THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Doctor of Philosophy (Horticulture)
DEGREE

Horticulture
FIELD

Horticulture
DEPARTMENT

TITLE: Regulation of Lignin Biosynthesis in Fruit Pericarp Hardening of Mangosteen (*Garcinia mangostana* L.) after Impact

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THESIS

REGULATION OF LIGNIN BIOSYNTHESIS IN FRUIT PERICARP HARDENING OF MANGOSTEEN (*GARCINIA MANGOSTANA* L.) AFTER IMPACT

CHANATTIKA KAMDEE

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Horticulture)
Graduate School, Kasetsart University
2014

Chanattika Kamdee 2014: Regulation of Lignin Biosynthesis in Fruit Pericarp Hardening of Mangosteen (*Garcinia mangostana* L.) after Impact. Doctor of Philosophy (Horticulture), Major Field: Horticulture, Department of Horticulture. Thesis Advisor: Professor Saichol Ketsa, Ph.D. 179 pages.

Pericarp hardening in fresh mangosteen (*Garcinia mangostana* L.) fruit is a rapid in response to mishandling during and after harvest. Firmness, lignin content and lignin composition (G and S lignin) increased rapidly, while total free phenolic contents decreased in damaged mangosteen pericarp following impact. Application of nitrogen atmosphere to the fruit after impact reduced these effects, compared with the fruit kept in ambient air. A full length mangosteen MYB transcription factor (R2R3 MYB) and all the lignin biosynthetic pathway genes were isolated, including phenylalanine ammonia lyase (*GmPAL*), cinnamate 4-hydroxylase (*GmC4H*), *p*-coumaroyl shikimate 3-hydroxylase (*GmC3H*), ferulate (coniferaldehyde) 5-hydroxylase (*GmF5H*), 4-coumarate: coenzyme A ligase (*Gm4CL*), hydroxycinnamoyl coenzyme A shikimate hydroxycinnamoyl transferase (*GmHCT*), cinnamoyl CoA reductase (*GmCCR*), caffeoyl CoA O-methyltransferase (*GmCCoAMT*), caffeic acid (5-hydroxyconiferaldehyde) O-methyltransferase (*GmCOMT*), cinnamyl alcohol dehydrogenase (*GmCAD*) and peroxidase (*GmPOD*). Expression analysis using qPCR showed that of the genes encoding enzymes in lignin biosynthesis, only *GmF5H* increased after impact and correlated highly with increases in firmness and lignin content. The transcript level of a stress-related R2R3 MYB transcription factor was significantly increased by impact. These results suggest that pericarp hardening of mangosteen after impact is due to rapid transcriptional activation of late steps of the lignin biosynthetic pathway, potentially via up-regulation of transcription factors such as R2R3 *GmMYB*30.

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Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Professor Dr. Saichol Ketsa, my supervisor for his valuable advice and supervision, critically reviewing the manuscript and encouragement throughout the course of my Ph.D. study. I also wish to express my sincere appreciation to Professor Dr. Ian B. Ferguson, Assistant Professor Dr. Wachiraya Imsabai, Dr. Parichart Burns, Dr. Rebecca Kirk and Associate Professor Dr. Andrew C. Allan for their kind suggestion, comment and assistance which were essential for the completion of this thesis. I greatly appreciate their advice, insight and perspectives.

I am indebted to the Royal Golden Jubilee Program, Thailand Research Fund grant number PHD/0317/2550 for awarding me a scholarship to undertake this study, partially financial supported by The Postharvest Technology Innovation Center and technical assistance provided by The Plant & Food Research Institute, Auckland, New Zealand.

I would like to thank Monthathip Thongkum, all graduate students and staff at Postharvest Technology Center, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand and also Khun Somnuke Promdaeng for his hospitality and facilitating instruments and materials for my research.

In addition, I would like to express my sincere thank to Dr. Aree Thunkijjanukij, my ex-boss and Ajarn Bhumitthavara Nimnuan who always encourage me all the time.

Finally, I express my special appreciation to my family, particularly my parent and Dr. Edmond Fleischer whose constant encouragement and support contributed enormously throughout my study.

Chanattika Kamdee

June 2014

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REGULATION OF LIGNIN BIOSYNTHESIS IN FRUIT PERICARP HARDENING OF MANGOSTEEN (*GARCINIA MANGOSTANA* L.) AFTER IMPACT

INTRODUCTION

Mangosteen (*Garcinia mangostana* L.) is an increasingly important economic fruit crop. However, its value is often affected by a number of internal postharvest disorders that appear in the flesh (e.g. translucence), calyx area (wrinkling and browning), or pericarp (cracking and hardening) (Jarimopas *et al*., 2009). Pericarp hardening is one of the most important and occurs during fruit handling. It can be elicited by low storage temperature, or compression and physical impact. This negatively affects fruit quality, and thus acceptability (Ketsa and Koolpluksee, 1993; Tongdee and Suwanagul, 1998). This disorder is associated with an increase in lignin biosynthesis (Ketsa and Atantee, 1998; Bunsiri *et al*., 2003; Dangcham *et al*., 2008), paralleled by a decrease in phenolic acid levels, principally in *p*-coumaric and sinapic acids (Bunsiri *et al*., 2003).

