CHARACTERIZATION OF ENZYMES AND PHENOLICS INVOLVED IN THE PERICARP HARDENING OF MANGOSTEEN FRUIT STORED AT LOW TEMPERATURE

INTRODUCTION

Mangosteen (*Garcinia mangostana* Linn.) is an important economic fruit of Thailand and widely grown in the east and the south of the country. In 2006, the amount of exports was 15,009.49 tons and the value was 262,774,419 baht (Thai Customs Department, 2007). The export of mangosteen fruit is very limited. Some of the limiting factors are associated with the very slow growth and poor fruit quality due to internal disorders such as flesh translucent disorder, gummosis (gumming), shriveling of calyx and stylar ends of the fruit, cracking and hardening of the pericarp (Sdoodee and Limpun-Udom, 2002; Siriphanich, 2002; Choehom *et al.*, 2003). The pericarp hardening occurs when the fruit is stored at low temperature for a prolonged period (Uthairatanakij and Ketsa, 1996; Choehom *et al.*, 2003) or after impact (Tongdee and Suwanagul, 1989; Ketsa and Koolpluksee, 1993; Bunsiri *et al*., 2003).

Generally, mangosteen fruit has a short shelf life at ambient temperatures (29-30^{o}C). Low temperature can increase the storage life, but it can also cause chilling injury (CI), of which pericarp hardening is a common symptom. Kosiyachinda (1986) reported that the fruit stored at 1^{o}C showed CI symptoms within 3-4 days and these symptoms became more prominent when fruit were transferred to room temperature.

Lignin contents and firmness have been shown to increase in damaged pericarp after impact, while total phenolics decreased (Atantee, 1995). Thus pericarp hardening may occur as a result of an increase in lignin contents. Lignin biosynthesis is correlated with the activity of some enzymes such as phenylalanine ammonia lyase (PAL), cinnamyl alcohol dehydrogenase (CAD), and peroxidase (POD) (Lewis and Yamamoto, 1990; Whetten and Sederoff, 1995; Boudet, 2000; Bunsiri, 2003; Cai *et al*., 2006b). Uthairatanakij (1995) investigated mangosteen fruit that were

stored at 5^{o}C for 9 days and then transferred to room temperature with or without O_2. Fruit stored in 20% O_2 had higher POD and polyphenol oxidase (PPO) activities, lignin contents, and firmness than those stored without O_2, but total phenolics were lower in 20% O_2.

Despite the above information, the mechanisms involved in the pericarp hardening of mangosteen fruit stored at low temperature are not yet clear. There is a need for a substantial study to elucidate the relationships between PAL, CAD, and POD activities, phenolic compounds and lignin contents in mangosteen pericarp and gene expression of the enzymes involved in pericarp hardening.

OBJECTIVES

1. To study the effect of fruit maturity on pericarp hardening of mangosteen fruit stored at low temperature.

2. To study phenolics metabolism and the key enzymes involved in lignin biosynthesis in mangosteen pericarp stored at low temperature.

3. To study the effect of low O_2 on pericarp hardening of mangosteen fruit stored at low temperature.

4. To characterize the expression of genes regulating lignin synthesis in mangosteen pericarp stored at low temperature.

LITERATURE REVIEW

Mangosteen

Mangosteen (*Garcinia mangostana* Linn.) fruit have been called the "Queen of Fruit" (Popenoe, 1974) due to their beautiful purple color and delicious flavor. The edible aril of mangosteen fruit is white, soft and juicy with a sweet, slightly acid taste and pleasant aroma (Martin, 1980). Mangosteen is an important economic fruit crop in Thailand and its production and export have been increasing over recent years. However, cultivation and export of the crop is very limited due to the slow growth and internal disorders of the fruit.

Mangosteen fruit are consumed at the edible or ripe stage when the pericarp has darkened to reddish purple, no latex remains in the pericarp and the aril segments separate easily from the pericarp. The fruit are usually 4-7 cm across, with a persistent calyx at the stem end. The pericarp is 6-10 mm thick and turns purple on ripening. The edible white aril has 4-8 segments, with one or two larger segments containing apomictic seeds; there is no true seed. Fruit development takes 100-120 days from anthesis and up to 180 days in cooler areas or at high elevations (Nakasone and Paull, 1998). Normally, the harvest time of mangosteen fruit growing in the east is between May to July, while the south is between August to October (Osman and Milan, 2006).

At present, there is no universal maturity index for mangosteen fruit. Several countries growing mangosteen plants have developed their own harvesting indices. Skin color is the major criteria used to judge maturity (Paull and Ketsa, 2004). In Thailand, Tongdee and Suwanagul (1989) have divided the maturity stage into 7 stages (stage 0-6) as follows: stage 0 (uniformly yellowish white or yellowish white with light green tinge on pericarp, severe yellowish latex, and aril is not separate from pericarp); stage 1 (light greenish yellow with scattered pinkish spot on pericarp, severe yellowish latex, and aril is not separate from pericarp); stage 2 (light pinkish yellow with distinct irregular pink-red spots covering the entire fruit, moderate yellowish latex, and aril separate moderately from pericarp); stage 3 (uniformly

pinkish on pericarp, spotting not as distinct as in stage 2, slight to very slight latex, and aril separate moderately from pericarp); stage 4 (red to reddish brown or some with purple tinge on pericarp, very slight latex in pericarp, and aril separate readily from pericarp); stage 5 (darkened to reddish purple on pericarp, no latex remains in pericarp, and aril separate completely and easily from pericarp); stage 6 (purple, dark purple or black with or without purple coloration remain on pericarp, no latex remains in pericarp and aril separate completely from pericarp).

