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THESIS

GENE EXPRESSION OF EXPANSINS AND CELL WALL DEGRADING ENZYMES DURING FRUIT GROWTH AND RIPENING OF SAPODILLA

(*Manilkara zapota* van Royen)

SUTIN KUNYAMEE

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Flesh firmness of sapodilla (*Manilkara zapota*) fruit cvv. Makok-Yai and Kra-Suay sharply decreased after harvest. The decrease in fruit firmness was hastened by ethylene treatment, and prevented by 1-methylcyclopropene (1-MCP) treatment. Two genes encoding expansins (called *MzEXP1* and *MzEXP2*) and three genes encoding cell wall-degrading enzymes [called *MzEG* (endo-β-1,4-glucanase), *MzPL* (pectate lyase) and *MzPG* (polygalacturonase)] were isolated. In both cultivars studied (Makok-Yai and Kra-Suay), *MzEXP1* and *MzEG* were transiently expressed early during fruit development on the plant. The transcript was not detectable after harvest and during fruit ripening. In contrast, *MzEXP2* was expressed between 1 day before harvest and day 4 after harvest in cv. Makok-Yai. In cv. Kra-Suay the expression of *MzEXP2* started 8 weeks before the harvesting stage, and ended on day 3 after harvest. When fruits of both cultivars were treated with ethylene just after harvest, the expression of *MzEXP2* became undetectable. After 1-MCP treatment, *MzEXP2* mRNA was highly abundant until day 5 after harvest, while the transcript abundance of the control was undetectable. The expression of *MzEXP2* ceased, both in controls and ethylene-treated fruits, when the fruit reached a rather low threshold firmness. The mRNA of the isolated *MzPL* and *MzPG* accumulated during fruit ripening. Ethylene treatment advanced the high transcript abundance in both genes. The expression of *MzPG* was well correlated with the decrease of fruit firmness throughout the treatments, whereas the expression of *MzPL* was not. The expression of the isolated *MzPG*, was correlated with the increase of PG activity, the loss of firmness and the increase of water-soluble pectin content.

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LIST OF ABBREVIATIONS

1-MCP	=	1-methylcyclopropene
BLAST	=	basic local alignment search tool
bp	=	base pairs
cDNA	=	complementary deoxyribonucleic acid
CDP-Star	=	disodium-2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′chloro) tricycle[3.3.1.13,7] decan}-4-yl)-1-phenyl phosphate
CTAB	=	hexadecyltrimethylammonium bromide
DEPC	=	diethyl pyrocarbonate
DIG	=	digoxigenin-11-dUTP
DNA	=	deoxyribonucleic acid
DNase	=	deoxyribonuclease
dNTPs	=	deoxynucleotide triphosphate (s)
EDTA	=	ethylene diamine tetraacetic acid
EGase	=	endo-β-1,4-glucanase
IPTG	=	isopropyl-β-D-thiogalactopyranoside
LB medium	=	Luria-Bertani medium
NCBI	=	National Center for Biotechnological Information
PCR	=	polymerase chain reaction
PG	=	polygalacturonase
PL	=	pectate lyase
PVP	=	polyvinyl pyrrolidone
RH	=	relative humidity
RNA	=	ribonucleic acid
RT-PCR	=	reverse transcriptase polymerase chain reaction
SDS	=	sodium dodecyl sulfate
X-Gal	=	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

GENE EXPRESSION OF EXPANSINS AND CELL WALL DEGRADING ENZYMES DURING FRUIT GROWTH AND RIPENING OF SAPODILLA (*Manilkara zapota* van Royen)

INTRODUCTION

Sapodilla (*Manilkara zapota* van Royen), a member of the Sapotaceae family, is native to Southern Mexico and some other parts of Central America. Fruit development follows a sigmoidal pattern. The maximum growth occurs between 5 and 7.5 months from fruit set (Mickelbart, 1996). The mature fruit peel of sapodilla is very thin which is susceptible to mechanical damage during handling (Suthumchai, 2003). After harvest, its respiration follows a climacteric fruit (Mickelbart, 1996). The fruit is fully ripe in 4-10 days, depending on the cultivar (Balerdi and Crane, 2005). It is rotten within two weeks (Morton, 1987). Exposure of the mature fruit to ethylene hastens ripening (Kader, 2005). Treatment with 1-methylcyclopropene (1-MCP), which blocks the ethylene receptor, delays both softening and ripening (Qiuping *et al*., 2006; Arevalo-Galarza *et al*., 2007). The data show that these processes in sapodilla fruit are regulated by endogenous ethylene.

Recently, mechanism of fruit growth and ripening in regarding to changes in cell wall composition, enzymes activity and gene expression involvement in cell wall modification have become more interested in fruit research. The primary cell wall is composed of numerous polymers which vary in structure somewhat between species, but eight polymeric components (cellulose, three matrix glycans composed of neutral sugars, three pectins rich in D-galacturonic acid, and structural proteins) are usually present (Brummell and Harpster, 2001).

Fruit growth and ripening is associated with changes in numerous cell wall components, executed by enzymes localised to the cell walls. An example is the expansin super family. Many expansins are involved in cell enlargement and

other developmental processes, such as abscission, in which cell wall modification occurs. Four families of expansins are currently recognised. They have been designated a-expansin (EXPA), like B (EXLB). EXPA and EXPB cause cell-wall loosening, but the function of action of expansin-like A and expansin-like B proteins are currently not known (Sampedro and Cosgrove, 2005; Choi *et al*., 2006; Carey and Cosgrove, 2007). Expansins involved in cell growth probably act by causing a reversible disruption of hydrogen bonds between cellulose microfibrils and matrix polysaccharides, resulting in a loosening of the wall and allowing turgor-driven movement of microfibrils relative to one another (Sampedro and Cosgrove, 2005). The expression patterns of several expansin genes have been correlated with the growth and development of various plant organs (Gookin *et al*., 2003; Sanchez *et al*., 2004; Muller *et al*., 2007). For example, the expression of several expansin genes was associated with fruit growth and that of other expansin genes with fruit ripening (tomato, Brummell *et al*., 1999*a*; Catala *et al*., 2000; pear, Hiwasa *et al*., 2003; strawberry, Dotto *et al*., 2006).

Degradation of cell wall components has been the primary focus of research aimed at understanding ripening-related tissue softening of fruit (Greve and Labavitch, 1991; Rose *et al*., 1998; Brummell *et al*., 1999b; Ali *et al*., 2004). Cell wall modification involves in activity of various cell wall enzymes including hydrolases enzymes (polygalacturonases, pectin methylesterase, β-galactosidases, and endo-β-1,4-glucanase or cellulase), transferases enzyme (xyloglucan endotransglycosylase) (Brummell and Harpster, 2001), and lyases enzyme (pectate lyases) (Marin-Rodriguez *et al.*, 2002). Softening of red tomato fruit has been found subsequently to depend on the action of both polygalacturonase (PG) and expansin, whereby expansin is apparently required to allow PG action (Cantu *et al*., 2008).

Even though a lot of mechanism of cell wall changes on many plants has been published, there is no study in mechanism of growth and ripening in sapodilla fruit which exhibit rapid softening after harvest. To understand this mechanism clearly, this study aimed to clarify the mechanism of fruit growth and ripening in regarding to changes in cell wall composition, enzymes and gene involvement in cell wall

modification in sapodilla fruit. Here, two ESTs of genes encoding expansins and three of genes encoding cell wall-modifying enzymes (an endo-β-1,4-glucanase, a pectate lyase and a polygalacturonase) were isolated. The transcript abundance of these genes in growing fruit and during ripening after harvest has been reported.

OBJECTIVES

1. To study the pattern of fruit growth

2. To examine changes in cell wall compositions during fruit ripening

3. To determine enzymes activities during fruit ripening

4. To characterize the expression of genes regulating proteins synthesis involved in fruit growth and softening

LITERATURE REVIEW

Sapodilla

The sapodilla, a member of the family Sapotaceae, is now known botanically as *Manilkara zapota* van Royen (syns. *M. achras* Fosb., *M. zapotilla* Gilly; *Achras sapota* L., *A. zapota* L.; *Sapota achras* Mill.) (Morton, 1987). Sapodilla is native to Central and South America, specifically from the Yucatan Peninsula of Mexico to Costa Rica, where the largest population of native trees still exists. It is now widespread throughout the tropical regions of the world (Mickelbart, 1996; Balerdi and Crane, 2005).

The fruit may be nearly round, oblate, oval, ellipsoidal, or conical; varied from 5 to 10 cm in width (Morton, 1987) and from 75 to 200 g in weight (Mickelbart, 1996). When immature it is hard, gummy and very astringent. Though smooth-skinned it is coated with a sandy brown scurf until fully ripe. The flesh ranges in color from yellowish to light- or dark-brown or sometimes reddish-brown; may be coarse and somewhat grainy or smooth. The flesh becomes soft and very juicy, with a sweet flavor resembling that of a pear (Morton, 1987). Seed number varies from 0 to 12. Seeds are dark brown to black, smooth, flattened, shiny, and 1.9 cm long (Balerdi and Crane, 2005). Fruit development follows a sigmoidal pattern. The initial growth phase is due to cell division and involves maturation of the embryo within the fruit. A phase of greatly reduced growth follows, until a second rapid growth phase occurs, during which time growth is due to cell enlargement. This second growth phase is the time when maximum growth occurs, between 5 and 7.5 months from fruit set. The fruits are suitable for harvesting after the first growth phase, although higher quality fruits are obtained if they are harvested following the second growth phase, when there is a dramatic increase in sugar content of the fruits. Fruit maturity occurs anywhere from 4 to 10 months following fruit set, depending on variety, climate, and soil conditions (Mickelbart, 1996).

Because immature sapodilla fruits contain latex, harvesting fruits at full maturity is critical to quality. Judging maturity in sapodilla is extremely difficult. Even within a single cluster, fruit maturity may vary greatly, although fruits generally mature from flowers produced at the base of the cluster to the tip (Mickelbart, 1996). Most people find it difficult to tell when a sapodilla is ready to pick. With types that shed much of the "sand" on maturity, it is relatively easy to observe the slight yellow or peach color of the ripe skin, but with other types it is necessary to rub the scurf to see if it loosens readily and then scratch the fruit to make sure the skin is not green beneath the scurf. If the skin is brown and the fruit separates from the stem easily without leaking of the latex, it is fully mature thought still hard and must be kept at room temperature for a few days to soften. It is best to wash off the sandy scurf before putting the fruit aside to ripen (Morton, 1987).

Suthumchai (2003) studied the affect of harvesting stage and postharvest handling by growers on quality of sapodilla (*Achras sapota* Linn.) in Thailand. Sapodillas cv. Ma-Kok harvested by growers was 54% bruised. After washing mechanically, there were 65% bruised fruit. More bruises occurred when sapodillas were washed, stained, graded, packed and transported to wholesale markets. Each step caused more bruising by about 5%. The brown fruits passing through the above postharvest handling steps had more external and internal bruises than the yellow-brown and green fruits. However, the brown fruits had higher acceptable scores, being sweeter and less astringent.

Respiration of sapodilla fruit follows a climacteric pattern after harvest (Mickelbart, 1996). Respiration peak ranges 25-35 mL CO_2 kg^{-1} h^{-1} and ethylene production peak ranges 2-4 μL kg^{-1} h^{-1} at 20˚C (Kader, 2005). Sapodilla fruit take about 4 to 10 days from picking to ripening (Balerdi and Crane, 2005), and rot within 2 weeks at normal summer temperature and relative humidity (Morton, 1987). Exposure of mature sapodilla and mamey sapote fruit to 100 ppm ethylene for 24 h at 20˚C hastens their ripening. Removal of ethylene from the storage environment delays deterioration (Kader, 2005). The application of Ethrel accelerated the loss of firmness of the pulp when the sapodilla fruits (Fino type) were transferred to 21±1˚C after 10

days of storage at 14±1˚C (Arévalo-Galarza *et al.*, 2007). Optimum temperature for sapodilla fruit storage is 14±1˚C which potential is 2-4 weeks depending on cultivar and ripeness stage. Storage in 5-10% CO_2-enriched atmospheres delays ripening. Higher CO_2 concentration may damage the appearance and taste of fruit (Kader, 2005). More than 50 years ago, sapodillas were shipped from Java to Holland, held at 40-50˚F (4.44-10˚C) for 3 days, and they ripened satisfactorily after arrival. Storage trials in Malaysia demonstrated that mature, hard sapodillas stored at 20˚C reached ripening stage within 10 days and remained in good condition for another 5 days. In Venezuela, mature fruits held at 20˚C and 90% relative humidity were in excellent condition at the end of 23 days. Lower temperatures, in efforts to prolong storage life, seriously retards ripening and lower fruit quality. Low relative humidity causes shriveling and wrinkling. Humid conditions promote sogginess. If long storage is necessary, the fruits may be kept at 15-20˚C in a controlled atmosphere of 85-90% relative humidity, 5-10% (v/v) CO_2, with total removal of C_2H_4 to delay ripening (Morton, 1987).

In addition, there is the study in mamey sapote (*Pouteria sapota* (Jacq.) H.E. Moore and Stearn) which is a member of Sapotaceae. Fruit physical and chemical changes during ripening and storage at various temperatures were evaluated. Ripening was associated with flesh softening, an increase in soluble solids content (SSC), and a change in flesh color from yellow or pale pink to a dark pink or red. No changes in fruit skin color or in flesh acidity were observed as ripening progressed. Ripe fruits had 30% or higher SSC, and flesh firmness (compression force) ≤ 50 N. Fruits held at 27, 25, or 20˚C ripened in 3.5, 5 or 7 days after harvest, respectively. Fruits kept at 10˚C showed minor changes in color and firmness and a slow rate of SSC increase. Fruits stored at 10 or 15˚C and then ripened at 20˚C had portions of the flesh with a much higher firmness and poorer development of red color compared to other parts of the fruit. This uneven ripening was probably a result of chilling injury. The number of fruit with injury was higher at 10˚C than at 15˚C, and increased with storage time. The rates of fruit weight loss relative to the initial fruit weight were 0.58, 0.98 and 1.83% per day at 10, 20 and 27˚C, respectively (Diaz-Perez *et al*., 2000). Moreover, a linear-plateau model was used to describe the changes in SSC of mamey sapote fruit

over time as affected by ripening temperature. The model assumed that, as fruit ripened, SSC increased at a linear rate reaching a maximum (30% SSC) at the ripe stage after which SSC changed little. The rate of fruit ripening and the time to reach the ripe stage were calculated from the model. The rate of ripening increased steadily with increase in storage temperature. Fruits kept at 28, 25, 20, 15 and 10˚C ripened 3.5, 5, 7, 15 and 30 days after harvest, respectively. Temperature quotients (Q_{10}) for fruit ripening decreased with increasing storage temperature. The model shows that shelf life of sapote mamey can be significantly extended by cool storage. The model can be used to estimate the time for fruit to reach the ripe stage, as long as the initial SSC and storage temperatures are known (Diaz-Perez *et al*., 2003).

1-Methylcyclopropene

Ethylene was first found to influence plant development more than a hundred years ago. Many different plant reactions to ethylene had been found before the molecular basis of these reactions was understood. The key protein responsible for these reactions to ethylene is the ethylene receptor, which is a family of membrane-bound proteins that bind ethylene (Serek *et al.*, 2006). 1-Methylcyclopropene (1-MCP) is an ethylene antagonist and active compound competes for ethylene receptor. 1-MCP shows competitive kinetics with ethylene before the compound is bound. After these "permanent" blocking compounds have bound, competition has not been demonstrated because the receptor has been inactivated for too long (Sisler and Serek, 1999). 1-MCP is an effective inhibitor of ethylene action because its affinity for the receptor is approximately 10 times greater than that of ethylene (Blankenship and Dole, 2003). However, the effectiveness of inhibition of ripening and/or senescence of fruit and vegetables is a function of the 1-MCP concentration applied, up to saturation of the binding sites. The extent and longevity of 1-MCP action is affected by species, cultivar, tissue and mode of ethylene biosynthesis induction. Some products such as pea require higher concentrations (40 nL L^{-1}) than carnations (0.5 nL L^{-1}) and banana (0.7 nL L^{-1}), suggesting that new receptors are produced in growing tissues or that a low affinity form of the receptors is present. Synthesis of new binding sites may be affected by temperature; in banana, temperatures between 30 and 40˚C results in

faster recovery of ripening, while application of 1-MCP at 2.5˚C is less effective than at 15 and 20˚C suggesting that binding of 1-MCP at low temperatures was incomplete (Watkins, 2006).

Application of 1-MCP can potentially extend shelf life and maintains quality of harvested fruits. The application of 1-MCP was an effective technology for ripening inhibitor and quality maintenance of harvested sapodilla fruits cv. Fupiguo. Exposure of sapodilla fruits to 1-MCP at 40 or 80 nL L^{-1} for 24 h at 20˚C inhibited markedly the rates of respiration and ethylene production, and ethylene-induced ripening such as softening and chlorophyll degradation. These treatments exhibited higher concentrations of TSS, TA and ascorbic acid by the end of storage (Qiuping *et al.*, 2006). The treatment 'Fino' sapodilla with 1-MCP (100 and 300 nL L^{-1}) permitted the prolongation of storage time at 14±1˚C up to 30 days, plus 6 days at ripening temperature (21±1˚C), retarding the production of ethylene, the degradation of phenolic compounds and maintaining firmness (Arévalo-Galarza *et al.*, 2007).

In addition, mamey sapote which is one of Sapotaceae family, its marketable life is significantly limited by abrupt softening during postharvest handling. Postharvest wax and 1-MCP treatments were evaluated for potential of extending mamey sapote postharvest life. Preclimacteric mamey sapote fruits treated with carnauba wax coating maintained good visual appearance and had the lowest water loss during storage at 20˚C. However, wax treatment accelerated fruit softening, reducing marketable life by 3 days compared with unwaxed (control) fruits. 1-MCP alone or in combination with wax coating extended the marketable life by 1 day over untreated fruits. 1-MCP has the potential to delay ripening of mamey sapote fruits and extend marketable life beyond 14 days at 20˚C with adequate decay control (Ergun *et al.*, 2005).

Cell wall component

The primary cell wall is composed of numerous polymers which vary in structure somewhat between species, but eight polymeric components (cellulose, three matrix glycans composed of neutral sugars, three pectins rich in D-galacturonic acid, and structural proteins) are usually present (Brummell and Harpster, 2001).

1. Cellulose is composed of (1→4)β-D-glucan chains assembled together by hydrogen bonding into very long crystalline microfibrils, each ca. 36 glucan chains in cross section but with many thousands of chains in total (Brummell and Harpster, 2001).

2. Xyloglucan possesses a (1→4) β-D-glucan backbone like cellulose, but is substituted with α- D-xylose in a regular fashion on three consecutive glucose residues out of four, xylose occasionally being extended with β-D-galactosyl-α-L-fucose (or α-L-arabinose in some species). Xyloglucan is susceptible to cleavage by *Trichoderma* endo-β-1,4-glucanases (EGases) on the reducing-end side of unsubstituted glucose residues to produce approximately equal amounts of heptasaccharide ($Glc_4.Xyl_3$) and nonasaccharide (Glc4.Xyl3.Gal.Fuc) xyloglucan subunit oligosacharides (Brummell and Harpster, 2001).

3. (Galacto) glucomannan has a backbone composed of regions of (1→4) β-D-glucan and (1→4) β-mannan in approximately equal amounts, with occasional side chains of single units of terminal α-D-galactose (Brummell and Harpster, 2001).

4. Glucuronoarabinoxylan has a backbone of (1→4) β-D-xylan, with side chains of single units of non-reducing terminal α-L-arabinose and α-D-glucuronic acid (Brummell and Harpster, 2001).

5. Homogalacturonan is composed of long chains of (1→4) α-D-galacturonic acid, and is initially highly methyl-esterified (Brummell and Harpster, 2001).

6. Rhamnogalacturonan I (RG I) is made of alternating α-D-rhamnose and α-D-galacturonic acid residues, with long side-chains attached to the rhamnose residues of either unbranched (1→4) β-D-galactan or branched α-L-arabinans or type I arabinogalactans (Brummell and Harpster, 2001).

7. Rhamnogalacturonan II (RG II) is made of a backbone of (1→4) α-D-galacturonic acid like homogalacturonan, but with complex side chains of several types of neutral sugar. It is a minor cell wall component but RG II monomers can dimerize together as boron di-esters and may affect the porosity of the wall (Brummell and Harpster, 2001).

8. Structural proteins, of four different types, some of which are heavily glycosylated (Brummell and Harpster, 2001).

Changes in cell wall composition in fruit ripening

In order to examine the ripening-related modifications in cell wall polymers which underly cell wall structural changes, cell walls must be isolated and sequentially extracted to produce fractions enriched in particular wall components. These extractions are usually with: 1) chelating agents, such as CDTA or EDTA, which remove calcium from the wall, solubilizing pectin held in the wall by ionic bonds, 2) sodium carbonate, which by de-esterification releases pectin held in the wall by covalent bonds (this treatment also breaks ester bonds between solubilized pectin molecules, resulting in extracted pectin of relatively low molecular weight), 3) weak alkali, such as 1 M or 4% (0.7 M) potassium hydroxide, which solubilizes matrix glycans loosely bound in the wall (these fractions usually contain only small amounts of xyloglucan, and are mainly matrix glycans such as glucomannan and glucuronoarabinoxylan) and 4) strong alkali, such as 4 M or 24% (4.3 M) potassium hydroxide (this releases matrix glycans tightly bound in the wall, both by breaking hydrogen bonds and by causing swelling, but not dissolution of cellulose microfibrils) (Brummell and Harpster, 2001).

Pectin is a major component of primary cell walls of all land plants and encompasses a range of galacturonic acid-rich polysaccharides (Willats *et al.*, 2001). More information, a reduction in cell-to-cell adhesion is caused by a breakdown and dissolution of the pectin rich middle lamella (Brummell and Harpster, 2001). Nowadays, the pectic network is clearly a target for specific developmental modifications such as cell wall swelling and softening during fruit ripening and cell separation during leaf and fruit abscission, pod dehiscence and root cap cell differentiation (Willats *et al.*, 2001).

The case of fruit softening is associated with cell wall disassembly and modifications to the pectin fraction are some of the most apparent changes that take place in the cell wall during ripening (Marin-Rodriguez *et al.*, 2002). The study in 'Charentais' melon showed evidence of modification of both pectin and hemicellulose polymers during ripening. Fruits were observed to undergo very rapid ripening, with the transition from the pre-ripe to over-ripe stage occurring within 24 to 48 h. During this time, the flesh first softened and then exhibited substantial disintegration. The total amount of pectin in the cell wall showed little reduction during ripening but its solubility changed substantially. Initial changes in pectin solubility coincided with a loss of galactose from tightly bound pectins. Depolymerization of polyuronides occurred predominantly in the later ripening stages. Depolymerization of hemicellulose was observed throughout ripening, and degradation of a tightly bound xyloglucan fraction was detected at the early onset of softening. Thus, metabolism of xyloglucan that may be closely associated with cellulose microfibrils may contribute to the initial stages of fruit softening (Rose *et al*., 1998). Softening of grape berry during veraison involved the depolymerization of pectin and xylogluuble can molecules and decrease in the amounts of hemicellulose and cellulose (Yakushiji *et al.*, 2001).

The study in banana fruit indicated that the modifications in polysaccharide compositions and glycosyl linkages, reduced molecular mass distributions and enhanced depolymerization of pectin fraction during banana ripening were responsible for fruit softening (Duan *et al.*, 2008). The similar result was detected in

avocado fruit which showed a rapid and extensive loss of firmness during ripening. Its water-soluble polyuronides increased dramatically and exhibited marked downshifts in molecular weight (Huber and O′Donoghue, 1993; Wakabayashi *et al.*, 2000). However, polyuronides from ripening tomato fruit extracted under condition identical with those used for avocado polyuronides exhibited markedly less rapid and less extensive downshifts in molecular weight during the transition from mature-green to fully ripe (Huber and O′Donoghue, 1993).

In addition, the differential softening phenomenon among the tropical fruits investigated cannot be attributed simply to differences in the modification of the wall pectin. According to results, time for 50% firmness loss was about 1.5 days for 'Beaumont' guava, 3 days for tomato, 'Mas' banana (AA group) and 'Eksotika' papaya, 4.5 days for 'Harumanis' mango, and 20 and 24 days for B10 carambola and 'Kampuchea' guava, respectively. Capacity to markedly modify cell walls as reflected by capability to impact increased pectin solubility and depolymerization of chelator-soluble pectin, and which varied markedly with the fruit types, may not necessarily be correlated with softening rate. Extensive pectin modifications occurred in ripening tomato, mango and papaya. 'Beaumont' guava and 'Mas' banana, though softened rapidly, experienced much limited pectin degradation, seemingly unable to match the modifications that occur in the slow-softening carambola and 'Kampuchea' guava (Ali *et al.*, 2004).

Enzyme activities involved in fruit ripening

Degradation of cell wall components has been the primary focus of research aimed at understanding ripening-related tissue softening of fruits (Greve and Labavitch, 1991; Ali *et al.*, 2004). Cell wall modification involved in activity of various cell wall enzymes including hydrolases (polygalacturonases, pectin methylesterase, β-galactosidases, and endo-β-1,4-glucanase or cellulase), transferases (xyloglucan endotransglycosylase) (Brummell and Harpster, 2001), lyases (pectate lyases) (Marín-Rodríguez *et al.*, 2002), and expansins, a cell wall localized proteins

that were originally identified by their ability to cause cell wall loosening in *in vitro* assays (McQueen-Mason *et al.*, 1992).

Polygalacturonases

Polygalacturonases [PGs, poly (1→4-α-D-galacturonide) glycanohydrolases] are enzymes that catalyze the hydrolytic cleavage of galacturonide linkages, and can be of the exo- or endo-acting types. The exo type (EC 3.2.1.67) removes single galacturonic acid units from the non-reducing end of polygalacturonic acid, whereas the endo type (EC 3.2.1.15) cleaves polymers at random (Brummell and Harpster, 2001). PGs were first identified over 35 years ago and have been suggested to be involved in the disassembly of pectin that accompanies many stages of plant development, particularly those that require cell separation. PG participates in many plant developmental processes, the majority of research has focused on PG in ripening fruits, abscission zones, or pollen (Hadfield and Bennett, 1998). An increase in the activity of PG has long been associated with fruit ripening, although the amount detected varies widely with species (Brummell and Harpster, 2001). The involvement of PG in the degradation of cell wall polyuronides during ripening has been studied in many fruits. It has been previously reported that durian softening was correlated with PG activity and pectin degradation (Imsabai *et al.*, 2002). Treatment of cell walls from pre-ripe fruit with purified avocado PG promoted the release and molecular mass downshift of polyuronides. The polyuronides released by PG were similar in size distribution to water-soluble polyuronides from fruit at intermediate stages of ripening (Wakabayashi *et al.*, 2000).

There are data supports a role for PG in softening of fruit. The activity of PG during ripening in banana fruit has been positively correlated with softening of the fruit tissue (Asif and Nath, 2005). Similarly, a high correlation was observed between an increase in PG activity and loss in flesh firmness during ripening process of control and ethylene-treated in banana fruit. Whereas 1-MCP- treatment maintained a high fruit firmness and exhibited low PG activity (Lohani *et al.*, 2004). The study in white- and pink-fleshed guava fruit types, PG activity increased

progressively during the ripening of both types with a high correlation between the increase in the enzyme activity and the loss of flesh fruit firmness (Abu-Goukh and Bashir, 2003). Same as the report in avocado, PG activity was not detectable in the preclimacteric stage, increased during the climacteric, and continued to increase during the postclimacteric phase (Awad and Young, 1979). However, there was varying levels of PG activities in unripe and ripe tropical fruits ('Beaumont' guava, 'Kampuchea' guava, tomato, 'Mas' banana (AA group), 'Eksotika' papaya, 'Harumanis' mango, and 'B10' carambola). PG activity in unripe fruits was ranging from 1.3 to 2.7 nkat g^{-1} FW, and increased with ripening in all fruits. The greatest, about 500% increase in PG activity, was recorded in tomato compared to about 300 and 250% increase in papaya and banana, respectively. These fruits recorded highest PG activity levels in ripe tissues ranging from about 6 in papaya to about 8 and 10 nkat g^{-1}FW in tomato and banana, respectively. The very rapid- and slow-softening Beaumont guava and carambola, respectively, had comparable PG levels and showed a small 150% increase compared to their initial activity of about 1.4 nkat g^{-1} FW. The slow-softening Kampuchea guava showed 20% increase in PG activity, almost comparable with the increase recorded in rapid-softening mango (Ali *et al.*, 2004).

For the previous study in gene expression, softening during ripening in banana fruit results from the concerted action of at least three PG genes, which are differentially expressed during ripening. *MAPG3* and *MAPG4* correlates with ripening and regulated by ethylene whereas *MAPG2* is associated more with senescence (Asif and Nath, 2005).This pattern of gene expression has been also shown in *CkPGC* gene of kiwifruit. *CkPGC* expression was observed in softening fruits, and reached maximum levels as fruit passed through the climacteric (Wang *et al.*, 2000). Similarly, the expression of pGDPG-1 in apple fruit correlated with the onset of ripening and remained high until the over-ripe stage (Goulao *et al.*, 2008). However, there is evidence have shown that PG is not necessary or sufficient for fruit softening. It has been shown in tomato fruit that the expression of antisence PG transgene suppressed 99% of PG mRNA accumulation, but the solubility of pectins remained at wild-type levels, while depolymerization of solubilized pectins was suppressed. The

transgenic fruits ripened normally and its softening was also comparable to wild-type fruits (Hadfield and Bennett, 1998).

In addition, there are reports suggest that the role of PG is required not only during periods of cell wall degeneration, but also during periods of cell wall turnover and expansion. In kiwifruit, *CkPGC* gene not only expressed in softening fruits but also expression detected during fruit development and in fruits harvested prior to the onset of softening. Expression of *CkPGA* and *CkPGB* was detected by northern analysis only in fruits producing endogenous ethylene, and by RT-PCR in other tissues including flower buds, petals at anthesis, and senescent petals (Wang *et al.*, 2000). *Pd-PG1* gene of damson plum was ubiquitously expressed in the reproductive organs, that was stimulated in sepals, petals and stamens during the flower-opening process (Iglesias-Fernández *et al.*, 2007).

Pectin methylesterase

Polygalacturonans are secreted to the cell wall in a highly methyl-esterified form. This is accomplished by pectin methylesterase (PME; EC 3.1.1.11), which de-esterifies polyuronides by removing methyl groups from the C6 position of galacturonic acid residues of high-molecular-weight pectin. Demethylation of pectin to their free carboxyl groups changes the pH and charge in the cell wall, allows the aggregation of polyuronides into a calcium-linked gel structure, and makes the polyuronides susceptible to degradation by PG (Brummell and Harpster, 2001). There was the evidence that PME activity supported the role of PG in avocado fruit. Polyuronides derived from fruits at intermediate stages of ripening were treated with PME, extensive molecular mass downshifts occurred in response to incubation with PG. These results suggest that PG plays the central role in polyuronide degradation in ripening avocado fruit cell walls and that partial de-esterification is necessary for the increase in the susceptibility of polyuronides to PG (Wakabayashi *et al.*, 2000).

PME protein and activity in tomato, are present throughout fruit development, increasing from the early stages of green fruit to the mature green stage

and then increasing again during ripening by two- to three-fold with a peak early in ripening, and then declining slightly. The abundance of PME mRNA showed a different pattern of accumulation, increasing to a maximum in mature green fruit and declining rapidly as ripening progresses (Harriman *et al.*, 1991). Contrastly, in grape berry, PME transcripts was present during fruit ripening (Nunan *et al.*, 2001).

The report in white- and pink-fleshed guava fruit types, activity of PME increased in both types up to the climacteric peak of respiration and subsequently decreased (Abu-Goukh and Bashir, 2003). However, the study in apricot showed PME activity decreased during the postharvest ripening although propylene increased the activity of this enzyme (Cardarelli *et al*., 2002). The study in ripening of tropical fruits (50% firmness loss of 'Beaumont' guava, 'Kampuchea' guava, tomato, 'Mas' banana (AA group), 'Eksotika' papaya, 'Harumanis' mango, and 'B10' carambola) showed low PME activity in mature fruits of both guava cultivars (ca. 10 activity units), quite low in carambola and papaya (30–60 units), high in banana and tomato (300–500 units), and very high in mango (>700 units). In ripening fruits, excepting mango that exhibited a substantial 40% decline in PME activity, the enzyme activity in all other fruits increased during ripening. The increase was dramatic in papaya (>600%), moderate in carambola (150%), low in 'Kampuchea' guava, tomato, and 'Beaumont' guava (30–80%), and very low in banana (Ali *et al.*, 2004).

β-Galactosidase

β-Galactosidases (EC 3.2.1.23) constitute a widespread family of enzymes characterized by their ability to hydrolyze terminal, nonreducing β-D-galactosyl residues from β-D-galactosides. Several β-galactosidases, sometimes referred to as exo-galactanases, have been purified from plants and shown to possess in vitro activity against extracted cell wall material via the release of galactose from wall polymers containing β(1→4)-D-galactan (Smith *et al.*, 1998). For example, the increase in β-Gal activity and mRNA was consistent with the observed decrease in

type-I arabinogalactan content of cell walls during ripening of grape berry (Nunan *et al.*, 2001).

In addition, northern-blot analysis revealed that the β-galactosidase II gene transcript was detectable at the breaker stage of ripeness, maximum at the turning stage, and present at decreasing levels during the later stages of normal tomato fruit ripening (Smith *et al.*, 1998). A cDNA fragment encoding a β-galactosidase from Japanese pear (*Pyrus pyrifolia*) fruit (*JP-GAL*). The results showed no *JP-GAL* mRNA was detected in the immature fruits. Increment of the mRNA level with fruit ripening coincided with the increase in the β-galactosidase III activity. The expression of of *JP-GAL* correlated with fruit softening (Tateishi *et al*., 2001). In mango, the β-D-galactosidase activity increased in parallel with increase in tissue softness during ripening. There were close correlation between changes in β-galactosidase activity, tissue softness, and increased pectin solubility and degradation suggests that β-galactosidase might play an important role in cell wall pectin modification and softening of mango fruit during ripening (Ali *et al.*, 1995).

The report in apple, an apple homolog (pABG1) was isolated that represents one member of an apple β-galactosidase gene family. Northern analysis during fruit development and ripening showed an accumulation of pABGl-homologous RNA during fruit ripening. Enzyme activity as measured in crude extracts increased during fruit development to a level that was maintained during ripening (Ross *et al.*, 1994). This result differed from the study of Goulao *et al.* (2007), reported that β-Gal activity increased after harvest as the apple fruit became over-ripe. Furthermore, they reported β-Gal transcripts accumulation that expressed at very high levels in ripening fruit and remained high until the over-ripe stage (Goulao *et al.*, 2008).

Endo-β-1,4-glucanase

The enzymes causing depolymerization of cell wall matrix glycans have not been unambiguously identified, but may include EGase (EC 3.2.1.4) or cellulase. These enzymes are often referred to as cellulases, but in higher plants most lack the cellulose binding domains found in microbial cellulases, and thus alone are probably not capable of degrading crystalline cellulose. EGases hydrolyze internal linkages of (1→4) β-D-linked glucan chains adjacent to unsubstituted residues. In the cell wall, their substrates probably include xyloglucan, integral and peripheral regions of non-crystalline cellulose (particularly the outer layers of cellulose microfibrils where glucan chains are interwoven with xyloglucan chains), and possibly glucomannan where sufficient consecutive (1→4) β-D-linked glucan residues occur for substrate binding (Brummell and Harpster, 2001).

EGase activity in avocado fruit was low in the preclimacteric fruits, started to increase just as respiration increased (Awad and Young, 1979). Its activity was found to be directly correlated with ripening processes such as climacteric rise of respiration, ethylene evolution, and softening (Pesis *et al.*, 1978). This correlation was also shown in white- and pink-fleshed guava fruit types. EGase activity increased progressively during the ripening of both types with a high correlation between the increase in the enzyme activity and the loss of flesh firmness (Abu-Goukh and Bashir, 2003).

There was evident that EGases were encoded by a multigene family in strawberry (Trainotti *et al.*, 1999) and peach (Trainotti *et al.*, 1997). It has been shown in tomato, two structurally divergent EGase cDNAs were cloned. Cell mRNA contributes significantly to total EGase mRNA accumulation within plant organs undergoing cell separation (abscission zones and mature anthers), whereas Cel2 mRNA is most abundant in ripening fruits (Lashbrook *et al.*, 1994). Similarly, in pepper, two EGase cDNAs were isolated, the cCel1 involved in fruit softening while the cCel2 involved in leaf and flower abscission (Ferrarese *et al.*, 1998). These data suggested EGase also associated with growing tissue. *EGL1* which encoding EGase of

pea (*Pisum sativum*) transcripted were detected abundantly in flowers and young pods undergoing rapid growth and in elongating epicotyls (Wu *et al.*, 1996). In addition, the expression of *Cel1* of *Arabodopsis thaliana* was present in the cell wall of elongating, young and developing tissue (Shani *et al.*, 2004; Shani *et al.*, 2006).

In the case of EGase associated with ripening process, EGase genes whose expression increased during ripening have been isolated (Lashbrook *et al.*, 1994; Llop-Tous *et al.*, 1999; Trainotti *et al.*, 1999). Strawberry fruit ripening is associated with a marked loss of firm texture, and EGase is believed to contribute to this loss process (Palomer *et al.*, 2004). Two cDNAs clones (*Cel1* and *Cel2*) encoding divergent EGases have been isolated from a cDNA library obtained from ripe fruit. Northern analysis showed that both EGases are highly expressed in fruits and that they have different temporal patterns of accumulation. The *Cel2* was expressed in green fruit, accumulating as the fruit turned from green to white and remaining at an elevated, constant level throughout fruit ripening. In contrast, the *Cel1* transcript was not detected in green fruit and only a low level of expression was observed in white fruit. The level of *Cel1* mRNA increased gradually during ripening, reaching a maximum in fully ripe fruit. The high levels of *Cel1* and *Cel2* mRNA in ripe fruit and their overlapping patterns of expression suggest that these EGases play an important role in softening during ripening. In addition, the early expression of *Cel2* in green fruit, well before significant softening begins, suggests that the product of this gene may also be involved in processes other than fruit softening, e.g. cell wall expansion (Llop-Tous *et al.*, 1999). Since, immunoblot analyses have demonstrated that Cel1 protein is related to ripening. Over-expression of *Cel1* in the yeast *Pichia pastoris* revealed that the recombinant protein acts against different β-(1,4) cell wall polymers, including carboxymethylcellulose and cellulose (Palomer *et al.*, 2004).