Lignin is a product of the phenylpropanoid metabolic pathway and its abundance in plants is only exceeded by cellulose (Whetten *et al*., 1998). It is an important component of the cell wall, imparting strength and rigidity during plant growth and development, and in plant response to biotic and abiotic stresses (Boerjan *et al*., 2003; Boudet *et al*., 2003; Li and Chapple, 2010). Lignification in fruit and vegetables occurs in response to different abiotic stresses, such as physical impact in mangosteen (Ketsa and Atantee, 1998; Bunsiri *et al*., 2003), wounding in bamboo shoots (Luo *et al*., 2007), and long-term low temperature storage in apple, custard apple, loquat, asparagus, and mangosteen (Cai *et al*., 2006; Liu and Jiang, 2006; Dangcham *et al*., 2008; Li and Chapple, 2010). Lignification has been also observed in response to biotic stress. For example, infection of *Brassica rapa* with *Erwinia carotovora* results in a significant increase of lignin content within 12 to 72 h following inoculation (Zhang *et al*., 2007). Similarly, increased flesh firmness was observed in apple fruit infected by *Penicillium expansum* (Valentines *et al*., 2005; Li *et al*., 2010).

The lignification process involves monolignol formation, transportation and polymerization. Monolignols (alcohol monomers), which via polymerization produce lignin, consist of *p*-coumaryl, coniferyl, and sinapyl alcohols (abbreviated as H,- G- and S- monolignols, respectively) (Boudet, 2000; Donaldson, 2001; Boerjan *et al*., 2003; Bonawitz and Chapple, 2010; Li and Chapple, 2010). Lignin synthesis involves the coordinated expression of many genes as well as the activity of at least ten enzymes, including: (i) phenylalanine ammonia lyase (PAL), (ii-iv) three different cytochrome P450-dependent monooxygenases [cinnamate 4-hydroxylase (C4H), *p*-coumaroyl shikimate 3-hydroxylase (C3H), and ferulate (coniferaldehyde) 5-hydroxylase (F5H)], (v-vi) two methyltransferases [caffeoyl CoA *O*-methyltransferase (CCoAMT), and caffeic acid (5-hydroxyconiferaldehyde) *O*-methyltransferase (COMT)], and (vii-viii) two oxidoreductases [cinnamoyl CoA reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD). In addition to these enzymes, (ix-x) two more [4-coumarate: coenzyme A ligase (4CL) and hydroxycinnamoyl coenzyme A: shikimate hydroxycinnamoyl transferase (HCT)] are required to synthesis pathway intermediates, which serve as substrates for subsequent reactions (Bonawitz and Chapple, 2010). The last major step in lignin synthesis involves monolignol dehydrogenation and polymerization, and different classes of oxidative enzymes (xi) have been shown to be implicated, including (a) class III peroxidase laccase, (b) ascorbate peroxidase, and (c) NADPH oxidase (Boerjan *et al*., 2003).

Most characterized transcriptional regulators of lignin biosynthetic genes have been members of the MYB family. Several subgroups of R2R3-MYB factors have been reported to either positively or negatively control lignin biosynthesis. Examples of these regulators includes *Pinus taeda PtMYB4*, poplar (*Populus* sp.) *PttMYB21a* and *Eucalyptus gunnii EgMYB2* (Zhao and Dixon, 2011). In *Arabidopsis,* overexpression of MYB85 leads to ectopic deposition of lignin in epidermal and cortical cells of stems (Zhong *et al*., 2008). In addition, MYB46 functions as a transcriptional switch that turns on the genes necessary for secondary wall biosynthesis, while in the same clade, MYB83 overexpression is able to activate a number of the biosynthetic genes of cellulose, xylem and lignin and concomitantly induce ectopic secondary wall deposition (Maldonado *et al*., 2002; Ko *et al*., 2009). It has recently been shown that MYB63 (and MYB58) are transcriptional regulators specifically activating lignin biosynthetic genes

in *Arabidopsis.* They are expressed in fibers and vessels undergoing secondary wall thickening and are able to activate directly lignin biosynthetic genes and a secondary wall-associated laccase (LAC4) (Zhou *et al*., 2009).

Peroxidase and laccase use H_2O_2 and molecular oxygen to oxidized monolignols. Therefore, by decreasing oxygen levels, modified atmosphere (MA) treatments of fruit can avoid these reactions. Keeping persimmon fruit in high nitrogen can maintain quality and firmness (Dorria *et al*., 2011), while MA treatment of bamboo shoots (2% O_2 and 5% CO_2) inhibits lignification (Shen *et al*., 2006). In mangosteen after impact, fruit held under N_2 had higher firmness, and lower lignin content than fruit held in air (Ketsa and Atantee, 1998; Bunsiri *et al*., 2003). Low O_2 storage is therefore likely to be beneficial in reducing these postharvest disorders.

In mangosteen, only two genes [PAL and lignin peroxidase (LgPOD)] have been identified during pericarp hardening resulting from low storage temperature (Dangcham *et al*., 2008). Consequently, the genes involved in lignification in this species remain largely unexplored. In the present study, fruit firmness, phenolic contents, and lignin content in relation to pericarp hardening were studied. All the genes encoding enzymes of the lignin biosynthesis pathway were isolated as well as a potential stress-related R2R3 MYB transcription factor. The expression of these genes was examined using real-time quantitative PCR (qPCR) during pericarp hardening. The study also reports the effect of nitrogen atmosphere on pericarp hardening and gene expression in terms of the mangosteen lignin biosynthetic pathway.