However, Kosiyachinda (unpublished data) has reported only 6 stages (stage 0-5) for mangosteen maturity as follows: stage 0 (pericarp has uniformly yellowish white or yellowish white with light green tinge, aril is not separate from pericarp and very poor qualities for edible); stage 1 (pericarp light pinkish yellow with scattered pinkish spots, aril is not separate from pericarp, severe yellow latex, but suitable for export to faraway market); stage 2 (pericarp has uniformly pinkish or pinkish with scattered pink-red spots, and moderate yellowish latex); stage 3 (pericarp has red to reddish brown, slight to very slight latex, and aril separate readily from pericarp); stage 4 (pericarp has reddish purple, no latex remains in pericarp, and aril separate completely and easily from pericarp); stage 5 (pericarp has purple, dark purple or black, and no latex remains in pericarp). Both maturity indices developed by Tongdee and Suwanagul (1989) and Kosiyachinda (unpublished data) overlap in terms of color surface and qualities of the fruit. But the minimum maturity stage for high eating quality is when the pericarp has distinct irregular, pink-red spots over the whole fruit (Paull and Ketsa, 2004).

Postharvest storage of mangosteen fruit at 4 or 8^{o}C can lead to significant pericarp hardening, although the aril may still be acceptable after 44 days (Augustin and Azudin, 1986). Storage can be enhanced by application of surface coatings which reduce weight loss and prevent calyx wilting (Choehom, 1997). Dipping the calyx and the stem of the fruit in various concentrations of hormones before storage at 12^{o}C has also been shown to delay shriveling and extend the storage period (Choehom *et al.*, 2003). Kader (2007) reported that the CI symptoms in mangosteen include darkening and hardening of the pericarp. When the fruit were moved to

higher temperatures following storage at lower than 10^{o}C for longer period than 15 days or at 5^{o}C for more than 5 days, decay susceptibility was also increased.

One particular feature of the fruit is an increase in firmness when the pericarp is damaged after impact. For instance, Bunsiri *et al*. (2003) reported that firmness and lignin contents in damaged mangosteen pericarp after impact increased while total phenolics decreased. This increase in firmness may be related to oxidative processes, since damaged pericarp held in a N_2 atmosphere was less firm and had lower lignin contents and more total phenolics than damaged pericarp held in air (Ketsa and Atantee, 1998). Bunsiri (2003) reported that lignin, carbohydrate and protein contents of the lignin carbohydrate complex were higher in damaged pericarp of more mature fruit, following a greater drop height and at higher O_2 levels. The results show that lignification of the pericarp increases after physical injury and may play a role in the increased firmness of damaged mangosteen pericarp after impact. A potential role for polyamines has been shown by Kondo *et al.* (2003) who investigated the effect of spermine on abscisic acid, pericarp hardening and browning in stored mangosteen fruit. They showed that the hardening and browning were observed only in the pericarp of fruit stored at 7^{o}C. The spermine treatment decreased browning and hardening and extended storage time. Pericarp hardening was not accompanied by moisture loss. Tongdee and Suwanagul (1989) reported that the pericarp hardening at the impact point is a common symptom of mechanical injury in mangosteen fruit, when fruit dropped at 10 cm level above the ground or higher drops caused significantly greater damage.

The previous reports on pericarp hardening in mangosteen fruit were conflicting. There has been no report on the actual temperature that CI will be occurring. Moreover, the physical and biochemical changes of this symptom are still not investigated.

Chilling injury

Storage of fruit at low temperature is commonly used to maintain quality and extend shelf life, because low temperature reduces the rate of respiration, ethylene production and other metabolic processes (Wills *et al*., 1989). However, in long term storage at low temperature, CI may develop in many tropical and subtropical fruit crops. CI is manifest as a range of physiological disorders that reduce the acceptability of commodities during and after storage (Jackman *et al*., 1988). This damage results from the exposure of many susceptible plants and tissues to temperatures below a critical temperature, but above freezing point (generally 15^{o}C down to 0^{o}C) (Raison and Lyons, 1986; Jackman *et al*., 1988; Wills *et al*., 1989; Shewfelt, 1993). Often, CI does not appear until the produce is transferred to ambient temperatures. However, severe CI symptoms such as pitting will often appear during low temperature storage, as shown in 'Fortune' mandarin fruit kept at temperatures below 8^{o}C where severe peel pitting occurred before the transfer to ambient temperatures (El-hilali *et al*., 2003). The severity of CI increased with decreasing temperatures and prolonged storage duration.

