CHANGES IN CELL WALL COMPOSITION AND ENZYME ACTIVITIES IN HUSK DEHISCENCE OF 'MONTHONG' DURIANS

INTRODUCTION

Durian is one of the most important fruit crops grown in Thailand, with. The main growing areas are in the East and in the South, and a combined export value of more than 2,600 million Baht (Office of Agricultural Economics, 2005). Postharvest problems of durian, however, do still exist, such as rapid ripening, fruit rot, as well as physiological disorders such as chilling injury, water core and uneven fruit ripening (Kader, 2004), all of which reduce the price of durian.

The short shelf life of durian fruits is due to their high respiration and ethylene production. Consumer preferences for different stages of ripening also cause postharvest problems. Thai consumers prefer to consume durian fruit that have just begun to ripe. Fruits at this stage are difficult to dehusk. Other consumers, such as Malaysians and Singaporeans, prefer fully ripe fruits that tend to dehisce easily (Kosiyachinda, 1986). If these postharvest processes could be controlled, consumer demand for fruit at different developmental stages could be met accordingly.

In the past, researchers reported various methods for extending shelf life of durian. Suggested treatment methods include low temperature (Praditdoung, 1986; Imsabai, 1999), modified atmosphere (Suwannakul and Tongdee, 1988) and waxing (Kuansongbhum, 1987; Chupmadee, 1989; Sriyook, 1990). These methods delayed both dehiscence and pulp softening. Husk dehiscence alone can be delayed using gibberellic acid (GA_3) treatments (Siriatiwat, 1988; Nampradit, 1991), while pulp development proceeded normally. On the other hand, dehiscence can be enhanced by allowing a high rate of water loss, while softening rates of the pulp proceeded normally. Moreover, durian may be treated with ethylene to enhance ripening which includes both, pulp softening and husk dehiscence. Although husk dehiscence can be manipulated somewhat, the physiological basis of these developmental processes are not yet well understood.

During fruit softening, it is known that cell wall components are degraded and solubilized. In tomatoes, the fruits whose ripening processes have been most thoroughly studied, the process is controlled by various cell wall enzymes including polygalacturonase (PG), pectin methylesterase (PME), β-galactosidase, endo-(1,4)β-D-glucanase (EGase), xyloglucan endotransglycosylase (XET) and expansin (Brummell *et al.*, 1999). The roles of these enzymes vary with stages of fruit development as well as among species. Often, different isoforms of these enzymes play tissue-specific roles in cell wall modification at specific stages of development. In durian pulp, it was found that the activities of PG and PME increase more rapidly than those of β-galactosidase and cellulase (Ketsa and Daengkanit, 1998).

Fruit dehiscence has been studied mostly in oilseed rape and arabidopsis. It was shown that the mechanism is similar to abscission processes. It is also regulated by cell wall degrading enzymes especially PGs and β-1,4-glucanase. In addition to the weakness of the cell wall in the dehiscence zone (DZ), the dehiscence process depends on tension developed in the pod as a result of water loss (Tiwari and Bhatia, 1995). The process also responds to ethylene produced during the development of various fruits (Sexton and Robert, 1982).

In durian, dehiscence is associated with ripening, and tends to occur when the fruit was fully ripened or over-ripened (Sriyook, 1990). Fruit dehiscence takes place along the suture on the back of the locules, which have a DZ (Praditkul, 1987). The blossom end of the fruit is usually first to dehisce, but any point along the suture is vulnerable.

Dehiscence is greater under low relative humidity storage conditions; water loss is promoted, resulting in shrinkage of the husk separating the locules from each other at the DZ (Sriyook, 1990). Exposing durians to ethylene enhances ripening and dehiscence of the fruit. Coating or waxing the surface of the fruit reduces weight loss, ethylene production, and delays dehiscence and other ripening processes such as pulp softening and conversion of starch to sugar (Sriyook *et al*., 1994).

At present, there is no report about the changes in cell wall compositions and the role of cell wall degrading enzymes involved in durian fruit dehiscence. This study was set to determine the role of cell wall enzymes in association with fruit dehiscence. The changes in cell wall composition during dehiscence processes were also determined.

The objectives of this study were:

1. To examine changes in cell wall composition and molecular size distribution of pectin and hemicellulose in the durian dehiscence zone (DZ) and it's surrounding husk during fruit dehiscence.