In tomato, *Cel7* mRNA abundance increased and remained constant during later stages of fruit growth. The expression of *Cel7* was undetectable at the onset of and during fruit ripening, which is consistent with a specific role of this gene in regulating cell wall loosening during fruit growth, not in ripening-associated cell wall disassembly (Catala *et al.*, 2000). Moreover, Cel1 protein appears in the pericarp

at the stage in which many ripening-related changes start, and remains present throughout fruit ripening. In locules, Cel1 protein is already present at the onset of fruit ripening and remains constant during fruit ripening. This pattern of expression supports a possible role for this EGase in the softening of pericarp tissue and in the liquefaction of locules that takes place during ripening (Real *et al.*, 2004).

Xyloglucan endotransglycosylase

The endo-cleavage of xyloglucans can be achieved not only irreversibly by EGases, but also reversibly by the activity of xyloglucan endotransglycosylase (XET, EC 2.4.1.207; also called endo-xyloglucan transferase or EXGT). These enzymes cleave internal linkages of the (1→4) β-D-glucan backbones of xyloglucan and transfer the newly formed potentially reducing end to the C-4 position of the glucose unit at the non-reducing end of another xyloglucan polymer or oligosaccharide, with net retention of the anomeric configuration of the glycosidic bond. (Brummell and Harpster, 2001).

In tomato, Total XET activity was higher during fruit growth, and decreased during fruit ripening (Miedes and Lorences, 2009). XET genes (*LeEXT1*) expressed their mRNA accumulation peaking during the highest growth stages of fruit. The expression of *LeEXT1* was undetectable at the onset of and during fruit ripening, which is consistent with a specific role of this gene in regulating cell wall loosening during fruit growth, not in ripening-associated cell wall disassembly (Catala *et al.*, 2000).

Contrastly, XET activity in apple fruit that is most important only after the fruit stopped growing and is maintained throughout ripening (Goulao *et al.*, 2007). The similar result was present in grape berry, XET gene expression was closely related to berry softening (Ishimaru and Kobayashi, 2002). Its expression was slightly detected before veraison and was markedly increased at veraison which was the stage of the onset of berry softening) (Nunan *et al.*, 2001; Ishimaru and Kobayashi, 2002).

In kiwifruit, at harvest, extractable XET activity per unit fresh weight in the inner pericarp (IP) and core tissue was 4.5 and 42 times higher, respectively, than in the outer pericarp (OP). Within 24 h of ethylene treatment there was an increase in the activity and specific activity of XET in all tissues that continued throughout softening. Activity increased most in the OP, xyloglucan, galactoglucomannan, and cell wall materials isolated and purified from kiwifruit OP were tested as donor substrates for kiwifruit XET. The enzyme showed activity against xyloglucan but was inactive against galactoglucomannan. XET was active against cell wall materials from unripe and ripe fruit, with swollen walls from the latter being the better substrate.The results indicate that XET may have a key role early in fruit ripening, loosening the cell wall in preparation for further modification by other cell wall-associated enzymes (Redgwell and Fry, 1993). Moreover, Schroder *et al.* (1998) reported kiwifruit core XET was capable of depolymerising xyloglucan in the absence of [^{3}H]XXXG-ol (a reduced heptasaccharide derived from kiwifruit xyloglucan) by hydrolysis, and in the presence of [^{3}H]XXXG-ol by hydrolysis and endotransglycosylation. Expression of the AdXET1-6 gene family was induced in ripening kiwifruit when endogenous ethylene production could first be detected, and peaked in climacteric samples when fruits were soft.

Pectate lyase

Pectate lyases (PL, EC 4.2.2.2), is known as pectate transeliminases, catalyse the eliminative cleavage of de-esterified pectin, which is a major component of the primary cell wall of many higher plants (Marin-Rodriguez *et al.*, 2002). After PL cleave internal glycosidic bonds by β-elimination, generate products with a 4, 5-unsaturated residue at the non reducing end which is a series of oligomeric products of pectin. The lyases have pH optima of ca. 8.5 and require divalent cations (Collmer *et al.*, 1988). PL activity was first discovered in 1962 in cultures of *Erwinia carotovora* and *Bacillus sp.* PL-like sequences from higher plants were first reported from pollen (Marin-Rodriguez *et al.*, 2002).

PL has been purified from ripe banana fruit. The molecular mass of the enzyme is 43 kDa. The pI of the enzyme is 8 with optimum activity at pH 8.5. Analysis of the reaction products, reveal that the enzyme releases several oligomers of unsaturated galacturonane from polygalacturonate. PL is sensitive to inhibition by different phenolic compounds, thiols, reducing agents, iodoacetate and N-bromosuccinimide. The enzyme has a requirement for Ca^{2+} ions (Mg^{2+} and Mn^{2+} can substitute equally well), for exhibiting maximum activity (Payasi *et al.*, 2006).

Roles of PL have also been reported in many fruit. Transcripts for PL in grape berry, was present during ripening (Nunan *et al.*, 2001). Whereas PL activity in apple fruit, increased from that in unripe fruit to fruit at harvest and was maintained at similar level thereafter, throughout the over-ripe stage (Goulao *et al.*, 2007). In ripe banana fruit, PL activity was not detected in preclimacteric banana fruits. PL activity increased progressively from early climacteric and reached maximum level at climacteric peak and declined in post climacteric and over ripened fruits (Payasi and Sanwal, 2003). The enhanced levels of PL activity corresponded with an increase in soluble polyuronides from banana pulp. Expression of two distinct cDNA clones was strongly ripening-related (Marın-Rodrıguez *et al.*, 2003). Furthermore, *Banl7* encoding a protein homologous to PL, showed mRNA is first detected in early climacteric fruit, reaches a steady-state maximum at the climacteric peak, and declines thereafter in overripe fruit (Dominguez-Puigjaner *et al.*, 1997). The similar trend was reported that transcripts of MWPL1 and MWPL2 were not detectable in unripe preclimacteric fruit. They began to accumulate as ripening progressed and remained high level thereafter in over-ripe fruit (Pua *et al.*, 2001). Exogenous ethylene stimulated transcripts accumulation of these genes in preclimacteric fruit (Dominguez-Puigjaner *et al.*, 1997; Pua *et al.*, 2001). The advancement of PL activity peak in fruits treated with ethylene or 2,4-D, which hastened banana ripening and delay in appearance of PL activity peak in fruits treated with GA, which delayed fruit ripening. The results suggested the role of PL in banana fruit ripening (Payasi *et. al.*, 2004).

In strawberry, gene expression of a PL like gene occurred predominantly in strawberry fruit, with little gene expression in unripe stages nor in other plant tissues. The removal of the achenes from unripe green fruits induced the expression of this gene (Medina-Escobar *et al.*, 1997). Three transcripts (*plA*, *plB*, and *plC*) of fruit specifically expressed only in fruit during the ripening stage (Benítez-Burraco *et al.*, 2003). Ripened fruit of transgenic strawberry showed a significant down-regulation of *FaplC* (Youssef *et al.*, 2009) and firmness of full ripen fruit was significantly higher than control fruit (Jiménez-Bermúdez *et al.*, 2002; Youssef *et al.*, 2009). PL plays an important degradative role in the primary wall and middle lamella in ripening strawberry fruit. Three independent transgenic lines were identified exhibiting a greater than 90% reduction in pectate lyase transcript abundance. Wall extracts from transgenic fruits showed a reduction in pectin solubility and decreased depolymerization of more tightly bound polyuronides. Moreover, microscopic studies revealed that the typical ripening-associated loss of cell-cell adhesion was substantially reduced in the transgenic fruits (Santiago-Doménech *et al.*, 2008).

Expansins

Not all cell wall-modifying enzymes act by cleavage of covalent glycosidic or ester bonds. Expansins are cell wall localized proteins that were originally identified by their ability to cause cell wall loosening in *in vitro* assays (McQueen-Mason *et al.*, 1992). They probably act by causing a reversible disruption of hydrogen bonding between cellulose microfibrils and matrix polysaccharides, particularly xyloglucan, resulting in a loosening of the wall and allowing the turgor-driven slippage of microfibrils relative to one another (Cosgrove, 2000;Whitney *et al.*, 2000). This event is associated with changes in primary cell wall and middle lamella structure, leading to fruit growth or softening.

Expansins are encoded by a superfamily of genes comprised of subfamilies that evolved from a common ancestor and encode the α–expansins (EXPAs), β–expansins (EXPBs), the expansin-like A (EXLA), and expansin-like B (EXLB) proteins (Choi *et al*., 2006). α-Expansins and β-expansins proteins are known

to have cell-wall loosening activity and to be involved in cell expansion and other developmental events during which cell-wall modification occurs. Whereas expansin-like A, and expansin-like B proteins are known only from their gene sequences. α-Expansin proteins and some β–expansin proteins are implicated as catalysts of 'acid growth', the enlargement of plant cells stimulated by low extracellular pH. β-Expansin proteins are expressed at high levels in the pollen of grasses (Sampedro and Cosgrove, 2005). Expansins are present as a multigene family during growth and ripening of fruit (Brummell *et al.*, 1999a; Catala *et al.*, 2000; Hiwasa *et al.*, 2003; Dotto *et al.*, 2006). Moreover, the accumulation of expansin mRNA for specific expansin gene family member has been correlated with the growth and development of various plant organs, including fruit, leaf, flower, tendril, root, and seed of grape (Ishimaru *et al.*, 2007), epicotyl, stem, radicle and pod of chickpea (Sanchez *et al.*, 2004), tobacco leaf (Pien *et al.*, 2001), maize leaf (Muller *et al.*, 2007), and flower of *Mirabilis jalapa* (Gookin *et al.*, 2003). Expansins have been proposed to disrupt hydrogen bonds between cellulose and hemicellulose microfibrils in the cell wall, thereby allowing movement and rearrangement of these cell wall polymers during expansive growth (Civello *et al.*, 1999). Whereas ripening-regulated expansins might contribute to cell wall polymer disassembly and fruit softening by increasing access of specific cell wall polymers to hydrolase action (Rose and Bennett, 1999). For example, the expression of several expansin genes was associated with fruit growth and that of other expansin genes with fruit ripening (tomato, Brummell *et al.*, 1999a; Catala *et al.*, 2000; pear, Hiwasa *et al.*, 2003; strawberry, Harrison *et al.*, 2001, Dotto *et al.*, 2006; banana, Asha *et al.*, 2007; grape, Ishimaru *et al.*, 2007). However, some expansin genes display a rather unexpected expression pattern. For example, the transcript accumulation of an expansin gene in loquat fruit that were stored at 0 °C was associated with visible chilling injury symptoms and with cell wall lignification. Interestingly, the transcript abundance of this expansin gene was reduced after treatment with 1-MCP, which also alleviated chilling injury (Yang *et al.*, 2008).

Recently, expansins have become more interested in fruit growth and softening research. In apple, *MdEXPA3* transcription was initiated at fruit set, increased progressively as the fruit grow and the highest level of signal was detected

in fruit at harvest stage. After that the expression was reduced to low level as ripening stage. For the study in tomato, *LeExp1* exhibited high levels of mRNA abundance and was specifically expressed in ripening fruits. In addition, expression of *LeExp1* was regulated by ethylene (Rose *et al.*, 1997). *LeExp2* gene expressed their mRNA accumulation peaking during the highest growth stages of fruit. The expression of *LeExp2* was undetectable at the onset of and during fruit ripening, which is consistent with a specific role of this gene in regulating cell wall loosening during fruit growth, not in ripening-associated cell wall disassembly (Catala *et al.*, 2000). mRNA of *Exp3* was present throughout fruit growth and ripening, with the highest accumulation in green expanding and maturing fruit, and lower, declining levels during ripening. *Exp4* mRNA was present only in green expanding fruit, whereas *Exp5* mRNA was present in expanding fruit but had highest levels in full-size maturing green fruit and declined during the early stages of ripening. mRNAs from each of these genes were also detected in leaves, stems and flowers but not in roots. *Exp6* and *Exp7* mRNAs were present at much lower levels than mRNAs of the other expansin genes, and were detected only in expanding or mature green fruit. The results indicate the presence of a large and complex expansin gene family in tomato, and suggest that while the expression of several expansin genes might contribute to green fruit development, only *Exp1* mRNA was present at high levels during fruit ripening (Brummell *et al.*, 1999a).

Functional analysis showed that at least one expansin is required for fruit softening. Overexpression of the ripening-specific *Exp1* in tomato fruit resulted in much earlier and more softening, whereas suppression of the gene resulted in firmer fruit. The results supported the idea that the Exp1 protein relaxes the cell wall directly, which regulates the rate of polyuronide depolymerization late in ripening. Additionally, very small amounts of Exp1 protein are apparently required for depolymerization of hemicelluloses (Brummell *et al.*, 1999b; Brummell and Harpster, 2001; Brummell *et al.*, 2002). Softening of red tomato fruit has been found subsequently to depend on the action of both PG and expansin, whereby expansin was apparently required to allow PG action (Cantu *et al.*, 2008). 1-MCP treatment

decreased the mRNA abundance of *EXP1* in mature green, breaker, orange, and red ripe fruit (Hoeberichts *et al.*, 2002).

In strawberry (*Fragaria x ananassa* Duch.), a full-length cDNA encoding a ripening-regulated expansin (*FaExp2*) was isolated from strawberry fruit. *FaExp2* mRNA was not detected in vegetative tissues (root, stem, leaves, and sepals), ovaries, or green achenes (Civello *et al.*, 1999). This pattern suggests that the expression of *FaExp2* is fruit specific, like the expression of *LeExp1* in tomato (Rose *et al.*, 1997). *FaExp2* mRNA abundance was very low in green fruit and increased from the white to the ripe stage (Civello *et al.*, 1999; Harrison *et al.*, 2001). While tomato fruit ripening and *LeExp1* gene expression are ethylene-regulated, the expression of *FaExp2* was ethylene insensitive and *FaExp2* expression was not strongly affected by auxin levels. This result indicates that endogenous signals other than ethylene and auxin must operate to regulate gene expression in ripening strawberry. The *FaExp2* gene may be a useful reporter gene to probe the nature of the non-climacteric signals that regulate its expression in ripening strawberry (Civello *et al.*, 1999). *FaExp3* mRNA was present at low level and was expressed in small green fruit and in ripe fruit. *FaExp4* mRNA was present throughout fruit development but was more strongly expressed during ripening. *FaExp5* gene showed fruit specific expression that was up-regulated at the onset of ripening. *FaExp6* and *FaExp7* expression were present at low levels in the fruit whereas showed highest expression in stolon tissue (Harrison *et al.*, 2001). In addition, Dotto *et al.* (2006) have also reported the involvement of expansins in strawberry fruit softening. They have found a correlation between mRNA expression levels and fruit firmness for *FaEXP1*, *FaEXP2*, and *FaEXP5*. These three mRNAs showed higher levels in the softest cultivar (Toyonaka) than in the other two firmer cultivars (Selva and Camarosa) at the beginning of ripening. However, this correlation was not found in the case of *FaEXP4* and *FaEXP6*.

During fruit ripening of apricot (*Prunus armeniaca* L. cv. Bergeron), *Pa-Exp1* and *Pa-Exp2* are mostly expressed in fruit. *Pa-Exp1* mRNA accumulated abundantly at the half-ripe stage of fruit development and decreased thereafter. *Pa-*

Exp2 mRNA level increased from the immature-green stage to the half-ripe stage where it peaked before declining. *Pa-Exp1* appeared to be developmentally down-regulated by ethylene while *Pa-Exp2* is not affected (Mbéguié-A-Mbéguié *et al.*, 2002). The multiple expansin genes of pear fruit showed differential expression and hormonal regulation during fruit development. Seven cDNAs, designated *PcExp1* to *PcExp7*, encoding expansin homologues, were isolated from mature pear fruit. Accumulation of *PcExp2*, *-3*, *-5* and *-6* mRNA increased markedly with fruit softening and then declined at the over-ripe stage. Treatment of fruit at an early ripening stage with 1-MCP, an inhibitor of ethylene action, suppressed ethylene biosynthesis, fruit softening and the accumulation of the expansin mRNAs. Conversely, propylene treatment at the preclimacteric stage induced accumulation of the same four expansin genes, as well as ethylene production and fruit softening. The expression patterns correlated with alteration in the rate and extent of fruit softening. The abundance of *PcExp1* mRNA increased at the late expanding phase of fruit development and further increased during ripening, whereas *PcExp4* mRNA levels were constant throughout fruit growth and ripening. 1-MCP and propylene treatments had little effect on *PcExp1* and *PcExp4* expression. *PcExp7* was expressed in young but not mature fruit (Hiwasa *et al.*, 2003).

In peach, *PchExp1* was isolated from ripe fruit. The expression of this gene was fruit-specific and ripening-regulated. Furthermore, it was enhanced abundantly by ethylene treatment. However, *PchExp1* may not regulate the changes in fruit firmness of peach. There were the data that pattern of *PchExp1* mRNA abundance in the ripe fruit of Akatsuki, which lost its firmness rapidly after harvest, and Yumyeong, which retained its firmness after harvest, were very similar during storage for eight days (Hayama *et al.*, 2000). For more study, the accumulation of *PpExp2* mRNA was study in peach fruit development. This gene was constitutively expressed throughout fruit development but was abundant when the fruit expanded exponentially and then matures (Hayama *et al.*, 2001; Hayama *et al.*, 2003). During storage, the ripe fruit of ‘Akatsuki’ cultivar rapidly softened as the level of ethylene increased significantly, while ‘Manami’ cultivar fruit remained firm and exhibited very low levels of ethylene production. The *PpExp1* and *PpExp2* mRNAs were

constitutively detectable during the 8-day storage of both cultivars, whereas *PpExp3* mRNAs were up-regulated at the onset of ripening. *PpExp3* mRNA was detectable in 'Akatsuki' but hardly detectable in 'Manami,' suggesting that *PpExp3* expression may be related to the changes in fruit firmness. To address the detailed role of *PpExp3* in the loss of fruit firmness, the fruit of 'Manami' was treated by ethylene to induce softening. The *PpExp3* mRNA accumulation in the ethylene-treated 'Manami' was detectable and similar to that observed in 'Akatsuki'. The results suggest that *PpExp3* is more likely to play a role in peach fruit softening than *PpExp1* or *PpExp2* (Hayama *et al.*, 2003).

The identification of four α-expansin genes, *MaEXPA2*, *MaEXPA3*, *MaEXPA4* and *MaEXPA5* from banana fruit which express differentially during fruit development and ripening. All the four genes were expressed during ripening. *MaEXPA2* was fruit specific and showed a strong ripening related and ethylene dependent expression in banana fruit. *MaEXPA4* expressed both during fruit growth as well as ripening and might be related to expansion. *MaEXPA3* and *MaEXPA5* were also expressed in tissues other than fruit (Asha *et al.*, 2007). Ethylene exposure to unripe mature banana fruit induced *MaExp1* expression, which increased with the progression in ripening and 1-MCP treatment prior to ethylene exposure inhibits expression (Trivedi and Nath, 2004). Moreover, there was the study that increased tolerance of banana fruit pre-treated with propylene to low temperature, was related to higher post-storage ethylene production rates and enhanced expression of *MaExp1* and *MaExp2*. According to the stimulation of ripening with propylene applied prior to storage at low temperature reduced chilling injury. RNA blotting analysis showed no accumulation of either *MaExp1* or *MaExp2* transcripts during low temperature storage. These expansin genes were expressed more intensively in propylene pre-treated fruits than in control fruits upon removal from cold storage for propylene-initiated ripening (Wang *et al.*, 2006).

The expression of expansin gene in mango, *MiExpA1* expression correlated with fruit softening and this expression was triggered by ethylene treatment. The result showed that a ripening associated peak in transcript

accumulation on the third day after ethylene treatment. Treatment with 1-MCP inhibited both ripening/softening as well as *MiExpA1* transcript and protein accumulation. These results suggest that *MiExpA1* expression is ethylene dependent and its expression increases with the progression of ripening (Sane *et al.*, 2005).

MATERIALS AND METHODS

Plant Materials

Fruits of sapodilla (*Manilkara zapota* van Royen), cvv. Kra-Suay and Makok-Yai, were obtained from a commercial orchard in Ratchaburi province, western Thailand, during 2005 and 2006 for duplicate experiments.

Experiment I. Physiological changes and gene expression of cell wall enzymes during fruit growth and after harvest

Experiments were carried out during two growth seasons. Flowers were tagged at anthesis for 600 fruits per cultivar. Fruits were harvested every two weeks after anthesis, until they reached the mature stage, i.e. when the peel color changed from dark green to light brown. The harvested fruits were separated into two groups. In one group (25 fruits) fresh and dry weight was measured. The other group (about 50 fruits) was frozen in liquid nitrogen and stored at -70˚C until use. Expanding leaves and flowers that had just opened were also collected, placed in liquid nitrogen and frozen at -70˚C until use.

Mature fruits were harvested at commercial maturity, i.e. when the peel color changed from dark green to light green. These fruits were transported to laboratory using a temperature-controlled truck (20˚C), and arrived within 2 h after harvest. Fruits were cleaned by washing the scruff on the skin using sponge. Fruits were then selected for uniformity of color and size. Fruits were placed into corrugated boxes and stored at 25 ± 0.5˚C and 80-85% relative humidity in the storage room. At daily intervals, 20 fruits were taken assessed for firmness. The equatorial part of peel and the outer flesh (until a depth of approximately 0.5 cm below the peel) was cut into 0.5 x 0.5 x 0.5 cm^3 pieces, immediately frozen in liquid nitrogen and stored at -70˚C until further use.

1.1 Fruit size

Diameter and length of each fruit was determined using a vernier caliper. The measurements comprised 25 fruits.

1.2 Fruit weight

Each fruit was determined the fresh weight for 0.01 g readability of a scale. The fresh weight of individual fruit was determined by weighing. After determination of fresh weight, each fruit was cut into small pieces (0.3 x 0.3 x 0.3 cm^3) and dried in an oven at 60˚C for two weeks. Then they were determined the dry weight for 0.01 g readability of a scale. The measurements comprised 25 fruits.

1.3 Fruit firmness

Firmness of 25 fruits was measured for five days after harvest. Firmness was determined at one place, at the middle between the pedicel and the remnants of the flower, using a penetrometer (Effegi FT-011, Alfonsine, Italy). A cylindrical plunger, 0.2 cm in diameter, was inserted to a depth of 0.5 cm and the force was recorded in Newtons (multiply by 9.807).

1.4 Fruit peel color

Change in color of sapodilla peel was determined using a colorimeter (Chroma meter CR-400, Konica Minolta Sensing, Inc., Japan). The color reading was taken twice at the equatorial region of each fruit and averaged to give a value for each fruit. Color measurements were recorded using the CIE L*, a*and b* color space [where: L = lightness, range from 0 (black) to 100 (white); a = bluish-green/red-purple hue component, range from -60 to 60 and b = yellow/blue hue component, range from -60 to 60].

1.5 Respiration and ethylene production

Two fruits were sealed in 0.65-liter glass jar for 30 min at 25˚C. A 1 mL gas sample was withdrawn from the sampling port by a hypodermic syringe and the gas composition analyzed by a Shimadzu GC 8A (Japan) a gas chromatograph. The CO_2 production was determined using a thermal conductivity detector, whilst the ethylene production was determined with a flame ionization detector. The respiration rate was expressed as mg CO_2 kg^{-1} h^{-1}. The ethylene production rate was expressed as μL kg^{-1} h^{-1}. Glass jars were opened after gas sampling and the fruits were placed at 25 ± 0.5˚C and 80-85% RH. Each value was calculated from the average of six jars.

1.6 Total soluble solids (TSS) content and titratable acidity (TA)

Total soluble solids (TSS) content of the pulp juice, SSC was measured with a hand refractometer (Atago, Tokyo, Japan) and calibrated with distilled water. Titratable acidity (TA) content of the pulp was determined by titrating the pulp juice with 0.1 N NaOH using 1% phenolphtalein as an indicator and expressed as percentage of malic acid. The TSS/TA ratio was calculated.

1.7 Genes expression of expansins, endo-β-1,4-glucanase, polygalacturonase, and pectate lyase

1.7.1 Total RNA extraction

Total RNA was extracted from sapodilla flesh, leaves and flowers following a modified protocol by Chang *et al.* (1993). Two grams of frozen ground tissue were added to 15 mL of RNA extraction buffer [containing 2% CTAB (hexadecyltrimethylammonium bromide), 2% PVP (polyvinyl pyrrolidone K30), 100 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, 2.0 M NaCl, and 500 μL L^{-1} of spermidine], which had been pre-warmed at 65˚C for 10 min. Before using the extraction buffer 300 μL of β-mercaptoethanol was added. The samples were mixed

by vortexing and homogenised at full speed before two times extraction using an equal volume of chloroform: isoamyl alcohol (24 : 1). The phases were separated at 4˚C (6,188*g* for 20 min). The aqueous phase was carefully removed, and the RNA was precipitated overnight using 1/3 volume 8 M of LiCl at 4˚C. RNA was collected after centrifugation at 4˚C (12,631*g* for 20 min). Precipitated RNA was dissolved into 300 μL of SSTE (containing1.0 M NaCl, 0.5% SDS, 10mM Tris–HCl pH 8.0, 1mM EDTA pH 8.0) then extracted once with an equal volume of chloroform: isoamyl alcohol (24 : 1). Two volumes of ethanol were added to the supernatant, which was held at -70˚C for 1 h, for precipitation. RNA was collected by centrifugation at 4˚C (18,190*g* for 20 min). The precipitated RNA was washed with 1mL of 70% cool ethanol and centrifuged at 4˚C (18,190*g* for 10 min). The pellet was allowed to air dry at room temperature and was resuspended in 30 μL of diethylpyrocarbonate-treated distilled water. After that RNA was quantified by measuring the absorbance at 260 nm. One absorbance unit at 260 nm corresponded to approximately 40 μg mL^{-1} (Sambrook and Russel, 2001). The RNA concentration of each sample was estimate in μg mL^{-1} by the following equation: RNA concentration = A_{260} x dilution factor x 40 μg mL^{-1}. Quality of the purified total RNA was determined by calculating the A_{260}/A_{280} ratio. The ratio between 1.8-2.0 indicated good quality of the RNA. The RNA quality was confirmed by gel electrophoresis on 0.8% agarose gel (Appendix Figure 1 and 2).

1.7.2 Isolation of *MzEXP1*, *MzEXP2*, *MzEG*, *MzPG*, and *MzPL* cDNA fragments

1) Primer design

Degenerated primers were designed from GenBank for RT-PCR. For expansins, a degenerate sense primer 5'-GGHGGHGCKTGTGGDTA-3' (H = A/C/T, K= G/T, D= A/G/T) and antisense primer 5'-CARTTYTGSCCCCARWTHC-3' (R=A/G, Y=C/T, S=C/G, W=A/T) were designed according to the conserved regions of plant expansin sequences from *Lycopersicon esculentum* Mill. (AF059487, AF059488, AF059489) and *Pyrus communis* L.

(AB093031, AB093032, AB093033, AB093034). A partial coding sequence of *MzEXP1* (accession number EU139436) (Kunyamee *et al.*, 2008) was isolated from the expanding fruit (16 weeks after anthesis). Another degenerate sense primer 5'-ACHATGGGNGGDGCDTGTG-3' (N = A/C/G/T) and antisense primer 5'-GATSARAACCARGTTGAAG-3' were designed according to the conserved regions of plant expansin sequences from *Malus_domestica* Borkh. (AY083166), *Mangifera indica* L. (AY600964), *Prunus avium* L. (AF297521), *Prunus cerasus* L. (AF448467), *P. mume* Siebold & Zucc. (AB218787), *P. persica* (L.) Batsch (AB029083), and *Pyrus communis* L. (AB093028). A partial coding sequence of *MzEXP2* (accession number EU251387) (Kunyamee *et al.*, 2008) was isolated from the ripening fruit (three days after harvest).

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'-AANCCNACCATRTANCWCAT-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 (Rungruchkanont *et al*., 2007). 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 (three 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 x ananassa* (U63550), *Malus x 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 (three days after harvest).

Actin gene was amplified using specific primers. The *Actin* primers were got from Miss Suganya Chidtrakul. The sequence of sense primer was 5'-ATGTTT GAG ACC TCC AAT GTA CCT G-3' and the sequence of antisense primer was 5'-GTT TCC ATA GAG ATC CTT CCT GAT A-3'.

2) RT-PCR

The total RNA from pulp of 'Makok-Yai' sapodilla fruit was used for the isolation of expansins (*MzEXP1*, *MzEXP2*), Endo-β-1,4-glucanase (*MzEG*), polygalacturonase (*MzPG*), pectate lyase (*MzPL*), and actin (*MzActin*) genes. One microgram of total RNA was pretreated with deoxyribonucleaseI (DNaseI, Rnase-free, Fermentas, Canada) to remove contaminating genomic DNA, then used to make cDNA using M-MLV reverse transcriptase (Promega, USA) as described by the supplier's instructions. The synthesized cDNA was used as a template to amplify the target genes by PCR. Sequences of the primers are described in Table 1.

The reaction components were used for RT-PCR as Table 2. The amplification reactions were initially denatured at 94˚C for 3 min and then subjected to 35 cycles at the following condition: 94˚C for 30s, 57˚C for 60 s (*MzEXP1*) or 50˚C for 60 s (*MzEXP2*) or 52˚C for 60 s (*MzEG*, *MzPG*, and *MzPL*) or 55˚C for 60 s (*MzActin*), and 72˚C for 60 s (*MzEXP1*, *MzEG*, *MzPG*, *MzPL*, and *MzActin*), or for 30 s (*MzEXP2*), with a final extension at 72˚C for 10 min.

Table 1 Sequences of primers used in RT-PCR analysis.

Gene	Primer	Tm (˚C)	Expected size (bp)
MzEXP1	F’ 5’-GGHGGHGCKTGTGGDTA-3’	52	482
	R’ 5’-CARTTYTGSCCCCARWTHC-3’	52	
MzEXP2	F’ 5’-ACHATGGGNGGDGCDTGTG-3’	58	400
	R’ 5’-GATSARAACCARGTTGAAG-3’	50	
MzEG	F’ 5’-TAYTAYGAYGCNGGNGAYAA-3’	52	1000
	R’ 5’-AANCCNACCATRTANCWCAT-3’	52	
MzPG	F’ 5’- TTWGGAGCYARAGSDRATGG-3’	52	651
	R’ 5’-CCAATRCTRATTCCRTGGCC-3’	58	
MzPL	F’ 5’-AYTGYTGGMGDTGYGACC-3’	52	878
	R’ 5’-NCCYTCHGAYCKCCARTTCC-3’	58	
MzActin	F’ 5’-ATGTTTGAGACCTCCAATGTACCTG-3’	52	512
	R’ 5’-GTTTCCATAGAGATCCTTCCTGATA-3’	61	

Table 2 Reaction of RT-PCR of each gene.

Components	*MzEXP1*	*MzEXP2*	*MzEG*	*MzPG*	*MzPL*	*MzActin*
cDNA template (μL)	1.0	1.0	1.0	1.0	1.0	1.0
10 μM F' primer (μL)	2.5	2.0	2.0	2.5	2.5	0.5
10 μM R' primer (μL)	2.5	2.0	2.0	2.5	2.5	0.5
10x PCR buffer (μL)	2.5	2.5	2.5	2.5	2.5	2.5
25 mM $MgCl_2$ (μL)	1.5	1.5	1.5	2.5	1.5	1.5
10 μM dNTPs (μL)	0.5	0.5	0.5	0.5	0.5	0.5
5 U μL^{-1} Taq DNA polymerase enzyme (μL)	0.125	0.125	0.125	0.125	0.125	0.125
DEPC treated water (μL)	14.375	15.375	15.375	13.375	14.375	18.375
total volume (μL)	25	25	25	25	25	25

3) PCR products cloning and DNA sequencing

The reaction product was separated with 1.2% agarose gel electrophoresis at 100 volts for 30 min. The gel was stained in 2.5 μg mL^{-1} ethidium bromide (EtBr) solution for 15 min. The RNA bands were visualized under UV transilluminator and photographed by SYNGENE BIO IMAGINE Gel Documentation (Syngene, England). 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 as figure 1. It was cut and used for gel extraction in order to purify the PCR product. The amplified cDNA fragments from each gene were purified using a gel extraction kit (QIAquick® Gel Extraction, Qiagen, Germany).

The purified product was ligated into the pGEM-T easy vector (pGEM®-T Easy Vector Systems, Promega, USA) as described in the supplier's instructions. The ligations were set up using 5 μL of 2x Rapid Ligation Buffer, 0.5 μL

of pGEM-T vector, 2 μL of PCR product, 1 μL of 10x T4 DNA Ligase and 1.5 μL of DEPC treated water to adjust a final volume of 10 μL. The reactions were mixed by pipetting and then incubated overnight at 4˚C.

E. coli DH5-α competent cell was used for the transformations. Two LB/ampicillin/IPTG/X-Gal plates for each ligation reaction were prepared. The ligation reaction (10 μL) was carefully put into 100 μL of frozen DH5-α tube then gently flicked to mix and placed on ice for 30 min. The cells were heat shocked for 1 min in a water bath at 42˚C and immediately returned on ice for 2 min. Then 800 μL of SOC medium was added to the tube and incubated for 1 h at 37˚C with shaking approximately 100 rpm. Each transformation culture (100 μL) was plated onto duplicate LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37˚C. Single white colonies would be selected for the next step.

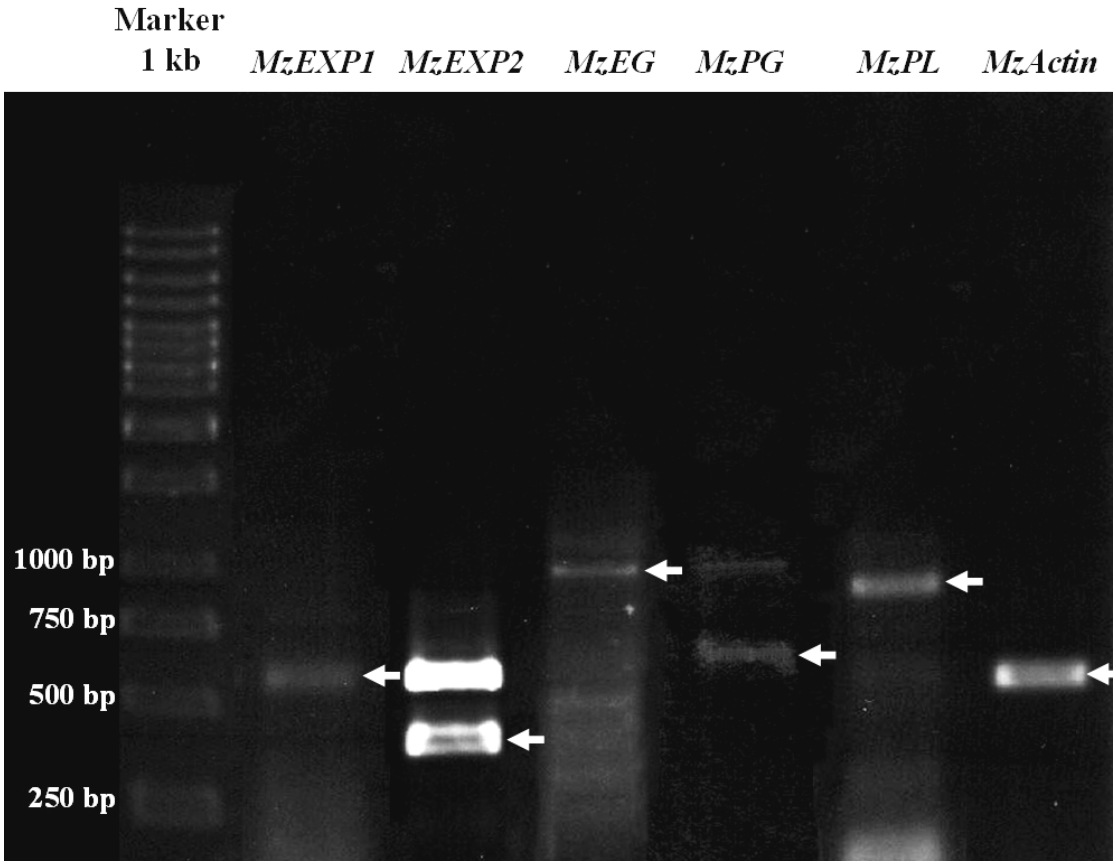

Figure 1 PCR products from each reaction with primers which were designed for *MzEXP1*, *MzEXP2*, *MzEG*, *MzPG*, *MzPL*, and *MzActin* fragments. Arrows indicate the expected size.

4) Purification of plasmid DNA using a miniprep kit (QIAprep® Spin Miniprep Kit, Quaigen, Germany).

Single white colonies containing the vector and inserted gene were cultured in 1 mL of LB medium overnight (in 1.5 mL-microtube). Cells were centrifuged at 7,000 rpm for 3 min and collected the pellet cells. The pellet cells were re-suspended in 250 µL of re-suspension buffer and transferred to 1.5 mL microtube. Then, 250 µL of lysis buffer was added and the tube gently inverted to mix. Neutralization buffer (350 µL) was added and the tube was inverted immediately then centrifuged at 13,000 rpm for 5 min. The supernatant was transferred to the spin column then centrifuged for 1 min. The flow-through was discarded. The spin column was washed by adding 750 µL of washing buffer and centrifuged again for 1 min. The flow-through was discarded and the column was centrifuged to remove the residual washing buffer. The plasmid DNA was eluted by adding 50 µL of elution buffer and centrifuged for 1 min. The plasmid DNA was kept at -20˚C until used for cutting with the *Eco*RI restriction enzyme (New England Biolabs, Inc., USA) and the right insert size was checked by gel electrophoresis as Figure 2.