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OBJECTIVES

1. To assess the total free phenolic and lignin contents, and relate them with changes in pericarp firmness following impact.

2. To determine the effect of air composition (by manipulating oxygen levels) on the speed and the degree of lignification process.

3. To characterize the expression of genes regulating protein synthesis, involved in the lignification process.

LITERATURE REVIEW

Origin, cultivation and economic importance

Mangosteen (*Garcinia mangostana* Linn., Family: Guttiferae) is believed to have originated from south-east Asia. Production initially occurred in the area of its origin (mainly Thailand, Malaysia and Indonesia), though the last two centuries, it has been spread to other tropical areas, including Sri Lanka and southern India, as well as to other continents including America and Australia (Janick and Paull, 2006).

Thailand is the largest exporter of mangosteen in the world, while its exportation has been steadily increasing during the last 10 years. In 2013, for example, Thailand's income from mangosteen exports was about 4,000 million baht (Office of Agricultural Economics, 2014). The planting areas are spread in both eastern and southern regions of the country, together covering an area of about 164 thousand hectares, and reaching an annual production of about 200 thousand metric tons on average. This production volume is spread over the year, since the eastern and southern regions have different harvesting seasons (April to June, and July to September, respectively) (Office of Agricultural Economics, 2011).

Mangosteen has been named as the "Queen of Fruits", because the four large green sepals are not abscised, but remain on the fruit calyx, and appear like a crown (Wieble *et al*., 1992; Choehom *et al.,* 2003). It develops parthenocarpically (i.e. no true seed) in 100-120 days from anthesis. In cooler areas or higher elevations, fruit development is prolonged for about 2 months (Janick and Paull, 2006).

Mangosteen fruit is roughly spherical shape, and it is classified as berry (indehiscent fruit with two or more seeds and a fleshy pericarp). At its upper pole, mangosteen bears a large stalk, which at the point of attachment is surrounded by two pairs of sepals. Inside the fruit, its base bears 4-8 star-shaped furrows, where a respective number of (edible) segments are situated. At harvest, fruit diameter ranges between 4 and 7 cm, while pericarp thickness is 0.6 to 1.0 cm. At that stage, fruit weight

is between 80 and 150 g, of which 25–30% consists of the edible part, called aril or pulp. The edible aril is white, soft and 4–8 pieces segmented (Martin, 1980).

Mangosteen is frequently harvested at an earlier stage than that of consumption. Following harvest, cell growth and metabolism continue within the fruit, and after a certain period it can be consumed. Pericarp color is the major criterion used as harvesting index to judge harvest stage maturity. The following color stages have been identified: stage I, green; stage II, occurrence of red lines (the so called "blood- lines"); stage III, pink; stage IV, red; stage V, dark red/red purple; and stage VI, black color (Figure 1) (Shaw *et al*., 1998). At commercial practice, the fruits are harvested at stage I. At ambient temperatures, each stage takes place within 1 day. For instance, fruits harvested at stage I are expected to be at stage IV within 3 days. About 8 days after stage VI (i.e. about 13 days following harvest), rot symptoms appear on the fruit at ambient temperatures.

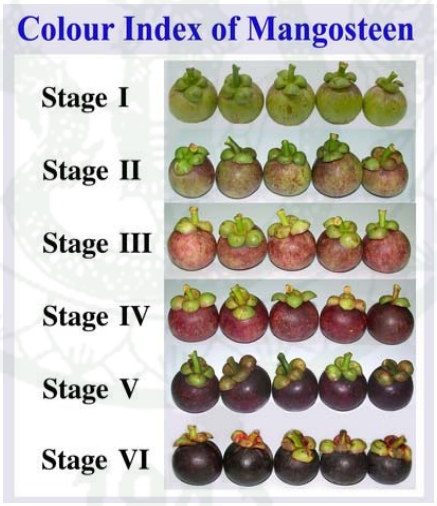

Figure 1 Pericarp color scale used to judge harvest stage maturity in mangosteen.

Source: Palapol *et al*. (2009)

Another means of gauging the maturity of the fruit is to observe the way in which the fruit separates from the stalk. At harvest maturity, no yellowish latex remains in the skin, and the aril can be easily separated from the skin (Tongdee and Suwanagal, 1989; Paull and Ketsa, 2004; Janick and Paull, 2006).

Pericarp hardening

Mangosteen is very susceptible to mechanical force (e.g. by means of drop, physical impact with other objects, or compression), which not only is present in almost every step of fruit handling, but also in the distribution chain. Consequently, unsuitable harvesting methods and/or inappropriate postharvest handling rapidly result in a number of symptoms (e.g. pericarp hardening, pulp browning and pulp texture loss) that deteriorate fruit quality (Shaw *et al.*, 1998). That explains careful handling (e.g. harvesting by individually plucking each fruit with pole from the tree; Shaw *et al*., 1998) is a common practice. Despite laborious harvesting methods and minimization of handling errors, Jarimopas *et al.* (2009) found that as much as 2% of the harvested fruits showed symptoms of impact within transport from the orchard to the nearby wholesalers in Thailand, which represents only a very small part of the postharvest chain. Mechanical force symptoms may appear both in the pericarp and the edible arils (Peeraprasompong, 1996).