2. To determine the activities of cell wall degrading enzymes in the DZ and the husk during fruit dehiscence.

3. To study the effect of gibberellic acid (GA_3) on durian fruit dehiscence, as well as its relation to cell wall composition and cell wall degrading enzymes.

LITERATURE REVIEW

Durian (*Durio zibethinus* Murray) is a tropical fruit of the Bombacaceae family. It is commercially important and often referred to as the 'King of Fruits' in South-East Asia. Growing areas in Thailand are in the East and in the South. The export value was more than 2,600 million Bahts in 2005 (Office of Agricultural Economics, 2005).

The most important postharvest problem was the fruits short shelf life. The rise in respiration and ethylene production coincides with ripening (Ketsa and Daengkanit, 1998). Climacteric respiration increased from 24°C to 33°C, while the ethylene production reached a maximum at 27°C and reduced rapidly as the temperature was increased from 30°C to 33°C (Ketsa and Pangkool, 1995). This indicates that ethylene production in durian appears to be more sensitive to high temperature than is respiration. The ripening process occurred rapidly in 3-4 days at room temperature (~30°C). Increases in soluble solids, total sugar, reducing sugar and β-carotene content were observed, while starch content declined (Suttraphun, 1993). Firmness declined during ripening and was found to be correlated with the increase in activities of PG and PME (Ketsa and Daengkanit, 1998).

In the past, researchers reported methods to extend shelf life by delaying husk dehiscence and pulp softening together. Smodmongkon *et al*. (1974) reported an extended shelf life of Kanyao durian (up to 9 days) by using $KMnO_4$ to remove ethylene, while non-treated durians could be kept for only 3-4 days. Praditdoung (1986) showed that Kanyao durian pulp stored at low temperature at 4°C and wrapped with LDP film could extend shelf life up to 40 days. Imsabai (1999) reported that Chanee durian stored at 12°C had a very low rate of pulp softening and pulp color development, while soluble solids increased normally. Modified atmosphere of about 5-7.5% O_2 at 20°C delayed ripening by 7 days (Suwannakul and Tongdee, 1988). Kuansongthom (1987) reported that coating durian with Semperfresh delayed ripening and reduced water loss by about 35%. Chupmadee (1989) found that waxing with FMC Sta-fresh # 7055 (dissolved in water at a ratio of 1:5, 1:7 and 1:9) could extend shelf life of Chanee durian held at room temperature for 4 days, reduce water loss by about 25%, and reduce husk dehiscence by 50-80% compared with the control. In addition, Sriyook (1990) reported that 1% Semperfresh reduced weight loss and fruit dehiscence, delayed flesh firmness and delayed the decrease in starch and ripening-related increases in soluble solids content. This coating material extended durian shelf life for 9 days while non-treated durian could be kept for only 3-4 days. Chaiprasart (1993) found that waxing with 20% FMC Sta-fresh # 7055, followed by storage at 15°C could extend durian shelf life to about 14 days. Applying a GA_3 solution at 100 ppm could delay color change and fruit dehiscence of Chanee durian for 2 days (Siriatiwat, 1988). However, Nampradit (1991) showed that using a 50 ppm GA_3 solution together with 1%Semperfresh was no better than using Semperfresh alone, delaying durian fruit dehiscence by 3 days.

The various methods above delayed durian fruit ripening by controlling the rate of respiration and ethylene production. In durian, most of the ethylene production is associated with the husk, as the pulp has only a very low ethylene production rate (Siriphanich, 1996). When the pulp and husk of durian were separated, the softening of the dehusked pulp was slower than pulp of intact fruit. This indicated that the ripening of the durian aril requires ethylene from the surrounding husk (Booncherm, 1990).

Structure of Plant Cell Wall

The plant's primary cell wall is a complex structure of networks composed of various polysaccharides, structural proteins and some phenolics (Carpita and McCann, 2000). Its highly hydrate (~65% water) and the aqueous component contains many kinds of dissolved solutes, ions and soluble proteins, including enzymes. Even though in general the idea of the primary cell wall structure is understood as that of cellulose microfibrils embedded in a varied matrix of pectin and glycan polysaccharides, the details about the interaction between the components are not clear.