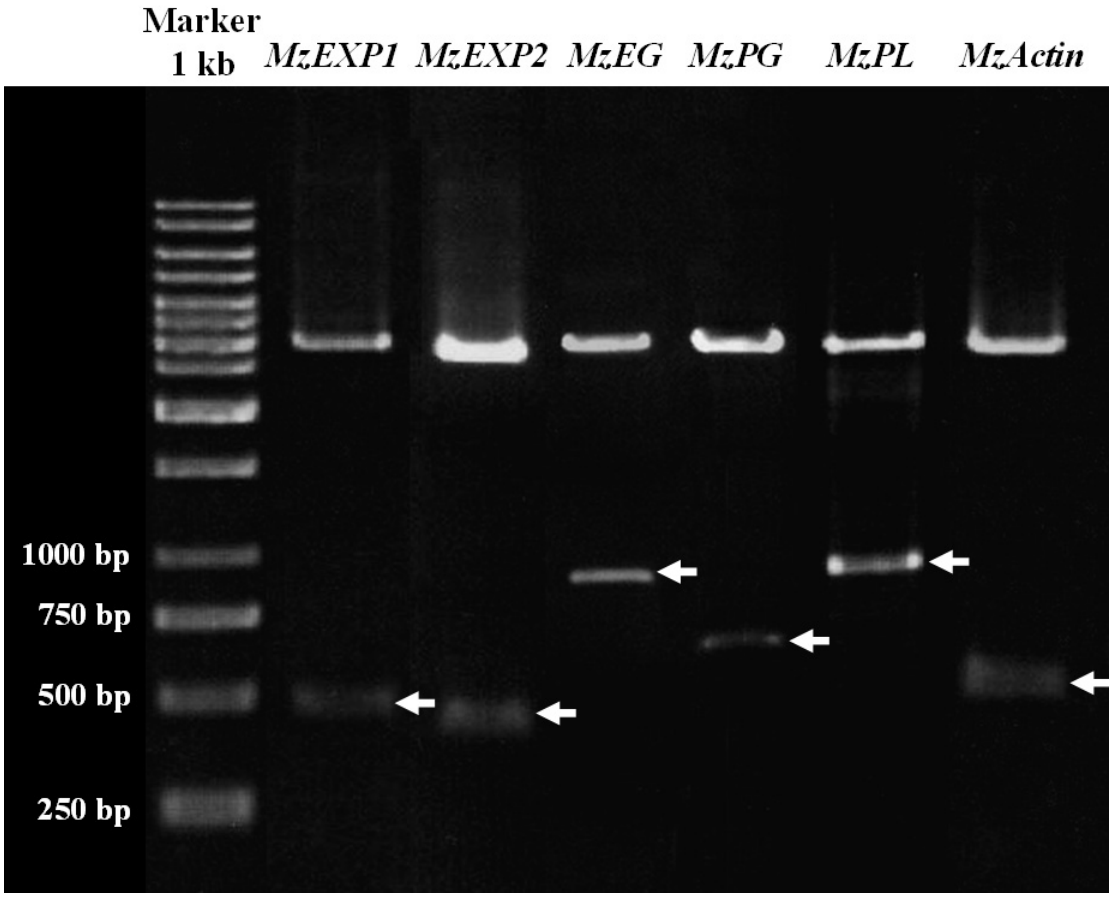

Figure 2 The plasmid DNA of *MzEXP1*, *MzEXP2*, *MzEG*, *MzPG*, *MzPL*, and *MzActin* were cut by using the *Eco*RI restriction enzyme and the right insert size was checked by gel electrophoresis. Arrows indicate the expected size.

5) DNA sequencing and analysis

Sequence analysis 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, CA). The fragment sequence was compared with information in the GenBank database using the BLAST program from National Centre for Biotechnological Information (NCBI). The sequences of *MzEXP1, MzEXP2, MzEG, MzPG, MzPL,* and *MzActin* genes were translated to deduced amino acid using Vector NTI 10.0 program (Invitrogen, USA).These sequences of amino acid were aligned using ClustalW program (http://www.ebi.ac.uk/clustalw) and submitted to GenBank (NCBI). The identified insert fragment was the template for probe synthesis, resulting in a specific mRNA probe.

1.7.3 Northern analysis using digoxygenin-11-UTP (DIG) labeling probes

1) DNA probe preparation

Plasmid DNA of *MzEXP1*, *MzEXP2*, *MzEG*, *MzPG*, *MzPL*, and *18S rRNA* (for mangosteen *18S rRNA*, which is the generous gift from Dr. Siriwan Dangcham; Dangcham *et al.*, 2008) clones were used as template for amplification with the sense and antisense primers followed as Table 1 (for *MzEXP1*, *MzEXP2*, *MzEG*, and *MzPL*). For *MzPG*, a sense primer 5'-TAGAGGTGATGGACGAAGTGAC-3' and antisense primer 5'-GTTGGGGCTCTTCTTTGGT-3' were used. For mangosteen *18S rRNA*, a sense primer 5'-CGGGGAGGTAGTGACAATAAATA-3' and antisense primer 5'-TAATGAAAACATCCTTGGCAAAT-3' were used (Dangcham, 2008). Templates were used for the PCR reaction as Table 3. The PCR mixture was initially denatured at 94˚C for 5 min and then subjected to 35 cycles at the following condition: 94˚C for 30 s, 57˚C for 60 s (*MzEXP1*) or 50˚C for 60 s (*MzEXP2*) or 52˚C for 60 s (*MzEG* and *MzPL*) or 51˚C for 45 s (*MzPG*) or 55˚C for 45 s (*18S rRNA*), and 72˚C for 30 s (*MzEXP1*, *MzEXP2*) or for 60 s (*MzEG*, *MzPG*, and *MzPL*) or for 45 s (*18s rRNA*), with a final extension at 72˚C for 10 min. PCR products of *MzEXP1*, *MzEXP2*,

MzEG, *MzPG*, *MzPL*, and *18S rRNA* (458, 392, 989, 490, 876, and 512 bp, respectively) were quantified by comparing with DNA low mass marker (Invitrogen, USA).

Table 3 The components of DIG labeling PCR reaction for *MzEXP1*, *MzEXP2*, *MzEG, MzPG*, *MzPL*, and *18S rRNA*.

Components	Volume
template (μL)	2
10 μM forward primer (μL)	1
10 μM reverse primer (μL)	1
10x PCR buffer (μL)	5
25 mM $MgCl_2$ (μLl)	4
PCR DIG labeling mix (μL)	5
5 U μL^{-1} Taq DNA polymerase enzyme (μL)	0.5
DEPC treated water (μL)	31.5
Total volume (μL)	50

2) Electrophoresis and blotting

Each total RNA sample aliquot (30 μg) was denatured at 65˚C and separated on 1.0% MOPs-formaldehyde agarose gel at 70V for 2 h, after which RNA loading was checked using ethidium bromide-stained gel The RNA quality was confirmed by gel electrophoresis on 0.8% agarose gel (Appendix Figure 3 and 4). The RNA was transferred to a positively charged nylon membrane (Roche, Mannheim, Germany) using 10× SSC (1.5 M NaCl, 150 mM trisodiumcitrate 2-hydrate, pH 7.0) (Sambrook *et al.*, 1989) for at least 15 h and the membrane was baked in an oven (Hybaid, Franklin, MA, USA) at 80˚C for 2 h. The membrane was pre-hybridised in hybridisation 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. Plasmid DNA of *MzEXP1*, *MzEXP2*, *MzEG*, *MzPG*, and *MzPL*

clones were used as a template for PCR DIG labeling using the PCR DIC labeling Mix (Roche), following the manufacturer's instructions. Denatured probe was added to the pre-hybridised buffer and following the hybridisation 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 hybridised 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, Indianapolis, IN, USA) for 1 min. The membrane was exposed to radiography film (Kodak medical X-ray film, Kodak, Rochester, NY, USA) suitable for chemiluminescence and the film was developed according to the manufacturers' recommendations. After placing the membrane in boiled 0.1% SDS stripping solution with vigorous agitation for 30 min, the membrane was ready for reprobing. Equal RNA loadings were checked by hybridisation with 18S rRNA probe (accession number EU032463) from mangosteen (Dangcham *et al.*, 2008) at 55˚C. Northern experiments were performed at least two times each.

Experiment II. Physiological changes, cell wall enzyme activity and gene expression of cell wall enzymes during fruit ripening

Mature fruits were harvested at commercial maturity, i.e. when the peel color changed from dark green to light green. These fruits were transported to laboratory using a temperature-controlled truck (20˚C), where they arrived within 2 h after harvest. Fruits were cleaned by washing the scruff on the skin using sponge. Fruits were then selected for uniformity of color and size. The experiment consisted of four treatments. Hundred fruits were placed in a sealed plastic container (71 L) and treated with 50 μL L^{-1} of ethylene at 25˚C for 20 h (ethylene treatment). Other fruits were treated with 1 μL L^{-1} of 1-MCP (EthylBloc, Floralife Inc., USA) for 20 h at 25˚C (1-MCP treatment). Release of 1-MCP occurred after 1.9 mL of deionized water to 114 mg powder, which was placed in a 10-mL vial. After treatment the container was opened to ventilate for 2 h, and again resealed and exposed to 50 μL L^{-1} of ethylene at 25˚C for 20 h (1-MCP + ethylene treatment). The control group was fruits without any treatment. Following treatments, fruits from all treatments were placed into corrugated boxes and stored at 25 ± 0.5˚C, with 80-85% RH 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 fruits were very soft. The equatorial part of peel and the outer flesh (until a depth of approximately 0.5 cm below the peel) was cut into 0.5 x 0.5 x 0.5 cm^3 pieces, immediately frozen in liquid nitrogen and stored at -70˚C until further use.

2.1 Fruit firmness

Changes in firmness of the fruits were studied for all the 5 days after treatments. Twenty fruits from each treatment were used to measure fruit firmness. Fruit firmness was determined on one point of each fruit with skin, at the middle between the pedicel and the remnants of the flower, using a penetrometer (Effegi FT-011, Alfonsine, Italy). A cylindrical plunger, 0.2 cm in diameter, was inserted to a depth of 0.5 cm and the force was recorded in Newtons (multiply by 9.807).

2.2 Genes expression of expansins, endo-β-1,4-glucanase, polygalacturonases, and pectate lyase during fruit ripening

The sapodilla pulp from each treatment was extracted for total RNA. The RNA quality was confirmed by gel electrophoresis on 0.8% agarose gel (Appendix Figure 5 and 6). Each total RNA sample aliquot (30 µg) was denatured at 65˚C and separated on 1.0% MOPs-formaldehyde agarose gel at 70V for 2 h, after which RNA loading was checked using ethidium bromide-stained gel (Appendix Figure 7 and 8), then followed the method for determination of gene expression by northern blotting as described in Experiment 1. Northern experiments were performed at least twice each.

2.3 The activity of cell wall degrading enzymes during fruit ripening

2.3.1 Endo-β-1,4-glucanase (EGase)

Extraction for EGase enzyme occurred according to Awad and Young (1979). One g of frozen pulp was homogenized in 8 mL of 40 mM sodium acetate buffer pH 5.5 and 2 mL of 0.2 M NaCl. The homogenate was centrifuged at 4˚C, 17,390 *g* for 20 min. The supernatant was held on ice. EGase activity was determined by measuring the release of reducing groups according to the assay of Mandels and Sternberg (1976) with slight modification. The reaction mixture contained 0.2 mL of crude enzyme extract and 1.3 mL of 50 mM sodium acetate buffer pH 4.8. A 0.5 x 3 cm (25 mg) of Whatman No. 1 filter paper strip was added to the reaction mixture and Vortex mixed. Incubation was carried out at 50˚C for 1 h, after which 3 mL of dinitrosalicylic acid reagent was added to stop the reaction. The samples were heated in boiling water for 5 min. The samples were removed from the hot water, after which 16 mL of distilled water was added, and were allowed to cool to room temperature. The absorbance at 550 nm was determined, in order to determine total reducing sugars. A calibration curve was obtained using D-glucose as a standard. One unit of EGase activity was defined as 1 µmole D-glucose released per mg protein per min. Protein content was measured using the standard Bradford method.

2.3.2 Polygalacturonase activity

Extraction occurred according to the protocol of Abu-Goukh and Bashir (2003), with slight modification. Six g of frozen pulp were homogenized in 25 mL of 100 mM sodium acetate buffer pH 6.6 containing 1% PVP. 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 times renewal of the water. All extraction steps were conducted at 4˚C. The dialysed sample was the crude enzyme extract.

Assay for PG activity followed the Anthon and Barrett (2002) method. 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^{-1} 3-methyl-2-benzothiazolinone hydrazone [MBTH] and 1 mg mL^{-1} dithiothreitol (DTT), 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% $FeNH_4(SO_4)_2$. $12H_2O$, 0.5% sulfamic acid, and 0.25 N HCl was added, after which the samples 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 µmole galacturonic acid released per mg protein per hour.

2.3.3 Pectate lyase (PL)

Extraction and assay method for PL enzyme were as described by Payasi and Sanwal (2003), with modification. Four g of frozen pulp were homogenized in 20 ml of 20 mM sodium phosphate buffer pH 7.0 containing 1% v/v Triton X-100. The homogenate was centrifuged at 15,000*g* for 30 min. The reaction mixture contained 2.5 mL of polygalacturonic acid solution (1% w/v in 0.05 M Tris-HCL buffer, pH 8.5), 1.0 mL of 10 mM $CaCl_2$, 1.5 mL of crude enzyme extract and 1.5 mL of water. It was incubated at 37˚C for 2 h. The reaction was stopped by addition of 0.3 mL of 9% $ZnSO_4.7H_2O$, followed by addition of 0.3 ml of 0.5 M NaOH. The mixture was centrifuged at 3,000*g* for 10 min. To the supernatant (2.5 mL) was added 1.5 mL of 40 mM thiobarbituric acid, then 0.75 mL of 0.1 M HCl, and 0.25 mL of water. The mixture was placed in boiling water for 30 min, was allowed to cool to room temperature and centrifuged at 3,000*g* for 20 min. The absorbance was determined at 550 nm. The control tubes received crude enzyme after addition of $ZnSO_4$ and NaOH. One unit of PL activity was defined as the amount of enzyme causing a change in absorbance of 0.01 under the condition of the assay.

2.4 Modification of cell-wall polymer

2.4.1 Cell wall preparation, fractionation, and analysis

Alcohol insoluble solids (AIS) of cell walls were prepared from frozen fruit tissue by immersing 20 g of sample in 150 mL of 95% alcohol and boiling for 20 min. Boiled samples were allowed to cool and then were homogenized (Polytron). Insoluble material was recovered by filtration through 8 layers of Miracloth, washed with 100 mL of 95% alcohol and with acetone until it was decolorized, and then air dried (Carrington *et al*., 1993). The weight of resulting AIS powder was recorded.

2.4.2 Cell wall fractionation and analysis

AIS weighing 100 mg was extracted sequentially twice with 19 mL of distilled water and 1 mL of 0.4% NaN_3 was added, shaked at 200 rpm for 10 h each, and then centrifuged at 17,390 *g* for 20 min. All two extracts were pooled and designated as water-soluble fraction. The insoluble residue were then extracted twice with 19 mL of 50 mM tran-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) in 50 mM sodium acetate pH 6.0 and 1 mL of 0.4% NaN_3 was added, shaked at 200 rpm for 10 h each, and then centrifuged at 17,390 *g* for 20 min. All two extracts were pooled and designated as a CDTA (chelating) soluble fraction. The insoluble residue was then extracted twice with 19 mL of 50 mM Na_2CO_3 in 20 mM $NaBH_4$ and 1 mL of 0.4% NaN_3 was added, shaked at 200 rpm for 10 h each, and then centrifuged at 17,390 *g* for 20 min. All two extracts were pooled and designated as Na_2CO_3 soluble fraction.

The hemicellulose fractions (1 and 4N KOH fractions) were obtained by sequentially extracting the residues from pectin fractions as described by Maclachlan and Brady (1994). The pellet from the Na_2CO_3 soluble fraction was extracted twice with 19 mL of 1N KOH and 1 mL of 0.4% NaN_3 was added, shaked at 200 rpm for 10 h each. The supernatant was collected by centrifugation at 17,390 *g* for 20 min and designated as 1N KOH (a weak alkali) soluble fraction or loosely bound matrix glycan fraction. The pellet was then extracted 2 times with 19 mL of 4N KOH and 1 mL of 0.4% NaN_3 was added, shaked at 200 rpm for 10 h each. The supernatant was collected as above and designated as 4N KOH (a strong alkali) soluble fraction or tightly bound matrix glycan fraction.

The Na_2CO_3, 1N KOH, and 4N KOH soluble fractions were neutralized using concentrated acetic acid before dialysis. The supernatant of each pectin and hemicellulose fraction was exhaustively dialyzed against cool distilled water overnight at 4˚C with two changes of water. The total volume of each dialyzed fraction was recorded (Carrington *et al*., 1993). Then the pectin contents in water, CDTA, and Na_2CO_3 soluble fraction were determined measuring the uronic acid

content according to the colorimetric method at 520 nm of Blumenkrantz and Asboe-Hansen (1973) with D-galacturonic acid as a standard. The hemicellulose content in 1N KOH, and 4N KOH soluble fractions was determined measuring the total sugars content by the anthrone assay (Dische, 1962) at 620 nm. Glucose was used as a standard.

Statistical Analyzes

Experiment data were subjected to analysis of variance (ANOVA) and significant differences between means were determined using the Least Significant Difference (LSD) test at the 95% significance level. All experiments were repeated at least once.

THE EXPERIMENTAL TIME AND PLACES

The experiments were carried out during June 2006-November 2008 and the research was conducted at the places as described below:

1. Sapodilla orchards in Ratchaburi province.

2. Postharvest Technology Center, Kasetsart University, Kamphaeng Saen, Nakhon Pathom.

RESULTS

Experiment I. Physiological changes and gene expression of cell wall enzyme during fruit growth and after harvest

1.1 Fruit growth

Diameter (Figure 3A), length (Figure 3B), fresh weight (Figure 4A) and dry weight (Figure 4B) of sapodilla fruits, when attached the tree, showed the typical sigmoidal growth pattern. Until week 18 after anthesis, fruits of both cultivars showed the same increase in fresh weight (fresh weight was ~10 g by that time). After week 18, the increase in fresh weight of cv. Kra-Suay was higher than in cv. Makok-Yai. By week 32, the average fruit fresh weight in cv. Kra-Suay (62 g) was almost twice as high as the fresh weight of cv. Makok-Yai (36 g). The period required for the fruits to develop from anthesis to maturity stage was 32-34 weeks (about seven months) in cv. Makok-Yai and 36 weeks (eight months) in cv. Kra-Suay. The size of mature fruits in cv. Makok-Yai was smaller than that in cv. Kra-Suay (Figure 4).

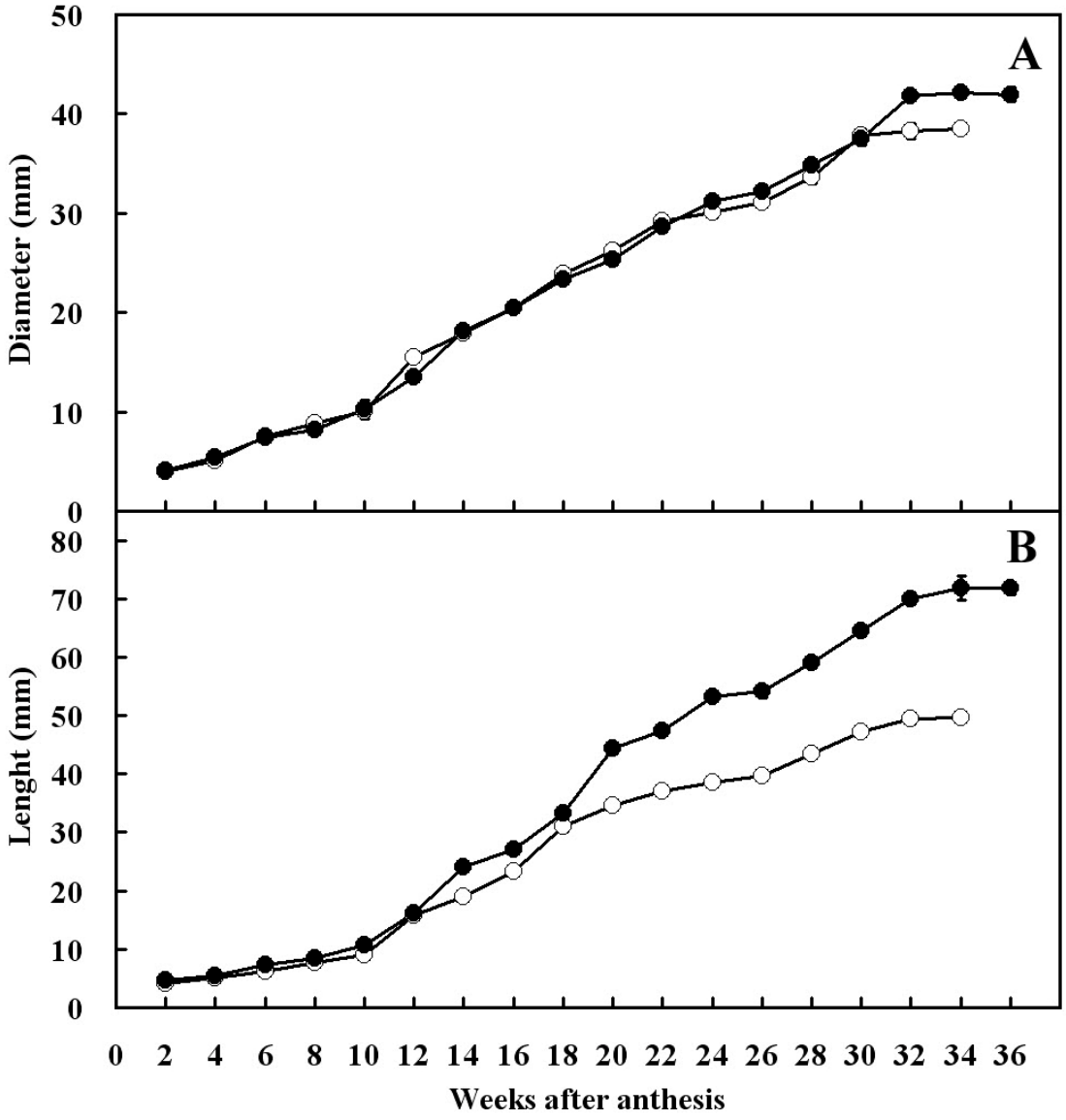

Figure 3 Changes in diameter (A) and length (B) of sapodilla fruits of cvv. Makok-Yai (○) and Kra-Suay (●) between anthesis and fruit maturity. Data are means ± SD (n = 25).

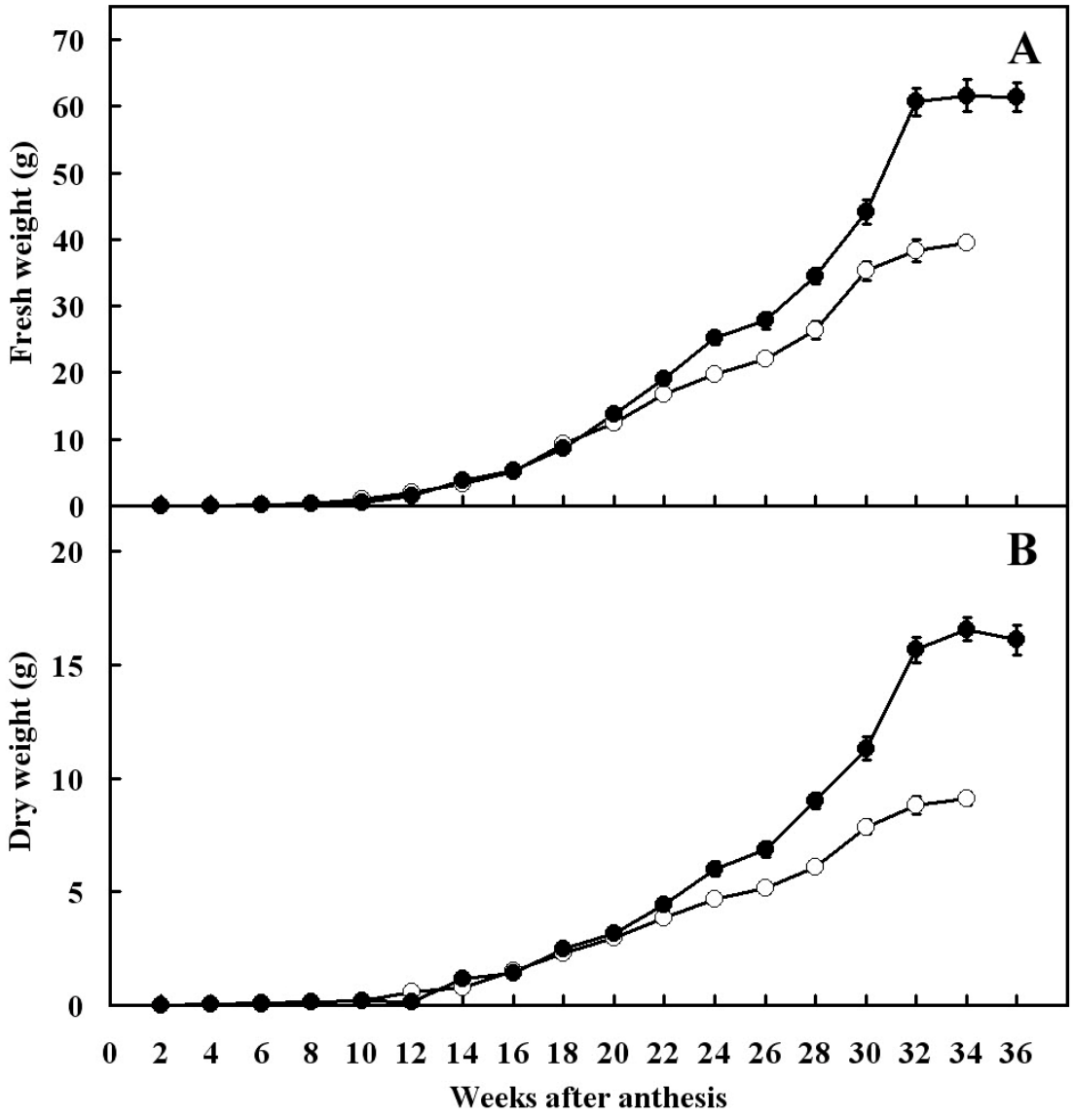

Figure 4 Changes in fresh weight (A) and dry weight (B) of sapodilla fruits of cvv. Makok-Yai (○) and Kra-Suay (●) between anthesis and fruit maturity. Data are means ± SD (n = 25).

1.2 Firmness, respiration rate and ethylene production of fruit after harvest

From day 1 after harvest, firmness of the peel and flesh of sapodilla fruits steadily decreased (Figure 5). The fruits were ripe (firmness about 10 N) on day 4, and overripe (firmness about 5 N) on day 5-6 (Appendix Table 1). The respiration rate of both cultivars showed a climacteric pattern (Figure 6). In cv. Makok-Yai, a rather broad maximum in respiration rate occurred on day 3-5 after harvest. The maximum of ethylene production was on day 3-4 (Figure 6A). In cv. Kra-Suay, a sharper maximum of both respiration rate and ethylene production was found on day 4 (Figure 6B).

1.3 Change in peel color of fruit after harvest

During ripening of sapodilla fruits, the peel color changed from light green to brown within 5 days after harvest (Figure 7). During color development of cv. Makok-Yai, the L* and b* values (Figure 8A and 8C) did not change, whereas these data in cv. Kra-Suay decreased slightly on day 5-6 (overripe stage). The increase in the a* value correlated well with color development in both cultivars (Figure 8B).

1.4 Change in total soluble solid and titratable acidity of fruit after harvest

During ripening of sapodilla fruits after harvested, total soluble solids (TSS) was quite stable in both cultivars, varied from 21 to 25% (Figure 9A). There was a decrease in titratable acidity (TA) and increase in TSS/TA ratio for both cultivars. Among the cultivars studied, the acidity (as malic acid) decreased from 0.3% (unripe fruits) to 0.1% (overripe fruits) and was higher in cv. Kra-Suay than those in cv. Makok-Yai. The ratio of TSS/TA in both cultivars ranged from 100 (unripe fruits) to 250 (overripe fruits) (Figure 9B and 9C).

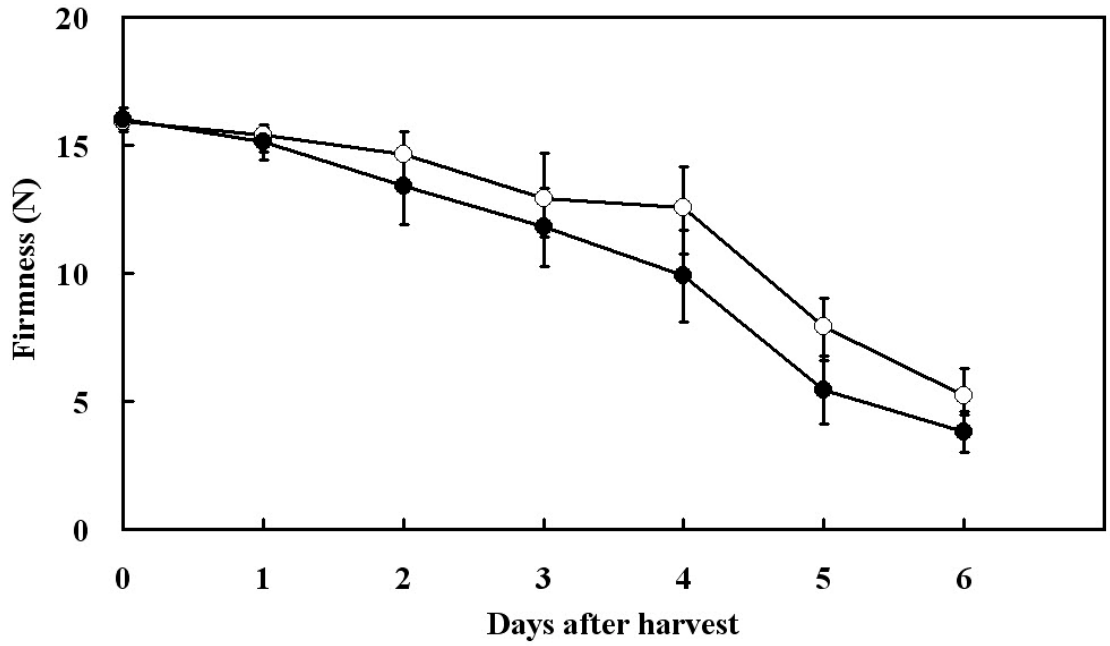

Figure 5 Changes in firmness of sapodilla fruits of cvv. Makok-Yai (○) and Kra-Suay (●) after harvest. Data are means ± SD (n = 20).

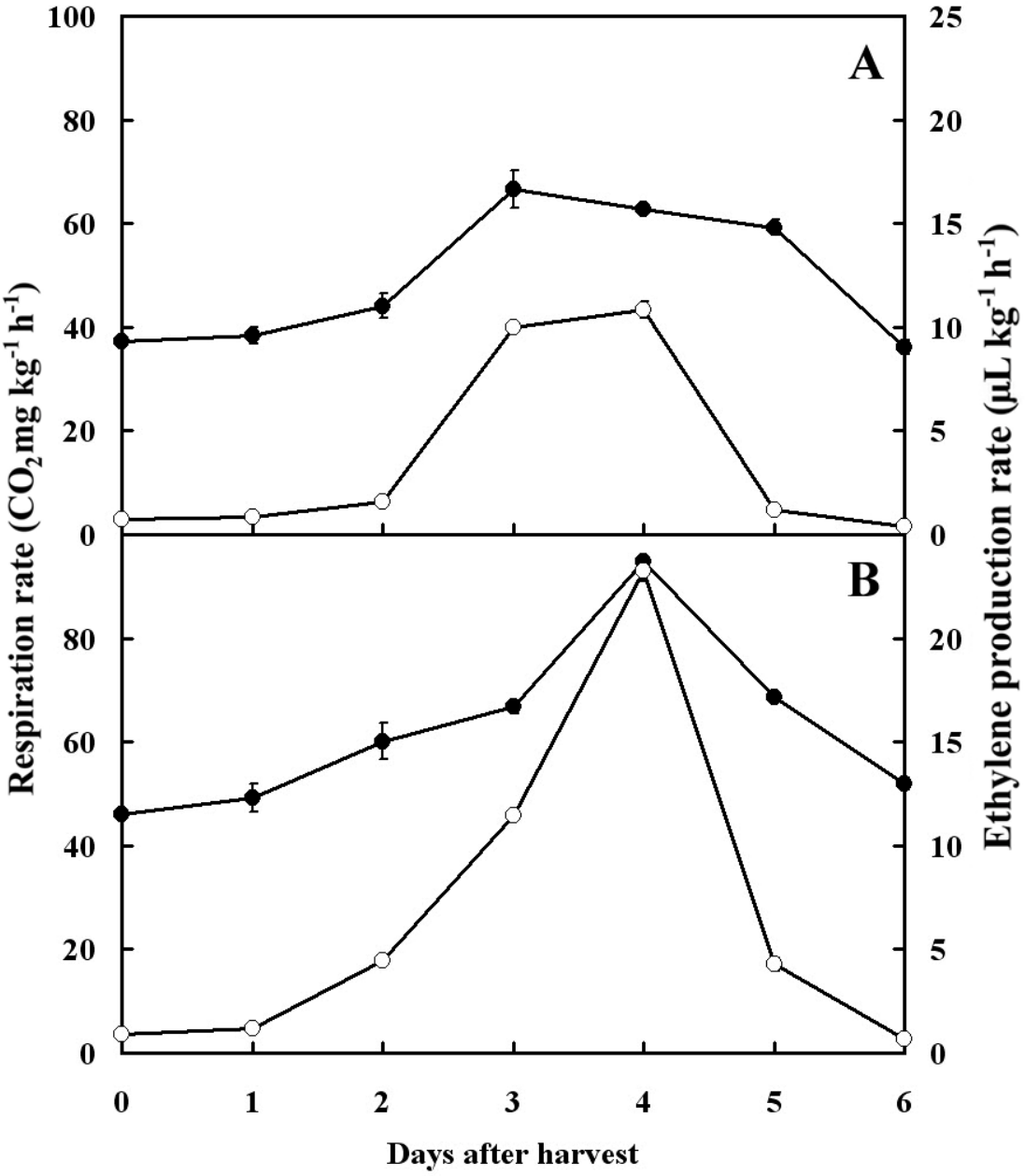

Figure 6 Changes in respiration rate (●) and ethylene production (○) of sapodilla fruits of (A) cvv. Makok-Yai and (B) Kra-Suay after harvest. Data are means ± SD (n = 6).

Figure 7 Changes in peel color of sapodilla fruits cvv. Makok-Yai (MY) and Kra-Suay (KS) after harvest.

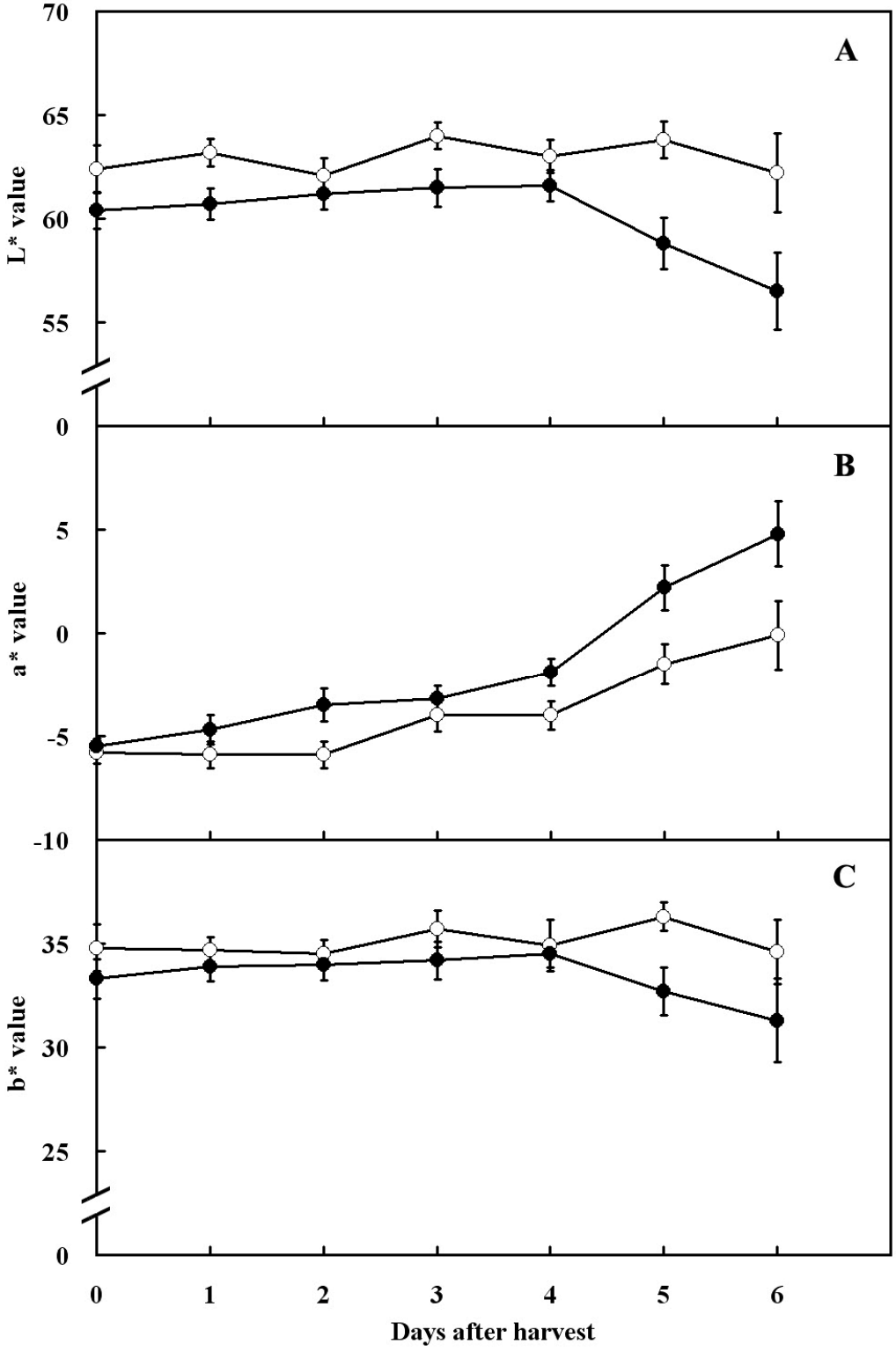

Figure 8 Changes in fruit peel color; L* value (A), a* value (B) and b* value (C) of sapodilla fruits cvv. Makok-Yai (○) and Kra-Suay (●) after harvest. Data are means ± SD (n = 20).