A mechanical force, as low as fruit drop from a 10 cm height, rapidly results in pericarp hardening (Tongdee and Suwanagal, 1989; Ketsa and Koolpluksee, 1993; Bunsiri *et al*., 2003). At the point of impact, the pericarp develops a hard core, at which nucleases are produced, and further assist the expansion of the hardening. Another frequent symptom of impact is the spread of latex into the edible arils, which deteriorates fruit quality and thus market acceptability (Peeraprasompong, 1996).

The extent of hardening depends on fruit maturity, applied mechanical force (i.e. drop height) and air composition. Pericarp hardening, following impact, increased from young through half-ripped to mature fruits (Ketsa and Koolpluksee, 1993; Bunsiri *et al*., 2003). Similarly, higher drop heights also caused significantly greater pericarp hardening (Ketsa and Atantee, 1998; Ketsa and Koolpluksee, 1993; Bunsiri *et al*., 2003). Surprisingly, a drop height of only 10 cm was able to induce pericarp damage (Tongdee and Suwanagal, 1989).

The mangosteen pericarp, following a drop by 1 m height, showed an increase in firmness within as short as 15 min (Bunsiri *et al*., 2003). Consequently, the pericarp

firmness dramatically increased within the following 3 h. This rate of pericarp hardening could be slowed down by eliminating oxygen (0.01%) in the ambient air (Tongdee and Suwanagal, 1989; Ketsa and Koolpluksee, 1993; Bunsiri *et al*., 2003).

The pericarp hardening, in other words the increase in firmness, following physical impact, has been shown to correlate with both an increase in lignin content and a decrease in total phenolic content (Tongdee and Suwanagal, 1989; Ketsa and Koolpluksee, 1993; Ketsa and Atantee, 1998; Bunsiri *et al*., 2003). Lignification, as a means to increase fruit firmness, also takes place naturally during fruit growth and development, while a role in response to a wide range of stresses (e.g. low temperature and dehydration) has also been suggested (Boerjan *et al*., 2003; Boudet *et al*., 2003).

Since pericarp firmness increases and correlates well with tissue lignin content, an increased activity of enzymes involved in its biosynthesis might be expected. Example of those enzymes include: 1) phenylalanine ammonia lyase (PAL), 2) peroxidase (POD), and 3) cinnamyl alcohol dehydrogenase (CAD). In the side of expectations, the activity of all three above-mentioned enzymes in pericarp tissue increased drastically within 15 min of impact, and subsequently decreased. Instead, total phenolics' content followed the opposite trend to that of lignin, and decreased during impact. Phenolic acids identified in mangosteen pericarp include the *p* - courmaric and sinapic acids. The latter phenolic acid had the highest concentration in the pericarp of mangosteen, following impact (Bunsiri *et al*., 2003).

Pericarp hardening does not only appear as a result of mechanical impact, but can also be induced by other abiotic stresses, such as low temperature and dehydration (Ketsa and Koolpluksee, 1993; Uthairatanakij and Ketsa, 1996). For example, increased pericarp thickness appears to be a typical symptom of chilling injury in mangosteen (Kader, 2007; Dangcham *et al*., 2008). Chilling injury and the concomitant pericarp hardening take place at temperatures of 4 to 8 ^{O}C, while the edible aril is less affected (Augustin and Azudin, 1986). These results were confirmed by a recent study where fruits stored at lower temperature (6 versus 12 ^{O}C) also showed greater pericarp firmness, as a result of lignin synthesis (Dangcham *et al*., 2008).

Increased tissue firmness, as a result of increased lignin biosynthesis, after storage at low temperature (2 ^{O}C) has also been shown in other species including *Phyllostachys praecox* (Luo *et al*., 2008) and *Eriobotrya japonica* (Shan *et al*., 2008; Yang *et al*., 2008). Interestingly, in *E. japonica* fruit there is variation depending on the genotype, as to of flesh lignification takes place during natural fruit senescence or in response to chilling injury. This change in firmness, in the genotype where lignification does take place, was also positively correlated with increased activities of PAL, POD and CAD.

Absence of O_2 (0.01%, by increasing N_2 in atmosphere) in mangosteen after impact resulted in reduced pericarp hardening, associated with lower lignin content and higher total phenolics' content compared to fruits held at ambient air (21% O_2) (Ketsa and Koolpluksee, 1993; Ketsa and Atantee, 1998), indicating that oxidative processes are involved. Similarly, *P. praecox* shoots, under low O_2 (2%) and high CO_2 (5%) atmosphere, had lower lignin content, compared to shoots held at ambient air, which correlated with decreased activities of three enzymes involved in its biosynthesis **(**Sheng *et al***.**, 2004).

Phenolic acids (a subclass of phenolics)

Phenolic acids consist of a subclass of a larger category of metabolites commonly referred as phenolics. The term phenolics encompasses approximately eight thousand naturally occurring compounds, all of which possess one common structural feature, a phenol (an aromatic ring bearing at least one hydroxyl substituent) (Figure 2). Current classification divides the broad category of phenolics into polyphenols and simple phenols, depending on the number of phenol subunits present. Polyphenols possess at least two phenol subunits. Naturally, phenolics contain two distinguishing constitutive carbon frameworks: the hydroxycinnamic (X_a), and hydroxybenzoic (X_b) structures (Figure 2). In many cases, aldehyde analogues (X_c) are also grouped in and referred as phenolics (Figure 2).