Cellulose microfibril are composed of 1,4-β-D-glucan chains which support each other by hydrogen bonds. The chains are large and similar to individual fibers of cotton thread. The internal region of microfibrils is crystalline and excludes water, whereas the outer layers are more amorphous (Pauly *et al*., 1999). Like cellulose, the matrix glycans known as hemicellulose, are composed of a 1,4-β-D-glucan backbone. They are neutral sugars or weakly acidic, but do not contain galacturonic acid (GalA). The major type of hemicellulose is xyloglucan, which has xylose side chains, and some are extended with either galactose-fucose or with arabinose. The other two major matrix glycans are glucuronoarabinoxylan and glucomannan. Pectin (poly 1,4-α-D-GalA) has high contents of GalA residues, which can be linear and unbranced, known as homogalacturonan. The side chain may be composed of a single xylose (xylogalcturonan) or of a complex containing numerous neutral sugars (rhamnogalacturonan I and II, or RG-I/II). RG-I or II has a backbone of alternating GalA and rhamnose residues, possessing large linear or branched arabinan and galactan side chains.

Beside these three components, there are several types of structural proteins which are heavily glycosylated. The ratio of these components varies between species, but is generally composed of approximately equal amounts of cellulose, matrix glycans and pectins (one-third of the dry weight of each). Structural proteins are much less abundant, comprising only 1-10% of the dry weight. However, fruit cell walls are generally enriched with pectin, which can form up to half of the polymeric content of the cell wall.

There are many types of bonds, interlinking with each other the various components of a primary cell wall. Hydrogen bonds are attached together hemicellulose to cellulose, and between each RG-II molecules through borate diesters. Homogalcturonan molecules are attached to each other through ionic calcium bridges. The ester bonds probably attach pectin molecules to other pectin,

glycan or phenolic molecules (Carpita and Gibeaut, 1993). Covalent bond links structural proteins, or structural protein and phenolics (Fry, 1986). A covalent bond also links between xyloglucan and the arabinan/galactan side chains of RG-I (Thompson and Fry, 2000), and between RG-I structural protein extensions (Qi *et al*., 1995).

At present, no model of a primary cell wall structure explains the measured physical properties. However, the most common models are referred to by Cosgrove (2000) as the sticky network and multi-coat models. In the sticky network models each XG and xylose molecules bonds to and spans between adjacent cellulose microfibrils; in addition, pectin forms a co-extensive but separate network (Hayashi, 1989; Carpita and Gibeaut, 1993). In the multi-coat models, layers of matrix glycans coat each microfibril, and extend into the regions between microfibrils (Talbott and Ray, 1992).

The study on cell walls compositions was normally carried out after fractionation based on solubility. The sequential extracts were provided by extracting with chelating agents (CDTA or EDTA), sodium carbonate, dilute and concentrated alkali (KOH or NaOH), and a residue composed mainly of cellulose (Brummell, 2006).

Mechanism of Fruit Dehiscence

Most studies on the process of dehiscence have been centered on oilseed rape, soybean and arabiopsis, where premature seed shedding is an economically significant problem. The DZ is located at the carpel margins adjacent to the septum along the length of the silique with a similar process in *Arabidopsis* (Ferrandiz, 2002). Dehiscence processes include tensions developed in the pod wall and a zone of weakness that was also found in other species, including sesame (Degan *et al.*, 2001) and soybean (Tiwari and Bhatia, 1995). Reducing tensions created in the pod wall and the DZ, reduced pod dehiscence.

Ethylene is believed to acts as a regulator of dehiscence. During oilseed rape pods development, a slightly delayed shattering was correlated with reduced ethylene production, and normal timing of cell separation could be restored by the application of exogenous ethylene (Child *et al*., 1998). However, *Arabidopsis* mutants with non-functional ethylene receptors exhibit a normal time-course of silique dehiscence, suggesting that ethylene might have a minor role in shatter induction (Ferrandiz, 2002).

In durian, dehiscence is associated with ripening, and tends to occur when the fruit is fully ripe. Fruit dehiscence takes place along the suture of the center of each locules, which contain a DZ (Praditkul, 1987). The blossom end of the fruit is usually first to dehisce, but any point along the suture is vulnerable. Dehiscence is greater in low humidity, rather than high humidity (Sriyook, 1990). As a result of dehydration, the tissue surrounding the DZ may shrink in opposite directions of the zone. Coating or waxing the surface of the fruit to lessen weight loss can reduce dehiscence (Sriyook *et al*., 1994). Ethylene is another factor controlling this process. The role of

ethylene was confirmed by using auxin which works as an ethylene inhibitor treatment and could reduce the timing of fruit dehiscence (Roberts *et al*., 2002). Moreover, a 1-methylcyclopropene (1-MCP) treatment, which is an ethylene perception treatment, also delayed anther dehiscence (Rieu *et al*., 2003).