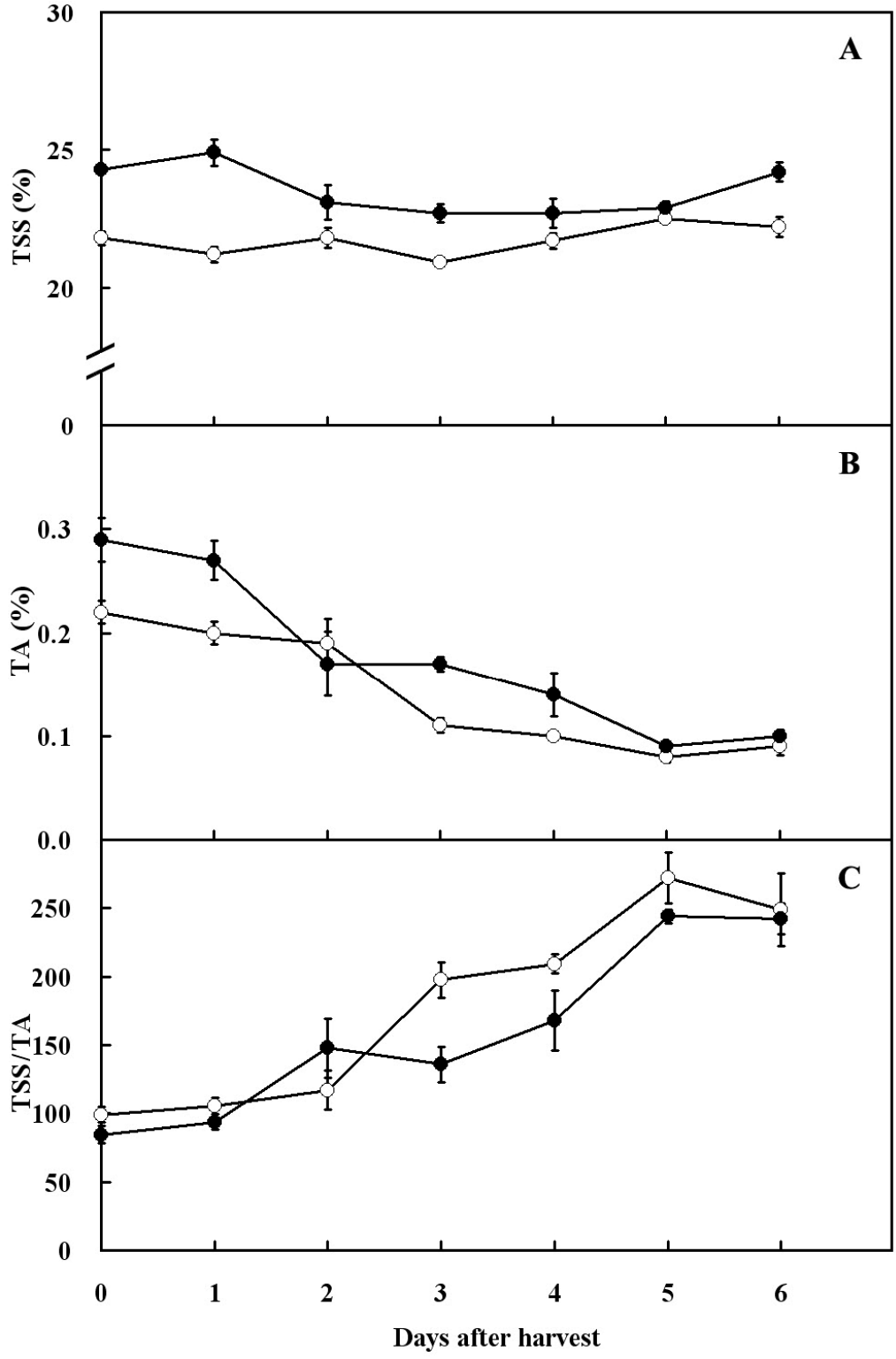

Figure 9 Changes in total soluble solids (TSS) (A), titratable acidity (TA) (B), and TSS/TA ratio (C) of sapodilla fruits cvv. Makok-Yai (○) and Kra-Suay (●) after harvest. Data are means ± SD (n = 4).

1.5 Isolation of genes encoding *MzEXP1, MzEXP2, MzEG, MzPG,* and *MzPL*

A partial coding sequence of *MzEXP1* (Table 4) was isolated from expanding fruits (16 weeks after anthesis), and a partial coding sequence of *MzEXP2* (Table 4) was isolated from ripening fruits (three days after harvest). A partial coding sequence of *MzEG* (Table 5) was isolated from mature fruits (32 weeks after anthesis), and a partial coding sequence of *MzPG* (Table 6), *MzPL* (Table 7), and *MzActin* (Table 8) were isolated from ripening fruits, three days after harvest.

MzEXP1 showed high homology, at the protein level, with expansins in many plant species, such as *Eucalyptus globulus, Vitis vinifera, Prunus persica, Capsicum annuum, Populus trichocarpa,* and *Malus hupehensis* whose were E-value 8e-77 to 3e-74 (Figure 10). It was also highly homologous with strawberry (*Fragaria x ananassa*) expansin 3 (accession AAK7274.1; e-value: 5e-74), and a tomato expansin precursor (called EXPA5; accession AAD13633.1; e-value: 2e-73). *MzEXP1* showed somewhat less but still high homology with some *Arabidopsis* expansins, for example with expansin A1 (accession NP_849869, expectation value: 4e-71). The partial sapodilla sequence *MzEXP1* showed homology with the predicted amino acids 45-204 of this 275 amino acid *Arabidopsis* protein. The predicted MzEXP1 sapodilla amino acid sequence contained the DPBB_1 conserved catalytic domain, followed by the conserved pollen allergy-1 (pollen_allerg_1) domain, which is also found in most expansins.

MzEXP2 also showed high homology with expansins in species in other families (Figure 11), such as *Sambucus nigra*, *Cicer arietinum*, *Litchi chinensis*, and *Gossypium hirsutum* (E-value 4e-67 to 5e-66). It was also homologous with *Arabidopsis* expansin A8 (accession NP_181593.1; expectation value: 2e-65). The partial sapodilla sequence *MzEXP2* showed homology with the predicted amino acids 46-175 of this 252 amino acid *Arabidopsis* protein. The predicted MzEXP2 sapodilla amino acid sequence contained the DPBB_1 conserved catalytic domain, which spanned most of the total length of sequence. It did not contain the pollen allergy-1 domain.

The isolated *MzEG* fragment showed high homology, at the protein level, with EGases in (the fruit of) several other species (Figure 12), such as *Glycine max, Fragaria x ananassa, Pyrus communis, Lycopersicon esculentum, Prunus persica, Gossypium hirsutum, Mangifera indica* and *Arabidopsis thaliana* (E-value: 3e-159, 5e-158, 1e-157, 2e-156, 6e-155, 4e-152, 4e-150, and 1e-154, respectively).

Similarly, the isolated *MzPG* fragment showed high homology, at the protein level, with PGs (in fruit) of other species (Figure 13), including *Actinidia deliciosa, Actinidia chinensis, Pyrus communis, Prunus persica, Capsicum annuum, Malus x domestica, Carica papaya, Citrus sinensis* and *Arabidopsis thaliana* (E-value: 8e-91, 7e-90, 1e-77, 1e-76, 2e-76, 6e-63, 3e-60, 4e-59, and 8e-64, respectively).

The isolated *MzPL* fragment also showed high homology, at the protein level, with PLs in (fruit of) other species (Figure 14), for examples *Fragaria x ananassa, Arabidopsis thaliana, Prunus persica, Fragaria chiloensis, Mangifera indica, Musa acuminata* AAA Group, *Prunus mume, Malus x domestica,* and *Vitis vinifera* (E-value: 7e-162, 5e-161, 7e-159, 5e-152, 2e-151, 9e-131, 2e-140, 2e-139, and 2e-132, respectively).

The isolated *MzActin* fragment also showed high homology, at the protein level, with Actins in other species (Figure 15), for examples *Cucumis sativus*, *Prunus salicina*, *Glycine max*, *Musa acuminata* x *Musa balbisiana* (ABB genome), and *Gossypium hirsutum* (E-value: 6e-96, 5e-95, 1e-94, 1e-94, and 3e-94, respectively).

Table 4 The partial nucleotide sequences of *MzEXP1* and *MzEXP2* of *Manilkara zapota* cDNA.

Name	Nucleotide sequences	Size (bp)	Genbank accession no.
MzEXP1	GGAGGTGCTTGTGGGTATGGAAACCTATACAGCCA AGGGTATGGGACAAACACAGCAGCATTGAGCACTG CACTATTCGACAATGGTTTGAGCTGCGGGTCTTGCT ATCAGATTAGGTGTGTGAACGATCCGCAGTGGTGCC TCCCCGGCGTCATTACCGTCACCGCCACCAACTTCT GCCCTCCCGGCGGTTGGTGCGACCCTCCAAACCTCC ACTTTGATCTCTCTCAGCCTGTCTTCCTCCACATTGC TCAATACAGGGCTGGGATTGTCCCCGTGGCTTTCAG AAGGGTACCCTGCAGGAGAAGGGGAGGTATTAGGT TCACCATCAATGGCCACTCCTACTTCAACCTTGTCCT TGTCACTAACGTTGGTGGTGCCGGGGATGTGCATAC TGTGGCGATCAAGGGGTCGAGAACAGCGTGGCAGC CAATGTCGAGGATCTGGGGCCAAAACTG	458	EU139436
MzEXP2	ACCATGGGGGGGGCATGTGGGTATGGAAACTTGTA CAGCCAAGGGTATGGGACAAATACTGCAGCACTCA GCACTGCTCTGTTCAACAATGGGCTGAGCTGTGGCT CATGCTATGAGATCAAGTGTGCAGACGACCCCAAA TGGTGCCTCCCGGGAACCATCACCGTCACTGCCACA AACTTCTGCCCCCCTAACCCTTCTCTGGCCAATGAC AATGGTGGTTGGTGCAACCCTCCCCTCCAGCACTTT GATCTTGCAGAGCCCGCTTTCTTGCAAATCGCCCAA TACAAAGCTGGGATTGTCCCTGTTACTTTCACGAGA GCGCCCTGCGCGAAGAAAGGAGGCATAAGGTTCAC AATCAATGGCCACTCTTACTTCAACCTGGTTCTGAT	392	EU251387

Table 5 The partial nucleotide sequences of *MzEG* of *Manilkara zapota* cDNA.

Name	Nucleotide sequences	Size (bp)	Genbank accession no.
MzEG	TATTACGATGCTGGTGATAACGTGAAGTTTGGGCTA CCCATGGCATTCACAGTGACGATGATGTCGTGGAGC ATAGTAGAGTATGGGAGGCAAATGGCTGCCAGTGG AGAGCTTGGTCATGCCATGGATGCTGTTAAGTGGGG GACTGATTATCTCCTTAAAGCTCACCCCTCTCCCAA TGTCTTCTATGGAGAGGTGGGGGATGGGAATACTG ATCACTACTGCTGGCAAAGGCCGGAGGATATGACG ACCCCACGGCAAGCCTACAAGATCGACCCCAACAA TCCTGAATCCGACCTCGCCGGGGAGAGCGCCGCCG CAATGGCCGCCGCCTCCATCGTCTTCCACCGCTACA ACCCTTCCTACGCTAGGAAGCTCCTCGCCCATGCCC AGCAGCTATTCGGCTTTGCAGATAAATACAGGGGC AAATACGACAGCAGCATCACGGTGGCTCAGAAGTA CTACCGATCCATCAGTGGATACGCCGATGAGTTGTT GTGGGCTGCAGCCTGGCTCTACAAAGCAACTGACA GCGAGTATTACTTGAGCTATCTGGGTAGGAATGGCG TTGCTCTGGGTGGGACCGGTTGGGCGATGACAGAGT TCGGGTGGGATGTTAAGTATGCCGGTGTGCAGACTC TTGTTGCCAAGATTCTAATGGGAGGCAAAGCCAGTC ACCATGCGCCAGTCTTCCAGGGGTACCAGCAGAAG GCAGAGTTTTTCATGTGTTCGTGCCTGGGGAAGGGC ACTCGGAATGTACGGAAGACTCCAGGAGGCCTTATT TTCCGACAGAGATGGAACAACATGCAGTTTGTTACC AGCGCTTCTTTCCTCCTCACTGTCTACTCCGACTACC TGACCACAGCGAGGAGAAACCTGAATTATGCTTCTG GCAGTGTCTCTCCATCTCAGATTCTTTCACTTGCAAA ATCTCAGGTGGACTATATTCTTGGCGACAACCCAAG AGCTATGAGATACATGGTAGGATT	989	EU819555

Table 6 The partial nucleotide sequences of *MzPG* of *Manilkara zapota* cDNA.

Name	Nucleotide sequences	Size (bp)	Genbank accession no.
MzPG	TTTGGAGCTAGAGGTGATGGACGAAGTGACGACAC TGAGGCATTTAAGAATGCATGGAAGAAAGCTTGTT CATCTACTGGTGCTGTTCTTTTGGTACCACAGAAGA ACTATCTTGTTAAACCAATCAGATTCTCAGGCCCTT GCAAATCTGATCTAACAGTGCAGGTTTATGGAACA ATTGACGCATCTGATAATCGAGCTGACTACAACGA AGATCAGAGGCATTGGCTTGTTTTTGATGGCGTTGA GAATTTAATGGTTGAGGGTGGTGGAACCATCAATG GAAATGGGGTGATATGGTGGAAAAATTCTTGCAAG ATCAATAAAGCTCTTCCTTGCAAGGACGCGCCAACG GCATTAACATTCTATAACTGCGAGAACTTGGTAGTA AGGAATTTGAAGATCCAAAACGCACAGCAAATTCA TGTTTCATTCGAGGAATGTGTGAATGTTCAGGCATC CAATCTGGTGGTAACTGCACCAAAGAAGAGCCCCA ACACTGATGGGATTCATGTCGCACGCACCCGAAAC ATCCAGATTTCCAGCTCTGTTATAGGAACAGGTGAT GATTGTATTTCAATTGCAAGTGGATCTCAGAAGGTG CAAGCCACGGATATAACCTGTGGTCCAGGCC	634	EU139437

Table 7 The partial nucleotide sequences of *MzPL* of *Manilkara zapota* cDNA.

Name	Nucleotide sequences	Size (bp)	Genbank accession no.
MzPL	TTGTTGGCGGTGTGACCCTAACTGGCACCGCAACCG CAAGCGGCTTGCTGACTGCGGCATTGGTTTTGGCCG GAACGCTATTGGAGGCCGCGATGGCCGCTTCTATGT TGTCACCGACCCAAGTGACGATGACCCTGTTAACCC ACGACCGGGCACTCTGCGCCATGCTGTCATTCAGGA CAGACCTCTCTGGATTGTGTTCAAGCGTGACATGGT GATACAATTGAAGCAGGAGCTCATCATGAACAGCT TCAAAACCATTGACGGTCGCGGGGCCAATGTCCAT ATTGCCAACGGAGGTTGCATTACAATCCAGTTTGTA ACCAATGTCATAATTCATGGTCTTCACATCCATGAC TGCAAACCCACTGGGAATGCCATGGTGAGGAGCTC GCATACACATTTTGGTTGGCGGACAATGGCTGATGG TGATGCCATCTCCATTTTTGGCTCGAGCCACATATG GGTTGATCACAATTCGCTTTCTAAATGTGCTGATGG CCTTGTTGATGCTGTCATGGGTTCAACTTCCCTTTCA ATTTCCAACAACCACCTCACCCACCACAATGAGGTG ATGCTGTTGGGTCATAGCGATTCCTATGTTAGAGAC AAGCAGATGCAAGTCACTATCGCCTACAACCATTTT GGGGAGGGTCTTATCCAGAGAATGCCAAGGTGCAG GCATGGGTATTTTCATGTGGTAAATAATGACTACAC TCACTGGGAAATGTATGCCATTGGTGGAAGTGCTGG TCCGACCATCAACAGCCAGGGTAACAGATACCTTG CTCCAACCAATGCATTCGCAAAGGAGGTCACAAAA AGAGTGGACGTAGCTACAAGTGAATGGCATGGTTG GAACTGGCGGTCTGAGGG	876	EU819554

Table 8 The partial nucleotide sequences of *MzActin* of *Manilkara zapota* cDNA.

Name	Nucleotide sequences	Size (bp)	Genbank accession no.
MzActin	ATGTTTGAGACCTTCAATGTACCTGCTATGTATGTT GCCATACAGGCTGTTCTTTCTCTATATGCCAGTGGT CGTACAACTGGTATTGTTCTTGACTCTGGAGATGGT GTCAGCCATACAGTCCCAATCTATGAAGGGTATGCG CTTCCACATGCTATTTTGCGTCTAGACCTTGCAGGTC GTGACCTCACGGATGCGCTGATGAAAATCCTTACAG AGCGTGGCTACTCATTTACCACCACCGCTGAACGTG AAATTGTGAGGGACATGAAGGAGAAGTTGGCGTAC ATTGCCCTTGATTACGAGCAGGAACTAGAAACTGCC AAGACAAGCTCTTCTGTTGAGGAGAGCTTTGAGTTA CCGGATGGGCAGGTAATAACCATTGGAGCTGAGCG TTTCCGATGTCCTGAAGTCCTTTTCCAGCCATCCATG ATCGGAATGGAATCTGCTGGAATTCATGAAACGAC ATACAACTCTATAATGAAGTGTGATGTCGATATCAG GAAGGATCTCTATGGAAAC	522	EU251386

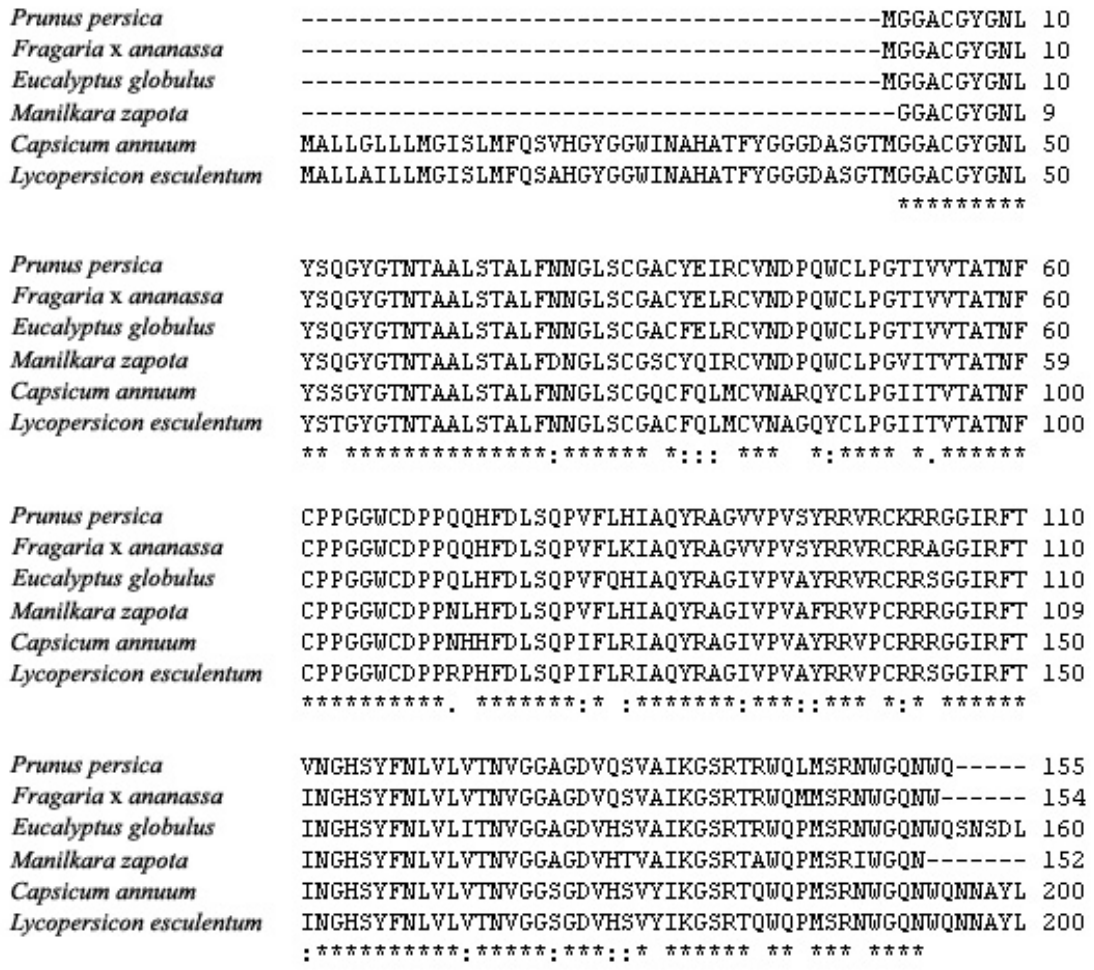

Figure 10 Alignment of partial deduced amino acid sequence of *MzEXP1* cDNA fragment with other plants. The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means the conserved substitutions have been observed. The dot (.) means that semi-conserved substitutions are observed.

Figure 11 Alignment of partial deduced amino acid sequence of *MzEXP2* cDNA fragment with other plants. The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means the conserved substitutions have been observed. The dot (.) means that semi-conserved substitutions are observed.

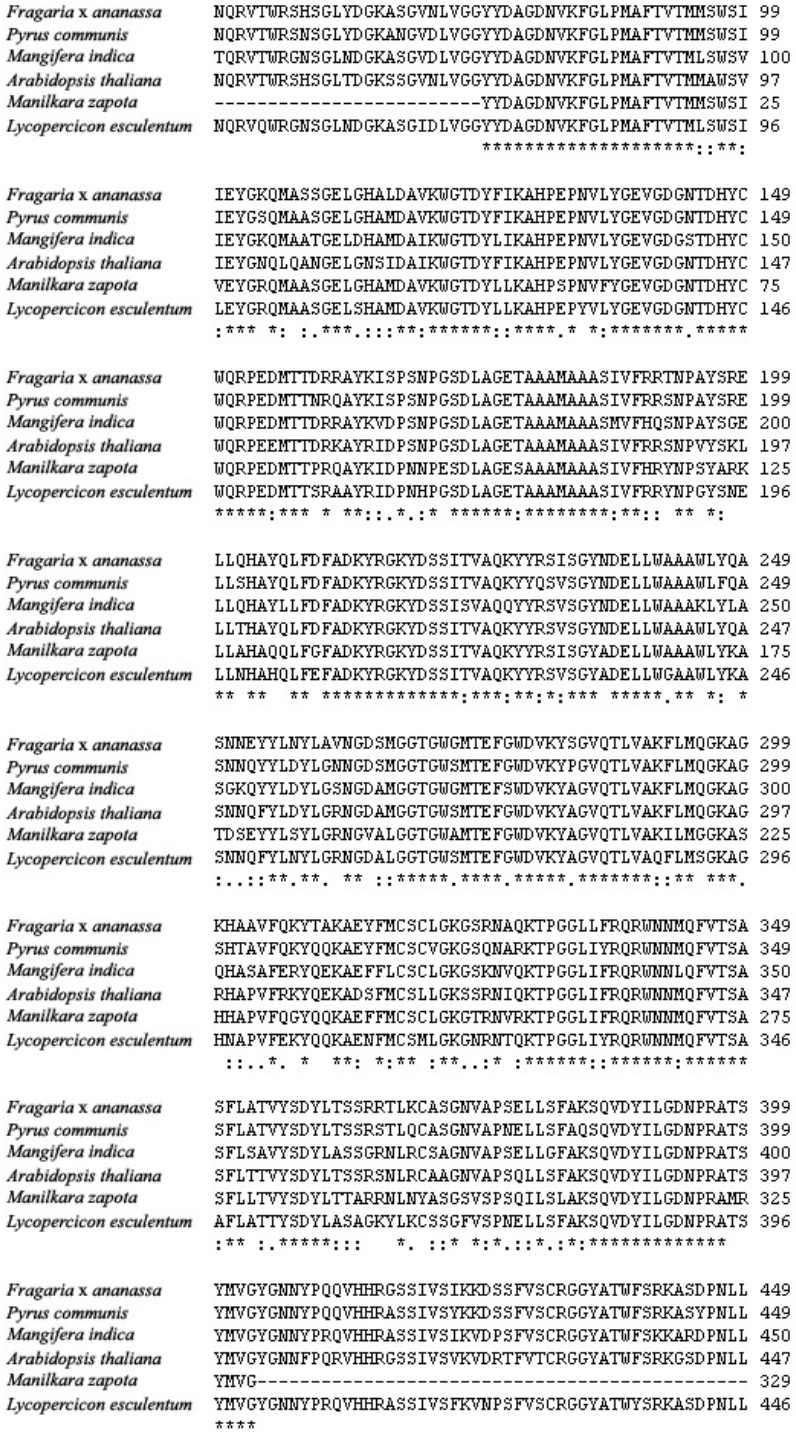

Figure 12 Alignment of partial deduced amino acid sequence of *MzEG* cDNA fragment with other plants. The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means the conserved substitutions have been observed. The dot (.) means that semi-conserved substitutions are observed.

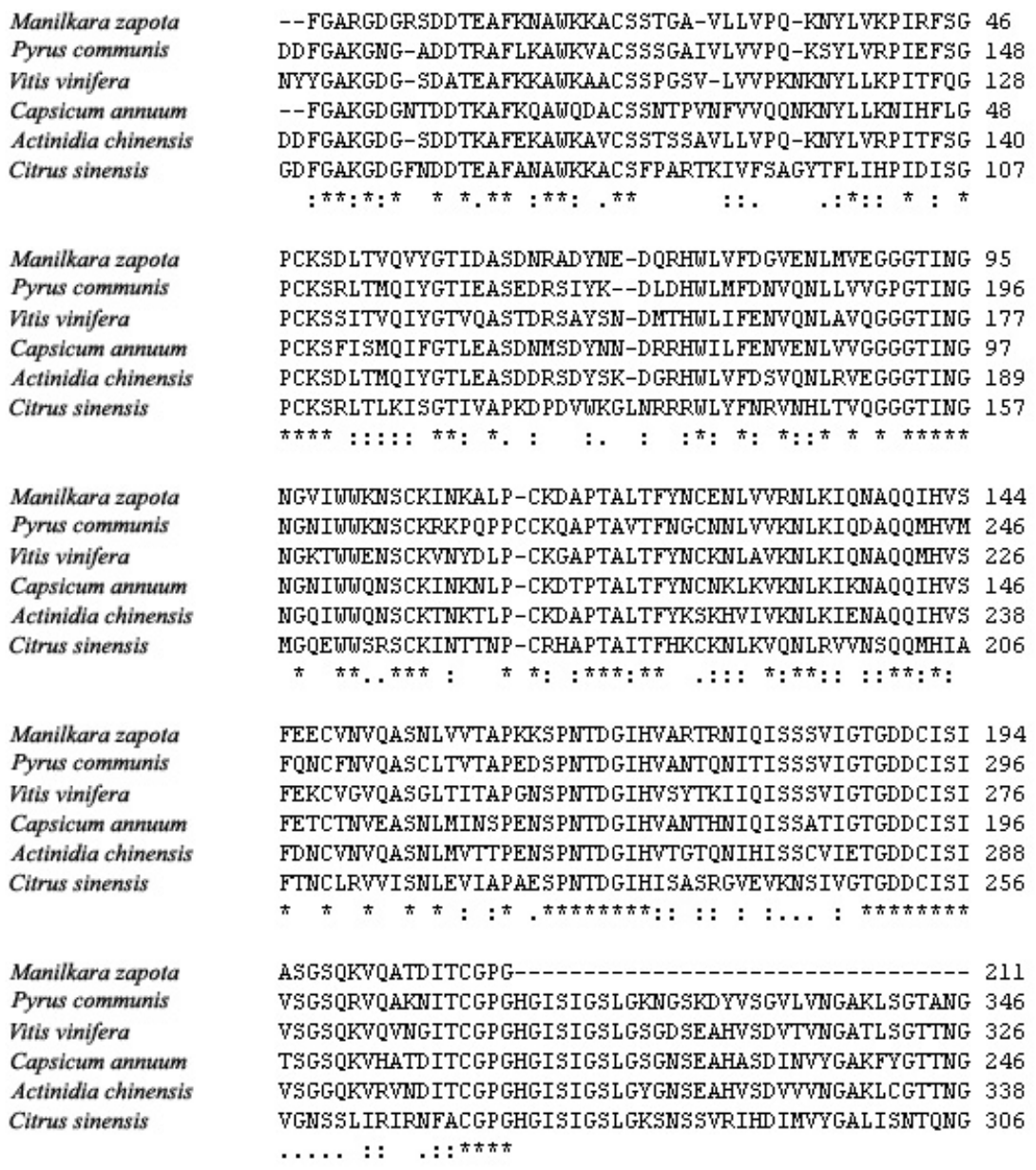

Figure 13 Alignment of partial deduced amino acid sequence of *MzPG* cDNA fragment with other plants. The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means the conserved substitutions have been observed. The dot (.) means that semi-conserved substitutions are observed.

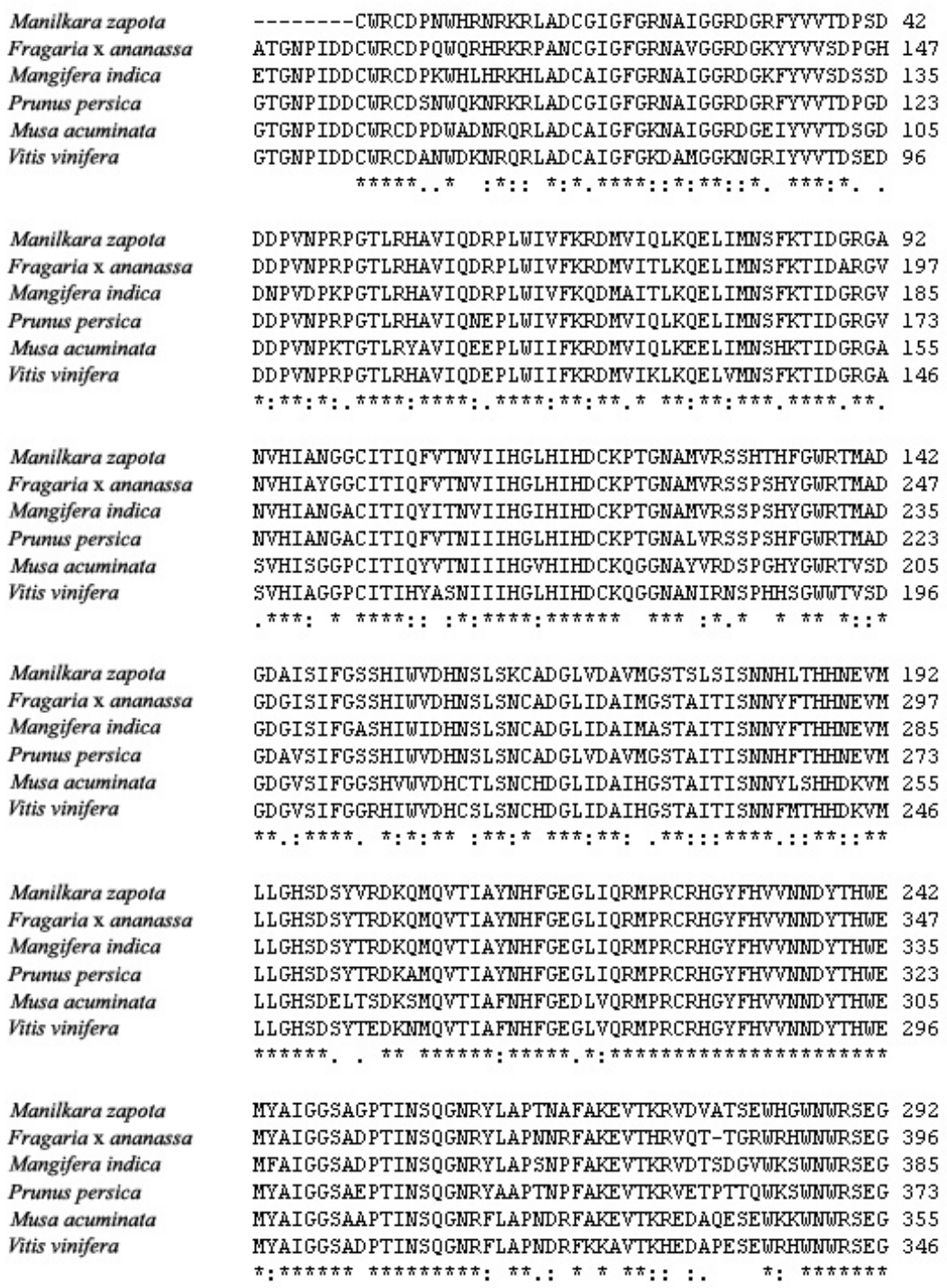

Figure 14 Alignment of partial deduced amino acid sequence of *MzPL* cDNA fragment with other plants. The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means the conserved substitutions have been observed. The dot (.) means that semi-conserved substitutions are observed.

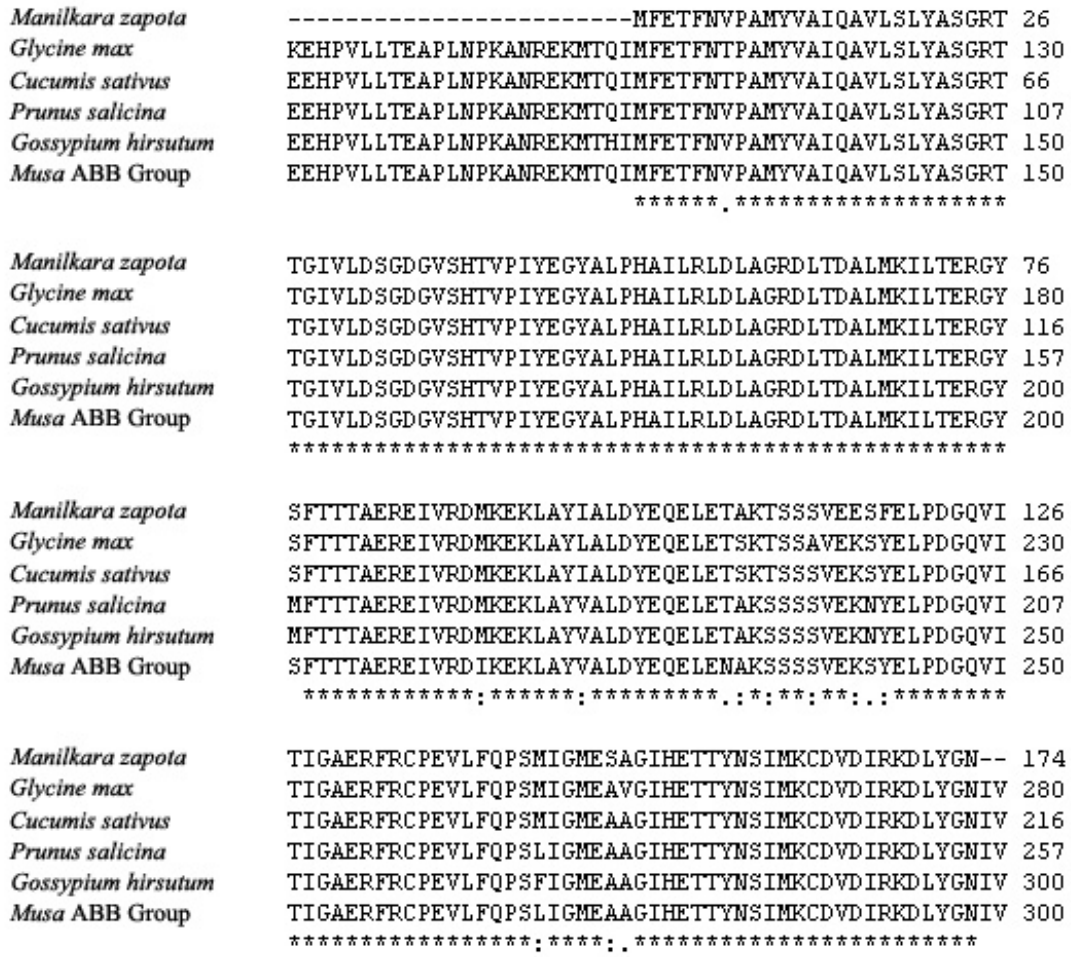

Figure 15 Alignment of partial deduced amino acid sequence of *MzActin* cDNA fragment with other plants. The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means the conserved substitutions have been observed. The dot (.) means that semi-conserved substitutions are observed.

1.6 mRNA accumulation during fruit growth and after harvest

The mRNA of *MzEXP1*, *MzEXP2*, *MzEG*, *MzPL*, and *MzPG* were detectable in fruits when using gel blot analysis. *MzEXP1* expressed during the early stage of growth. The expression pattern was very similar in both cultivars tested (Figure 16 and 17). The expression profile of *MzEXP2* was different in both cultivars investigated. In cv. Makok-Yai a very low transcript abundance was observed in mature fruits at 32 weeks after anthesis. Much higher abundance was found during the first four days after harvest while day 5 after harvest the transcript level was no longer detectable (Figure 16). In cv. Kra-Suay a low abundance was found at week 24 after anthesis. The transcript abundance showed some apparent variation during the subsequent weeks, and was high just before and after harvest. In cv. Kra-Suay *MzEXP2* mRNA was no longer detectable on day 4 and 5 (Figure 17). *MzEXP1* and *MzEXP2* genes were also expressed in opening flowers of both sapodilla cultivars studied. Furthermore, *MzEXP2* was detectable in expanding leaves of both cultivars, whereas *MzEXP1* was not detectable in expanding leaves (Figure 18).

The expression pattern of the isolated *MzEG* was similar in both cultivars. Transcript of the isolated *MzEG* expressed during the early stage of fruit growth as well as in mature (unripe) fruits (Figure 16 and 17). It was not detectable in mature fruits after harvest. The transcript level of *MzPG* was high during the later stage of fruit ripening, in both cultivars (Figure 16 and 17). The transcript of the isolated *MzPL* was not detectable in developing fruits. After harvest, *MzPL* mRNA accumulated during the early stage of ripening. It became undetectable in overripe fruits of both cultivars (Figure 16 and 17).

