containing the vector and inserted gene were cultured and plasmid DNA was obtained from them. The plasmid was cut using *Eco*RI restriction enzyme. A single band of the right insert size was observed again after gel electrophoresis. DNA sequencing of the identified clone was carried out on both strands (using T7 and SP6 primers) using the ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City). The fragment sequence was compared with information in the GenBank database using the BLAST program from NCBI. The identified insert fragment was used as a template for probe synthesis.

For endo-β-1,4-glucanase, a degenerate sense primer, 5′-TAYTAYGAYGCNGGNGAYAA-3′ (Y= C/T, N=A/C/G/T), and antisense primer, 5′-AAN-CCNACCATRTANCWCAT-3′ (R= A/G, W=A/T), were designed according to the conserved regions of endo-β-1,4-glucanases sequences from various plant species[13]. A partial coding sequence of *MzEG* (accession number EU819555) was isolated from mature sapodilla fruit (32 weeks after anthesis).

For polygalacturonase, a degenerate sense primer, 5′-TTWGGAGCYARAGSDRATGG-3′ (S=C/G, D=A/G/T), and antisense primer, 5′-CCAATRCTRATTCCRTGGCC-3′, were designed according to conserved regions of plant polygalacturonase sequences from *Persea americana* (L06094) and *Pyrus communis* (AB084461, AB084462). A partial coding sequence of *MzPG* (accession number EU139437) was isolated from the ripening fruit (3 days after harvest).

For pectate lyase, a degenerate sense primer, 5′-AYTGYTGGMGDTGYGACC-3′ (M=A/C), and antisense primer, 5′-NCCYTCHGAYCKCCARTTCC-3′ (H=A/C/T, K=G/T), were designed according to the conserved regions of plant pectate lyase sequences from *Fragaria* × *ananassa* (U63550), *Malus* × *domestica* (AY376878), *Mangifera indica* (AY987389), *Musa acuminata* (AF206319, AF206320), and *Prunus mume* (AB218786). A partial coding sequence of *MzPL* (accession number EU819554) was isolated from the ripening fruit (3 days after harvest).

RNA gel blot analysis

Each total RNA sample (30 µg/lane) was denatured at 65 °C and separated on 1.0% MOPs-formaldehyde agarose gel at 70 V for 2 h. The RNA was transferred to a positively charged nylon membrane (Roche)

by capillary transfer with 10× SSC (1.5 M NaCl, 150 mM trisodium citrate 2-hydrate, pH 7.0) for at least 15 h and the RNA was fixed by cross-linking with a hybridization oven (Hybaid oven, Thermo Fisher, Waltham, MA) at 80 °C for 2 h. The membrane was pre-hybridized in hybridization buffer (7% SDS, 50% formamide, 5× SSC, 0.1% N-lauroylsarcosine, 2% blocking solution and 50 mM sodium phosphate, pH 7.0) at 55 °C for 45 min using gentle agitation.

The plasmids containing the partial sequences of *MzEG*, *MzPG*, and *MzPL* clones were used as templates for PCR DIG labelling using the PCR DIG Labeling Mix (Roche), following the manufacturer's instructions. Denatured probe was added to the pre-hybridized buffer. After hybridization at 55 °C overnight, the membrane was washed twice in 2× SSC containing 0.1% SDS with gentle shaking for 10 min at room temperature and 0.5× SSC containing 0.1% SDS with gentle shaking for 15 min at 55 °C (pre-warmed). The hybridized membrane was equilibrated in 1× maleic acid washing buffer with gentle shaking for 5 min, and then blocked with 1× blocking solution with gentle shaking for 45 min at room temperature. Alkaline phosphatase antibody solution (Anti-Digoxigenin-AP, Roche) was added to the 1× blocking solution and incubated with gentle shaking for 30 min at room temperature. After being washed twice with 1× maleic acid washing buffer with gentle shaking for 15 min at room temperature, the membrane was equilibrated in detection buffer (0.1 M Tris, pH 9.5 and 0.1 M NaCl) for 5 min, and then equilibrated in CDP-Star substrate (Roche) for 1 min. The membrane was exposed to radiography film (Kodak medical X-ray film) suitable for chemiluminescence. The film was developed according to the manufacturer's recommendations. After placing the membrane in boiling 0.1% SDS stripping solution for 30 min, the membrane was ready for reprobing. Equal RNA loadings were checked by hybridization with *18S rRNA* probe (accession number EU032463) from mangosteen[14]. RNA gel blot experiments were performed at least twice.