The first study into the anatomy of the DZ in durian fruit was reported by Koksungnoen (2005). The results of that study showed that the DZ in durian fruit consisted of small parenchyma cells. The outer DZ had a cell size smaller than the inner DZ and had more taniferous cells than the inner DZ, arranged into 3-4 rows. As the results of differences in cell size and taniferouse cell numbers, the inner DZ separated earlier than the outer DZ. However, on both outer and inner DZ, the number of cell layers and cell sizes in the DZ did not change before or after harvesting and until fruit dehiscence.

Changes in cell wall composition in fruit dehiscence have not been studied in the past. Instead, the scientist had primarily focused on the activity of enzymes and the expression of many genes, which were also identified and characterized during the dehiscence process (Roberts, 2002). The mechanism of the dehiscence process was believed to be similar to abscission. By means of an anatomical study, Patterson (2001) reported a rearrangement in cellulose microfibrils and the conversion of insoluble pectin to soluble pectic acid in the abscission zone. These studies suggested that enzymes associated with cell wall disassembly modifications including PG, PME, PL, EGase, and expansins might play more of a role during the abscission process.

Enzyme Activities Involved in Fruit Dehiscence

Endoglucanase (EGase) and endopolygalacturonase (endo-PG) are cell wall hydrolases that have been shown to be involved in abscission and pod dehiscence (Koehler *et al*., 1999; Jenkins *et al*., 1996). Pedersen *et al*. (1996) showed that the activity of EGase increased specifically in the DZ tissue prior to oilseed rape pod shatter. Application of auxins to the pods not only delayed increased in EGase activity but also cell separation in the DZ, confirming the role of this enzyme. In soybean, EGase and endo-PG activities increased concomitantly, suggesting a role for these enzymes in pod dehiscence of soybean (Koehler *et al*., 1999). In oilseed rape, it was shown that endo-PG was specifically expressed in the DZ and was involved in pod dehiscence and shatter (Pedersen *et al.*, 1996).

Other reports to support a role for both PG and EGase in pods dehiscence were provided by studies of infected oilseed rape pods (Jenkins *et al.*, 1996). A molecular study, performing RT-PCR on DZ RNA, showed that PG mRNA was identified and separated to SAC 66 and RDPG1 groups, respectively. They both encode and appear to homologous transcripts of 1.7 kb with 97% similarity to fruit endo-PGs genes. Northern analysis indicates that these transcripts are expressed specifically in the DZ of oilseed rape pods. GUS activity was also seen in the junction between the seed and funiculars, which suggests that the DZ PG plays a role in pod dehiscence and seed abscission (Jenkins *et al.*, 1996).

The role of another enzyme related to wall loosening and exhibiting activities specifically in the DZ of oilseed rape is xyloglucan endotransglycosylase (XET) (Fry *et al*., 1992). A XET-encoding gene is specifically up-regulated in the DZ of at the final stages of pod development (Roberts *et al*., 2000). In *Arabidopsis*, a study on GUS-expression showed that XET plays more of a role in the sepal and petal abscission zones (Ostergaard *et al*., 2001).

Other Physiological Processes Involving Cell Wall Breakdown

1. Abscission

Abscission is the process of shedding in parts of plants such as leaves, flowers, seeds, and fruits. This process occurs after the formation of an abscission zone at the point of separation which consists of a thin layer of cells, the abscission layer (Taylor and Whitelaw, 2001). Its can be divided into three major stages; 1) the stimulus stage, resulting from natural senescence or external factors; 2) the signal stage, characterized by several internal parameters such as an increase in ethylene production and 3) the response stage, marked by the production of hydrolytic enzymes such as cellulase and PG which are responsible for the degradation of the cell wall and the middle lamella (Sexton and Roberts, 1982). Another enzyme that may play a role in the abscission process is β-galactosidase. That activity was also expressed in the abscission zone during citrus fruit abscission (Wu and Burns, 2004).