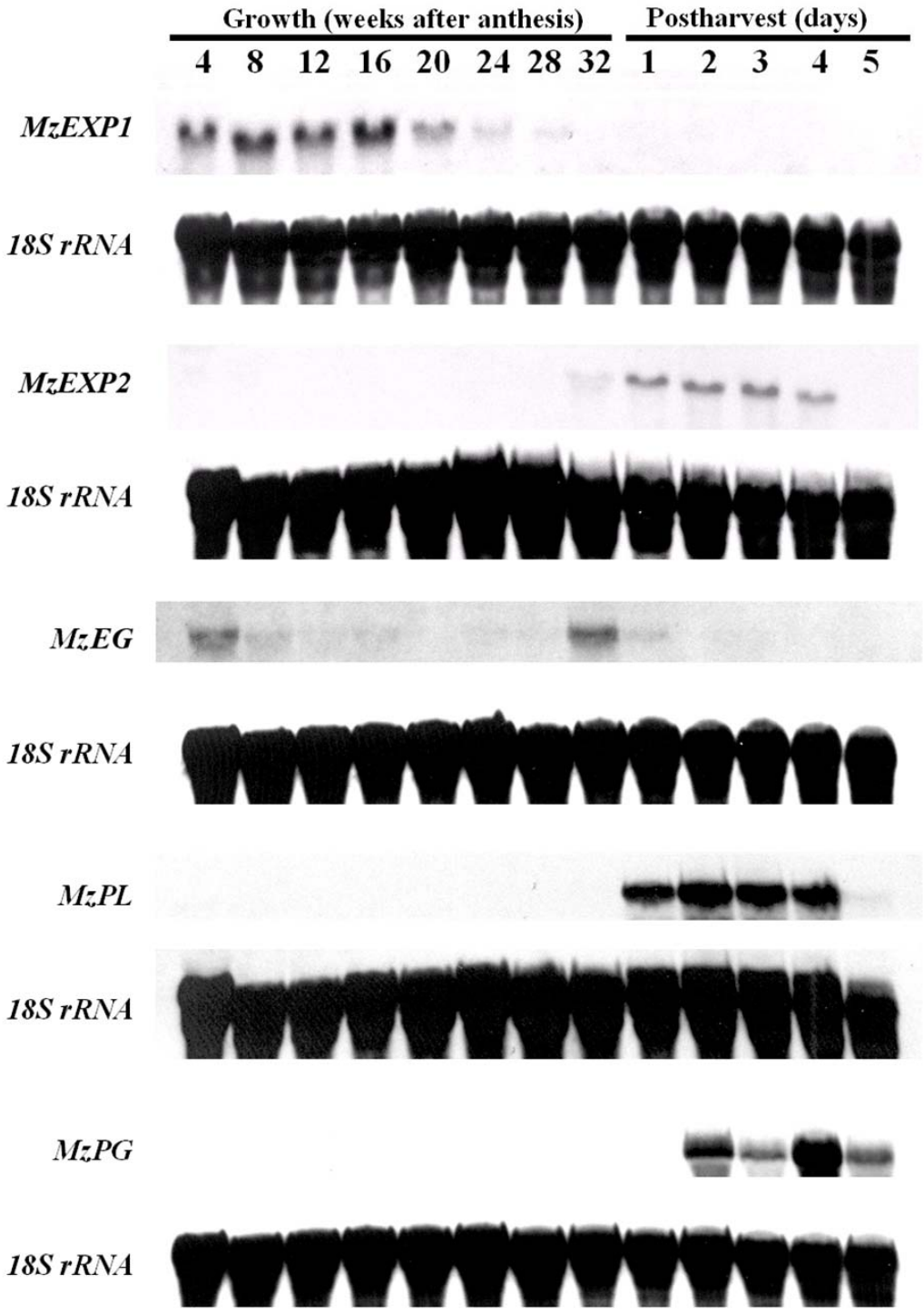

Figure 16 RNA gel blot analysis of *MzEXP1*, *MzEXP2, MzEG, MzPL* and *MzPG* mRNA abundance in sapodilla (*Manilkara zapota*) fruits cv. Makok-Yai during growth after anthesis until fruit maturity and in fruits after harvest.

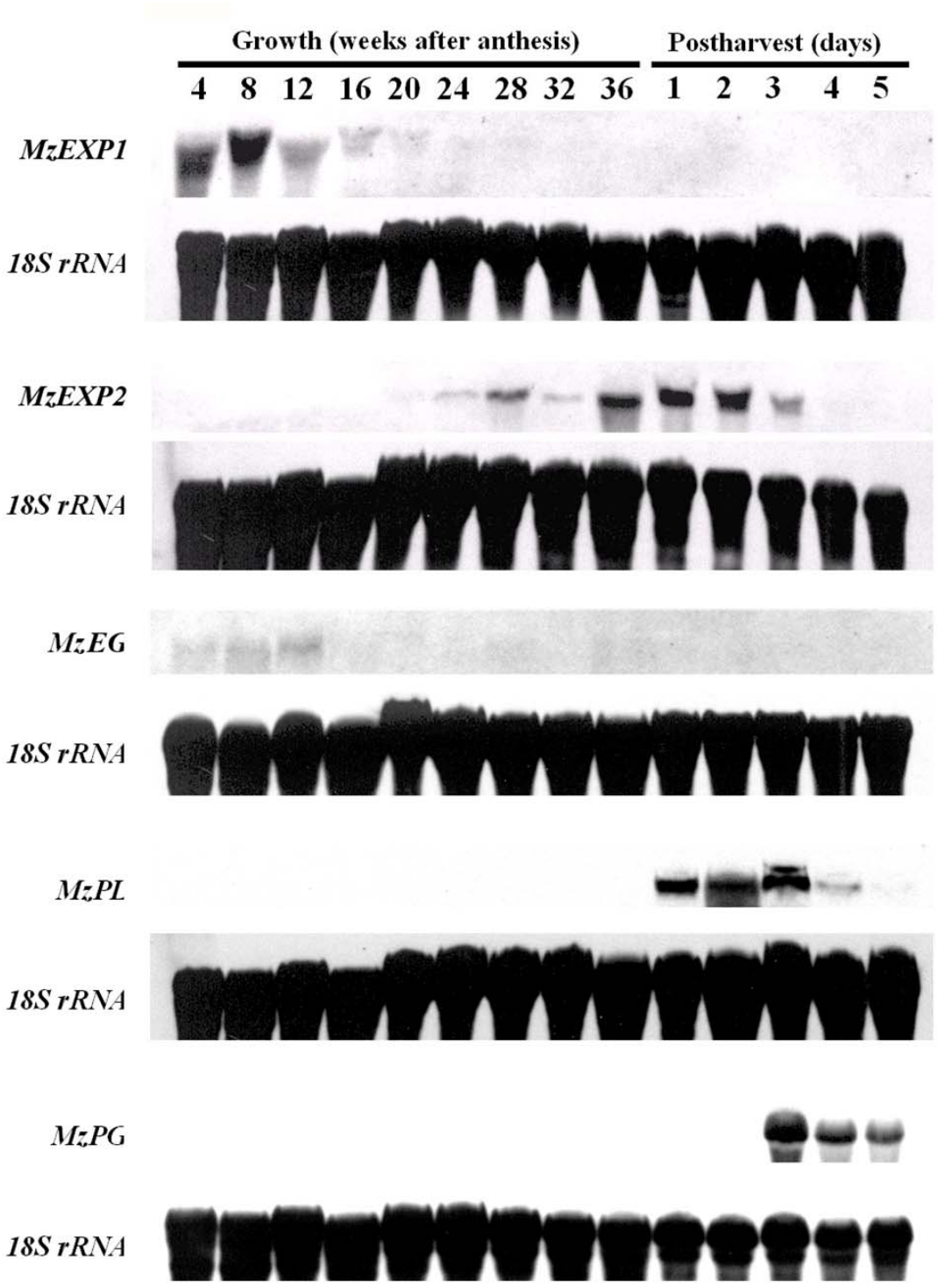

Figure 17 RNA gel blot analysis of *MzEXP1*, *MzEXP2, MzEG, MzPL* and *MzPG* mRNA abundance in sapodilla (*Manilkara zapota*) fruits cv. Kra-Suay during growth after anthesis until fruit maturity and in fruits after harvest.

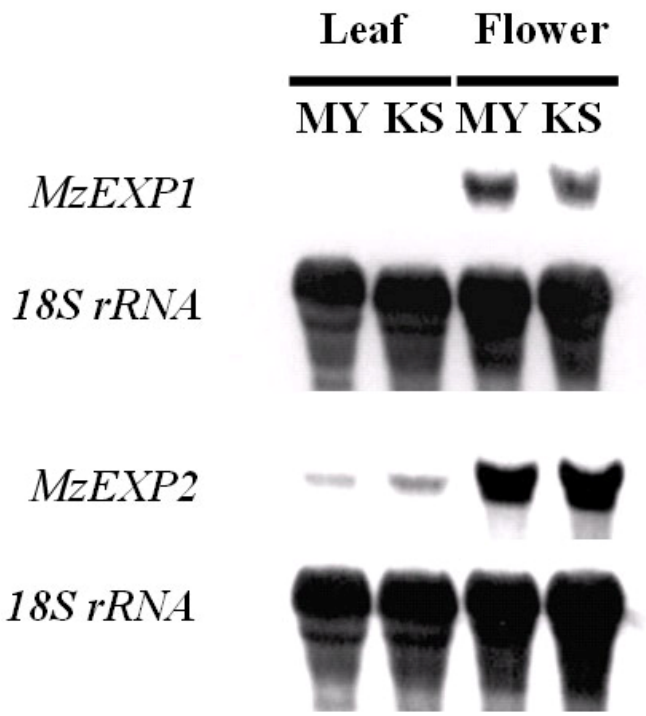

Figure 18 RNA gel blot of *MzEXP1* and *MzEXP2* mRNA abundance in expanding leaf (Leaf) and opening flowers (Flower) of sapodilla cvv. Makok-Yai (MY) and Kra-Suay (KS).

Experiment II. Physiological changes, cell wall enzyme activity and gene expression of cell wall enzyme during fruit ripening

2.1 Change in fruit firmness

From day 1 after harvest firmness of the peel and flesh of control fruits steadily decreased (Figure 19). The fruits were ripe (firmness about 10 N) on day 4 and overripe (firmness about 5 N) on day 5. Treatment with ethylene for 20 h immediately after harvest resulted in a drastic decrease in firmness, already during the period of ethylene application (day 0-1 in Figure 18). On day 2 the fruits had about the same firmness as overripe in control fruits. Treatment with 1-MCP prevented the decrease in fruit firmness. By day 5 no softening had occurred, in both cultivars (Figure 19). Ethylene, applied after the 1-MCP treatment, did not reduce the effectiveness of 1-MCP, at least until day 6 after harvest (Figure 19, Appendix Table 2 and 3).

2.2 Change in fruit peel color

The color of sapodilla fruits changed from light green to brown after harvest as the control fruits ripened and ethylene treatment, while 1-MCP treatment and ethylene applied after the 1-MCP treatment maintained the light green color (Figure 20). The control group, L* and b* values (Figure 21 and 23) did not change. Treatment with 1-MCP and ethylene applied after the 1-MCP treatment, showed the same pattern of L* and b* values as the control fruits. Treatment with ethylene for 20 h immediately after harvest resulted in a decrease in L* and b* values on day 4-5 (overripe fruits) (Appendix Table 4, 5, 8, and 9). In both cultivars, the increase in the a* value correlated well with color development in control fruits and ethylene treatment during ripening. Treatment with 1-MCP and ethylene applied after the 1-MCP treatment did not show the increase in a* value (Figure 22, Appendix Table 6 and 7).

2.3 Change in total soluble solid and titratable acidity of fruit

When fruit ripening occurred in control and ethylene treatment, total soluble solids (TSS) and titratable acidity (TA) content decreased (Figure 24 and 25) whilst TSS/TA ratio of control fruits increased sharply with fruit ripening (Figure 26). Treatment with ethylene immediately after harvest resulted in a drastic increase level in TSS/TA ratio throughout the experiment (Figure 26). TSS content of fruits with 1-MCP treatment and ethylene applied after 1-MCP treatment slightly increased (Figure 24). These treatments maintained high TA content (0.2% for cv. Makok-Yai and 0.4% for cv. Kra-Suay) (Figure 25). There was no change for TSS/TA ratio in both cultivars, 1-MCP treatment and ethylene applied after the 1-MCP treatment maintained the low TSS/TA (about 100) throughout the experiment period (Figure 26, Appendix Table 10, 11, 12, 13, 14, and 15).

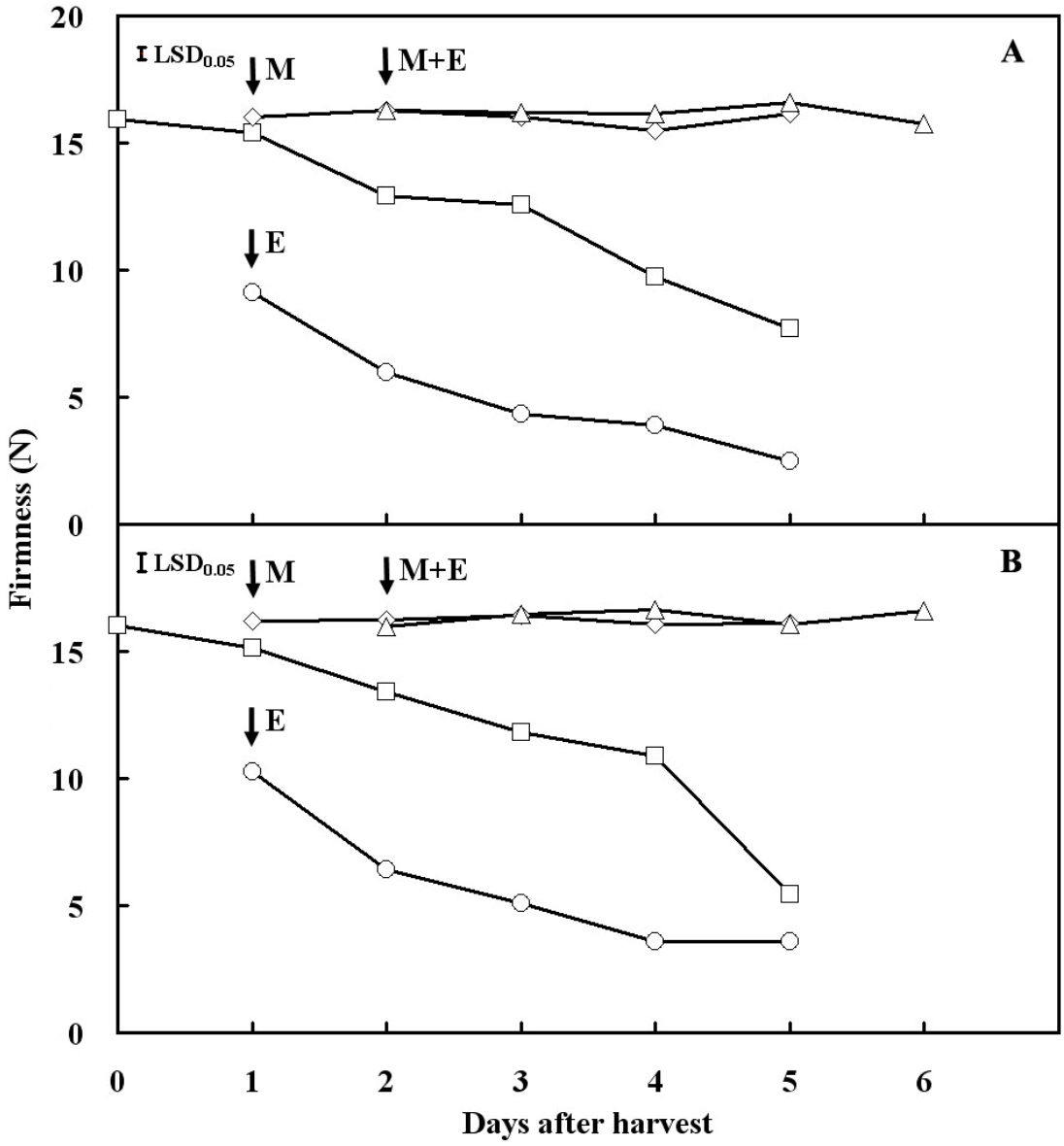

Figure 19 Changes in firmness of sapodilla fruits during ripening after harvest as a control fruits (□) and fruits treated with ethylene (○),1-MCP (◇) and 1-MCP + ethylene (△). Arrows indicate the day 0 after ethylene (E), 1-MCP (M), and 1-MCP + ethylene (M+E) treatment. Control fruits were kept for 5 days without treatment and treated fruits were kept for 4 days after treatment for cvv. Makok-Yai (A) and Kra-Suay (B). Data were the average of 20 fruits. Differences between means were analysed using the Least Significant Difference (LSD) at the 95% significance level.

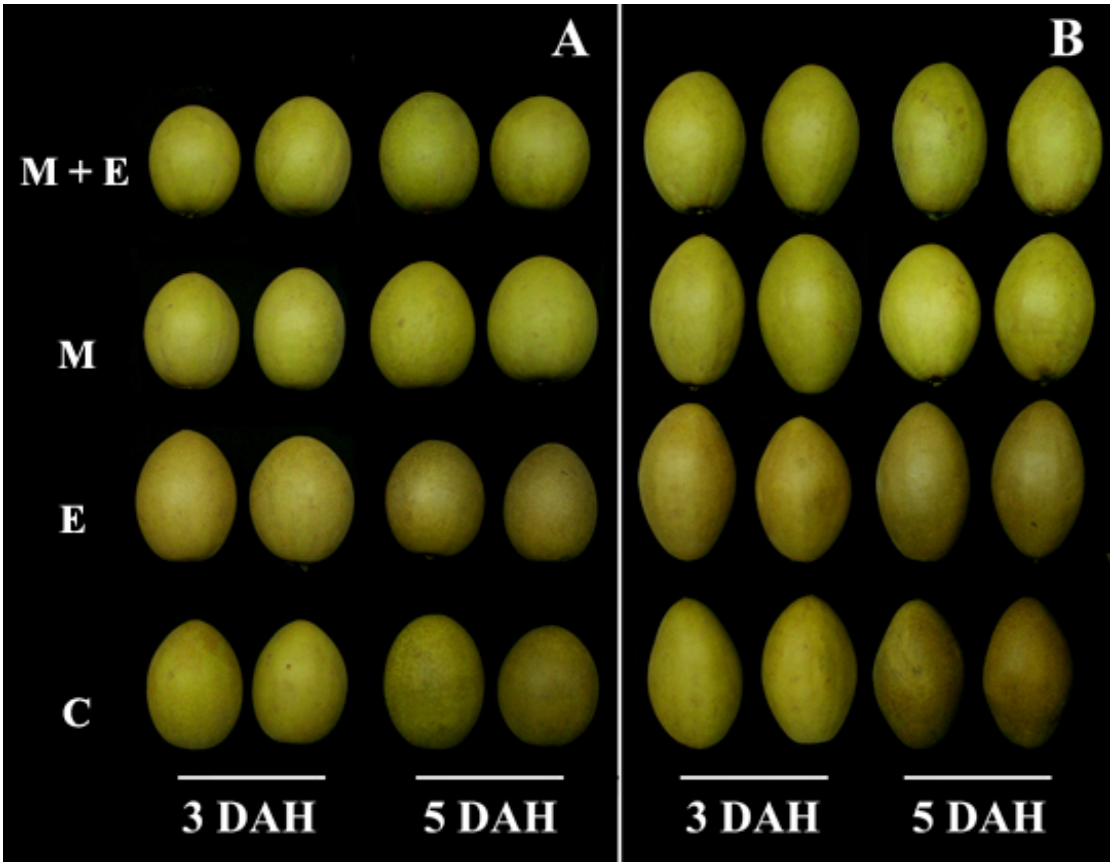

Figure 20 Changes in fruit peel color during ripening after harvest as a control fruits (C) and fruits treated with ethylene (E), 1-MCP (M) and 1-MCP + ethylene (M+E) in cvv. Makok-Yai (A) and Kra-Suay (B).

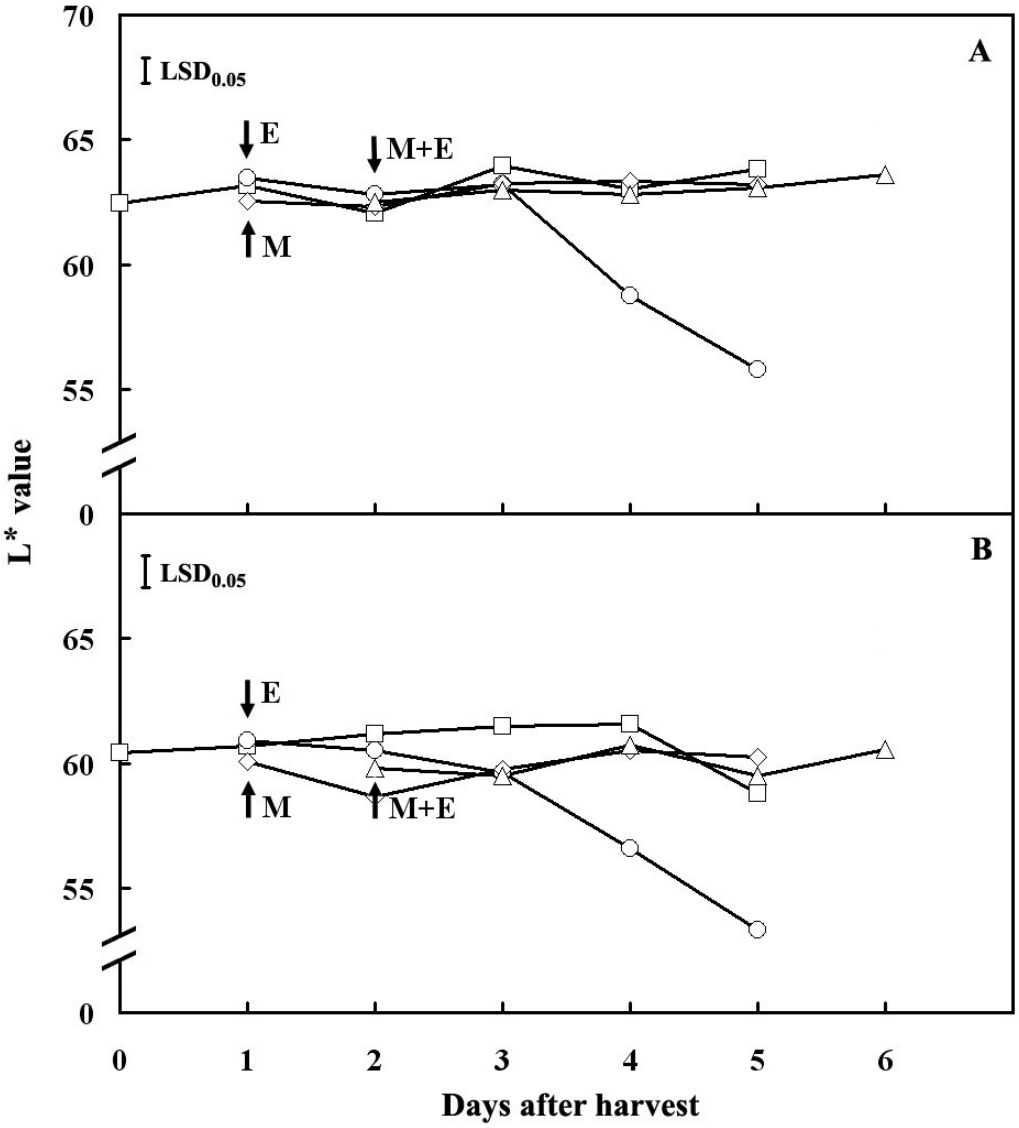

Figure 21 Changes in L* value of sapodilla fruit peel color during ripening after harvest as a control fruits (□) and fruits treated with ethylene (○),1-MCP (◇) and 1-MCP + ethylene (△). Arrows indicate the day 0 after ethylene (E), 1-MCP (M), and 1-MCP + ethylene (M+E) treatment. Control fruits were kept for 5 days without treatment and treated fruits were kept for 4 days after treatment for cvv. Makok-Yai (A) and Kra-Suay (B). Data were the average of 20 fruits. Differences between means were analysed using the Least Significant Difference (LSD) at the 95% significance level.

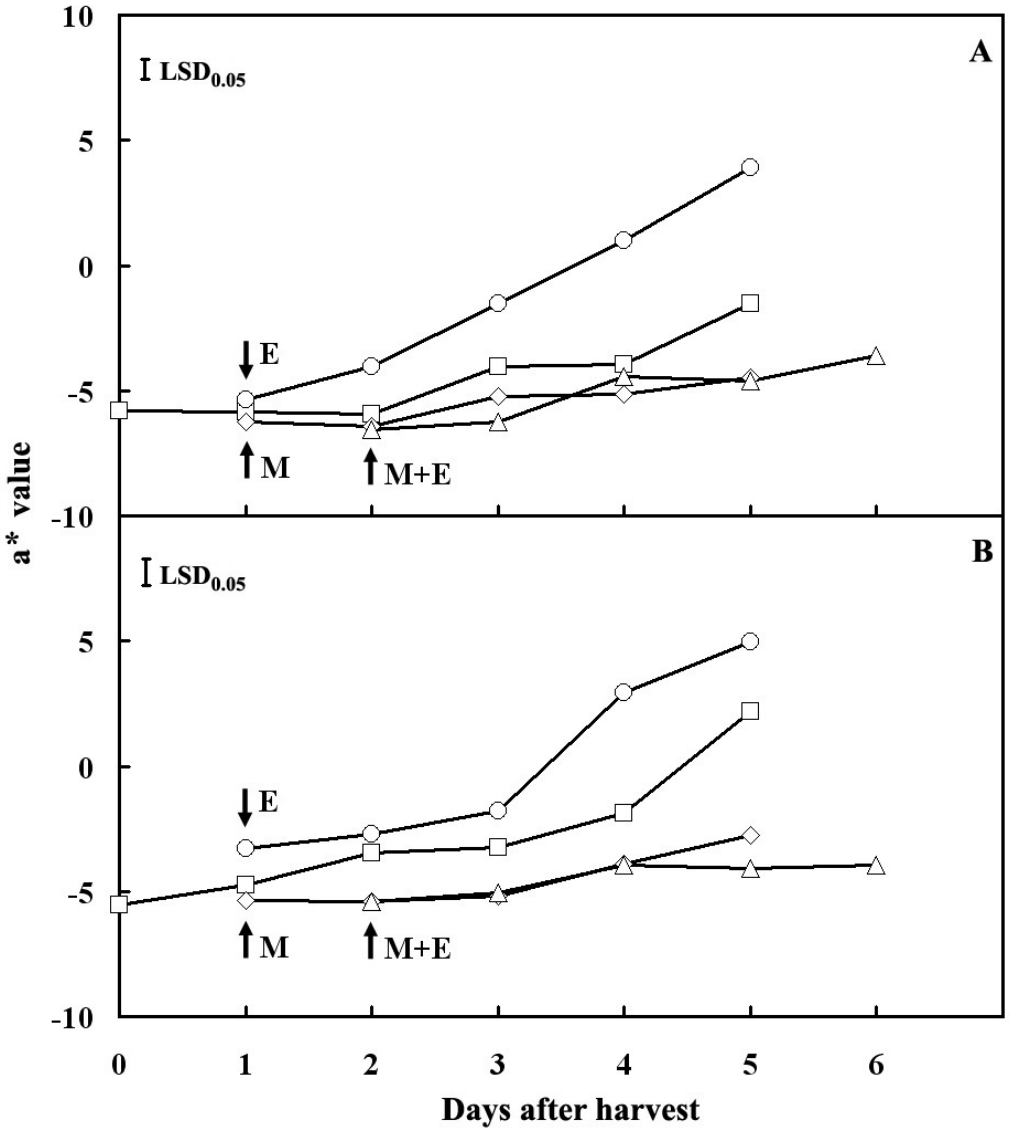

Figure 22 Changes in a* value of sapodilla fruit peel color during ripening after harvest as a control fruits (□) and fruits treated with ethylene (○),1-MCP (◇) and 1-MCP + ethylene (△). Arrows indicate the day 0 after ethylene (E), 1-MCP (M), and 1-MCP + ethylene (M+E) treatment. Control fruits were kept for 5 days without treatment and treated fruits were kept for 4 days after treatment for cvv. Makok-Yai (A) and Kra-Suay (B). Data were the average of 20 fruits. Differences between means were analysed using the Least Significant Difference (LSD) at the 95% significance level.

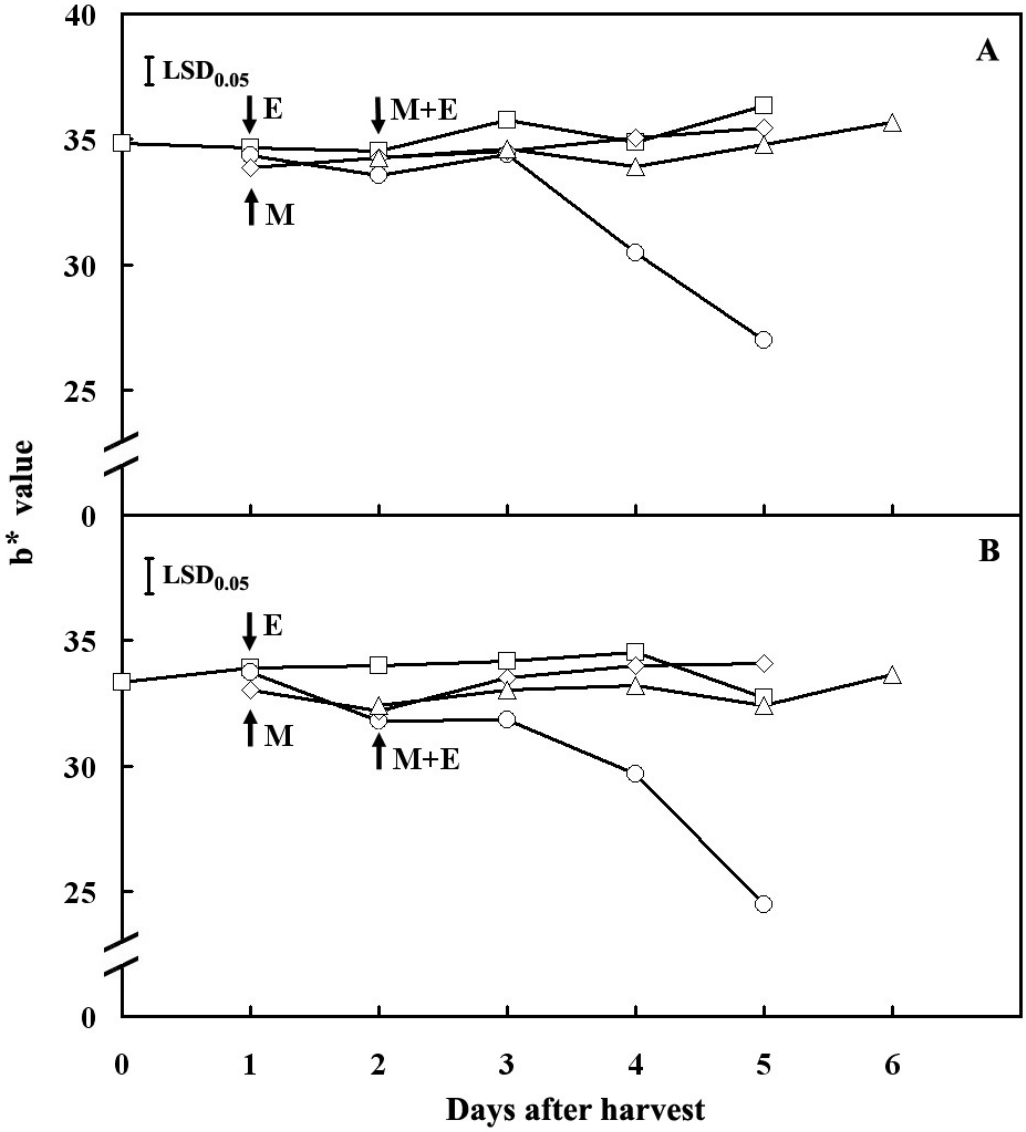

Figure 23 Changes in b* value of sapodilla fruit peel color during ripening after harvest as a control fruits (□) and fruits treated with ethylene (○),1-MCP (◇) and 1-MCP + ethylene (△). Arrows indicate the day 0 after ethylene (E), 1-MCP (M), and 1-MCP + ethylene (M+E) treatment. Control fruits were kept for 5 days without treatment and treated fruits were kept for 4 days after treatment for cvv. Makok-Yai (A) and Kra-Suay (B). Data were the average of 20 fruits. Differences between means were analysed using the Least Significant Difference (LSD) at the 95% significance level.

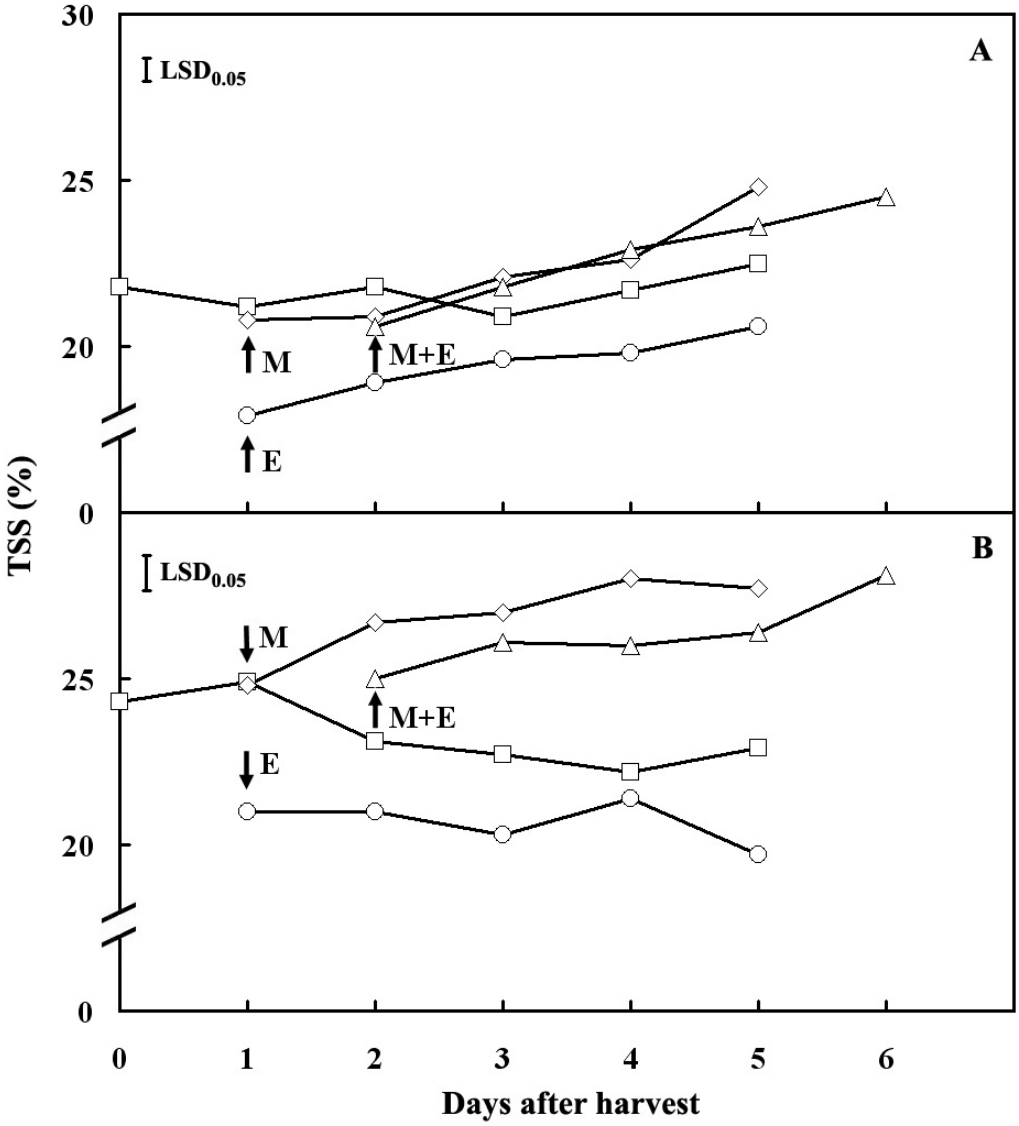

Figure 24 Changes in total soluble solids (TSS) of sapodilla fruits during ripening after harvest as a control fruits (□) and fruits treated with ethylene (○),1-MCP (◇) and 1-MCP + ethylene (△). Arrows indicate the day 0 after ethylene (E), 1-MCP (M), and 1-MCP + ethylene (M+E) treatment. Control fruits were kept for 5 days without treatment and treated fruits were kept for 4 days after treatment for cvv. Makok-Yai (A) and Kra-Suay (B). Data were the average of 4 replications. Differences between means were analysed using the Least Significant Difference (LSD) at the 95% significance level.

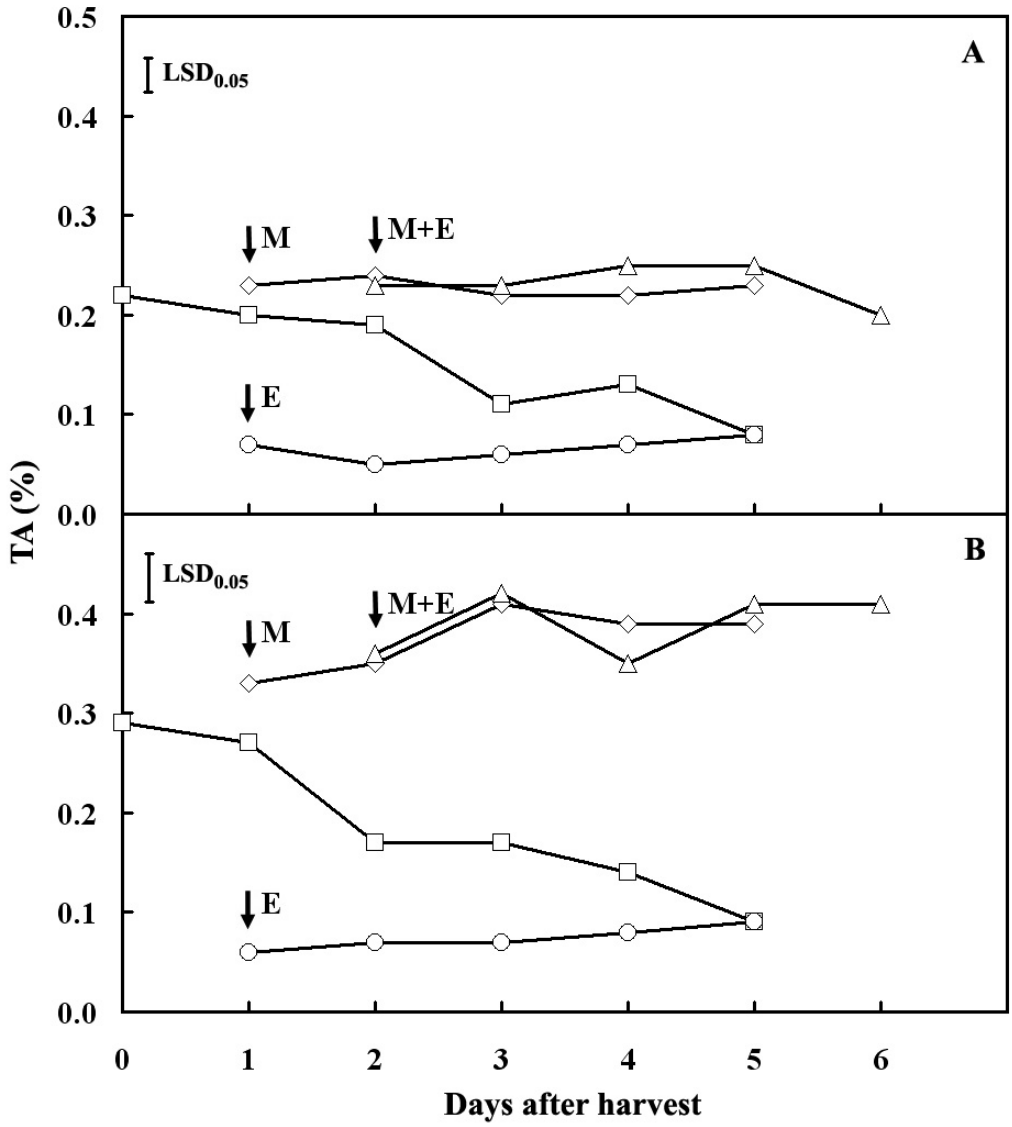

Figure 25 Changes in titratable acidity (TA) of sapodilla fruit during ripening after harvest as a control fruits (□) and fruits treated with ethylene (○),1-MCP (◇) and 1-MCP + ethylene (△). Arrows indicate the day 0 after ethylene (E), 1-MCP (M), and 1-MCP + ethylene (M+E) treatment. Control fruits were kept for 5 days without treatment and treated fruits were kept for 4 days after treatment for cvv. Makok-Yai (A) and Kra-Suay (B). Data were the average of 4 replications. Differences between means were analysed using the Least Significant Difference (LSD) at the 95% significance level.