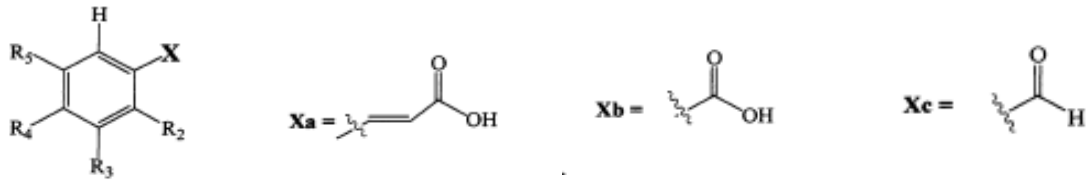

Figure 2 Phenol structure consisting of an aromatic ring bearing at least one hydroxyl substituent (-H), positions where hydroxyl groups (-OH) might be situated ($R_{2\text{-}5}$, see examples in Table 1) and a carbon framework (X) that can be either hydroxycinnamic (X_a)/hydroxybenzoic (X_b) structure, or an aldehyde analogue (X_c).

Source: Robbins, (2003)

Table 1 The common name, the positions where hydroxyl groups (-OH) might be situated ($R_{2\text{-}5}$, see Figure 2) and the carbon framework ($X_{a\text{-}c}$, see Figure 2) of the most common naturally occurring phenolics.

Common name	R_2	R_3	R_4	R_5	X
cinnamic acid	H	H	H	H	a
o-coumaric acid	-OH	H	H	H	a
p-coumaric acid	H	H	-OH	H	a
m-coumaric acid	H	-OH	H	H	a
ferulic acid	H	$-OCH_3$	-OH	H	a
sinapic acid	H	$-OCH_3$	-OH	$-OCH_3$	a
caffeic acid	H	-OH	-OH	H	a
benzoic acid	H	H	H	H	b
salicylic acid	-OH	H	H	H	b
p-hydroxybenzoic acid	H	H	-OH	H	b
vanillic acid	H	$-OCH_3$	-OH	H	b
syringic acid	H	$-OCH_3$	-OH	$-OCH_3$	b
protocatechuic acid	H	-OH	-OH	H	b
gentisic acid	-OH	H	H	-OH	b

Source: Robbins (2003)

The basic skeleton remains the same, while the number and positions of hydroxyl groups (R_{2-5}, see Figure 2 in combination with Table 1) on aromatic ring create the variety (Robbins, 2003).

In general, the principal ecological functions of plant polyphenols (i.e. phenolics possessing at least two phenol subunits) are to assist a) plant adaptation to abiotic changes (e.g. mechanical or chemical stress), b) plant defense against biotic factors (e.g. herbivore and pathogen attack), and c) interorgan communication by acting as signaling molecules (Walter *et al*., 1990; Rupasinghe, 2008). For example, phenolics are involved in the lignification of cell wall around the wounded zone to assist healing. Another example can be seen during mechanical damage, where plants create a physical barrier to prevent tissue destruction, including the synthesis of polyphenols such as lignin and suberin (Moura *et al*., 2010).

Plant phenolics may be present in either free or bound form. Bound phenolics, such as hydroxycinnamic acid (simple phenolic) and lignin, are ester-linked to cell wall polysaccharides (Nara *et al*., 2006; John and Shahidi, 2010). They contribute to the mechanical strength of the cell walls they are bound to, playing a regulatory role in plant growth and morphogenesis as well as in response to biotic and abiotic stresses (Naczk and Shahidi, 2004).

Phenolic acids' biosynthesis

Phenolics are synthesised through two major pathways: the shikimic acid and the malonic acid pathway (see examples in Figure 3). The latter pathway, although an important source of secondary phenolic products in fungi and bacteria, is of less significance in higher plants (Taiz and Zeiger, 2002). The former pathway is involved in biosynthesis of most plant phenolics. For example, the phenolic acids (a subclass of phenolics), widely spread in planta, are derived from phenylpropanoid metabolism through the shikimic pathway (Singh *et al*., 2010). The shikimic acid pathway is initiated with the reaction of erythrose-4 phosphate (an intermediate of the pentose phosphate pathway, PPP) and phosphoenol pyruvic acid (an intermediate of glycolysis),

that produces deoxy-arabino-heptulosonate -7- phosphate, which is subsequently converted to shikimic acid (Figure 3).

Following the shikimic acid pathway, L-phenylalanine is produced (Figure 3). The most abundant classes of plant phenolics are derived from L-phenylalanine via the elimination of ammonia molecule yielding to cinnamic acid (Figure 4). This reaction is catalyzed by the enzyme phenylalanine ammonia lyase (PAL). PAL is situated at a branch point between the primary and secondary metabolisms (Taiz and Zeiger, 2002).