2. Fruit Softening

Softening in many kinds of fruits are involved in the textural changes occurring during fruit ripening. It is also involved in the structural and composition of cell wall carbohydrates, mainly in pectin fractions by the action of cell wall degrading enzymes (Fischer and Bennett, 1991). Besides pectin, hemicellulose and cellulose are also modified during fruit softening (Maclachlan and Brady, 1994). However, most commonly reported are the changes in the pectin fractions. Pectin increases solubilization and depolymerization by the action of cell wall enzymes. Polygalacturonase (PG) activity is largely responsible for pectin depolymerization and solubilization, but the substrate of this enzyme requires pectin to be de-methylesterified by pectin methylesterase (PME). The removal of pectic galactan side-chains due to the activity of β-galactosidase is an important factor in cell wall changes and is related to firmness loss. So far it is uncertain how Endo-(1→4) beta-D-glucanase (EGase) activity is involved in fruit softening or in the depolymerization of matrix glycans. Further studies in the functioning of this enzyme are required to determine its role. Equally, additional research into XET activity is required to identify its role during fruit softening. A direct correlation has been established between expansin protein and fruit softening, and this has shown indirect effects on pectin depolymerization, confirming that this protein is involved in the fruit softening process (Brummell and Harpster, 2001).

Changes in Cell Wall Compositions during Cell Wall Degradation

During fruit ripening, cell wall polymers are progressively modified. The structures of cell walls become increasingly hydrated and are easily separated from one another (Jarvis, 1984). Mostly studies concerning such changes have focused on pectin, which is produced early in soft fruit such as tomato (Crookes and Grierson, 1983), but later in crisp fruit such as apple (Ben-Arie *et al*., 1997).

A general study on cell wall compositional changes during fruit softening has determined sequential extraction. The extraction was sequent with chelating agents such as trans-1,2-cyclohexanediamine N,N,N',N'-tetra-acetic acid (CDTA) or ethylenediamine-tetraacetic acid (EDTA), which removes calcium from the cell wall and extracts pectin held by ionic bonds. Sodium carbonate (Na_2CO_3) was then used to release the pectin that was held in the cell wall by covalent bonds, and the low molecular weight of pectin was also mostly extracted by this solution. The weak (1 M) alkali, followed by a strong alkali (4 M), were then used to extract loosely and tightly bound matrix glycans which were referred to hemicellulose fractions from the wall (Brummell and Harpster, 2001).

In many fruit, the major changes that occur during fruit ripening are produced by pectin and matrix glycans. Pectin becomes increasingly solubilized, depolymerized and de-esterified. In tomato, these changes occur at the mature green stage throughout fruit ripening (Carpita and Gibeaut, 1993). However, the fraction of both loosely-and tightly bound matrix glycans showed little changes in the molecular weight profile and depolymerization is limited (Brummell *et al*., 1999).

The fraction of matrix glycans loosely bound to cellulose, which is probably mainly glucomanan and xylans, shows little changes in the molecular weight profile during ripening. Depolymerization of matrix glycans is limited to those polymers, generally xyloglucans, tightly bound to cellulose (Brummell *et al.*, 1999).

Enzyme Activities during Cell Wall Degradation

1. Polygalacturonase (PG)

PGs [poly (1,4-α-D-galacturonide) glycanohydrolase] are enzymes that catalyze the hydrolytic cleavage of galacturonide linkages which can be exo-and endo acting. The exo type removes single galacturonic acid from the non-reducing end of polygalacturonic acid, whereas the endo type cleaves internal linkages of polygalacturonic acid at random. The general substrate for PG is homogalacturonan (HGA), which must be de-esterified (Carpita and Gibeaut, 1993).

The activity of PG is believed to be associated with softening in many fruits such as avocado, tomato and peach, which showed very high levels of PG activity (Huber and O'Donoghue, 1993). To understand better the role of PG, many studies used molecular techniques, and commonly tomato was employed as the model. Suppression of PG genes by using antisense, which can lower the genes' activity to about 1% of the wild type, did not affected the rate of fruit ripening (Kramer *et al.*,

1992), even though the results showed a reduction of solubilization in pectin (Brummell and Labavitch, 1997).

Giovannoni *et al.* (1989) examine the role of PG by using a non-softening *rin* mutant which the accumulation of the PG gene was reduced. Ethylene used to recover the action of the PG gene, did not increase the PG accumulation. In addition, fusion of the PG gene to an E8 promoter (ethylene or propylene responsive promoter) and then transformed it into *rin*, showed that the PG gene was expressed when the *rin* fruit was treated with ethylene or propylene. Even though this enhanced the depolymerization of polyuronides, the fruit did not soften (Della Penna *et al*., 1990), suggesting that PG alone may not be sufficient to initiate fruit softening and that change in pectin may occur normally without fruit softening.