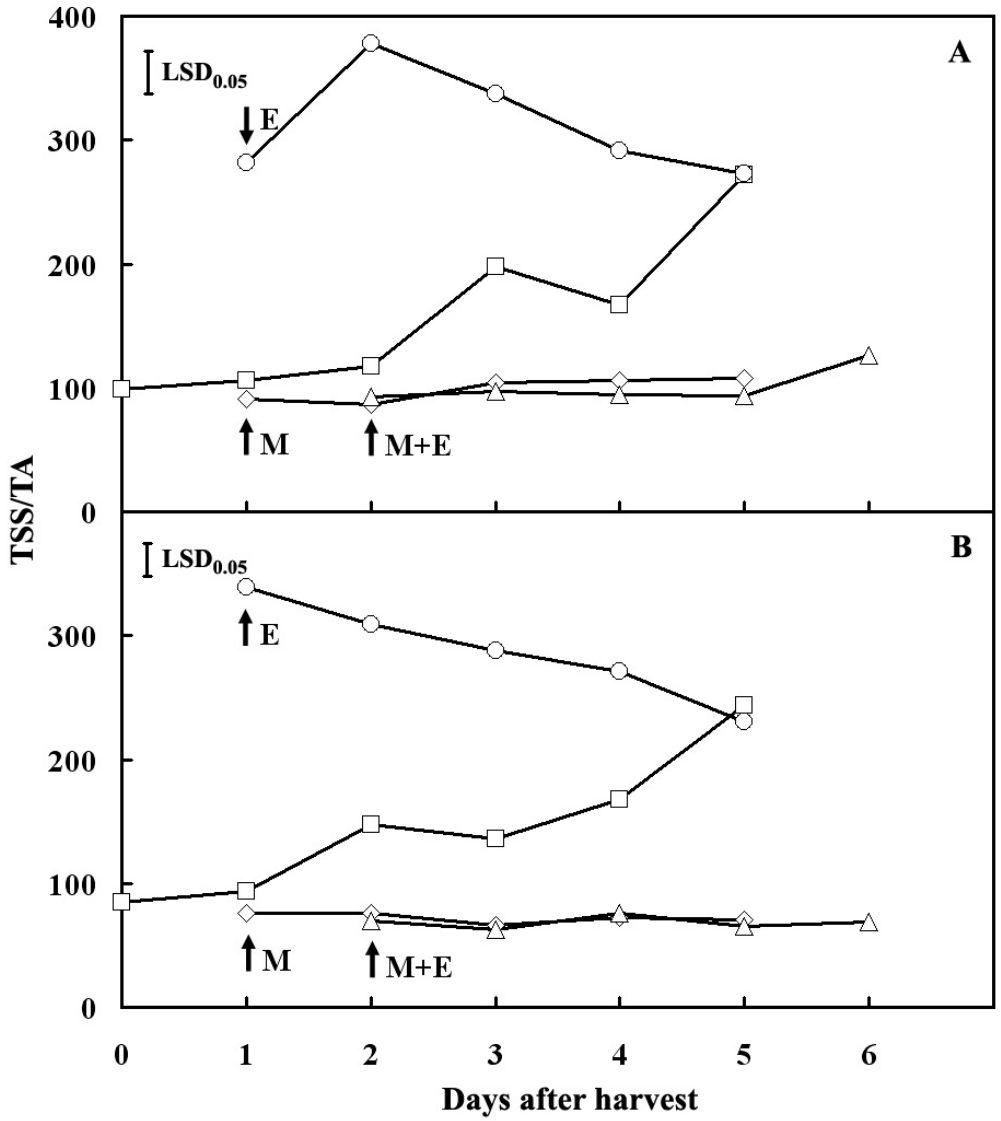

Figure 26 Changes in TSS/TA ratio of sapodilla fruit during ripening after harvest as a control fruits (□) and fruits treated with ethylene (○),1-MCP (◇) and 1-MCP + ethylene (△). Arrows indicate the day 0 after ethylene (E), 1-MCP (M), and 1-MCP + ethylene (M+E) treatment. Control fruits were kept for 5 days without treatment and treated fruits were kept for 4 days after treatment for cvv. Makok-Yai (A) and Kra-Suay (B). Data were the average of 4 replications. Differences between means were analysed using the Least Significant Difference (LSD) at the 95% significance level.

2.4 The activity of cell wall degrading enzymes

The EGase activity in control fruits increased during ripening in both cultivars (Figure 27). Ethylene treatment accelerated the increase in enzyme activity especially in cv. Makok Yai (Figure 27A) but not much in cv. Kra-Suay (Figure 27B). 1-MCP treatment prevented the increase in enzyme activity (Figure 27). In both cultivars, ripening fruits (control and ethylene treatment) exhibited higher activity than those in 1-MCP treatment (Appendix Table 16 and 17).

On day 1 after harvest the PG activity in the flesh of control fruits was higher than that of mature fruits (M, Figure 28), in both cultivars. In cv. Makok Yai the PG activity in control fruits further increased by day 5. In cv. Kra-Suay the activity was lower on day 5, compared with day 3. Ethylene treatment hastened the increase in activity, in both cultivars. 1-MCP treatment resulted in lower activities than in the control fruits in both cultivars (Figure 28, Appendix Table 18 and 19).

The PL activity of sapodilla fruits was not detectable in both mature and ripening fruits, in both cultivars, in controls and after ethylene or 1-MCP treatment (data not shown). The relationship between EGase and PG activities on the one hand and flesh firmness on the other is shown in Figure 29.

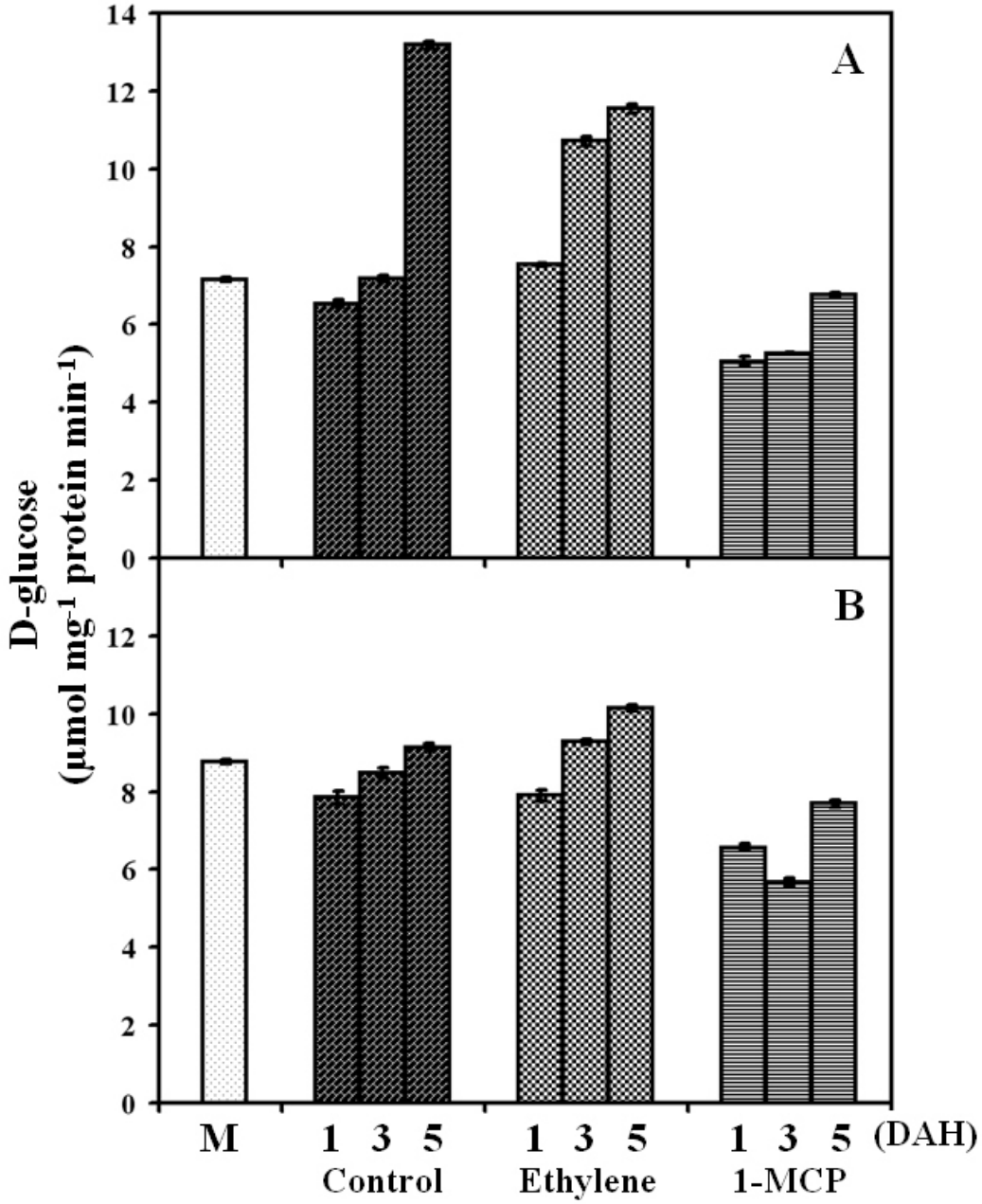

Figure 27 Changes in endo-β-1,4-glucanase activities of mature sapodilla fruits (M) cvv. Makok-Yai (A) and Kra-Suay (B) at 1, 3, and 5 days after harvest (DAH) for control, ethylene and 1-MCP treated fruits. Data are means ± SD (n = 3).

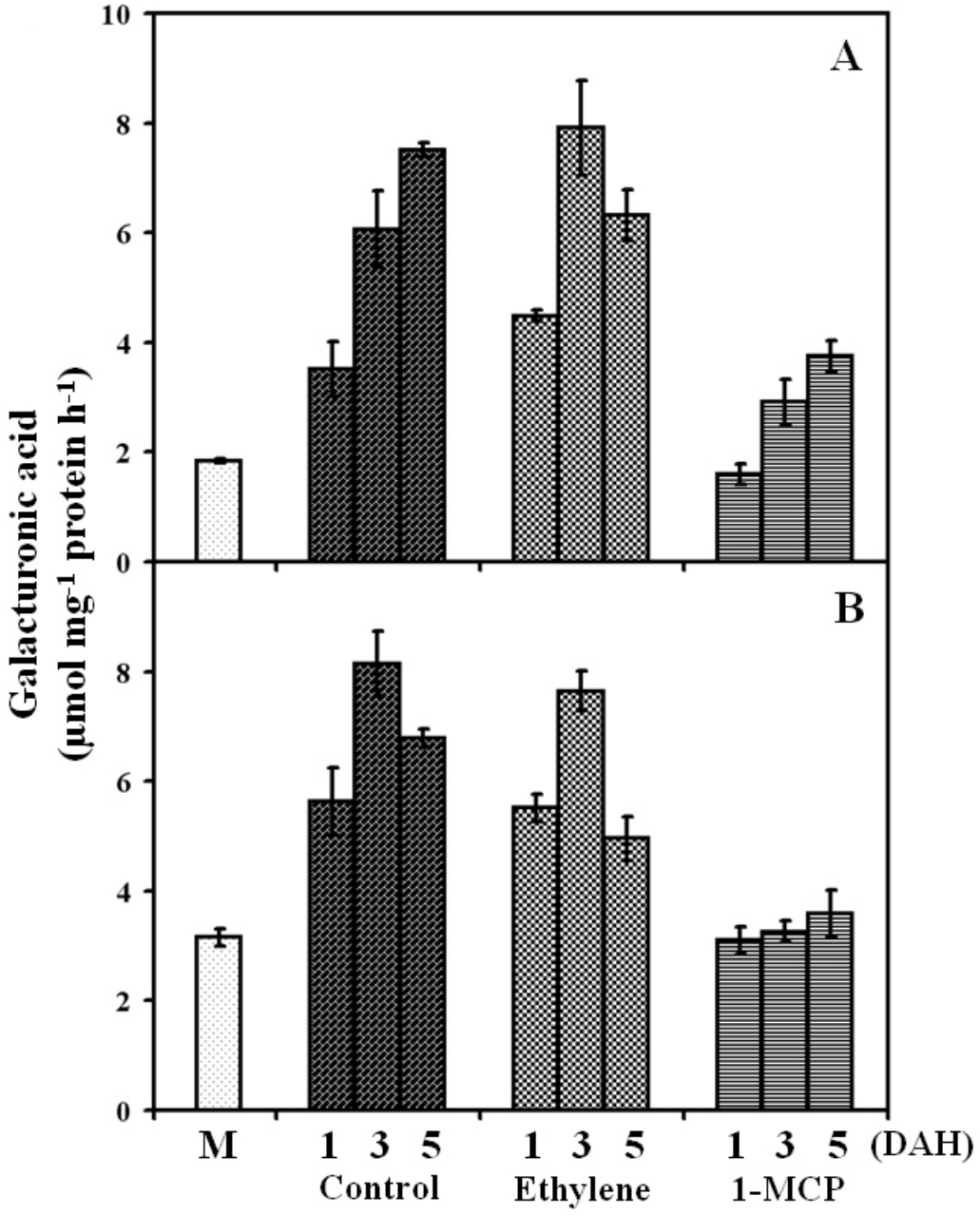

Figure 28 Changes in polygalacturonase activities of mature sapodilla fruits (M) cvv. Makok-Yai (A) and Kra-Suay (B) at 1, 3, and 5 days after harvest (DAH) for control, ethylene and 1-MCP treated fruits. Data are means ± SD (n = 3).

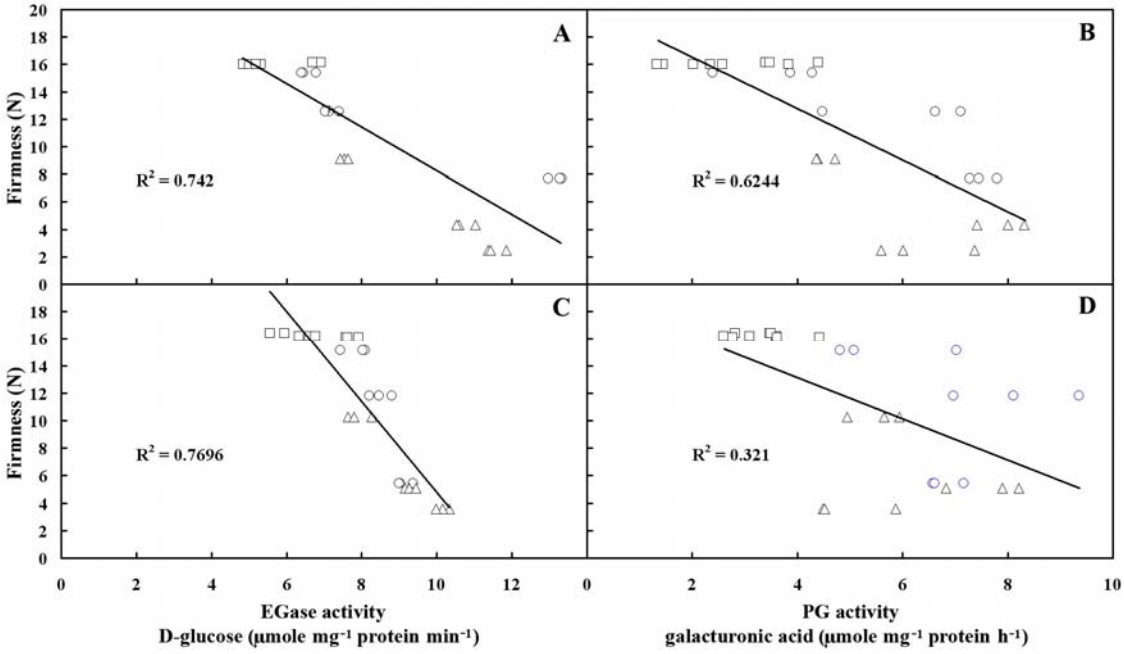

Figure 29 Relationship between the activities of EGase and PG and fruit firmness, after harvest. Some fruits were controls (○), others were treated with ethylene (Δ), and still others with 1-MCP (□). (A) relationship between the activity of EGase and firmness in cv. Makok-Yai. (B) relationship between the activity of PG and firmness in cv. Makok-Yai (C) relationship between the activity of EGase and firmness in cv. Kra-Suay. (D) relationship between the activity of PG and firmness in cv. Kra-Suay.

2.5 mRNA accumulation during fruit ripening

In both cultivars, the mRNA of *MzEXP1* was not detectable in sapodilla fruits after harvest (Figure 30 and 31). The effects of ethylene and 1-MCP on *MzEXP2* transcript abundance is shown in Figure 30 and 31. In both cultivars ethylene drastically reduced the mRNA abundance. In cv. Makok-Yai some transcript was still observed at the end of the one day ethylene treatment (day 1) (Figure 30), but in cv. Kra-Suay the mRNA was by then already below the detection limit (Figure 31). On day 3 and 5 after harvest there was no detectable transcript, in both cultivars. In contrast, fruits treated with 1-MCP resulted in high transcript abundance, in both cultivars. 1-MCP resulted in high transcript levels on day 5, whereas in controls no detectable transcript was found. When treatment with 1-MCP was followed by a treatment with ethylene, the high transcript abundance of *MzEXP2* was even found as late as day 6 (Figure 30 and 31).

Both the ethylene and 1-MCP treatments had little effect on the accumulation of *MzEG* mRNA in flesh of sapodilla fruits (Figure 30 and 31). A small signal was only found in cv. Makok-Yai, after 1-MCP treatment (Figure 30). Ethylene treatment drastically increased the transcript abundance of *MzPG*, in both cultivars, but no *MzPG* transcript was detectable in fruits treated with 1-MCP (Figure 30 and 31). Ethylene treatment induced high *MzPL* transcript abundance, in both cultivars. 1-MCP treatment also resulted in high *MzPL* transcript abundance in both cultivars (Figure 30 and 31).

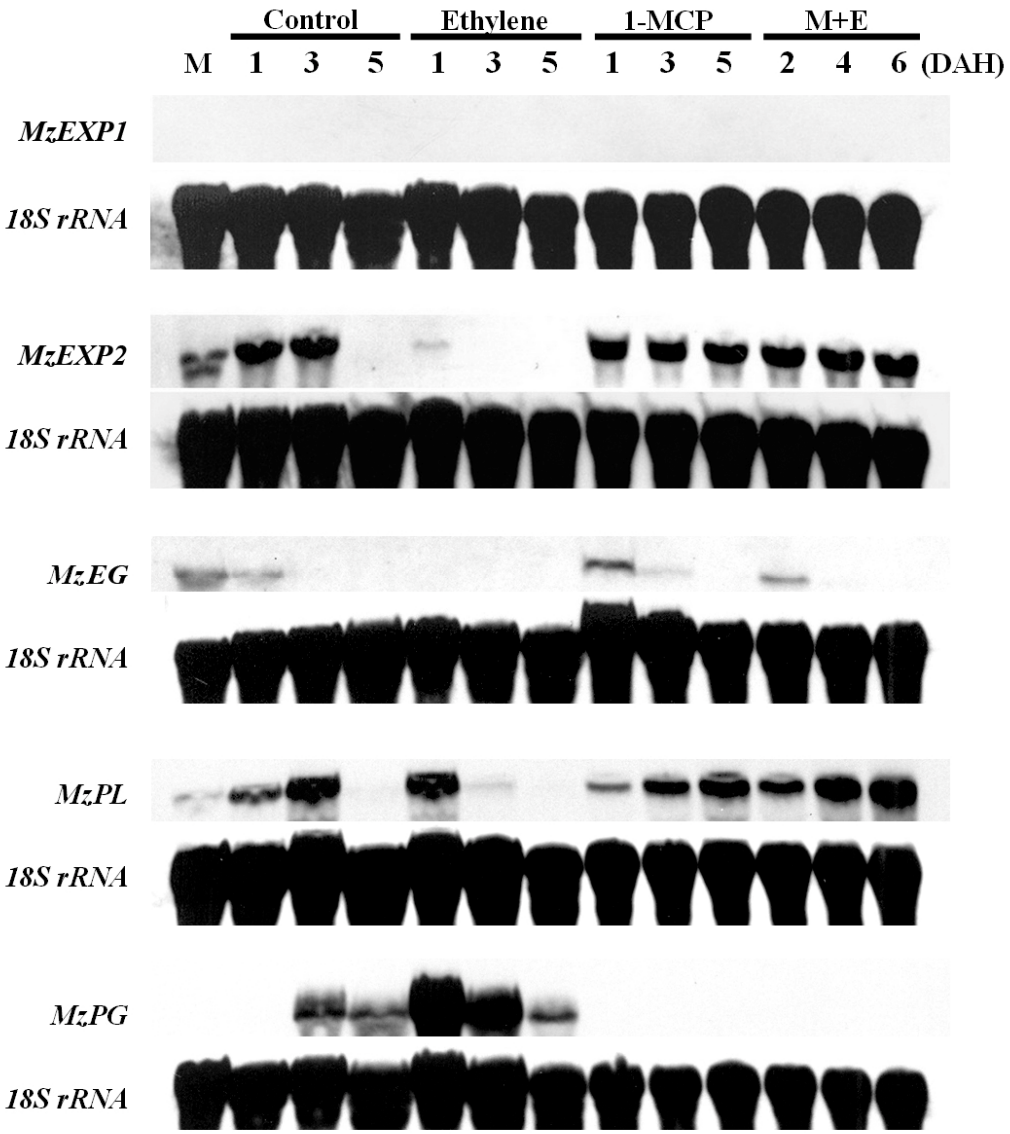

Figure 30 RNA gel blot analysis of *MzEXP1*, *MzEXP2*, *MzEG*, *MzPL*, and *MzPG* mRNA abundance in mature sapodilla fruits (M) cv. Makok-Yai at 1, 3, and 5 days after harvest (DAH). Treatment with ethylene (one day) started immediately after harvest, thus the data on day 1 were at the end of ethylene treatment, and those of day 3 and 5 referred to day 2 and 4 after the treatment. Similarly, 1-MCP was applied for one day, immediately after harvest. In the case of 1-MCP + ethylene (M+E) the 1-MCP treatment (one day) preceded the ethylene treatment (one day).

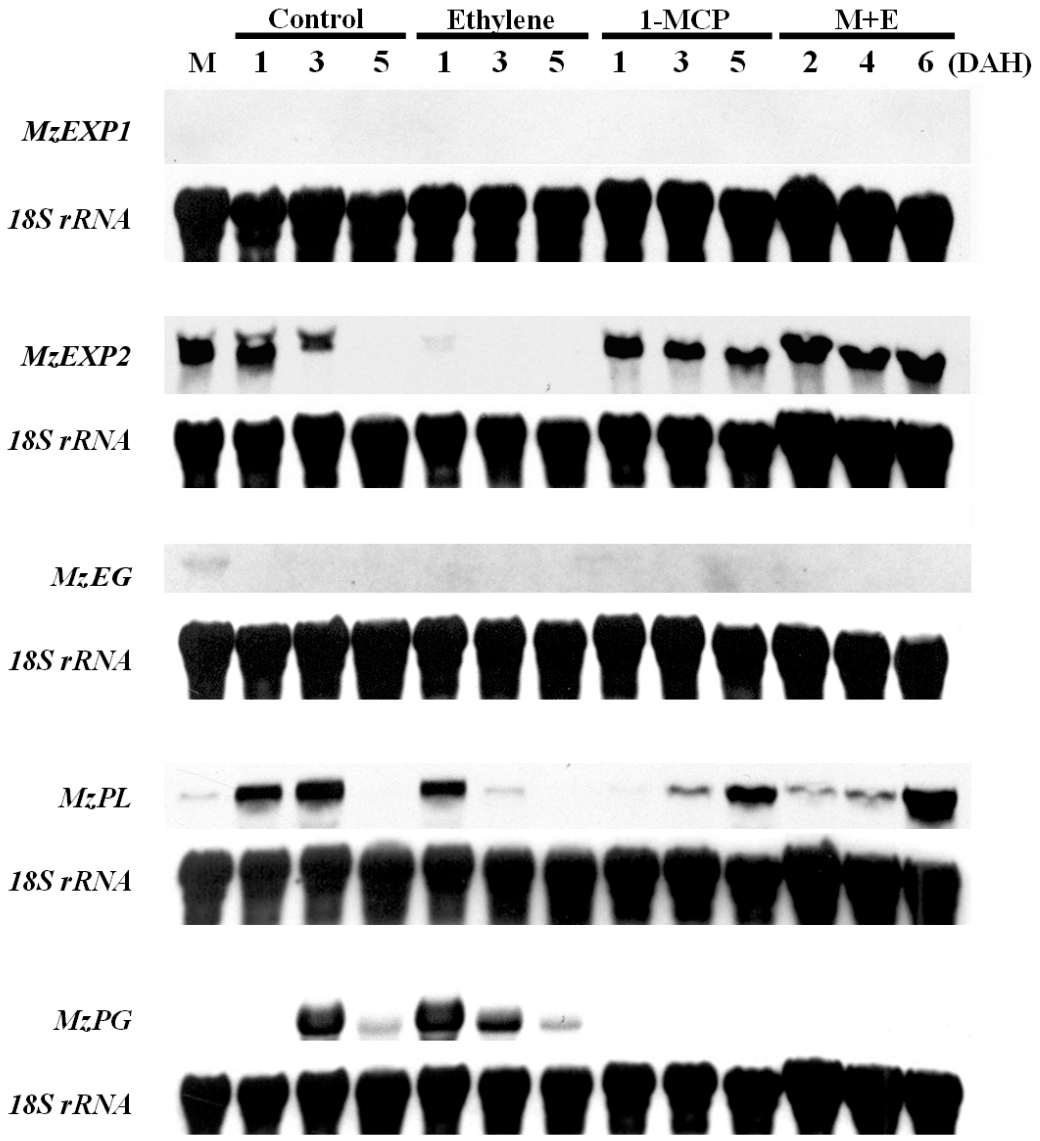

Figure 31 RNA gel blot analysis of *MzEXP1*, *MzEXP2*, *MzEG*, *MzPL*, and *MzPG* mRNA abundance in mature sapodilla fruits (M) cv. Kra-Suay at 1, 3, and 5 days after harvest (DAH). Treatment with ethylene (one day) started immediately after harvest, thus the data on day 1 were at the end of ethylene treatment, and those of day 3 and 5 referred to day 2 and 4 after the treatment. Similarly, 1-MCP was applied for one day, immediately after harvest. In the case of 1-MCP + ethylene (M+E) the 1-MCP treatment (one day) preceded the ethylene treatment (one day).

2.6 Fractionation of Cell Wall Polysaccharides

2.6.1 Change in pectin fraction

In cv. Makok Yai water-soluble pectin increased from about 3 μg galacturonic acid mg^{-1} AIS at harvest to about 12 and 26 μg galacturonic acid mg^{-1} AIS on day 5 in the control fruits and ethylene-treated fruits, respectively (Figure 32A). The same pattern was detected in cv. Kra-Suay with the ripened fruits (Figure 33A). Water soluble pectin content of 1-MCP treated fruits, remained low level in both cultivars during the first 5 days after harvest. There was a significantly higher amount of water-soluble pectin in the control fruits and ethylene treatment than those in the 1-MCP treated fruits in both cultivars (Figure 32A and 33A, Appendix Table 20 and 21). CDTA-soluble pectin content in the 1-MCP treated fruits was slightly higher than those in the ripening fruits (control and ethylene-treated fruits) as showed in Figure 32B and 33B (Appendix Table 22 and 23). Na_2CO_3-soluble pectin in the flesh of 1-MCP treated fruits was higher than those in mature and ripening fruits (control and ethylene-treated fruits) as showed in Figure 32C (Appendix Table 24), especially in cv. Kra-Suay (Figure 33C, Appendix Table 25).

2.6.2 Change in matrix glycan (hemicellulose) fractions

Neutral sugar content in 1N KOH-soluble fraction of the 1-MCP treated fruits, was slightly lower than those in the ripening fruits (control and ethylene-treated fruits) in both cultivars (Figure 34A and 35A, Appendix Table 26 and 27). Changes in amount of neutral sugar in 4N KOH-soluble fraction were not different between ripening fruits (control and ethylene-treated fruits) and 1-MCP treatment in both cultivars (Figure 34B and 35B, Appendix Table 28 and 29).

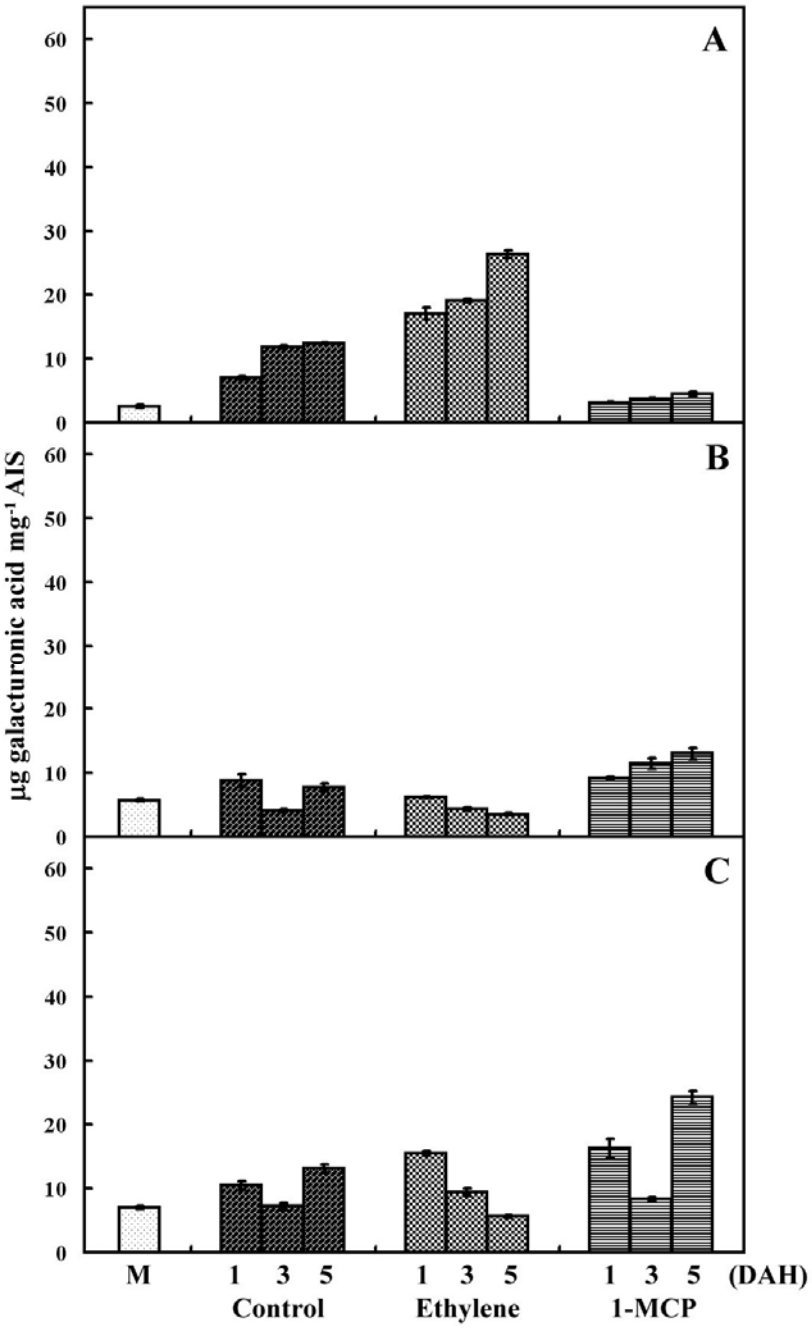

Figure 32 Changes in amounts of polyuronide in pectin extracts of cell wall materials (alcohol insoluble solids; AIS) from mature sapodilla fruits (M) cv. Makok-Yai at 1, 3, and 5 days after harvest (DAH) for control, ethylene and 1-MCP treated fruits. The AIS were extracted sequentially with water (A), CDTA (B) and Na_2CO_3 (C) to produce extracts of pectins. Data are the average of 3 replications.

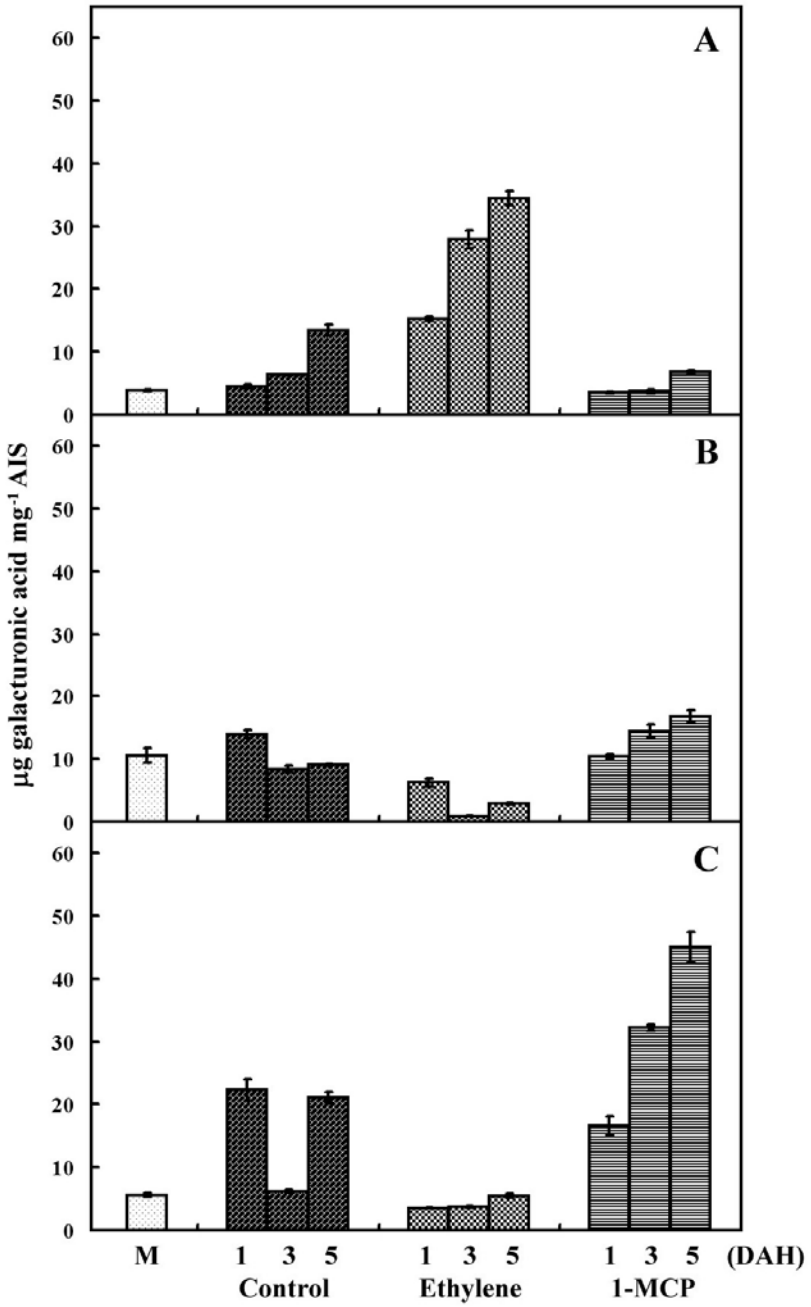

Figure 33 Changes in amounts of polyuronide in pectin extracts of cell wall materials (alcohol insoluble solids; AIS) from mature sapodilla fruits (M) cv. Kra-Suay at 1, 3, and 5 days after harvest (DAH) for control, ethylene and 1-MCP treated fruits. The AIS were extracted sequentially with water (A), CDTA (B) and Na_2CO_3 (C) to produce extracts of pectins. Data are the average of 3 replications.