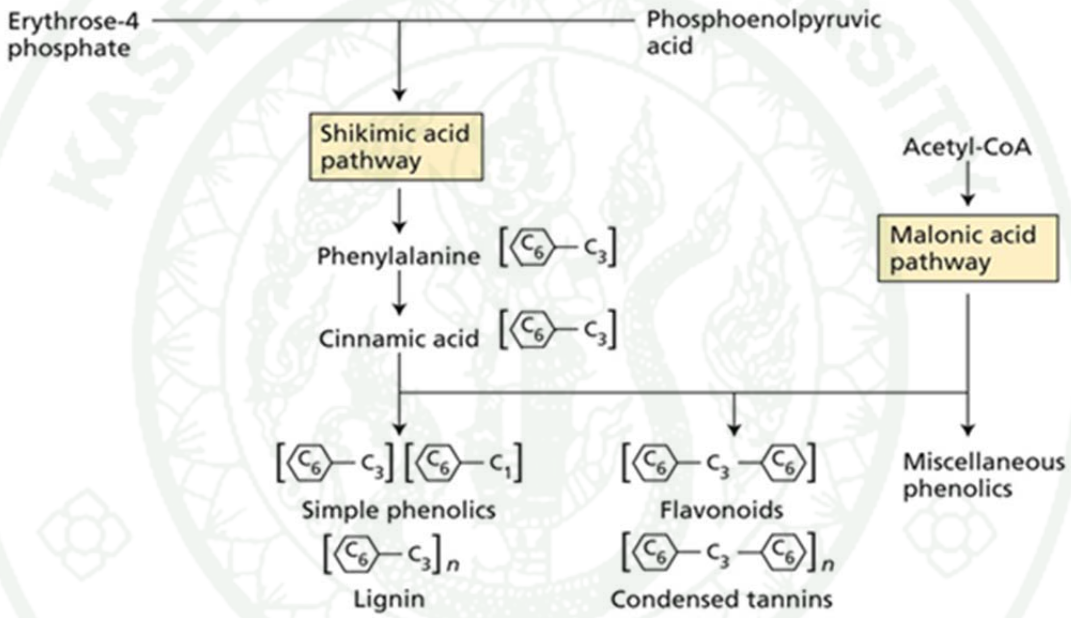

Figure 3 Phenolics' biosynthesis pathways consist of the shikimic acid and the malonic acid pathways. In higher plants, the vast majority of phenolics are derived, at least in part, from phenylalanine, which is a product of the former pathway. The latter pathway is typical for fungi and bacteria, and is of less significance in higher plants.

Source: Taiz and Zeiger (2002)

At the gateway from primary metabolism, PAL plays an important role in phenolics' synthesis and the correlation between increases in the corresponding PAL gene/protein expression/activity and increases in phenolics' content in response to a wide range of stimuli has been repeatedly shown (Boudet, 2007).

The conversion of L-phenylalanine to the various hydroxycinnamic acids involves a three-step reaction that is referred to as the "general phenylpropanoid metabolism", named after its almost universal appearance in synthesis of aromatic secondary metabolites. The first step in the phenylpropanoid metabolic pathway is the deamination of L-phenylalanine, catalyzed by PAL, generating the *trans*-double bond in the *trans*-cinnamic acid backbone (Figure 4). The produced *trans*-cinnamic acid might (i) act as substrate for the benzoic acid derivatives, or (ii) enter the second step of the phenylpropanoid metabolic pathway (Figure 4) (Robbins, 2003). In the latter case (second step of the phenylpropanoid metabolic pathway), hydroxylation of the aromatic ring at position 4 takes place, generating the *p*-coumaric acid (Figure 4). The third step of the phenylpropanoid metabolic pathway is the formation of the *p*-coumaroyl coenzyme A (CoA) catalyzed by 4- courmarate CoA ligase (4CL) (Figure 4). Apart from entering the phenylpropanoid metabolic pathway, *p*-coumaric acid may undergo hydroxylation and methylation reactions, yielding to a number of derivatives (i.e. caffeate, ferulate, and sinapate) (Figure 4). The final product of the phenylpropanoid metabolic pathway (*p*-coumaroyl CoA) may be subsequently converted to biosynthesis precursors of a diverse class of compounds (named phenylpropanoids, see explanation below), including lignin, flavonoids, anthocyanins, isoflavones, and tannins (Figure 4) (Taiz and Zeiger, 2002; Boudet, 2007).

Trans-cinnamic acid, *p*-coumaric acid, and their derivatives are simple phenolics (i.e. possessing one phenol subunit) called phenylpropanoids. Their name is derived from the six-carbon aromatic phenol group and the three-carbon side chain (Taiz and Zeiger, 2002).