CI symptoms vary among different commodities. They may appear as discoloration, pitting or water-soaked areas (Wills *et al*., 1989), lack of juiciness (mealiness, wooliness, leatheriness), failure to ripen, lack of characteristic aromas, and flesh translucency (Mitchell and Kader, 1989). A summary of symptoms by Wang (1989) shows that the CI symptoms most commonly seen in commodities are surface lesions such as pitting on cucumbers and squash, scald on papaya and citrus, large sunken areas on peppers, and internal discoloration in avocado, tomato seeds and pineapple fleshy tissues.

Many factors affect the development of CI including cultivar, harvest maturity and storage temperature. Sometimes cultivars that ripen later in the season are generally more susceptible to CI and susceptibility decreases as fruit maturity advances (Von Mollendorff, 1987). Mature-green and pink tomato fruit were most susceptible to CI (Autio and Bramlage, 1986). Salvador *et al*. (2005) found that

persimmon fruit harvested at early time were sensitive to CI when stored at 1^{o}C, but not in lately harvested. CI is also affected by genetics, growth conditions and cultural practices, and duration of chilling exposure. Morris (1982) showed that there is a genetic component to chilling sensitivity. For instance, tomato species *Lycopersicon esculentum* was susceptible to CI, but *Lycopersicon hirentum* was not (Patterson and Reid, 1990). Crisosto *et al*. (1999) also reported that nectarine cultivars were generally less susceptible to CI than peach cultivars. The severity of response to CI is linked to the exposure time (Paull, 1990). Shewfelt (1993) summarized some generalizations on CI as follows:

1. Susceptibility only appears in some species.

2. Some genotypes are more susceptible than others within a susceptible species.

3. Some organs are more susceptible than others within a genotype.

4. The response in an organ does not tend to be uniform and often is localized.

5. Damage may not appear at low temperatures until the plant or organ is exposed to non-chilling temperatures.

Several techniques have been shown to reduce CI symptoms or increase resistance of the tissue to CI. These include heat treatment, step–wise reduction of temperature, intermittent warming, controlled atmosphere storage (increased CO_2 or decreased O_2 in the atmosphere during chilling), waxing or coatings, packaging and application of chemical compounds such as calcium, jasmonic acid, polyamines or fungicides (Wang, 1993). For instance, preconditioning of papaya fruit for 4 days at 12.5^{o}C reduced chilling sensitivity, while the pulp around the vascular bundles showed water soaking and off-flavors after prolonged exposure to temperatures below 7.5^{o}C (Chen and Paull, 1986). Houck *et al.* (1990) reported that lemons stored

for 1 week at 5 or 10°C before cold treatment at 0-2.2°C developed less CI than non-cold treated fruit during 4 weeks of storage at 10°C after cold treatment. In mature-green tomatoes, conditioning for 3 days at 36-40°C prevented CI development at 2°C storage (Lurie and Klein, 1991). Prestorage heating effects have been associated with accumulation of heat shock proteins. Pepper fruit treated with hot water (53°C) for 4 min resulted in delayed CI and decay of the fruit (Gonzalez-Aguilar *et al*., 2000). Treatment with 200 mg/l imazalil (IMZ) in hot water (50°C) reduced CI in grapefruit, and IMZ was more effective than hot water alone in decay control. Treatment with lower doses of heated fungicide proved to be more effective in reducing CI of grapefruit (Schirra *et al.*, 2000).

Low O_2 and controlled atmosphere storages have been shown to be effective in reducing CI in many fruit crops. Low O_2 levels have also been shown to delay CI development and reduce the severity of CI symptoms in 'Fairtime' peaches stored at 5°C (Ke *et al.*, 1991). Storage life of 'Choke Anan' mango fruit could be extended for longer than 4 weeks when the fruit were stored at 15°C, 2% and 5%O_2 (Shukor *et al.*, 2000). However, papaya fruit stored in low O_2 (1.5-5%) with or without high CO_2 (2% or 10%) did not show any delay in development of CI symptoms (Chen and Paull, 1986). Thus the efficacy of controlled atmospheres to alleviate CI needs to be evaluated for each individual fruit and storage situation.

Another way of modifying atmospheres within a fruit is to use coatings and packaging, and these have also been shown to affect chilling responses. Paull and Rohrbach (1985) showed that waxing pineapple fruit either before or immediately after exposure to chilling temperatures was equally effective in reducing CI symptoms. Waxing papayas also reduced chilling (Chen and Paull, 1986). Plastic film packaging of fruit and vegetables helped to maintain high relative humidity (RH) and modify the O_2 and CO_2 concentration in the atmospheres surrounding the commodities (Forney and Lipton, 1990). With grapefruit, wrapping fruit individually in plastic heat-shrinkable film reduced pitting and scald during low temperature storage (Miller *et al*., 1990). Pesis *et al.* (2000) have also reported that storage of

'Tommy Atkins' and 'Keitt' mangoes in modified atmosphere packaging (MAP) using polyethylene (PE) film or Xtend® film (XF) at 12°C reduced CI symptoms.