Polygalacturonase activity

Extraction occurred according to the protocol of Abu-Goukh and Bashir[15] with slight modification. 6 g of frozen pulp were homogenized in 25 ml of 100 mM sodium acetate buffer pH 6.6 containing 1% polyvinyl pyrrolidone. The homogenate was centrifuged at 17 390 g for 20 min. The residue was suspended in 10 ml of 1 M sodium acetate buffer, pH 6.0, containing 6% NaCl. The pH of the suspension was adjusted to 8.2 with 2 N NaOH and then centrifuged. The supernatant was filtered using Whatman No. 1 filter paper. The filtrate (salt extract) was dialysed against distilled water for 24 h with two renewals of the water. All extraction steps were conducted at 4 °C. The dialysed sample was the crude enzyme extract.

Assay for PG activity was performed using the method of Anthon and Barrett[16]. The reaction mixture containing 100 µl of crude enzyme extract, 100 µl of 0.1% polygalacturonic acid, 100 µl of 0.4 M NaCl, and 100 µl of sodium acetate buffer pH 4.5 was incubated at 37 °C for 2 h. To the mixture was added 100 µl of 0.5 N NaOH and 100 µl of MBTH reagent (consisting of equal volumes of 3 mg/ml 3-methyl-2-benzothiazolinone hydrazone and 1 mg/ml dithiothreitol which was mixed immediately before use). The samples were then heated at 80 °C for 15 min. The samples were removed from the hot water and 200 µl of a solution containing 0.5% $FeNH_4(SO_4)_2 \cdot 12\,H_2O$, 0.5% sulphamic acid, and 0.25 N HCl was added, after which the samples were allowed to cool to room temperature. Finally, 4 ml of distilled water was added and the absorbance at 620 nm was determined. A calibration curve was obtained using D-galacturonic acid as a standard. One unit of PG activity was defined as 1 µmol galacturonic acid released per mg protein per hour.

Statistical analysis

Data of firmness were the means of 20. All experiments were repeated at least once at a later date, with similar results. The data of one series of experiments are shown.

RESULTS

Firmness, treatment with ethylene or 1-MCP

The flesh firmness of the control fruit decreased by day 3 after harvest and was low by day 5 in both cultivars (Fig. 1). Fruit tasting suggested an over-ripe stage on day 5 (results not shown). Application of ethylene after harvest resulted in a more rapid decrease of fruit firmness in both cultivars tested (Fig. 1). Treatment with 1-MCP, in contrast, prevented the decrease in fruit flesh firmness at least until day 5 after harvest. This was found in both cultivars (Fig. 1).

Isolation of genes encoding an EGase, a PG, and a PL

A partial coding sequence of *MzEG* (989 bp, accession number EU819555) was isolated from mature fruit (32 weeks after anthesis), and a partial coding sequence of both *MzPG* (634 bp, accession number EU139437) and *MzPL* (876 bp, accession number EU819554) were isolated from ripening fruit 3 days after harvest.