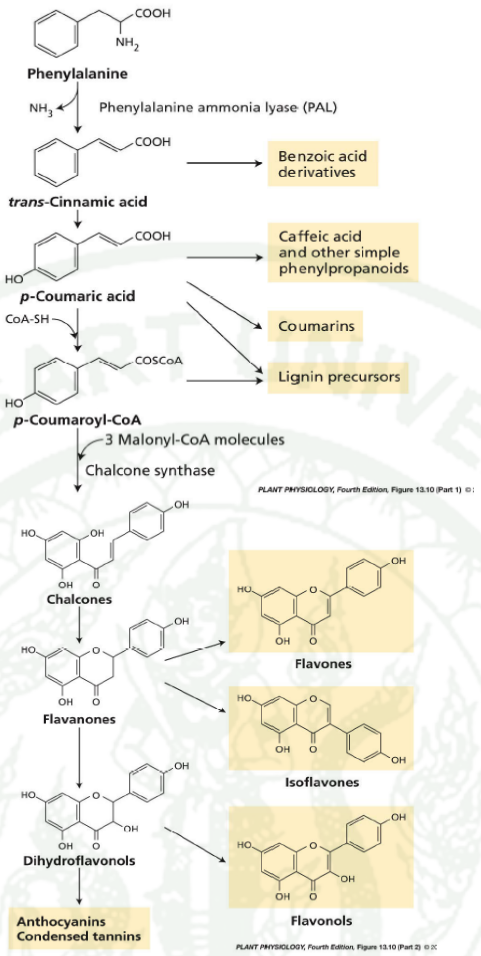

Figure 4 A general outline of phenolic biosynthesis is presented. Starting from phenylalanine, the three-step vertical reaction is named phenylpropanoid metabolic pathway and yields to *p*-coumaroyl coenzyme A (CoA). Intermediate products of this pathway may also act as precursors of several metabolites, of which examples are presented. The produced *p*-coumaroyl CoA also acts or it is converted to biosynthesis precursors of a diverse class of phenolic compounds, of which examples are given.

Source: Taiz and Zeiger (2002)

Phenylpropanoids contribute to fruit pigmentation that takes place naturally during fruit development, but also in response to biotic and abiotic stresses. In case of stress, they are not only indicators, but they also act as key-mediators of plant resistance (Vogt, 2010).

Phenolics' change in fruits

Mechanical or physical stress, causing damage to the plant tissue, alters the phenolics' metabolism (Rhodes and Wooltorton, 1978). For instance, the hardening of damaged mangosteen pericarp, as a result of physical impact, paralleled with the decrease in the total phenolics' content (Ketsa and Koolpluksee, 1993; Bunsiri *et al.*, 2003). It was also shown that this increase in firmness of mangosteen pericarp was accompanied by an increase of lignin content (Atantee, 1995). Combining the finding of those studies (Ketsa and Koolpluksee, 1993; Atantee, 1995; Bunsiri *et al.*, 2003), it might be expected that the phenolics' content decreased, because phenolics acted as substrate for lignin synthesis (note that lignin is also a phenolic compound). Two phenolics (*p*-courmaric and sinapic acids) were isolated in mangosteen pericarp after impact or low temperature storage (Bunsiri *et al*., 2003; Dangcham *et al*., 2008). Instead, Zadernowski *et al.* (2009) did not detect these acids in the free phenolics' fraction (phenolics that are not bound in the cell walls), isolated by either the peel or the (edible) aril. In the same study, it was shown that protocatechuic acid was the major phenolic acid in the peel, while *p*-hydroxybenzoic acid was the predominant phenolic acid in the aril.

Phenolics have various functions in plants. An enhancement of phenylopropanoid metabolism and the amount of phenolic compounds can be observed under different environmental factors and stress conditions. The induction of phenolic compound biosynthesis was observed in many plants responded to stresses. Increase in phenolics such as intermediates in lignin biosynthesis can reflect the typical anatomical change induced by stressors: increase in cell wall endurance and the creation of physical barriers (Michalak, 2006).

Two *Citrus sinensis* cultivars, the increase in total phenolics, induced by gamma radiation, paralleled with both the activation of PAL, and a rise in lignin (Dubery *et al*., 1992). PAL activity, total phenolics and lignin formation also showed the same pattern in the peel of injured/infected *Citrus limon* (L.) Burm (Nafussi *et al*., 2001), while these phenomena also took place in flavedo and albedo of *Citrus madurensis* fruits following wounding (Mulas *et al.*, 1996). Similarly, mechanical damage to *Pachyrhizus erosus*

pieces at low temperatures (5 and 10 ^{O}C) caused surface browning associated with increased phenolic content and activity of PAL (Aquino-Bolaños and Mercado-Silva, 2000). The eggplant were treated with four elicitors such as, chitosan (CHT), salicylic acid (SA), methyl salicylate (MeSA) and methyl jasmonate (MeJA) resulting increase in total phenolic in 0 - 48 h post elicitation and then decrease whereas the total lignin gradually increased during the post elicitation time. Accumulation of phenolics, it may effective resistance against the devastating wilt pathogen in eggplant (Mandal, 2010).

There are two differences of the phenolic patterns in plant stresses. First pattern, the phenolic increases while lignin decreases. This turnover of phenolic may be more rapid than synthesis, resulting a decrease in total phenolics (Bunsiri *et al*., 2003). Second, the turnover of phenolics metabolism is less than synthesis, resulting an increase in total phenolics. The accumulation of total phenolics in other responses such as radiation, wounding and low temperature storage are direct responses to disruption of cell compartmentation with tissue damage or part of defense mechanisms (Mazid *et al*., 2011).

Lignin

Lignin is a product of the phenylpropanoid metabolic pathway (Figure 4; see also Figure 3), and its abundance in planta is only exceeded by cellulose. It is a complex polymer composed of phenyl propane units derived from the following three alcohol monomers (also called monolignols): *p*-coumaryl (4-hydroxycinnamyl), coniferyl (3-methoxy 4-hydroxycinnamyl) and sinapyl (3,5-dimethoxy 4-hydroxycinnamyl) alcohols (Whetten *et al.*, 1998). Upon their incorporation into the lignin polymer, these monomers are referred to as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively (Bonawitz and Chapple, 2010). These monomers differ only in their degree of methoxylation. The H-monomer is not methoxylated, the G-monomer is methoxylated (-OCH_3 group) on position 3, while the S-monomer is methoxylated on positions 3 and 5 (Figure 5) (Parijs *et al.*, 2010).