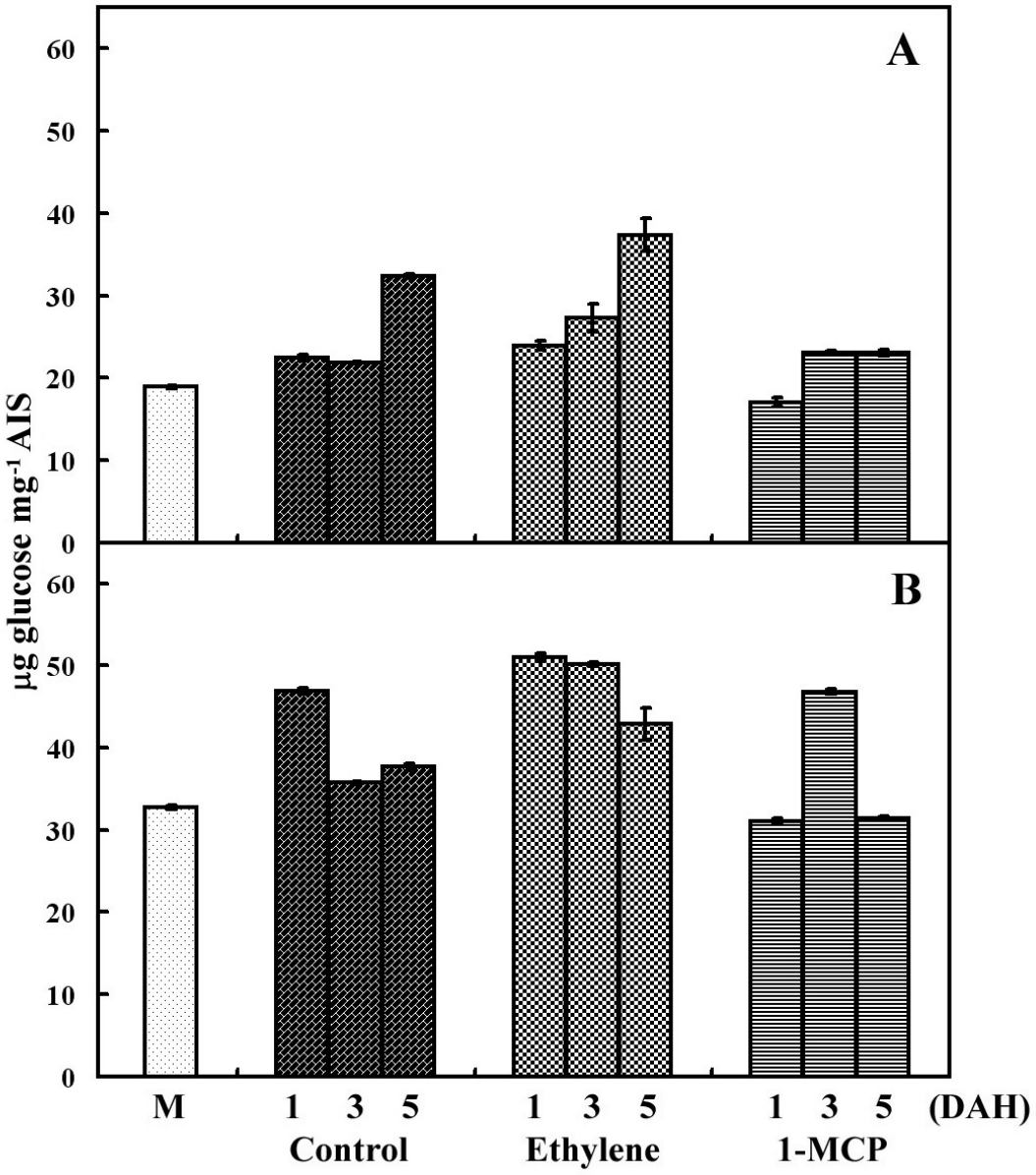

Figure 34 Changes in amounts of neutral sugar in matrix glycans extracts of cell wall materials (alcohol insoluble solids; AIS) from mature sapodilla fruits (M) cv. Makok-Yai at 1, 3, and 5 days after harvest (DAH) for control, ethylene and 1-MCP treated fruits. The AIS residues were extracted with 1N KOH (A) and 4N KOH (B) to produce extracts of matrix glycans. Data are the average of 3 replications.

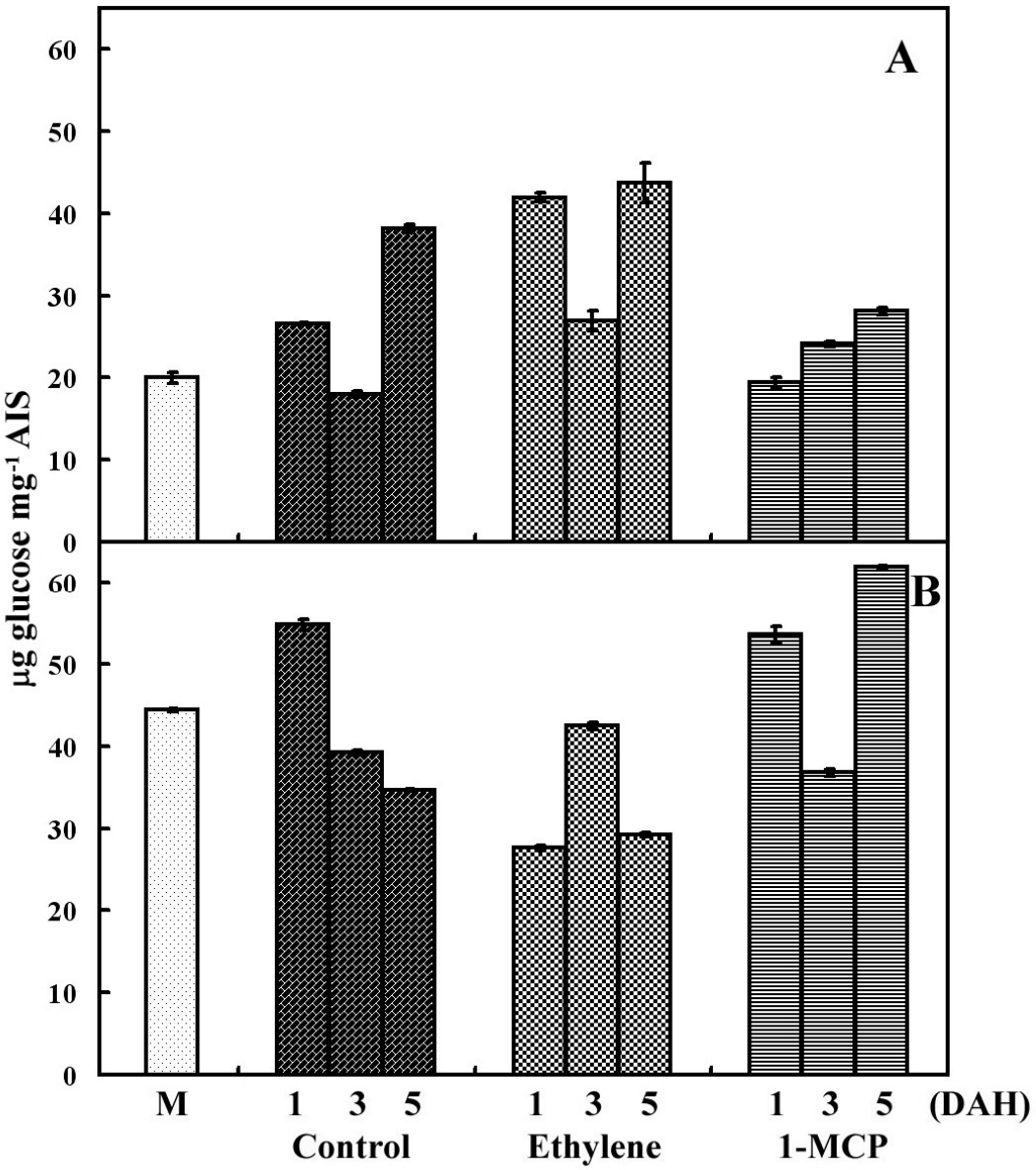

Figure 35 Changes in amounts of neutral sugar in matrix glycans extracts of cell wall materials (alcohol insoluble solids; AIS) from mature sapodilla fruits (M) cv. Kra-Suay at 1, 3, and 5 days after harvest (DAH) for control, ethylene and 1-MCP treated fruits. The AIS residues were extracted with 1N KOH (A) and 4N KOH (B) to produce extracts of matrix glycans. Data are the average of 3 replications.

DISCUSSION

I. Physiological changes and gene expression of cell wall enzyme during fruit growth and after harvest

Sapodilla cvv. Kra-Suay and Makok-Yai showed that fruit size and fruit weight (Figure 3 and 4) during growth followed a sigmoidal pattern similar to growth pattern of sapote mamey fruits (Alia-Tejacal *et al.*, 2007). Time required for the sapodilla fruits to completely develop, from anthesis to the fully mature stage whose peel color changed from dark green to light green, taken 7 months for cv. Makok-Yai and 8 months for cv. Kra-Suay. In contrast, the development of sapote mamey fruits varied from 18 to 20 months depending of variety and environmental conditions (Alia-Tejacal *et al.*, 2007).

During ripening of sapodilla fruits after harvest, the peel color changed from light green to brown within 5 days. To monitor the color level change of the fruit peel, in both cultivars the L* and b* values did not change (Figure 8A and 8C). The a* values of the fruits were around -5 at day 0, in agreement with the fact that negative a* values indicate green color. After six days, the a* values of fruit peel increased to around 5, that indicate red color tone in positive a* value (Figure 8B). This was similar to the increase a* value of banana peel during ripening, the a* values of the fruits were about -5 at day 0 changed to 0 on day 6. Because of chlorophyll degradation, degreening of fruit peel occurred during ripening (Yang *et al.*, 2009). After harvest, sapodilla fruits showed the climacteric pattern of respiration (Figure 6). Firmness of sapodilla fruits rapidly decreased within 5 days after harvest in both cultivars (Figure 5). Indeed, the same result was reported in sapote mamey held at ambient temperature (25˚C), ripened and softened within 5 days after harvest (Diaz-Perez *et al.*, 2000; Diaz-Perez *et al.*, 2003). Organic acids usually accumulate at the early stage of fruit development and are used as respiratory substrates during fruit ripening. In most fruits, the two major acids are malic and citric acids (Moing *et al.*, 2000; Chen *et al.*, 2009). During ripening of sapodilla fruits after harvest, TSS (total soluble solids) was quite stable, while TA (titratable acidity) decreased. These results

led to increase in TSS/TA ratio (Figure 9). This agrees with the general phenomenon found that organic acids were used as respiratory substrates during ripening at the later stages of loquat fruit development (Chen *et al.*, 2009).

The two expansin genes isolated (*MzEXP1*and *MzEXP2)* showed high homology, at the amino acid level, with *Arabidopsis* ATEXPA1 and ATEXPA8, respectively, thus with α-expansins. These two *Arabidopsis* expansins are rather closely related, but do belong to two subgroups, which branch at posterior probability 56 in the phylogenetic tree published by Choi *et al.* (2006).

Several expansins of the α-expansins type have been shown to be involved in cell wall loosening, both during fruit growth and softening (Sampedro and Cosgrove, 2005; Choi *et al.*, 2006; Carey and Cosgrove, 2007). Detectable levels of *MzEXP1* mRNA were found only during the early stage of fruit development (Figure 16 and 17). This suggests that *MzEXP1* plays a role in cell extension. *MzEXP1* transcript was also detectable in flowers that were opening (Figure 18), which might also suggest a role in growth processes. The expression profile of *MzEXP1* is reminiscent that of *Exp4* (Brummell *et al.*, 1999a) and *LeEXP2* (Catala *et al.*, 2000), both in tomato fruits, and that of *PcExp7* in pear fruits (Hiwasa *et al.*, 2003). The transcript levels of the similar *Arabidopsis EXPA1* responded to GA3 and red light, both known to promote elongation growth (Oh *et al.*, 2007). This also suggests a link between these types of expansins and cell wall changes relating to growth. In addition to fruit, *MzEXP1* was expressed in opening sapodilla flowers. Although some cell death processes occur in opening flowers (Wu and Cheung, 2000), there is also growth and the data on *MzEXP1* expression might therefore be taken to support the idea that its expression is associated with growth.

In contrast, the transcript abundance of *MzEXP2* was low until a late stage of fruit development (Figure 16 and 17). It was found, depending on the cultivar studied, about 16 weeks before the normal time of harvest, or just before harvest. After harvest its transcript abundance was also high, but depending on the cultivar the transcript levels became undetectable in ripe (day 4) and overripe (day 5) fruits. The expression

profile of *MzEXP2* was somewhat similar to that of *LeEXP1* of tomato fruits (Rose *et al.*, 1997; Brummell *et al.*, 1999b), and *PcEXP2* and *PcEXP3* in pears (Hiwasa *et al.*, 2003).

The isolated partial coding sequences of genes of cell wall degrading enzymes were differentially expressed. However, *MzEG* became mainly expressed during the early stages of growth and was expressed (above the detection limit) in unripe but mature fruits in one of the two cultivars. No expression was observed during fruit softening after harvest (Figure 16 and 17). During fruit ripening in a few other species some genes encoding an EGase (often called *Cel*) showed an increased expression during ripening (tomato: Lashbrook *et al.*, 1994; strawberry: Trainotti *et al.*, 1999; Llop-Tous *et al.*, 1999). In strawberry, it was inferred that an isolated EGase was causal in loss of firmness (Palomer *et al.*, 2004). EGases are encoded by a multigene family (strawberry: Trainotti *et al.*, 1999; peach: Trainotti *et al.*, 1997), so the absence of a relationship between gene expression and fruit firmness in sapodilla might also relate to the expression of other EGases that are responsible for the loss in fruit firmness. 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 (Wu *et al.*, 1996). It is also reminiscent of the expression pattern of *Cel1* in *Arabidopsis thaliana* in elongating tissue (Shani *et al.*, 2004; Shani *et al.*, 2006). The present data, therefore, might be interpreted to confirm that the expression of genes encoding EGases plays a role in cell wall modifications that are important in cell wall elongation.

MzPL mRNA accumulated abundantly during the early stage of ripening. The transcript level became undetectable in overripe fruits of both cultivars (Figure 16 and 17). This was similar to the expression of three transcripts (*plA*, *plB*, and *plC*) encoding PL in strawberry fruits (Benítez-Burraco *et al.*, 2003) and two genes encoding PLs in the pulp of ripe banana fruits (Domínguez-Puigjaner *et al.*, 1997; Marín-Rodríguez *et al.*, 2003). *MzPG* expression in sapodilla fruits was highly associated with softening. The transcript level of *MzPG* was high during the later stage of fruit ripening, in both cultivars (Figure 16 and 17). This finding was also

similar in kiwifruit (Wang *et al*., 2000) and apple fruit (Goulao *et al*., 2008). The expression of *PG* gene was observed in softening fruits and remained high level until the over-ripe stage. Some reports showed that PG activity was important at the later stage of fruit ripening (avocado: Awad and Young, 1979; durain: Imsabai *et al*., 2002; guava: Abu-Goukh and Bashir, 2003).

II. Physiological changes, biochemical changes and gene expression during fruit ripening after harvest

Application of ethylene to sapodilla fruits after harvest resulted in reduction of non-uniform ripening and firmness sharply decreased within 1 day after treatment. This case was similar to the report in sapote mamey fruits (Alia-Tejacal *et al.*, 2007). 1-MCP which is a powerful inhibitor of ethylene action (Blankenship and Dole, 2003), delayed fruit ripening and retained firmness in sapodilla fruits. 1-MCP inhibited fruit ripening and softening of both cultivars with and without ethylene treatment (Figure 19). This suggested that both sapodilla fruits cvv. Kra-Suay and Makok-Yai require ethylene to trigger ripening processes including softening similar to other climacteric fruits (Hofman *et al.*, 2001). The result in our study with 1-MCP was also similar to Fupiguo sapodilla (Qiuping *et al.*, 2006), Fino sapodilla (Arevalo-Galarza *et al.*, 2007), and sapote mamey (Ergun *et al.*, 2005). Although 1-MCP delayed fruit ripening but the experiment was stopped when the control fruits reached the overripe stage, in order to compare gene expression among treatments. The experiment was not continued until fruits treated with 1-MCP developed to ripe stage. Therefore, it was not known if treated fruit ripened normally or not. This requires further to study a suitable concentration of 1-MCP for sapodilla fruits prior to commercial application.

Ethylene hastened fruit softening as expected, but depending on the cultivar, it largely or wholly prevented the expression of *MzEXP2,* from the end of ethylene treatment onward. The drastic down-regulation of *MzEXP2* expression by ethylene, and the prevention of its normal down-regulation by 1-MCP suggest that in the control fruits the decrease (Figure 30 and 31) of its transcript abundance might be due

to ethylene (Figure 6). Indeed, in control fruits the rate of ethylene production had just increased by the time of the decrease in *MzEXP2* transcript abundance. Conversely, ethylene does not seem to induce the onset of *MzEXP2* expression as in both cultivars the rate of ethylene production had not increased by the time the first transcript became detectable.

The data on the effect of ethylene on the transcript abundance of *MzEXP2* are counter to the ethylene effects on several other expansin genes in ripe fruits. For example, ethylene treatment increased the transcript abundance of *LeEXP1* of tomato (Rose *et al.*, 1997), *MaEXP1* of banana (Trivedi and Nath, 2004), *MiExpA1* of mango (Sane *et al.*, 2005) and a gene called *PchExp1* in peach (Hayama *et al.*, 2000). The expression of several other expansin genes was not affected by ethylene (Civello *et al.*, 1999; Mbéguié-A-Mbéguié *et al.*, 2002; Hiwasa *et al.*, 2003).

Only one example other than sapodilla has apparently been reported of an expansin gene that was down-regulated by ethylene: *Pa-EXP1* of apricot. In fruits that were left on the tree *Pa-EXP1* became expressed when the fruits were between the breaker stage (light orange skin) and the half ripe stage (orange skin). The expression of this gene decreased again when the fruits were overripe (Mbéguié-A-Mbéguié *et al.*, 2002). After harvest of fruits at the breaker stage and 2-day treatment with ethylene, the transcript abundance of this expansin gene was lower than in control fruits. Treatment with 1-MCP, in contrast, resulted in higher transcript abundance than in control fruits. These data were similar to those found for *MzEXP2*. The amino acid sequence of sapodilla MzEXP2 partial protein sequence (accession number ABX11269) and that apricot *Pa-EXP1* (U93167) shows considerable similarity. The 130 amino acid sequence known of the sapodilla gene was homologous to amino acids 48-177 of the 253 amino acids full apricot sequence. BLASTp of the sapodilla MzEXP2 sequence, using NCBI, gave a high score (246 of 268) and expectation (e-value 4e-64). However, some expansins isolated from fleshy fruits such as litchi, papaya, grape, longan, loquat, apple and pear showed an even higher homology with the sapodilla MzEXP2 amino acid sequence. This might indicate the presence of a group of similar genes in these fruits.

Functional analysis suggests that some fruit expansins are involved in softening after harvest. For example, *LeExp1* from tomato was specifically expressed in softening fruits, when growth had ceased but when selective disassembly of cell wall components took place. The expression of *LeExp1* was increased by ethylene, which also induced early softening. Furthermore, *LeExp1* expression was absent in the ripening-impaired tomato mutants *rin* and *nor*. These data show that some expansins may contribute to cell wall disassembly in non-growing tissue (Rose *et al.*, 1997). *rin* is a member of the *SEPALLATA* subfamily of MADS-box genes, and *nor* is an as yet unknown transcription factor. Both apparently act upstream in the ripening/ethylene pathway (Barry and Giovannoni, 2007). However, *LeExp1* expression was not affected in the never ripe (*Nr*) tomato mutant (Rose *et al.*, 1997), which is a lesion in one of the ethylene receptor genes (Barry and Giovannoni, 2007). These data suggest a causal connection between the softening-related *LeExp1*gene, and the action of early transcription factors that leads to the onset of an increase in ethylene production in tomato.

At the present it is not clear what the functional role of *MzEXP2* might be. In the work of Hayama *et al.* (2000) evidence was found for the idea that an expansin gene in peach fruits (*PchExp1*) was not related to changes in fruit firmness. Ethylene treatment increased the transcript abundance of this gene. However, the expression pattern of *PchExp1* in the ripe fruits of cv. Akatsuki, which lost its firmness rapidly after harvest, was very similar to that in cv. Yumyeong, which retained its firmness for a long time after harvest. It was therefore suggested that the gene may play an important role in some aspect of peach fruit ripening other than softening. The expression of another expansin gene, *Pa-Exp2* of apricot, was not affected by exogenous ethylene, whereas fruit softening was very much hastened by ethylene. This suggested that *Pa-Exp2* was also not involved in apricot fruit softening. It was hypothesized that both genes may be associated rather with fruit growth, which goes on even after harvest (Mbéguié-A-Mbéguié *et al.*, 2002). In sapodilla, in contrast, the relation between expansin gene expression and fruit growth was more complex. In cv. Kra-Suay the expression of *MzEXP2* occurred concomitant with the growth of fruits from week 24 to week 32. During week 32 and 36, the gene was still expressed but

growth had stopped (Figure 17). In cv. Makok-Yai the gene was expressed only by the time of harvest and after harvest (Figure 16). Since after harvest no growth occurred, the data suggested only a weak correlation with fruit growth. Nonetheless, *MzEXP2* was also expressed in expanding leaves and in opening flowers (Figure 18). This might suggest an association with growth.

Expansins have also been related to pathogen infection. Loosening of the cell wall is required for plant growth and for fruit softening, but may also make the plant vulnerable to biotic intruders. In red tomato, the decrease of fruit firmness and the associated cell wall disassembly depends on cooperative action of both polygalacturonase (PG) and expansin. Susceptibility to the necrotrophic pathogen *Botrytis cinerea* was assessed in transgenic tomato fruits with suppressed polygalacturonase (*LePG*) and expansin (*LeExp1*) expression. Suppression of either *LePG* or *LeExp1* alone did not reduce cell wall disassembly, softening, and susceptibility to the pathogen, but simultaneous suppression of both proteins dramatically inhibited these parameters. Thus in tomato fruits, *LeEXP1* is instrumental in increasing the chances of grey mold infection, because of its effect on cell wall disassembly (Cantu *et al.*, 2008). These data do not show that expansins affect pathogen infection other than by changing cell wall composition. A role of *MzEXP2* in defence against sapodilla pathogens, at a time of a large decrease in fruit firmness, is therefore not likely.

However, *MzEXP2* might be important for fruit softening. As described, the softening of red tomato fruits did not occur by PG alone nor by expansin alone. The expansin was required to allow the action of PG. Similarly, in sapodilla, the protein encoded by *MzEXP2* might prepare the wall for the action of enzymes that cleave various chemical bonds. If so, the down-regulation of *MzEXP2* by ethylene, might indicate that the gene is no longer required for the preparation of the cell walls for the action of hydrolytic enzymes. The data of Figure 30 and 31 seem to support this contention. The absence of a detectable level of transcript is associated with low fruit firmness values. In control fruits of cv. Makok-Yai, *MzEXP2* transcript was no longer detectable by day 5 after harvest (Figure 30), when fruit firmness had become lower

than about 7.5 N (Figure 19A). In ethylene-treated fruits, no transcript was found on day 3 and 5 after harvest (Figure 30), when fruit firmness had also become lower than about 4.3 N (Figure 19A). A relatively small amount of transcript was detectable at the end of ethylene treatment (day 1) (Figure 30) when firmness was about 9 N (Figure 19A). Similarly, in cv. Kra-suay, no transcript was found in controls (Figure 31) with fruit firmness below 10 N (Figure 19B). In ethylene-treated fruits the same relationship was observed: no transcript on day 3 and 5 (Figure 31), when firmness was below 10 N (Figure 19B). In controls in which firmness was still higher than 10 N (day 3 after harvest) (Figure 19B) some transcript was still detectable (Figure 31). These results show that the expansin gene was no longer expressed by the time the fruits were already quite soft. This can be interpreted by assuming that by then the gene had done its job of producing a protein that aided cell wall softening. According to this interpretation *MzEXP2* seems important for the process of cell wall disassembly during fruit ripening.

Since fruit softening has been attributed to the activity of cell-wall modifying enzymes, we analyzed the total activity of EGase, PL and PG. The EGase activity in control and ethylene-treated fruits progressively increased during fruit ripening in a similar manner in both cultivars (Figure 27). In control fruits a temporal relationship was observed between the increase in EGase activity and loss in flesh firmness. Ethylene treatment promoted both EGase activity and fruit softening. The EGase activity of fruits treated with 1-MCP remained the same as in the control mature fruits, and these fruits did not show softening during the period of the investigation. Thus a close relationship was found between the two parameters (Figure 29A and 29C). A similar relationship was shown, for example, in avocado (Pesis *et al.*, 1978) and guava (Abu-Goukh and Bashir, 2003). The expression of an isolated gene encoding an EGase (*MzEG*) was not correlated with EGase activity and fruit ripening. No expression was observed during fruit softening after harvest. This suggests the idea that EGase activity during softening was due to one or more other genes encoding EGase or that EGase is activated posttranscriptionally. In other fruits EGases are encoded by a multigene family (for example, strawberry: Trainotti *et al.*, 1999; peach: Trainotti *et al.*, 1997), 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 (tomato: Lashbrook *et al.*, 1994; and strawberry: Trainotti *et al.*, 1999, and Llop-Tous *et al.*, 1999). 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 (Palomer *et al.*, 2004).

In several species a role of PL activity in fruit softening has been suggested (Payasi and Sanwal, 2003). We used the same protocol as they used in banana determination of PL activity. However in our study, the PL activity of sapodilla fruits remained below the detection limit. They reported an increase in PL activity in banana pulp during fruit ripening (Saengpook *et al.*, 2007). Others also measured an increase in PL activity in banana pulp (Payasi and Sanwal, 2003; Marín-Rodríguez *et al.*, 2003). Jiménez-Bermúdez *et al.* (2002) measured PL activity in strawberry fruits, using a number of protocols, but found low reproducibility because of strong extract interferences and low assay sensitivity. Similarly, the PL activity in sapodilla fruits might be too low, compared to banana, or might be affected by interfering substances in the extract (Jiménez-Bermúdez *et al.*, 2002; Deng *et al.*, 2005; Goulao *et al.*, 2007). This requires further study.

Exogenous ethylene induced accumulation of *Banl7* transcript, encoding a PL, in green banana fruits (Domínguez-Puigjaner *et al.*, 1997), similar to the hastened *MzPL* expression in ethylene-treated sapodilla fruits (Figure 30 and 31). 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 process (Jiménez-Bermúdez *et al.*, 2002). However, contrary to expectation, we found that treatment of sapodilla fruits with 1-MCP (which prevented softening) did not affect *MzPL* transcript abundance, in both cultivars (Figure 30 and 31). Thus *MzPL* transcript abundance was not well correlated, when considering all treatments, with firmness loss in sapodilla fruits.

In the pulp of ripening banana, the activity of PL increased earlier and showed an earlier peak, compared with the respective increase in activity and peak activity of PG (Payasi and Sanwal, 2003). The earlier expression of *MzPL* compared with *MzPG* in sapodilla might suggest that the respective enzyme activities show a pattern similar to that of banana pulp. However, testing of this hypothesis requires optimization of PL activity measurement in sapodilla.

Our data can be interpreted to support a role for PG in softening of sapodilla fruits. A good relationship was observed between an increase in PG activity and loss in flesh firmness in control fruits and in ethylene-treated fruits. Additionally, 1-MCP-treated fruits retained high firmness and low PG activity (Figure 29B and 29D). This relationship is similar to banana fruits treated with ethylene and 1-MCP (Lohani *et al.*, 2004). It also has been reported previously that durian softening was correlated with PG activity and pectin degradation (Imsabai *et al.*, 2002).

MzPG expression in sapodilla fruits was highly associated with softening. In both cultivars with ethylene treatment (which promoted fruit softening) dramatically increased the *MzPG* mRNA abundance**,** while 1-MCP treatment suppressed *MzPG* expression (Figure 30 and 31). A similar expression pattern has been shown in the *CkPGC* gene of kiwi fruits (Wang *et al.*, 2000) and in *MAPG3* of banana fruits (Asif and Nath, 2005). These results might indicate that *PG* gene expression is limiting PG activity and softening, in the fruit of at least some species. In tomato fruits a gene encoding PG was also up-regulated, and its transcript abundance was increased after ethylene treatment. The PG activity in control ripening tomato fruits 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 fruits) showed that PG alone was not sufficient for fruit softening (Hadfield and Bennett, 1998; Brummell and Harpster, 2001; Giovannoni, 2004; Saladié *et al.*, 2007). 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, and stayed low in 1-MCP-treated fruits (Figure 29B and 29D). 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 was up-regulated by ethylene, and the prevention of its normal up-regulation by 1-MCP. Similarly, *OsPG* expression of prickly pear (Rosas-Cárdenas *et al*., 2007) and PG activity of banana (Lohani *et al*., 2004) 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 of 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 (Brummell and Harpster, 2001). In addition, measurement of total PG activity and *FaPG1* expression of strawberry were performed in three cultivars with contrasting softening rates (Villarreal *et al*., 2008).

Moreover, previous research reported that pectin methylesterase (PME) and PG cooperated to degrade methylesterified pectins (Wakabayashi *et al*., 2003). PME de-esterifies polyuronides by removing methyl groups from the C6 position of galacturonic acid residues of high-molecular-weight pectin. Demethylation of pectin makes the polyuronides susceptible to degradation by PG (Brummell and Harpster, 2001). There was the evidence that PME activity supported the role of PG in avocado fruits. Polyuronides derived from fruits at intermediate stages of ripening were treated with PME, extensive molecular mass downshifts occurred in response to incubation with PG. These results suggested that PG plays the central role in polyuronide degradation in ripening avocado fruit cell walls and that partial de-esterification was necessary for the increase in the susceptibility of polyuronides to PG (Wakabayashi *et*

al., 2000; Wakabayashi *et al*., 2003). The *PME* expression and/or PME activity in sapodilla fruits is required for further study, to find out the mechanism of pectin degradation by PME during fruit softening.

Pectin is one of the major components of the primary cell walls and middle lamellae between primary cell walls in plant tissue. Pectin encompasses a range of galacturonic acid-rich polysaccharides. Three major pectic polysaccharides (homogalacturonan, rhamnogalacturonan-I and rhamnogalacturonan-II) are thought to occur in all primary cell walls (Willats *et al.*, 2001). Fruit softening is associated with cell wall disassembly and modifications to the pectin fraction are some of the most apparent changes that take place in the cell wall during ripening (Marin-Rodriguez *et al.*, 2002). Water-soluble pectin of sapodilla fruits increased with loss of firmness in ripening process, while this content of 1-MCP treatment (which prevented softening) remained low level in both cultivars (Figure 32A and 33A). This was similar to the increase in the water-soluble pectin content, while fruit firmness of banana decreased rapidly during ripening (Duan *et al.*, 2008). The same pattern was reported in avocado fruits during ripening. Moreover, treatment of cell walls from pre-ripe avocado fruits with purified PG promoted the polyuronide degradation (Wakabayashi *et al.*, 2000).

Finally, we can suggest that the expression of *MzEXP1* and *MzEG* were correlated with fruit growth and not with loss of fruit firmness after harvest. The expression of an *MzEXP2* in sapodilla flesh started on day 1 after harvest in cv. Makok-Yai and was already high just prior to harvest in cv. Kra-Suay. The gene thus showed a pattern similar to, or earlier than that now found in *MzPL*. The previous and present data, taken together, might suggest a concerted activity of several enzymes in cell wall polymer modification before and during fruit softening. However, it was previously found that the transient expression of the sapodilla expansin gene (*MzEXP2*) was down-regulated by ethylene treatment. Whereas in control fruits no more expression occurred on day 5, after treatment with 1-MCP the expression was still high on day 5. The ethylene regulation of this expansin gene was thus opposite to the one now found in *MzPL* and *MzPG*. The expression of *MzPL* occurred during the early stage of fruit softening after harvest. The expression of this gene was correlated

with fruit softening both in control fruits and in ethylene-treated fruits, but such a correlation was absent in fruits 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. The expression of this gene encoding a PL was therefore not sufficient for loss of firmness. Lastly, the expression of *MzPG* was also restricted to the postharvest stage. The expression of this gene was well correlated with changes in fruit firmness and water soluble pectin content 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. This suggests the hypothesis that the expression of this gene plays an important role in rapid softening of sapodilla fruits after harvest.

CONCLUSION

The results of experiments during fruit growth and ripening of sapodilla cvv. Kra-Suay and Makok-Yai are concluded as the following:

1. Fruit growth of sapodilla followed a sigmoidal pattern and fruits showed the climacteric pattern of respiration after harvest.

2. Application of ethylene to sapodilla fruits after harvest induced ripening process and resulted in reduction of non-uniform ripening, while 1-MCP treatment maintained flesh firmness and delayed fruit ripening.

3. *MzEXP1* and *MzEG* had a specific role for fruit growth and development, while *MzEXP2* had a role with ripening-associated cell wall disassembly at the early stage of ripening and expression of *MzEXP2* was down-regulated by ethylene.

4. The expression of *MzPL* occurred during the early stage of fruit softening after harvest. The expression of this gene was not sufficient for firmness loss.

5. PG activity in the fruit flesh was well correlated with the increase in transcript abundance of *MzPG* and the decrease of flesh firmness.

6. The increase of water-soluble pectin content was concomitant with firmness loss.

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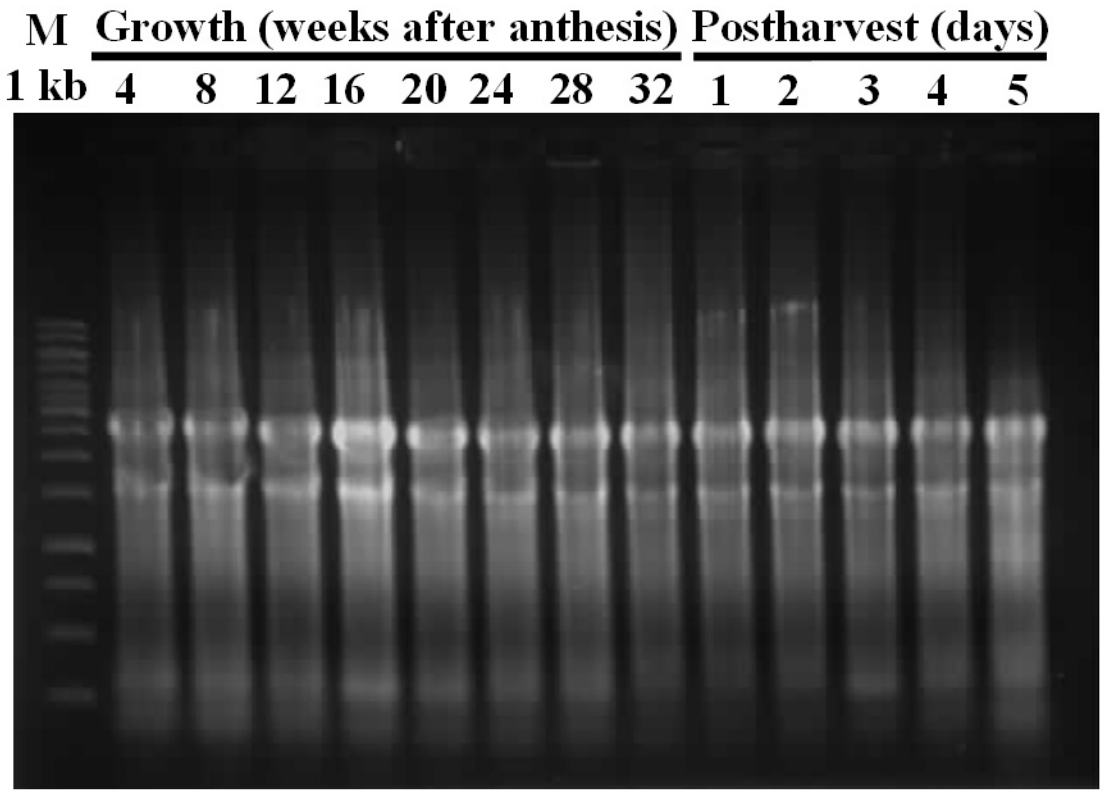
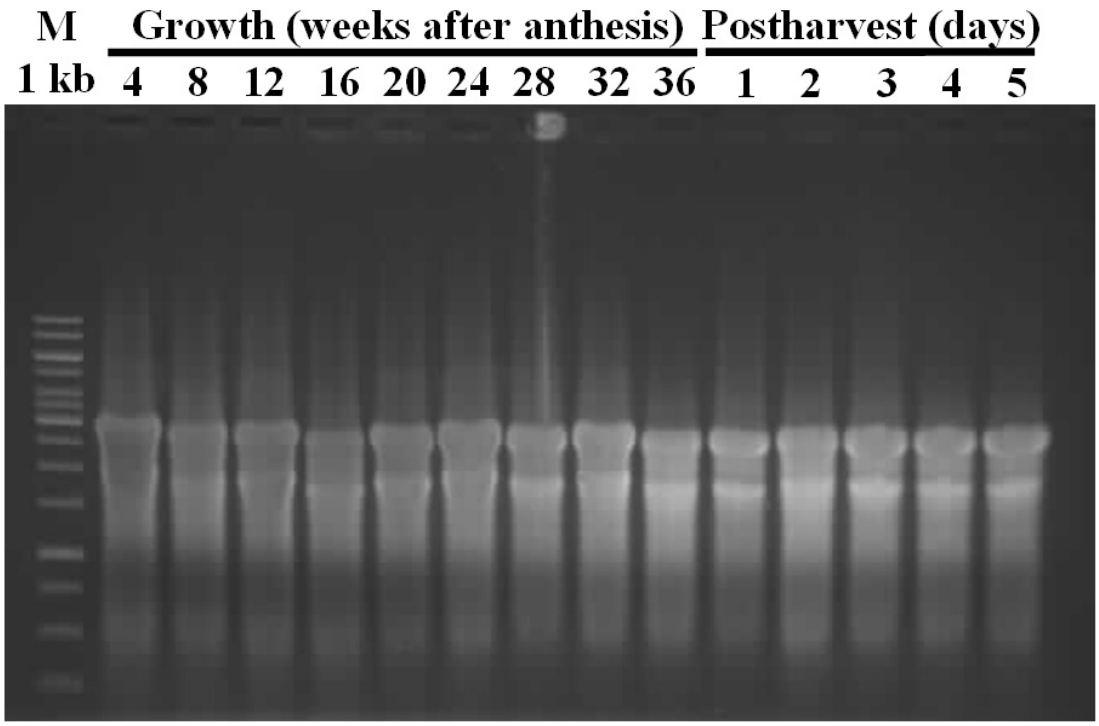
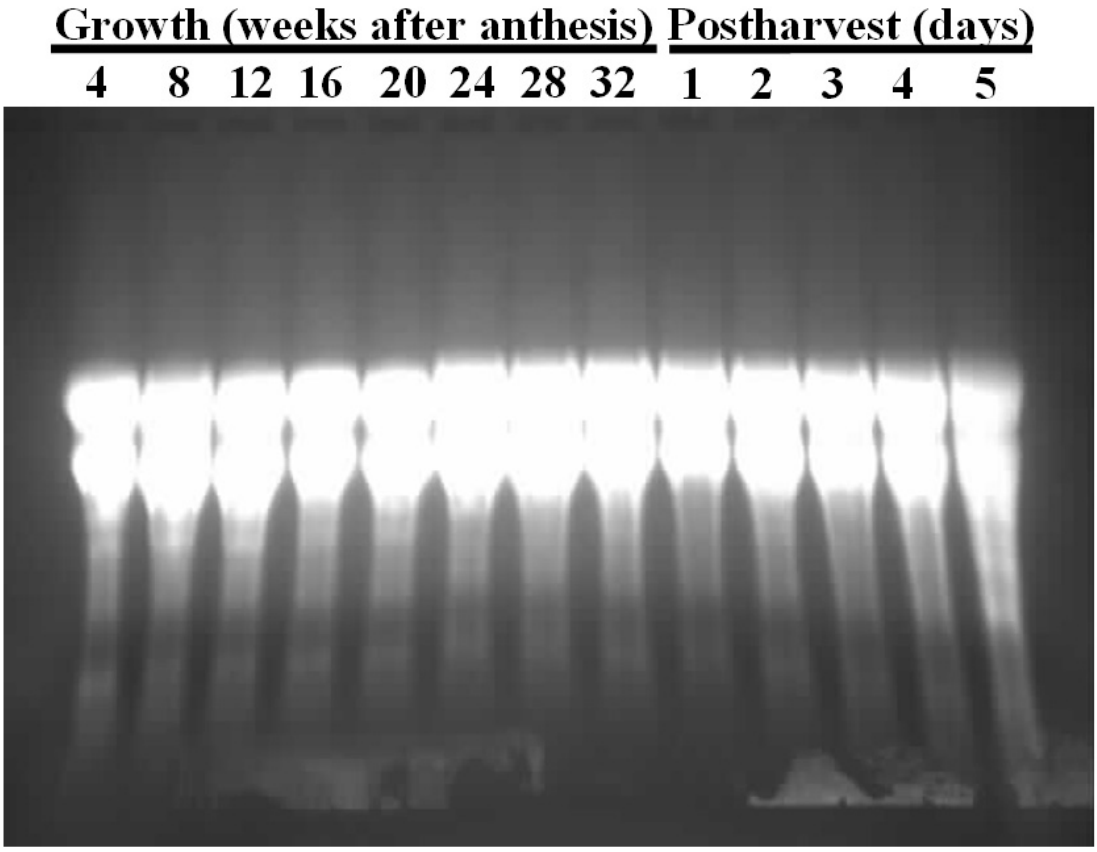
APPENDIX



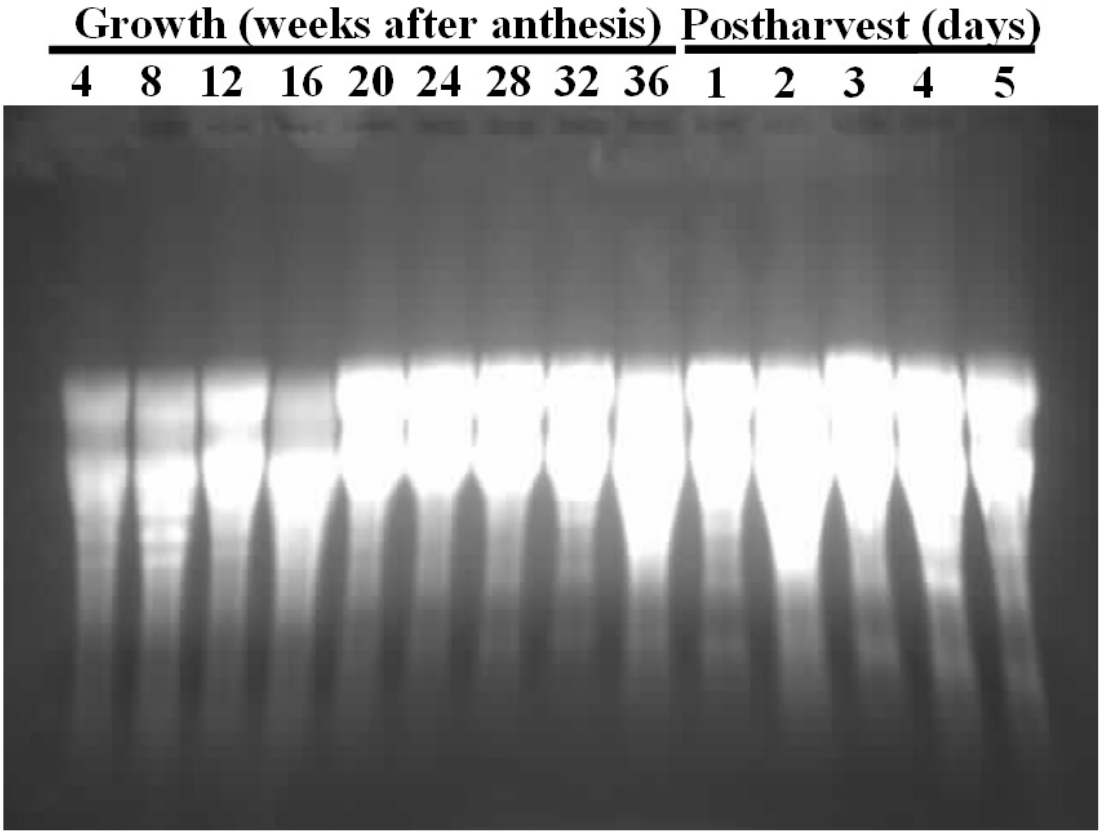
Appendix Figure 1 The RNA (2 μg) of sapodilla (*Manilkara zapota*) fruits cv. Makok-Yai during growth after anthesis until harvest maturity and in fruits after harvest, was run by gel electrophoresis on 0.8% agarose gel.