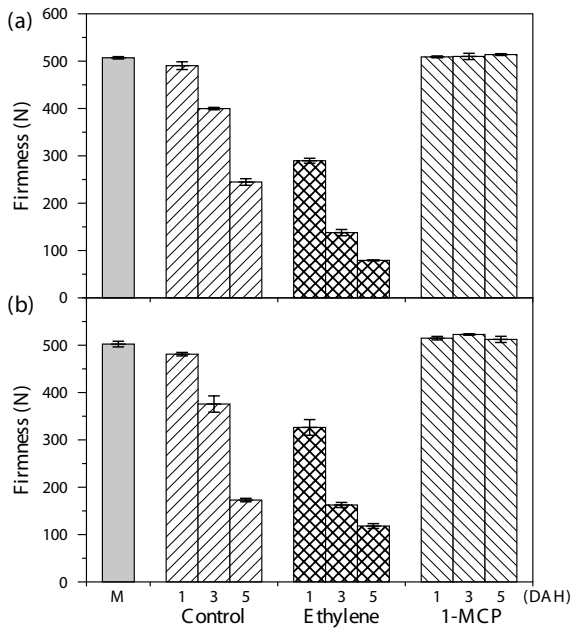

Fig. 1 Fruit firmness of sapodilla fruit during ripening process after harvest in a control fruit and treated fruit with ethylene and 1-MCP. Control fruits were kept for 5 days without any treatment and treated fruits were kept for 4 days after treatment. Firmness accessed at maturity stage (M), at 1, 3, and 5 days after harvest for control, ethylene and 1-MCP treated fruit in cvv. Makok-Yai (a) and Kra-Suay (b). Data are means $\pm$ SD ($n = 20$).

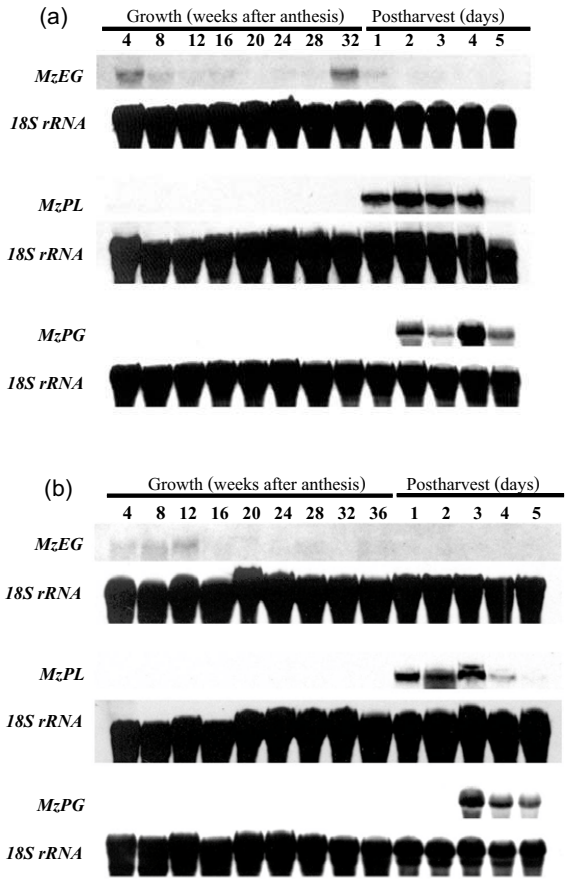

Fig. 2 RNA gel blot analysis of *MzEG*, *MzPL* and *MzPG* showing the adundance of mRNA in sapodilla during fruit growth after anthesis until reach maturity and in mature fruit during ripening process after harvest in (a) cvv. Makok-Yai and (b) Kra-Suay.

The isolated *MzEG* fragment showed high homology, at the protein level, with EGases in several other species such as *Glycine max*, *Fragaria* × *ananassa*, *Lycopersicon esculentum*, *Pyrus communis*, *Prunus persica*, *Gossypium hirsutum*, *Arabidopsis thaliana*, and *Mangifera indica* (% identities: 82, 81, 81, 80, 79, 78, 78, and 76, respectively). The predicted MzEG sapodilla amino acid sequence contained the glyco_hydro_9 conserved domain.

Similarly, the isolated *MzPG* fragment showed high homology, at the protein level, with polygalacturonases of other species, including *Actinidia deliciosa*, *Actinidia chinensis*, *Capsicum annuum*, *Malus* × *domestica*, *Pyrus communis*, *Prunus persica*, *Carica papaya*, and *Arabidopsis thaliana* (% identities: 76, 75, 71, 71, 70, 69, 63, and 63, respectively). The predicted MzEG sapodilla amino acid sequence contained the glyco_hydro_28 conserved domain.