Various chemicals have been reported to delay the development of CI symptoms including calcium, mineral or vegetable oils, fungicides and some free radical scavengers. Application of these chemicals can reduce CI by retarding the secondary events such as suppressing the oxidative processes and reducing moisture loss (Wang, 1993). Methyl jasmonate (MJ) application before exposure to low temperatures decreased CI symptoms in zucchini squash (Wang and Buta, 1994). Jasmonic acid (JA) in the skin of mangosteen fruit stored at 7°C increased when compared with fruit stored at 13°C. Exogenous application of *n*- propyl dihydrojasmonate (JA derivative) effectively decreased CI symptoms. Hence, low temperature-induced JA may play a protective role against CI (Kondo, 2006). Kondo *et al.* (2001) have reported that a major polyamine (putrescine) may be associated with the CI in rambutan. CI symptom in the skin of 'Rongrien' and 'Seechompoo' rambutan fruit stored at 8°C for 4 days or longer was detected and putrescine content in fruit stored at 8°C was higher than those at 13°C. Kondo *et al.* (2003) have also reported that mangosteen fruit treated with spermine decreased pericarp hardening and extended storage time. Recently, 1-methylcyclopropene (1-MCP) has been reported to reduce CI in many fruit such as pineapple (Selvarajah *et al*., 2001), avocado (Pesis *et al*., 2002) and peach (Fan *et al*., 2002), suggesting that CI symptoms in these fruit are associated with ethylene response.

The first symptom of CI in mangosteen fruit is the hardening of the pericarp and browning of the cortex. This occurs after about 6 weeks in the fruit stored at 4 and 8°C (Paull, 1990). CI symptoms can be more prominent when fruit are transferred to room temperature (Kosiyachinda, 1986). Srivastava *et al.* (1962) found that the optimum storage conditions of mangosteen fruit were 4-6°C with 85-90% RH to obtain a maximum shelf life of 49 days. Hardening of the fruit increased, particularly at storage temperatures lower than 4°C. Uthairatanakij and Ketsa (1996) reported that mangosteen fruit stored at 5°C for 9 days and then transferred to room temperature (25-35°C) showed pericarp hardening, water loss, lignin contents and

activities of POD and PPO increased, while total phenolics decreased with time. Moreover, Choehom *et al.* (2003) reported that the unacceptable CI symptoms of mangosteen fruit such as hardening of the pericarp, browning of the edible aril and off-flavor were found within 5 days at 3 and 6^{o}C.

Low O_2 storage

The composition of gases in the storage atmosphere can affect the storage life of horticultural produce (Wills *et al*., 1998). Exposing fresh fruit and vegetables to low O_2 and/or high CO_2 can either be beneficial or harmful, depending on concentration of these gases, temperature and exposure duration. During storage, the physiological effects of low O_2 are as follows: decreased in respiration rate and ethylene production, delayed ripening of climacteric fruit, retained of chlorophyll contents, texture quality and sensory attributes of leafy vegetables, and prolonged shelf life (Richardson and Meheriuk, 1982; Kader, 1986). Most commodities require a minimum of 1-3% O_2 in CA or MA storage to avoid anaerobic metabolism; O_2 levels as low as 0.2% in the plant cell may result in anaerobic respiration. Low O_2 storage can reduce fruit sensitivity to ethylene at O_2 levels below about 8%. Atmospheric modification (less than 1% O_2 or 40% CO_2) can be an useful tool for insect control (Kader, 2002) and suppress development of green mold fungi at ultra-low O_2 refrigerated storage (Shellie, 2002). A 1 kPa O_2 atmosphere also reduced decay incidence in 'Clemenules Clementine' mandarin fruit during storage at 5^{o}C for up to 4 weeks (Luengwilai *et al.*, 2007). Burdon *et al.* (2007) have also been reported that low O_2 levels (<0.5% O_2) resulted in a rapid accumulation of acetaldehyde and ethanol in 'Hass' avocado fruit.

Choehom *et al*. (2004) found that 'Sucrier' banana fruit covered with 'Sun wrap' polyvinyl chloride film prevented the senescent peel spotting at early time due to low O_2 effect in the package. Low O_2 at 3-8% atmospheres also retarded pericarp browning of litchi (*Litchi chinensis* Sonn.) fruit cv 'Hong Huay' (Techavuthiporn *et al*., 2006). In fresh cut produce, low O_2 or pure N_2 atmospheres have been shown to be useful in delaying apple slice softening and browning (Toivonen, 1997). The

maintaining higher flesh firmness of avocado fruit after low O_2 treatments has been reported (El-Mir *et al*., 2001). MA treatment at 2% O_2 and 5% CO_2 could also inhibit the lignification of bamboo shoots (Shen *et al.*, 2006). Initial low O_2 stress (ILOS) with 0.25% O_2 for 2 weeks prior to CA storage at 1.5% O_2 and 3% CO_2 can complete control of scald in several cultivars of apples (Wang and Dilley, 2000). Zanella (2003) reported that superficial scald of 'Granny Smith' apple fruit was controlled after long-term storage under 0.7 kPa O_2 or with ILOS at 0.4 kPa O_2 for 2 weeks followed by 1.0 kPa O_2. Low O_2 treatment at 1 and 3% O_2 delayed leaf yellowing of Chinese chive and retarded chlorophyll and protein degradation after storage at 20^{o}C for 7 days (Imahori *et al*., 2004).