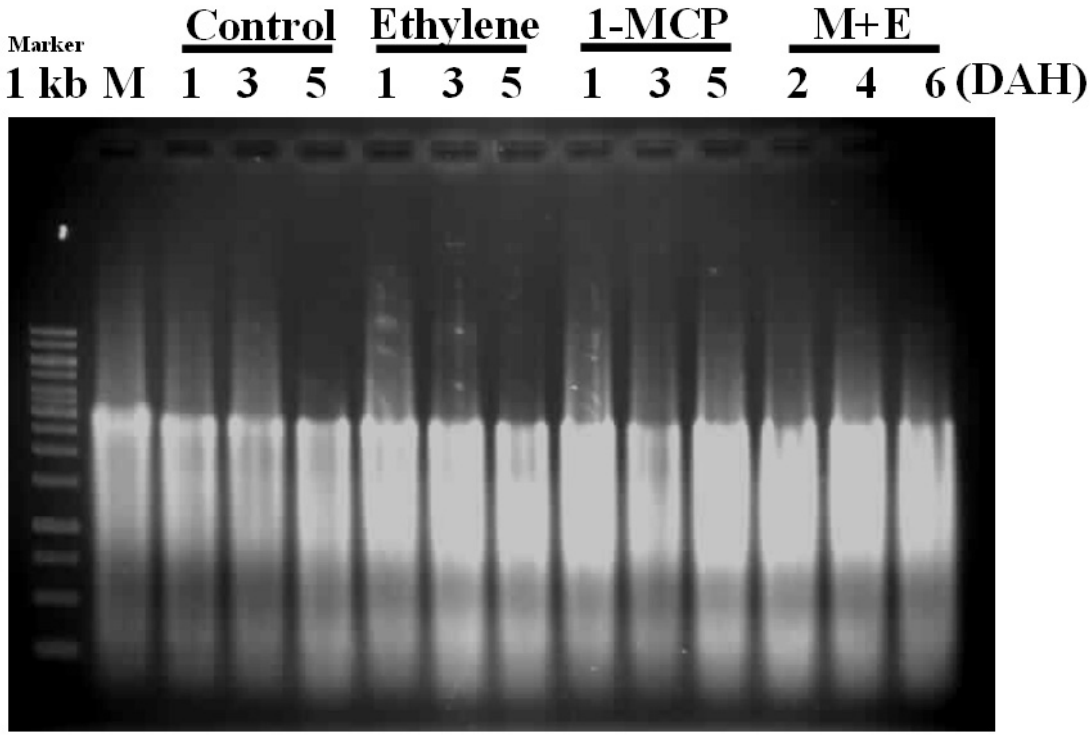
Appendix Figure 2 The RNA (2 μg) of sapodilla (*Manilkara zapota*) fruits cv. Kra-Suay during growth after anthesis until harvest maturity and in fruits after harvest, was run by gel electrophoresis on 0.8% agarose gel.



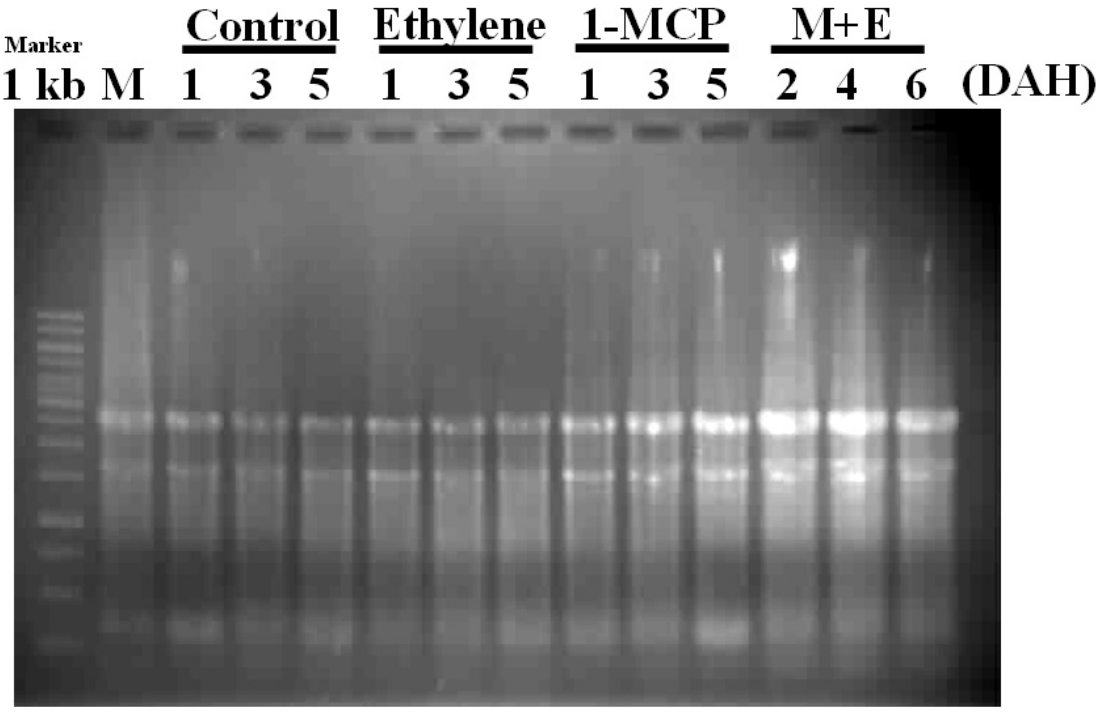
Appendix Figure 3 RNA (30 μg) loading on 1.0% MOPs-formaldehyde agarose gel before transferring to a positively charged nylon membrane**,** in sapodilla (*Manilkara zapota*) fruits cv. Makok-Yai during growth after anthesis until harvest maturity and in fruits after harvest.



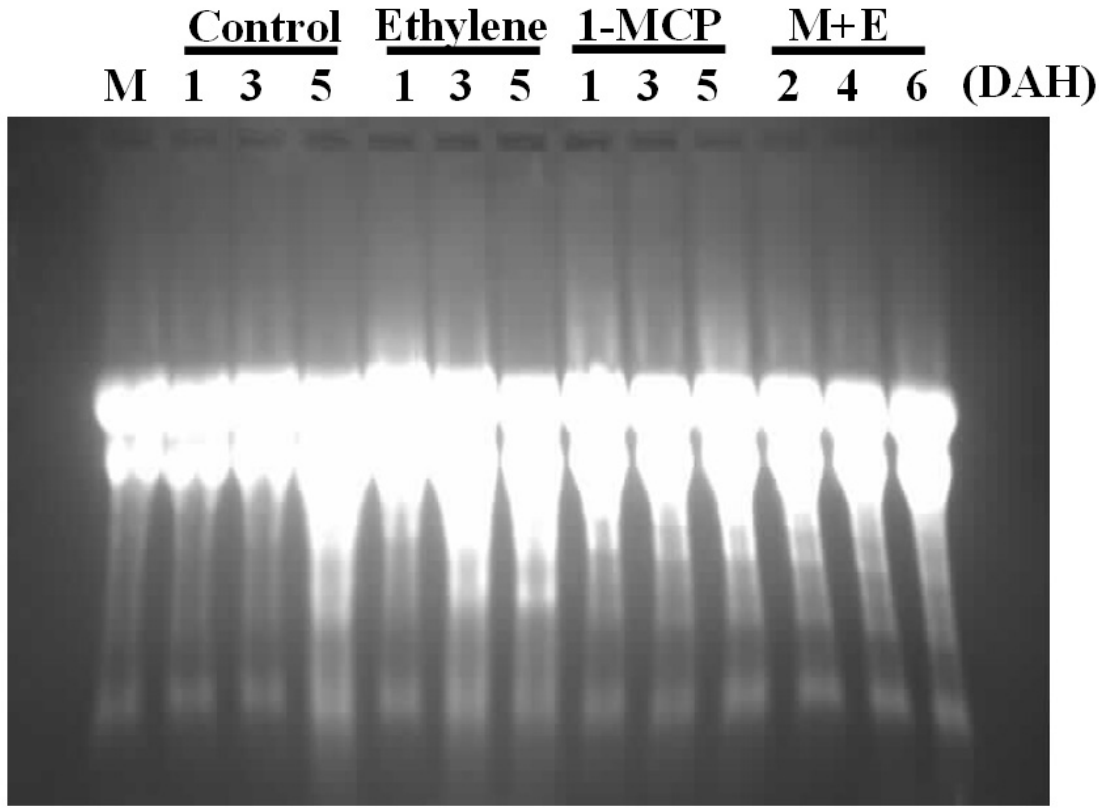
Appendix Figure 4 RNA (30 μg) loading on 1.0% MOPs-formaldehyde agarose gel before transferring to a positively charged nylon membrane**,** in sapodilla (*Manilkara zapota*) fruits cv. Kra-Suay during growth after anthesis until harvest maturity and in fruits after harvest.



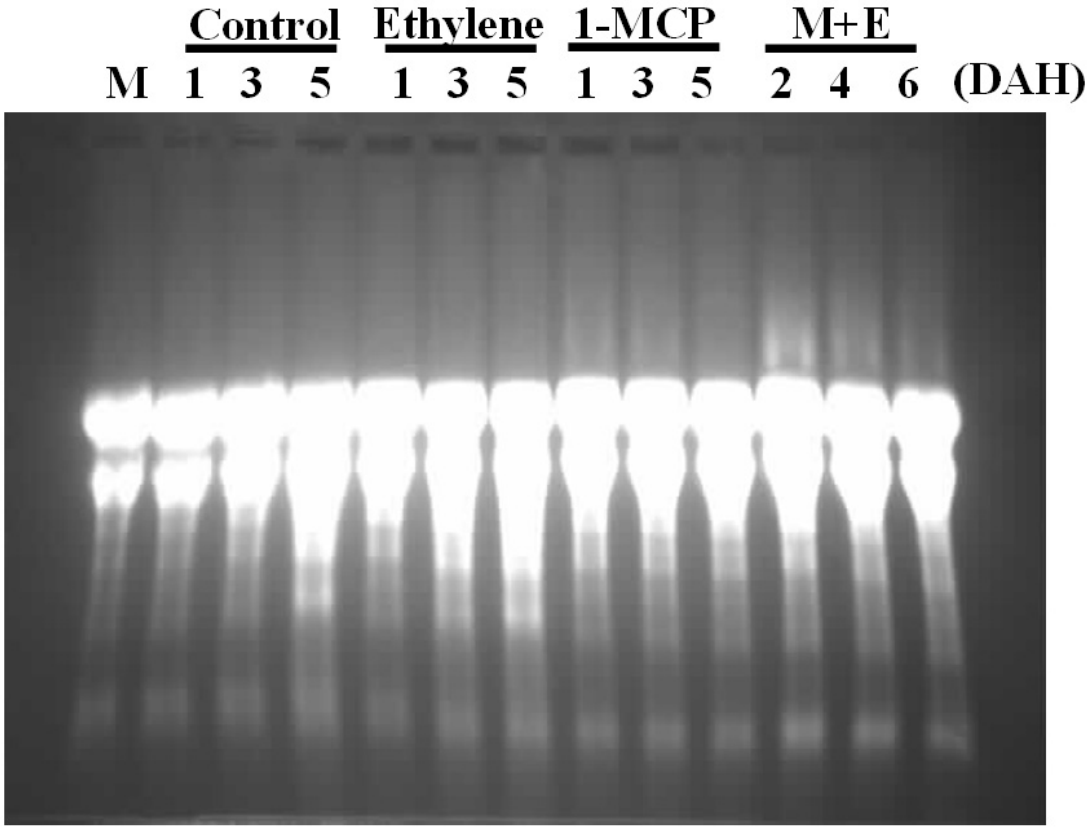
Appendix Figure 5 Gel electrophoresis on 0.8% agarose gel of the RNA (2 μg) of sapodilla (*Manilkara zapota*) fruits cv. Makok-Yai at harvest maturity (M), and at 1, 3, and 5 days after harvest (DAH) in controls. Treatment with ethylene (one day) started immediately after harvest, thus the data on day 1 are at the end of ethylene treatment, and those of day 3 and 5 refer to day 2 and 4 after the treatment. Similarly, 1-MCP was applied for one day, immediately after harvest. In the case of 1-MCP + ethylene (M+E) the 1-MCP treatment (one day) preceded the ethylene treatment (one day).



Appendix Figure 6 Gel electrophoresis on 0.8% agarose gel of the RNA (2 μg) of sapodilla (*Manilkara zapota*) fruits cv. Kra-Suay at harvest maturity (M), and at 1, 3, and 5 days after harvest (DAH) in controls. Treatment with ethylene (one day) started immediately after harvest, thus the data on day 1 are at the end of ethylene treatment, and those of day 3 and 5 refer to day 2 and 4 after the treatment. Similarly, 1-MCP was applied for one day, immediately after harvest. In the case of 1-MCP + ethylene (M+E) the 1-MCP treatment (one day) preceded the ethylene treatment (one day).



Appendix Figure 7 RNA (30 μg) loading on 1.0% MOPs-formaldehyde agarose gel before transferring to a positively charged nylon membrane**,** in sapodilla fruits cv. Makok-Yai at harvest maturity (M), and at 1, 3, and 5 days after harvest (DAH) in controls. Treatment with ethylene (one day) started immediately after harvest, thus the data on day 1 are at the end of ethylene treatment, and those of day 3 and 5 refer to day 2 and 4 after the treatment. Similarly, 1-MCP was applied for one day, immediately after harvest. In the case of 1-MCP + ethylene (M+E) the 1-MCP treatment (one day) preceded the ethylene treatment (one day).



Appendix Figure 8 RNA (30 μg) loading on 1.0% MOPs-formaldehyde agarose gel before transferring to a positively charged nylon membrane**,** in sapodilla fruits cv. Kra-Suay at harvest maturity (M), and at 1, 3, and 5 days after harvest (DAH) in controls. Treatment with ethylene (one day) started immediately after harvest, thus the data on day 1 are at the end of ethylene treatment, and those of day 3 and 5 refer to day 2 and 4 after the treatment. Similarly, 1-MCP was applied for one day, immediately after harvest. In the case of 1-MCP + ethylene (M+E) the 1-MCP treatment (one day) preceded the ethylene treatment (one day).

Appendix Table 1 Changes in firmness (Newtons) of sapodilla fruits after harvest.

Treatment	Days after harvest						
	0	1	2	3	4	5	6
cv. Makok-Yai	15.94	15.40	14.83	12.95	12.55	7.64	5.07
cv. Kra-Suay	16.02	15.12	13.40	11.81	9.89	5.43	3.79
t-test	ns	ns	ns	ns	ns	*	ns

ns = non-significantly different at $p \leq 0.05$

* = significantly different at $p \leq 0.05$

Appendix Table 2 Changes in fruit firmness (Newtons) of sapodilla fruits of cv. Makok-Yai during ripening process after harvest as control fruits and treated fruits with ethylene,1-MCP and 1-MCP + ethylene.

Treatment	Days after harvest					
	0	1	2	3	4	5
Control	15.94	15.42 b	12.93 b	12.57 b	9.72 c	7.69 c
C_2H_4	-	9.10 c	5.97 c	4.33 c	3.88 d	2.49 d
1-MCP	-	16.00 a	16.27 a	16.03 a	15.48 b	16.15 b
1-MCP + C_2H_4	-	-	16.30 a	16.21 a	16.15 a	16.58 a
F-test	-	**	**	**	**	**
C.V. (%)	-	2.65	2.75	2.95	3.55	2.49

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 3 Changes in fruit firmness (Newtons) of sapodilla fruits of cv. Kra-Suay during ripening process after harvest as control fruits and treated fruits with ethylene,1-MCP and 1-MCP + ethylene.

Treatment	Days after harvest					
	0	1	2	3	4	5
Control	16.02	15.12 b	13.40 b	11.81 b	10.87 b	5.43 b
C_2H_4	-	10.26 c	6.42 c	5.11 c	3.60 c	3.59 c
1-MCP	-	16.18 a	16.26 a	16.23 a	16.06 a	16.10 a
1-MCP + C_2H_4	-	-	15.98 a	16.47 a	16.63 a	16.06 a
F-test	-	**	**	**	**	**
C.V. (%)	-	4.48	1.79	3.37	5.91	6.08

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 4 Changes in L* value of sapodilla fruit peel color of cv. Makok-Yai during ripening process after harvest as control fruits and treated fruits with ethylene, 1-MCP and 1-MCP + ethylene.

Treatment	Days after harvest					
	0	1	2	3	4	5
Control	62.44	63.16	62.05	63.97	63.05 a	63.83 a
C_2H_4	-	63.47	62.80	63.19	58.74 b	55.78 b
1-MCP	-	62.56	62.34	63.23	63.34 a	63.19 a
1-MCP + C_2H_4	-	-	62.50	62.98	62.79 a	63.07 a
F-test	-	ns	ns	ns	**	**
C.V. (%)	-	1.11	1.21	1.16	1.36	1.05

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

ns = non-significantly different at $p \leq 0.05$

** = significantly different at $p \leq 0.01$

Appendix Table 5 Changes in L* value of sapodilla fruit peel color of cv. Kra-Suay during ripening process after harvest as control fruits and treated fruits with ethylene, 1-MCP and 1-MCP + ethylene.

Treatment	Days after harvest					
	0	1	2	3	4	5
Control	60.40	60.70	61.19 a	61.46 a	61.57 a	58.79 a
C_2H_4	-	60.89	60.52 ab	59.61 b	56.59 b	53.32 b
1-MCP	-	60.07	58.64 c	59.75 b	60.52 a	60.23 a
1-MCP + C_2H_4	-	-	59.78 bc	59.51 b	60.74 a	59.49 a
F-test	-	ns	**	**	**	**
C.V. (%)	-	1.48	1.47	1.16	1.24	2.29

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

ns = non-significantly different at $p \leq 0.05$

** = significantly different at $p \leq 0.01$

Appendix Table 6 Changes in a* value of sapodilla fruit peel color of cv. Makok-Yai during ripening process after harvest as control fruits and treated fruits with ethylene, 1-MCP and 1-MCP + ethylene.

Treatment	Days after harvest					
	0	1	2	3	4	5
Control	-5.80	-5.86 b	-5.92 b	-4.02 b	-3.96 b	-1.55 b
C_2H_4	-	-5.37 a	-4.04 a	-1.55 a	1.01 a	3.91 a
1-MCP	-	-6.24 b	-6.40 b	-5.24 c	-5.16 c	-4.49 c
1-MCP + C_2H_4	-	-	-6.54 b	-6.23 d	-4.43 bc	-4.60 c
F-test	-	**	**	**	**	**
C.V. (%)	-	6.53	14.65	10.24	8.09	7.48

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 7 Changes in a* value of sapodilla fruit peel color of cv. Kra-Suay during ripening process after harvest as control fruits and treated fruits with ethylene, 1-MCP and 1-MCP + ethylene.

Treatment	Days after harvest					
	0	1	2	3	4	5
Control	-5.53	-4.75 b	-3.46 a	-3.25 b	-1.89 b	2.19 b
C_2H_4	-	-3.27 a	-2.71 a	-1.78 a	2.94 a	4.95 a
1-MCP	-	-5.38 b	-5.43 b	-5.18 c	-3.89 c	-2.76 c
1-MCP + C_2H_4	-	-	-5.40 b	-5.06 c	-3.97 c	-4.08 d
F-test	-	**	**	**	**	**
C.V. (%)	-	12.57	14.25	9.60	9.62	7.84

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 8 Changes in b* value of sapodilla fruit peel color of cv. Makok-Yai during ripening process after harvest as control fruits and treated fruits with ethylene, 1-MCP and 1-MCP + ethylene.

Treatment	Days after harvest					
	0	1	2	3	4	5
Control	34.85	34.67	34.52	35.75	34.88 a	36.34 a
C_2H_4	-	34.37	33.57	34.41	30.46 b	26.96 c
1-MCP	-	33.85	34.26	34.54	35.07 a	35.47 ab
1-MCP + C_2H_4	-	-	34.28	34.63	33.91 a	34.79 b
F-test	-	ns	ns	ns	**	**
C.V. (%)	-	1.62	2.05	2.39	2.74	2.31

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

ns = non-significantly different at $p \leq 0.05$

** = significantly different at $p \leq 0.01$

Appendix Table 9 Changes in b* value of sapodilla fruit peel color of cv. Kra-Suay during ripening process after harvest as control fruits and treated fruits with ethylene, 1-MCP and 1-MCP + ethylene.

Treatment	Days after harvest					
	0	1	2	3	4	5
Control	33.35	33.89	33.99 a	34.19 a	34.51 a	32.70 a
C_2H_4	-	33.74	31.77 b	31.84 b	29.67 b	24.44 b
1-MCP	-	33.02	32.17 b	33.49 a	33.99 a	34.10 a
1-MCP + C_2H_4	-	-	32.40 b	33.04 ab	33.22 a	32.40 a
F-test	-	ns	*	*	**	**
C.V. (%)	-	2.40	2.93	2.97	3.51	3.88

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

ns = non-significantly different at $p \leq 0.05$

* = significantly different at $p \leq 0.05$

** = significantly different at $p \leq 0.01$

Appendix Table 10 Changes in TSS (%) of sapodilla fruits of cv. Makok-Yai during ripening process after harvest as control fruits and treated fruits with ethylene,1-MCPand 1-MCP + ethylene.

Treatment	Days after harvest					
	0	1	2	3	4	5
Control	21.75	21.20 a	21.80 a	20.90 b	21.65 b	22.45 c
C_2H_4	-	17.85 b	18.90 c	19.60 c	19.80 c	20.55 d
1-MCP	-	20.75 a	20.90 b	22.00 a	22.55 a	24.75 a
1-MCP + C_2H_4	-	-	20.60 b	21.80 a	22.85 a	23.60 b
F-test	-	**	**	**	**	**
C.V. (%)	-	1.99	2.21	2.10	2.64	1.93

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 11 Changes in TSS (%) of sapodilla fruits of cv. Kra-Suay during ripening process after harvest as control fruits and treated fruits with ethylene,1-MCPand 1-MCP + ethylene.

Treatment	Days after harvest					
	0	1	2	3	4	5
Control	24.25	24.85 a	23.10 c	22.65 b	22.20 c	22.85 c
C_2H_4	-	20.95 b	20.95 d	20.25 c	21.35 c	19.65 d
1-MCP	-	24.80 a	26.65 a	27.00 a	27.95 a	27.65 a
1-MCP + C_2H_4	-	-	25.00 b	26.10 a	25.95 b	26.40 b
F-test	-	**	**	**	**	**
C.V. (%)	-	2.71	3.13	3.14	4.01	2.69

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 12 Changes in TA (%) of sapodilla fruits of cv. Makok-Yai during ripening process after harvest as control fruits and treated fruits with ethylene,1-MCPand 1-MCP + ethylene.

Treatment	Days after harvest					
	0	1	2	3	4	5
Control	0.22	0.20 a	0.20 b	0.11 b	0.13 b	0.08 b
C_2H_4	-	0.07 b	0.05 c	0.06 c	0.07 c	0.07 b
1-MCP	-	0.23 a	0.24 a	0.22 a	0.22 a	0.23 a
1-MCP + C_2H_4	-	-	0.23 ab	0.22 a	0.24 a	0.25 a
F-test	-	**	**	**	**	**
C.V. (%)	-	15.28	16.59	14.41	14.79	11.20

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 13 Changes in TA (%) of sapodilla fruits of cv. Kra-Suay during ripening process after harvest as control fruits and treated fruits with ethylene,1-MCPand 1-MCP + ethylene.

Treatment	Days after harvest					
	0	1	2	3	4	5
Control	0.29	0.27 b	0.17 b	0.17 b	0.14 b	0.09 b
C_2H_4	-	0.06 c	0.07 c	0.07 c	0.08 b	0.09 b
1-MCP	-	0.33 a	0.35 a	0.41 a	0.40 a	0.40 a
1-MCP + C_2H_4	-	-	0.37 a	0.42 a	0.35 a	0.41 a
F-test	-	**	**	**	**	**
C.V. (%)	-	12.81	17.38	10.98	17.12	11.02

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 14 Changes in TSS/TA ratio of sapodilla fruits of cv. Makok-Yai during ripening process after harvest as control fruits and treated fruits with ethylene,1-MCPand 1-MCP + ethylene.

Treatment	Days after harvest					
	0	1	2	3	4	5
Control	99.20	105.5 b	117.1 b	197.6 b	167.2 b	272.3 a
C_2H_4	-	281.9 a	377.5 a	337.3 a	291.0 a	272.8 a
1-MCP	-	90.9 b	86.7 b	104.4 c	105.8 c	107.4 b
1-MCP + C_2H_4	-	-	93.1 b	97.0 c	94.1 c	93.6 b
F-test	-	**	**	**	**	**
C.V. (%)	-	19.86	12.16	13.87	16.00	11.04

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 15 Changes in TSS/TA ratio of sapodilla fruits of cv. Kra-Suay during ripening process after harvest as control fruits and treated fruits with ethylene,1-MCPand 1-MCP + ethylene.

Treatment	Days after harvest					
	0	1	2	3	4	5
Control	84.53	93.9 b	147.8 b	135.8 b	167.8 b	244.0 a
C_2H_4	-	339.0 a	308.7 a	288.2 a	271.1 a	230.3 a
1-MCP	-	76.0 b	75.9 c	66.5 c	72.1 c	70.6 b
1-MCP + C_2H_4	-	-	69.6 c	62.5 c	76.3 c	65.5 b
F-test	-	**	**	**	**	**
C.V. (%)	-	9.03	19.77	6.70	16.61	6.16

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 16 Changes in endo-β-1,4-glucanase activity (μmol D-glucose mg^{-1} protein min^{-1}) of sapodilla fruits of cv. Makok-Yai accessed after harvest for control, ethylene and 1-MCP treated fruits.

Treatment	Days after harvest			
	0	1	3	5
Control	7.16	6.54 b	7.18 b	13.18 a
C_2H_4	-	7.54 a	10.71 a	11.55 b
1-MCP	-	5.06 c	5.26 c	6.77 c
F-test	-	**	**	**
C.V. (%)	-	2.76	2.27	1.71

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 17 Changes in endo-β-1,4-glucanase activity (μmol D-glucose mg^{-1} protein min^{-1}) of sapodilla fruits of cv. Kra-Suay accessed after harvest for control, ethylene and 1-MCP treated fruits.

Treatment	Days after harvest			
	0	1	3	5
Control	8.76	7.85 a	8.49 b	9.13 b
C_2H_4	-	7.90 a	9.29 a	10.16 a
1-MCP	-	6.57 b	5.68 c	7.70 c
F-test	-	**	**	**
C.V. (%)	-	3.74	2.69	2.01

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 18 Changes in polygalacturonase activity (μmol galacturonic acid mg^{-1} protein h^{-1}) of sapodilla fruits of cv. Makok-Yai accessed after harvest for control, ethylene and 1-MCP treated fruits.

Treatment	Days after harvest			
	0	1	3	5
Control	1.84	3.51 a	6.07 a	7.51 a
C_2H_4	-	4.49 a	7.91 a	6.33 a
1-MCP	-	1.60 b	2.92 b	3.75 b
F-test	-	**	**	**
C.V. (%)	-	19.48	17.18	10.95

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 19 Changes in polygalacturonase activity (μmol galacturonic acid mg^{-1} protein h^{-1}) of sapodilla fruits of cv. Kra-Suay accessed after harvest for control, ethylene and 1-MCP treated fruits.

Treatment	Days after harvest			
	0	1	3	5
Control	3.16	5.63 a	8.14 a	6.79 a
C_2H_4	-	5.51 a	7.64 a	4.96 b
1-MCP	-	3.10 b	3.26 b	3.60 b
F-test	-	*	**	**
C.V. (%)	-	17.10	13.20	13.40

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

* = significantly different at $p \leq 0.05$

** = significantly different at $p \leq 0.01$

Appendix Table 20 Changes in water-soluble pectin content (μg galacturonic acid mg^{-1} AIS) in control, ethylene and 1-MCP treatment of sapodilla fruits of cv. Makok-Yai after harvest.

Treatment	Days after harvest			
	0	1	3	5
Control	2.54	6.99 b	11.83 b	12.45 b
C_2H_4	-	17.01 a	19.09 a	26.36 a
1-MCP	-	3.09 c	3.76 c	4.46 c
F-test	-	**	**	**
C.V. (%)	-	13.30	3.19	5.84

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 21 Changes in water-soluble pectin content (μg galacturonic acid mg^{-1} AIS) in control, ethylene and 1-MCP treatment of sapodilla fruits of cv. Kra-Suay after harvest.

Treatment	Days after harvest			
	0	1	3	5
Control	3.83	4.52 b	6.35 b	13.45 b
C_2H_4	-	15.25 a	27.92 a	34.46 a
1-MCP	-	3.47 c	3.70 b	6.82 c
F-test	-	**	**	**
C.V. (%)	-	6.40	13.91	9.30

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 22 Changes in chelator (CDTA)-soluble pectin content (μg galacturonic acid mg^{-1} AIS) in control, ethylene and 1-MCP treatment of sapodilla fruits of cv. Makok-Yai after harvest.

Treatment	Days after harvest			
	0	1	3	5
Control	5.74	8.80 a	4.07 b	7.68 b
C_2H_4	-	6.16 b	4.40 b	3.53 c
1-MCP	-	9.16 a	11.45 a	13.03 a
F-test	-	*	**	**
C.V. (%)	-	14.04	15.91	16.73

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

* = significantly different at $p \leq 0.05$

** = significantly different at $p \leq 0.01$

Appendix Table 23 Changes in chelator (CDTA)-soluble pectin content (μg galacturonic acid mg^{-1} AIS) in control, ethylene and 1-MCP treatment of sapodilla fruits of cv. Kra-Suay after harvest.

Treatment	Days after harvest			
	0	1	3	5
Control	10.56	13.94 a	8.37 b	9.03 b
C_2H_4	-	6.24 c	0.88 c	2.94 c
1-MCP	-	10.38 b	14.43 a	16.81 a
F-test	-	**	**	**
C.V. (%)	-	11.86	16.70	11.90

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 24 Changes in Na_2CO_3-soluble pectin content (μg galacturonic acid mg^{-1} AIS) in control, ethylene and 1-MCP treatment of sapodilla fruits of cv. Makok-Yai after harvest.

Treatment	Days after harvest			
	0	1	3	5
Control	7.00	10.47 b	7.21	13.14 b
C_2H_4	-	15.43 a	9.44	5.63 c
1-MCP	-	16.29 a	8.34	24.24 a
F-test	-	*	ns	**
C.V. (%)	-	13.76	12.14	10.09

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

ns = non-significantly different at $p \leq 0.05$

* = significantly different at $p \leq 0.05$

** = significantly different at $p \leq 0.01$

Appendix Table 25 Changes in Na_2CO_3-soluble pectin content (μg galacturonic acid mg^{-1} AIS) in control, ethylene and 1-MCP treatment of sapodilla fruits of cv. Kra-Suay after harvest.

Treatment	Days after harvest			
	0	1	3	5
Control	5.61	22.32 a	6.12 b	21.09 b
C_2H_4	-	3.54 c	3.76 c	5.44 c
1-MCP	-	16.71 b	32.25 a	45.12 a
F-test	-	**	**	**
C.V. (%)	-	18.67	4.16	12.49

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 26 Changes in neutral sugar content (μg glucose mg^{-1} AIS) of 1 N KOH-soluble fraction in control, ethylene and 1-MCP treatment of sapodilla fruits of cv. Makok-Yai after harvest.

Treatment	Days after harvest			
	0	1	3	5
Control	18.92	22.50 a	21.91 b	32.33 b
C_2H_4	-	23.97 a	27.30 a	37.36 a
1-MCP	-	17.08 b	23.07 b	23.09 c
F-test	-	**	*	**
C.V. (%)	-	4.56	8.16	7.57

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

* = significantly different at $p \leq 0.05$

** = significantly different at $p \leq 0.01$

Appendix Table 27 Changes in neutral sugar content (μg glucose mg^{-1} AIS) of 1 N KOH-soluble fraction in control, ethylene and 1-MCP treatment of sapodilla fruits of cv. Kra-Suay after harvest.

Treatment	Days after harvest			
	0	1	3	5
Control	20.02	26.58 b	17.97 b	38.19 a
C_2H_4	-	41.98 a	26.96 a	43.73 a
1-MCP	-	19.47 c	24.15 a	28.13 b
F-test	-	**	**	**
C.V. (%)	-	3.13	6.49	7.87

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 28 Changes in neutral sugar content (μg glucose mg^{-1} AIS) of 4 N KOH-soluble fraction in control, ethylene and 1-MCP treatment of sapodilla fruits of cv. Makok-Yai after harvest.

Treatment	Days after harvest			
	0	1	3	5
Control	32.73	46.82 b	35.72 c	37.64 b
C_2H_4	-	50.99 a	50.10 a	42.85 a
1-MCP	-	31.05 c	46.81 b	31.41 c
F-test	-	**	**	**
C.V. (%)	-	1.87	1.12	6.24

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

Appendix Table 29 Changes in neutral sugar content (μg glucose mg^{-1} AIS) of 4 N KOH-soluble fraction in control, ethylene and 1-MCP treatment of sapodilla fruits of cv. Kra-Suay after harvest.

Treatment	Days after harvest			
	0	1	3	5
Control	44.41	54.80 a	39.22 b	34.63 b
C_2H_4	-	27.64 b	42.51 a	29.24 c
1-MCP	-	53.63 a	36.80 c	61.93 a
F-test	-	**	**	**
C.V. (%)	-	3.19	1.82	0.99

Mean values followed by different letters in the same column were significantly different at $p \leq 0.05$ by LSD.

** = significantly different at $p \leq 0.01$

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