The isolated *MzPL* fragment also showed high homology at the protein level with PLs in other species: *Fragaria* × *ananassa*, *Arabidopsis thaliana*, *Vitis vinifera*, *Prunus persica*, *Fragaria chiloensis*, *Musa acuminata*, *Mangifera indica*, *Prunus mume*, and *Malus* × *domestica* (% identities: 91, 91, 90, 89, 86, 85, 84, 78, and 78, respectively). The predicted MzEG sapodilla amino acid sequence contained the pec_lyase_C conserved domain.

Transcript abundance

The expression pattern of the isolated *MzEG* was similar in the two cultivars tested. Transcript of the isolated *MzEG* was expressed during the early stage of fruit growth (Fig. 2). It was not detectable in mature fruit after harvest.

The transcript level of *MzPG* was high during the later stage of fruit ripening in both cultivars (Fig. 2). The transcript of the isolated *MzPL* was not detectable in developing fruit (Fig. 2). After harvest, *MzPL* mRNA accumulated during the first stage of ripening. It became undetectable in overripe fruit of

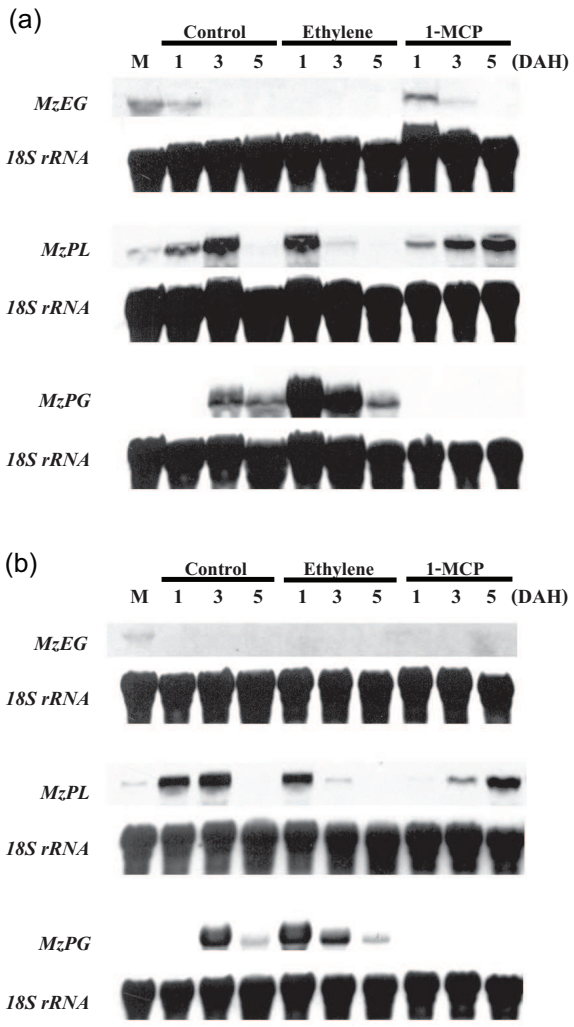

Fig. 3 RNA gel blot analysis of *MzEG*, *MzPL*, and *MzPG* mRNA abundance in sapodilla fruit at maturity stage (M), at 1, 3, and 5 days after harvest for control, ethylene, and 1-MCP treated fruit in (a) cvv. Makok-Yai and (b) Kra-Suay.