Phenolic compounds

Phenolic compounds are classified as secondary metabolites derived from the shikimate pathway and phenylpropanoid metabolism (Ryan and Robards, 1998; Ryan *et al.*, 2002). The metabolic pathways are particularly complex with multiple alternative routes. Phenolic compounds are also associated with lignin synthesis.

L-phenylalanine ammonia lyase (PAL) is a key enzyme that converts L-phenylalanine to *trans*-cinnamic acid, a precursor of various phenylpropanoids, such as lignins, coumarins and flavonoids (Schuster and Retey, 1995). PAL is the first step in the pathway of phenylpropanoid biosynthesis and it is a potential site for regulation. PAL catalyses the deamination of L-phenylalanine, and the product, *trans*-cinnamate, is converted in plants to various phenylpropanoid compounds such as chlorogenic acid, lignin monomers and flavonoids (Assis *et al*., 2001).

Accumulation of phenolic compounds in plant and fruit tissues is associated with browning of tissues. For example, accumulation of phenolic compounds in the Japanese radish pith can result in internal browning (IB). IB susceptible cultivars tend to have higher levels of PAL and PPO activities (Fukuoka and Enomoto, 2002). In litchi fruit, Zhang *et al.* (2000) have shown that flavan-3-ol monomers and dimers represent about 87.0% of the total phenolic compounds and decline with storage time

or browning of fruit. Heat shock treatment (90 s, 45^{o}C) can prevent an increase in PAL activity and browning in fresh cut lettuce; the lettuce also had low phenolic contents (Saltveit, 2000).

With mangosteen fruit, Uthairatanakij (1995) showed that mangosteen fruit stored at 5^{o}C for 9 days and then transferred to ambient conditions for 2 and 4 days, had reduced total phenolic contents in the pericarp while hardening increased. Phenolic compounds of fruit with and without low temperature treatments can be separated by thin-layer chromatography and are associated with the same maximum wavelengths. Ketsa and Koolpluksee (1993) showed that when hardening of damaged pericarp increased after impact, the total phenolics levels decreased. It is probable that the phenolic compounds were used for lignin synthesis, resulting in increased firmness. In another study, Atantee (1995) found that lignin contents and firmness of damaged pericarp increased, while total phenolics decreased in the damaged pericarp after impact, when compared with non-damaged pericarp. The hardening of the damaged pericarp may be due to an increase in lignin contents after impact. Bunsiri *et al.* (2003) have also reported that the decreased phenolic contents occurred concomitantly with increased firmness and lignin contents. Phenolic acids in mangosteen pericarp after impact have been identified as *p*-coumaric and sinapic acids. *p*-Coumaric and sinapic acid levels in the damaged pericarp of reddish brown mangosteen fruit were less than those in dark purple fruit. *p*-Coumaric acid levels decreased more rapidly than those of sinapic acid. The decrease in both phenolic acids in damaged pericarp occurred more rapidly in air than under the N_2.

Lignin synthesis

Lignin is a major plant cell wall component that is formed through oxidative polymerization of cinnamyl alcohols. It is an important process in higher plants, especially in woody tissues where lignin concentrations reach up to 30% of dry weight. Lignification occurs under special conditions such as pathogen attack or fungal elicitor treatment (Vance *et al*., 1980). With genetic engineering for papermaking, there are attempts to (a) lower lignin content and (b) alter lignin

composition for better pulping (Stasolla *et al.*, 2003). In the pulp of fleshy fruit, lignin is generally not formed, except in the sclereids or stone cells of some fruit such as pear. In contrast, lignin is abundant in the integuments of dry fruit, seeds and stones of drupes (Macheix *et al.,* 1990).

Lignin biosynthesis is associated with the activities of many enzymes such as PAL, CAD, and POD (Lewis and Yamamoto, 1990). Lignin biosynthesis starts from phenylalanine, which is converted to *trans*-cinnamic acid by PAL. Cinnamic acid is then converted to *p*-coumaric, caffeic, ferulic and sinapic acids. Each of these can be converted to cinnamoyl CoA esters, cinnamyl aldehydes, or cinnamyl alcohols and can be polymerized to the lignin residues by POD. The last enzyme of the monolignol pathway is CAD. It catalyzes the reduction of cinnamaldehyde to cinnamyl alcohols (Figure 1). Conversion of the aldehydes to the alcohols is via an aldehyde intermediate and this conversion is catalysed by an aromatic alcohol dehydrogenase (Davies *et al.*, 1973). Generally, lignins contain three aromatic alcohols: coniferyl, sinapyl and *p*-coumaryl alcohols. Lignin from hardwood trees and herbaceous dicotyledons and grasses contain less coniferyl alcohol and more of the other alcohols (Salibury and Ross, 1985). Lignin composition in gymnosperms is different from that of angiosperms; in gymnosperms, lignin is made up mainly of coniferyl alcohol (guaiacyl lignin), whereas lignin from angiosperms (dicotyledons) originates from both coniferyl and sinapyl alcohols (guaiacyl-syringyl lignin). Lignin in gymnosperms contains guaiacyl units, while in angiosperms contains both syringyl and guaiacyl units (Whetten *et al.*, 1998).