2. Pectin methylesterase (PME)

PME (EC 3.1.1.11) acted as de-esterifier of polyuronides, removing methyl groups at the C6 (carboxyl) position from galacturonic acid residues of pectic polysaccharides (Pressey and Avants, 1982). This might indicate that PME may play an important role in determining the extent to which pectin is accessible to degradation by PG (Fischer and Bennett, 1991).

The PME enzyme was found to be present throughout fruit development (Tieman *et al*, 1992). In tomato, the accumulation of PME mRNA was at a maximum in mature green tomato, and declined as ripening proceeded (Harriman *et al*., 1991). Antisense of the PME gene showed that the fruit failed to de-esterified pectin, resulting in reduced polyuronide depolymerization with a normal processing of fruit ripening (Tieman *et al*., 1992). The antisense PME also maintained the resistance of methyl-esterified pectin to be a substrate for PG (Carrington *et al*., 1993), suggesting that without the action of PME, pectin solubilization could not occur. Suppression of this enzyme did not affect fruit softening (Tieman and Handa, 1994).

3. Pectate lyase (PL)

PL [(poly (1,4-α-D-galacturonide) lyase; E.C. 4.2.2.2)] catalyzes the cleavage of 1,4-α-D-galacturonan linkages of pectate by β-elimination reaction in de-esterified pectin which is a major component of the primary cell walls of many higher plants (Carpita and Gibeaut, 1993) and generates oligosaccharides with unsaturated galacturonosyl residues at their non-reducing ends. This enzyme has been characterized in several microorganisms (Ried and Collmer, 1986). Recent reports about the activity of this enzyme during fruit ripening in banana have been published (Pua *et al*., 2001; Lohani *et al*., 2004; Imsabai *et al*., 2006), indicating that this enzyme might be involve in the early stages of fruit ripening. In transgenic tomato, the PG gene was suppressed, but the solubilization and depolymerization of pectin was still observed, suggesting that fruit softening progressed normally. The softening in this transgenic experiment was also associated with an increase in PL activity (Smith *et al*., 1989). In some fruit such as strawberry (Medina-Escobar et al., 1997), banana (Dominguez-Puigjaner et al., 1997) and grape berry (Nunan et al., 2001), in

which PG activity was very low or absent, a the progressive increased in the PL activity has been reported during ripening.

4. β-galactosidase (β-gal)

β-Gal (EC 3.2.1.23) is a family of enzymes characterized by their ability to hydrolyze terminal, nonreducing beta-D-galactosyl residues from beta-D-galactosides. The loss of galactosyl residues from the cell wall polymer by the action of β-galactosidase has been observed during fruit ripening (Gross and Sams, 1984). The galactan are found as side chains of the rhamnogalacturonan (RG) pectin and arabinogalactan protein (Seymour *et al*., 1990).

β-gal purified from tomato fruit can be separated into types I, II and III, which are active against the ρ-nitrophenyl-β-D-galactopyranoside substrate, and only β-galactosidase II is active against a (1,4)β-galactan-rich pectin polymer (Pressey, 1983). These enzymes are mostly of type II, being exo-(1,4) β-galactanase (Smith and Gross, 2000). Type I and III are found to be high in green fruit but declining during ripening, whereas type II is low in green fruit and show a 7 fold increase in activity during ripening (Pressey, 1983). In fig, the β-gal gene expression known as *Fc-Gal*1, increased accumulatively from the onset of ripening to it's over ripe stage. This enzyme acts synergistically with other enzymes to reduce the molecular size of pectic polymers, leading to polymerization and increased solubility of pectic polysaccharides (Owino *et al*., 2004).

5. Endo-(1,4)β-D-glucanase (EGase or cellulase)

EGase (EC 3.2.1.4) hydrolyzes internal linkages of (1,4)β-D-glucans. The substrate may be the non-crystalline regions of cellulose, xyloglucan or glucomanan. A reduction of the EGase activity during heat treatment was observed to delay hemicellulose degradation (Vicente *et al*., 2005). EGase activity varies between species. Based on fresh weight, in avocado, the activity was 160 times more than in peach, and 770 times more than in tomato (Lewis and Varner, 1974). Unlike PG (whose highest activity was found at the over ripe stage), EGase activity is present throughout fruit development, with the highest level in young and still expanding green fruit, as well as and during ripening (Lashbrook *et al*., 1994).