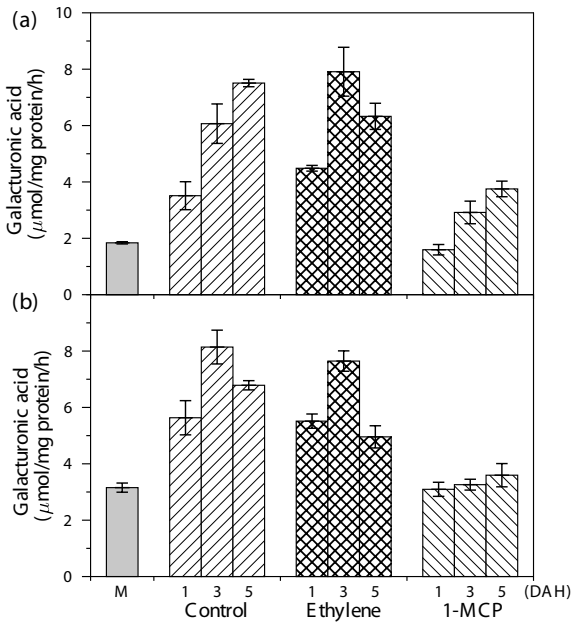

Fig. 4 Pattern of polygalacturonase activity in sapodilla fruit accessed at maturity stage (M), at 1, 3, and 5 days after harvest for control, ethylene, and 1-MCP treated fruit in (a) cvv. Makok-Yai and (b) Kra-Suay. Data are means $\pm$ SD ($n = 3$).

both cultivars (Fig. 2).

Both the ethylene and 1-MCP treatments had little effect on the accumulation of *MzEG* mRNA in sapodilla fruit flesh. A small signal was found only in cv. Makok-Yai after 1-MCP treatment (Fig. 3). Ethylene treatment drastically increased the transcript abundance of *MzPG* in both cultivars but no *MzPG* transcript was detectable in fruit treated with 1-MCP (Fig. 3). Ethylene treatment induced high *MzPL* transcript abundance in both cultivars. 1-MCP treatment also resulted in high *MzPL* transcript abundance in both cultivars (Fig. 3).

PG activity

On day 1 after harvest the PG activity in the fruit flesh of controls was higher than in mature fruit (Fig. 4) in both cultivars. In cv. Makok Yai the PG activity in controls had further increased by day 5. In cv. Kra-Suay the activity was lower on day 5 than on day 3. Ethylene hastened the increase in activity in both cultivars. 1-MCP treatment in both cultivars resulted in activities that were lower than in the controls.

DISCUSSION

The transcripts putatively encoding a PG, an EGase, and a PL were differentially expressed. However, *MzEG* became mainly expressed during the early stages of fruit growth on the plant. It was expressed (above the detection limit) in unripe but mature fruit of one of the two cultivars. No expression was observed during fruit softening after harvest. In other fruits EGases are encoded by a multigene family (for example: strawberry[17], peach[18]), so the absence of a relationship between the expression of a gene encoding an EGase and fruit firmness in sapodilla might indicate that the expression of other EGase-encoding genes is responsible for the loss in fruit firmness. In other species, genes encoding an EGase

(often called *Cel*) showed increased expression during fruit ripening (strawberry[17,19] and tomato[20]). It was inferred that the increased expression of a gene encoding an EGase was the cause of at least part of the loss of strawberry fruit firmness[21].

The isolated *MzEG* gene might be involved in growth processes. Its early expression is similar to the expression pattern of *EGL1* which encodes an EGase of pea[22]. It is also reminiscent of the expression pattern of *Cel1* in *Arabidopsis thaliana* in elongating tissue[23,24]. The present data, therefore, might be interpreted as confirming that the expression of genes encoding EGases play a role in cell wall modifications that are important in cell wall elongation.

MzPL mRNA accumulated abundantly during the early stage of sapodilla fruit ripening. The transcript level then rapidly became undetectable in overripe fruit of both cultivars. This is similar to the expression of three transcripts (*plA*, *plB*, and *plC*) encoding PLs in strawberry fruit[25] and two genes encoding PLs in the pulp of ripe banana fruit[26,27]. Exogenous ethylene induced accumulation of *Banl7* transcript encoding a PL in green banana fruit[26] is similar to the hastening of *MzPL* expression now found in ethylene-treated sapodilla fruit. In strawberry, the expression of a gene encoding a PL was reduced by antisense expression. Fruit softening was inhibited, showing a causal role of the enzyme in the softening processes[28]. However, contrary to expectation, we found that treatment of sapodilla fruit with 1-MCP (which prevented softening) did not affect *MzPL* transcript abundance in both cultivars. *MzPL* transcript abundance was therefore not well correlated, when considering all treatments, with loss of firmness in sapodilla fruit.