The differences in lignin composition are also reflected in the substrate specificity of enzymes involved in the biosynthesis of lignin precursors. In angiosperms, *O*-methyl transferases (OMTs) catalyse methylation of both caffeic and 5-hydroxyferulic acids, a precursor of sinapic acid, but in gymnosperms, 5-hydroxyferulic acid is only a very poor substrate for OMTs. The reduction of cinnamyl-CoA esters from angiosperm tissues can reduce ferulic and sinapic acids to alcohols, whereas those from gymnosperms can reduce only ferulic acid (Nakamura *et al.*, 1974). Luderitz and Grisebach (1981) found differences in substrate

specificities between the enzymes from spruce (gymnosperms) and soybean (dicotyledonous angiosperms); feruloyl-CoA is the preferred substrate for the reductase from both sources whereas sinapoyl-CoA is a substrate only for the soybean reductase and sinalpyldehyde is a substrate only for the soybean dehydrogenase. 4-Coumaroyl-CoA is a poor substrate for the reductase from both spruce and soybean, resulting in a low content of 4-coumaryl alcohol units in gymnosperm and angiosperm lignins.

Lignin accumulation during development of maize internodes has been associated with activity of CAD, but not with PAL (Morrison *et al*., 1994). An increase in lignin contents has also been correlated with a reduction in phenolic compounds and enhanced PAL and POD activities during pollen germination in maize (Zin-Huang and Mang-Jue, 1997). Lignification could be affected by regulation of PAL levels or phenylalanine production, or both (Camm and Towers, 1973). Lignin levels can be reduced as a result of action of specific inhibitors of PAL such as (1-amino-2-phenylethyl) phosphonic acid (APEP) (Laber *et al.*, 1986) or increased by fungal elicitors (Maule and Ride, 1976; Moerschbacher *et al.*, 1988). Chen and McClure (2000) showed that lignin formation can be inhibited by 2-aminoindan-2-phosphonic acid (AIP), a competitive inhibitor of PAL activity. CAD is important because it is required for lignin synthesis not only of monolignols and lignin, but also of lignans and related phenolics such as didehydroconiferyl alcohol and suberin (Lewis and Yamamoto, 1990). In wheat leaf, treatment of wounds with a partially acetylated chitosan hydrolysate or spores of the nonpathogen *Botrytis cinera* elicited lignification at wound margins. Treatment increased significantly in PAL, POD and CAD activities (Mitchell *et al*., 1994).

Lignin contents in damaged mangosteen pericarp after impact have been shown to be higher in the more mature fruit, following a greater drop height and at higher O_2 levels (Bunsiri, 2003). These results support the concept of lignification being involved in cells undergoing physical injury and playing a role in the increases in firmness of mangosteen pericarp after impact. In loquat fruit, fruit firmness increased and showed a positive correlation with the lignin accumulation in the flesh,

this increase in firmness being associated with increases in PAL, CAD and POD activities (Cai *et al*., 2006b). Shen *et al.* (2006) have also reported that PAL and POD activities which attributed to the lignification were inhibited in MA storage of bamboo shoots.

Gene expression in relation to lignin synthesis

A number of studies have explored the relationship between expression of genes of enzymes associated with lignin synthesis and increased lignin in plant tissues. Transgenic tobacco plants with suppressed levels of the PAL enzyme exhibited more rapid and extensive lesion development than wild-type plants after infection with *Cercospora nicotianae* (Maher *et al*., 1994). Atanassova *et al.* (1995) studied the effects of sense and antisense of 10 constructs of OMT cDNA compared with wild types. The results showed that expression of only the full-length sense construct led to an increase in OMT activity and the suppression of OMT activity led to a change only in lignin composition with a decrease in syringyl (S) units.
In poplar, an antisense construct reduced by 50-95% of caffeic/5-hydroxyferulic acid *O*-methyltransferase (COMT) activity. The syringyl/guaiacyl ratio (S/G) was reduced six-fold due to a decrease in S units and an increase in G units (van Doorsselaere *et al*., 1995). To confirm that OMTs were essential for lignin biosynthesis, both caffeoyl-coenzyme A (CCoA) *O*-methyltransferase (CCoAOMT) and COMT antisenses resulted in a reduction in total lignin contents (Zhong *et al*., 1998). Moreover, Meyermans *et al.* (2000) found that down-regulation of CCoAOMT in poplar resulted in a 12% reduction in lignin contents, although the composition of lignin was similar to that of the wild type.