EGase was derived from a large gene family. Some types (L*eCel*1, *LeCel*2 and *LeCel*3) were expressed at early ripening whereas the other such as *LeCel7* was expressed later (Catala *et al*., 2000). *LeCel1* and *LeCel2* exhibited a ripening-related accumulation of mRNA in fruit pericarp with large increases at the onset of ripening. In *rin* tomato, *LeCel*1 mRNA was present at low levels, and strongly increased with ethylene treatment while *LeCel*2 mRNA was barely detectable either with or without ethylene treatment. The *rin* mutant showed the differentially expressed of *LeCel1* and *LeCel2* mRNA. By itself, the ethylene induced accumulation of *LeCel1* was not sufficient to cause fruit softening; however, the accumulation of *LeCel2* mRNA in *rin* might also play a role in softening (Woolley *et al*., 2001).

6. Xyloglucan endotransglycosylase (XET)

XET was observed in many kinds of fruit, mostly during fruit expansion. These enzymes cleave internal linkages of the (1,4)β-D-glucan backbones of xyloglucan and transfer the reducing end at C-6 to the C-4 of the glucose unit at the non-reducing end of another xyloglucan (Sulova *et al.*, 1998). However, the characteristics of XET enzymes vary considerably and can be divided into two types based on their biochemical properties. One type possesses hydrolase activities, whereas and the other type does not.

In fruit of tomato, kiwi and apple, XET activity was very high in young expanding fruit, declining during fruit maturation and rising again slightly during ripening (Maclachlin and Brady, 1994). Also, XET mRNA in tomato accumulated to a maximum at the pink stage and declined slightly at the red ripe stage (Arrowsmith and de Silva, 1995). Ripening-related XET activities and mRNA were positively regulated by ethylene in kiwi fruit (Redgwell and Fry, 1993). In *rin* tomato fruit, the XET activity was reduced as compared to the wild-type. It seems likely that the ripening-related XET gene expression is positively regulated by ethylene in climacteric fruit (Maclachlan and Brady, 1994).

XET is believed to be involved in the synthesis of new xyloglucan into the wall during growth. High levels of this enzyme were found in young fruit during cell division and expansion, but the enzyme's role during fruit softening is still unclear. In kiwi, the XET activity increased during ripening, but not so in tomato. This enzyme could play a role during fruit ripening by generating the new xyloglucan (Schroder *et al*., 1997). In tomato, the XET activity from ripe fruit lacks hydrolase activity (. This activity could not bring about a net depolymerization of xyloglucan by transglycosylation, unless xyloglucan oligosaccharides were present. However, XET activity could still rearrange xyloglucan crosslinks in the wall, and so contribute to wall loosening. Such XET activity could also play a maintenance role during ripening, by incorporating new xyloglucan into the wall (de Silva *et al*., 1994).

7. Expansins

Expansins are cell wall proteins that cause cell wall loosening and are involved in cell wall modification during development. These proteins are believed to disrupt the non-covalent bonding between matrix glycans and cellulose microfibrils (Cosgrove, 2000). Several genes are expressed during the expanding and mature green stage in tomato (Catala *et al*., 2000). In tomato, mRNA of *LeExp*1 accumulated during ripening and was regulated by ethylene (Rose *et al*., 1998). Suppression of *LeExp*1 mRNA and expansin reduced fruit softening. Modifying this protein was found to result in changes of the cell wall polymer metabolism, but did not affect matrix glycan depolymerization. In contrast, over expression of this *LeExp1* protein to 3-fold of wild type did not affect polyuronide depolymerization but increased fruit softening and the depolymerization of matrix glycans (Brummell *et al*., 1999). *LeExp*2 and *LeExp*4 genes are present at the early stages of fruit development, while *LeExp*5 and *LeExp*6 genes also accumulated early in development, rising to their highest levels later. *LeExp*7 is detected at low levels in young green fruit. *LeExp*3 is

present throughout green fruit development and persists into ripening (Rose *et al*., 2000). Analysis of transgenic tomato fruit with suppressed expansin gene expressions or with suppressed PG gene expressions supports the general model of sequential disassembly of xyloglucan and pectin that control the early and late phases of fruit softening, respectively. This information indicates that two distinct genetic strategies, the control of the expansin gene expression or the control of PG gene expression, may be useful to control early or late phases of fruit softening.