MzPG expression in sapodilla fruit was strongly associated with softening. In both cultivars, ethylene treatment (which promoted fruit softening) dramatically increased the *MzPG* mRNA abundance, whereas 1-MCP treatment suppressed *MzPG* expression. A similar expression pattern has been shown in the *CkPGC* gene of kiwi fruit[29] and in *MAPG3* of banana fruit[30]. These results might indicate that *PG* gene expression is limiting PG activity and causing softening in the fruit of at least some species. In tomato fruit a gene encoding PG was also up-regulated, and its transcript abundance was increased after ethylene treatment. The PG activity in ripening control tomato fruit increased, which was associated with fruit softening. Ethylene advanced fruit softening and resulted in high PG activity. However, experiments with transgenic tomato plants in which fruit PG activity was very much reduced (through antisense repression) or much increased (ectopic expression in young fruit) showed that PG alone was not sufficient for fruit softening[9,31–33]. A note of caution is therefore appropriate when interpreting the possible role in fruit softening of genes encoding PG until experiments have been carried out in which gene expression and enzyme activity have been considerably inhibited or increased.

Because of the high correlation between fruit flesh softening and the expression of the isolated PG gene, we also measured the PG activity in the fruit flesh. Total PG activity in the fruit flesh was well correlated with fruit softening as it increased during ripening in the control fruits and in ethylene-treated fruits but stayed low in 1-MCP-treated fruits. This means that there is also a good correlation between the expression of the isolated gene encoding a PG (*MzPG*) and PG activity. The data indicated that *MzPG* expression and PG activity were up-regulated by ethylene, and their normal up-regulation was prevented by 1-MCP. Similarly, *OsPG* expression of prickly pear[34] and PG activity of banana[35] were stimulated by ethylene. However, the increase in transcript abundance of *MzPG* might therefore be partially responsible for the increase in PG activity and the decrease in flesh firmness. Nonetheless, these data should be interpreted with caution, as the relative contribution of the isolated gene in the increase of PG activity might be small, and PG activity might, as in tomato fruits, not be the real cause of fruit softening[9]. In addition, measurement of total PG activity and *FaPG1* expression of strawberry were performed in three cultivars with contrasting softening rates[36].

Finally, our previous data on the expression of an expansin gene (*MzEXP2*) in sapodilla fruit flesh showed an increase on day 1 after harvest in cv. Makok-Yai, whilst the expansin gene was already highly expressed just prior to harvest in cv. Kra-Suay[5]. The gene thus showed a pattern similar to, or was expressed earlier than, the expression now found of *MzPL*. However, it was previously found that the transient expression of the sapodilla expansin gene (*MzEXP2*) was down-regulated by ethylene treatment. Whereas in controls no more expression occurred on day 5, after treatment with 1-MCP the expression was still high on day 5[5]. The ethylene regulation of this expansin gene was thus opposite to the one now found in *MzPL* and *MzPG*.

In conclusion, the expression of an isolated gene encoding an EGase was correlated with fruit growth and not with loss of fruit firmness after harvest. The expression of an isolated gene that encoded a PL, in contrast, occurred during the early stage of fruit softening after harvest. The expression of this gene was correlated with fruit softening both in controls

and in ethylene-treated fruit, but such a correlation was absent in fruit treated with 1-MCP (no loss of firmness and high gene expression). This lack of correlation questions the role of this gene in fruit softening. Lastly, the expression of a gene encoding a PG was also restricted to the post-harvest stage. The expression of this gene was well correlated with changes in fruit firmness in all treatments. The PG activity measurements showed a good correlation between expression of the isolated gene encoding a PG and total PG enzyme activity. These data suggested that *MzPG* plays an important role in rapid softening of sapodilla fruits after harvest.

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