In the phenylpropanoid pathway, ferulate 5-hydroxylase (F5H) catalyzes the hydroxylation of ferulate to 5-hydroxyferulate. But Humphreys *et al.* (1999) found that F5H also used coniferaldehyde and coniferyl alcohol as substrates and suggested that the S monomers may be derived from their G analogues by hydroxylation and methylation, respectively. Antisense suppression of 4-coumarate CoA ligase (4CL) activity in *Arabidopsis* revealed a significant decrease in G units, but the level of

S units did not change (Lee *et al.*, 1997). Three antisense constructs of cinnamoyl-CoA reductase (CCR) in tobacco showed an increase in the S/G ratio. The line with the most severely depressed CCR activity exhibited the strongest reduction in lignin content together with altered plant development such as reduced plant size, abnormal morphology of the leaves and collapsed vessels (Piquemal *et al*., 1998).

In modified tobacco plants (antisense for CAD), CAD activity was reduced. They had approximately the same levels of lignin, but they contained more cinnamylaldehydes and fewer cinnamyl alcohol residues (Halpin *et al*., 1994). Antisense CAD in poplars had reduced CAD activity, but both the amount of lignin and the lignin composition did not change (Baucher *et al*., 1996). Lapierre *et al.* (1999) reported that the lignin content of antisense CAD poplars was slightly lower than that of the wild types, but unchanged in the frequency of G units. Antisense COMT also did not change lignin content but it increased the frequency of G units. In mutant loblolly pine (*Pinus taeda* L.), where the expression of the gene encoding CAD was severely reduced, due to the presence of a recessive (*cad-n1*) allele (MacKay *et al*., 1997), CAD activity and *cad* RNA transcription were low, free CAD substrate (coniferaldehyde) accumulated to a high level, and there was a lower lignin content. The lignin quantity in transgenic alfalfa (*Medicago sativa* L.) with down-regulation of the CAD enzyme remained unchanged but the lignin composition had a lower of S/G ratio, mainly because of a decreased amount of S units (Baucher *et al*., 1999). Furthermore, *Arabidopsis null* mutants of CAD (*Atcad-D*) showed a slight reduction in lignin content, together with a significantly reduced proportion of S units (Sibout *et al.*, 2003). Furthermore, Sitbon *et al.* (1999) have been reported that in transformed tobacco plants, POD activity increased and this was accompanied by an increase in lignin deposition. From these reviews, the suppression of some enzymes in lignification pathway can reduce lignin contents or/and change the composition of lignin. There were only a few reports on the lignification of fruit. Therefore, this needs further study.

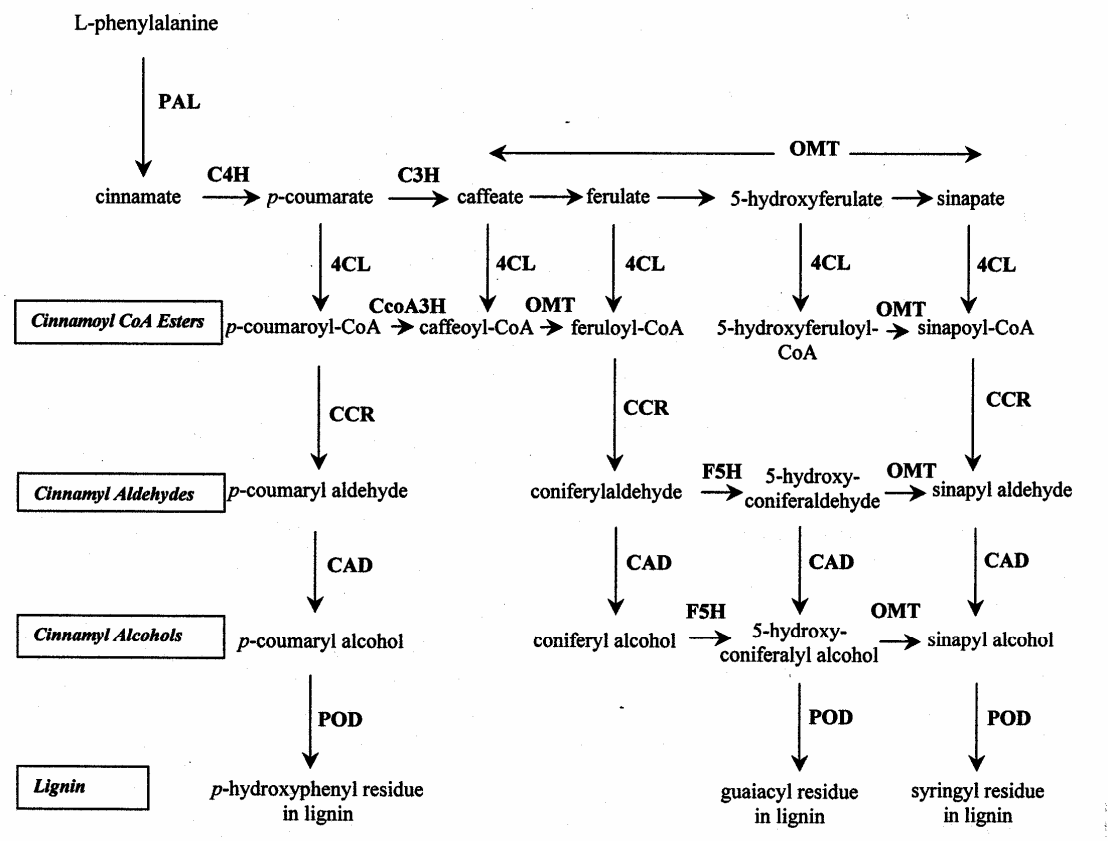

Figure 1 The pathway of lignin synthesis [modified from Whetten and Sederoff (1995); Campbell and Sederoff (1996); Boudet (2000); Meyermans *et al*. (2000)]

PAL : phenylalanine ammonia-lyase
C4H : cinnamic acid 4-hydroxylase
C3H : coumaric acid 3-hydroxylase
OMT : *O*-methyltransferase
4CL : 4-coumaric acid CoA ligase
CCoA3H : coumaroyl-CoA 3-hydroxylase
CCR : cinnamoyl-CoA reductase
F5H : ferulic acid 5-hydroxylase
CAD : cinnamyl alcohol dehydrogenase
POD : peroxidase

Analysis of gene expression

Gene expression can be divided into two main phases; transcription and translation. The methods developed to study the gene expression are analysis of specific RNA levels or specific protein expression (Dale and von Schantz, 2003).

1. Northern blotting

Northern hybridization is used to measure the amount of RNA transcription. This method is a standard part of the repertoire of molecular biology. Although many variations and improvements have been published during the succeeding 20 years, the basic steps in northern analysis are as follows:

1. Extraction of intact RNA from the cells and tissues

2. Electrophoresis of RNA through a denaturing agarose gel

3. Transfer and fixation of denatured RNA from agarose gel to the solid support (usually a nylon membrane)

4. Hybridization of the fixed RNA to a specific probe complementary to the sequences of interest

5. Removal of the nonspecific probe that bound to the membrane

6. Detection and analysis of an image of the specifically bound probe

RNA samples that have been transferred and fixed to a membrane may be hybridized with a specific probe to locate the RNA species of interest. After treating the membranes with blocking agents that suppress nonspecific absorption of the probe, the membrane is incubated under conditions favoring hybridization of the labeled probe to the immobilized target RNA. The membrane is then washed

extensively to remove adventitiously bound probe and finally manipulated to yield an image of the distribution of the tightly bound probe on the membrane. After analysis of the hybridization results, the probe may be stripped from the membrane, and the membrane used again in other hybridization experiments (Sambrook and Russell, 2001).

The northern blot method is analogous to southern blot analysis but utilizes RNA instead of DNA (Fütterer *et al*., 1995; Ignacimuthu, 1997). This method is used to screen the expression of a gene. It involves analysis of transcribed mRNA. Labeled DNA or a probe will hybridize only with RNA transcribed from a gene complementary to that DNA (Ignacimuthu, 1997).

2. Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR is a common, sensitive and powerful tool for highly sensitive RNA expression level. RT-PCR permits analysis of gene expression from very small amounts of RNA. It is possible to detect a specific mRNA in a single cell or to analyze gene expression in cells that are difficult to obtain in large amounts (Dale and von Schantz, 2003). Furthermore, this method can be conducted on a large number of samples and/or many different genes in the same experiments (Freeman *et al*., 1999).

For gene quantification, northern blotting works well, but it requires a large amount of total RNA and time-consuming. PCR greatly amplifies both the targets and errors. The combination of reverse transcription and PCR led to the establishment of RT-PCR for mRNA quantification (Becker-André and Hahlbrock, 1989).

3. Real-time PCR

Real-time PCR differs from classical PCR by the measurement of the amplified PCR product at each cycle throughout the PCR reaction. Compared with

other techniques, it allows the detection of the target in a rapid, specific and very sensitive way (Gachon *et al*., 2004). The times used in real-time PCR allow rapid production of data. It ranges from 20 min to 2 h. Real-time PCR provides a high sensitivity for the detection of DNA or RNA due to a combination of the amplification performed by the PCR step and the system of detection (Bustin, 2000). Basically, real-time quantitative PCR may be used for quantifying DNA or RNA abundance, leading to some applications such as detection and quantification of foreign DNA (e.g. micro-organisms contamination, transgenic plants and gene expression studies).

The detection technology in real-time PCR is based on the measurement of the fluorescence emitted by probes incorporated into the newly formed PCR product, or alternatively released into the buffer during the amplification of the PCR product. The end-point product of real-time PCR is measured through the use of fluorescent intercalating agents such as ethidium bromide or SYBR®Green or through radioactivity by autoradiography or phosphor imaging. It also uses solid-state approaches in which a bond enzyme produces fluorescence or luminescence or measured following by HPLC or capillary electrophoresis.