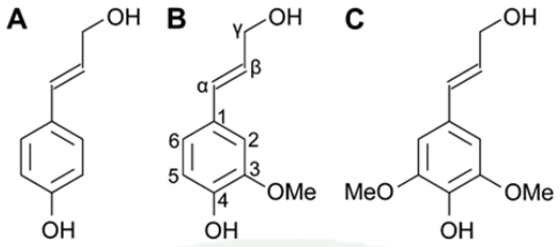

Figure 5 Structure of the three monolignols (alcohol monomers), of which phenyl propane units are derived and by polymerization produce lignin. A, *p*-coumaryl alcohol (H-monomer). B, coniferyl alcohol (G-monomer). C, syringyl alcohol (S-monomer). Note that the monomers differ only in their degree of methoxylation.

Source: Parijs *et al.* (2010)

Lignin plays critical roles during plant growth and development, as well as in response to biotic and abiotic stress (Li *et al*., 2010). Lignin is an important cell wall component, which fills in the spaces between cellulose, hemicellulose, and pectin components. It is also deposited in terminally differentiated cells of supportive and water-conducting tissues (i.e. tracheid, sclereid and xylem cells), imparting them the capacity to withstand the force of gravity and the negative pressure (i.e. water potential) generated by transpirational water loss. Lignification is also been triggered at specific sites in response to a wide range of biotic (e.g. low temperature, physical impact and mechanical injury) and abiotic (pathogen infection) stresses (Boudet, 2000; Donaldson, 2001; Hatfield and Vermerris, 2001; Boerjan *et al.*, 2003; Bonawitz and Chapple, 2010; Li and Chapple, 2010; Moura *et al*., 2010). For instance, in order to counteract mechanical damage, plants create a physical barrier to prevent tissue destruction, including the synthesis of lignin and other polyphenols such as suberin (Walter *et al*., 1990).

Interestingly, different ripening patterns depending on the cultivar has been noted in *E. japonica* fruits, where one cultivar (LYQ) showed lignification during ripening, while the other studied one (BS) not (Li *et al*., 2010). Besides natural ripening and senescence, lignification in fruits and vegetables also occurs in response to different

abiotic stresses, such as physical impact in mangosteen (Ketsa and Atannee, 1998; Bunsiri *et al*., 2003), wounding in *P. praecox* (Luo *et al*., 2007), long-term low storage temperature in *Malus domestica* (Li *et al*., 2010), *Annona cherimola* (Maldonado *et al*., 2002), *E. japonica* (Cai *et al*., 2006a,b), *Asparagus officinalis* (Liu and Jiang, 2006) and mangosteen (Dangcham *et al.*, 2008). Lignifications have been also observed in response to biotic stress. For example, infection of *Erwinia carotovora* on *Brassica rapa* resulted in a significant increase of lignin content within 12 to 72 hours following inoculation (Zhang *et al*., 2007). Similarly, increased flesh firmness was observed in *M. domestica* fruits infected by *Penicillium expansum* (Valentines *et al*., 2005; Li *et al*., 2010).

Monolignol biosynthesis

The three monolignols (alcohol monomers), that by polymerization produce lignin, are: the *p*-coumaryl, coniferyl, and sinapyl alcohols (Figure 6; for structure see Figure 5), consisting the H-, G-, and S- monomers, respectively. Upon their incorporation into the lignin polymer, these monomers are referred as *p*-hydroxyphenyl (H-), guaiacyl (G-), and syringyl (S-) lignin, respectively (Figure 6).

The monolignol synthesis from L-phenylalanine requires several steps including a) deamination, b) hydroxylation at one, two, or three positions of the aromatic ring, c) methylation of one or two of these hydroxyl groups, and d) two successive reductions of the monolignol side chain. The carboxylic acid of the monolignol side chain is first converted to aldehyde, and subsequently to alcohol (Figure 6). This reaction is mediated by PAL (Whetten *et al*., 1998; Singh *et al.*, 2010). The following seven enzymes: i-iii) cytochrome P450-dependent mono-oxygenases [cinnamate 4-hydroxylase (C4H), *p*-coumaroyl shikimate 3-hydroxylase (C3H), and ferulate (coniferaldehyde) 5-hydroxylase (F5H)], iv-v) methyl-transferases [caffeoyl CoA *O*-methyltransferase (CCoAMT), and caffeic acid (5-hydroxyconiferaldehyde) *O*-methyltransferase (COMT)], and vi-vii) oxido-reductases [cinnamoyl CoA reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD)], play critical roles in the final pathway products (Bonawitz and Chapple, 2010). For instance, CAD (the last enzyme in the list) is one of the more specific enzymes in the pathway and catalyzes the reduction of cinnamaldehydes to cinnamyl alcohol in the last step of monolignol biosynthesis. In

addition to those eight enzymes (PAL and the list of seven), two more [4-coumarate: CoA ligase (4CL), and hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT)] are required to synthesis pathway intermediates that serve as substrates for subsequent reactions.

The 4CL enzyme is an ATP-dependent CoA ligase that catalyzes the synthesis of *p*-coumaroyl CoA. This *p*-coumaroyl CoA is then used as an acyl donor by the BAHD family acyltransferase. The family acyltransferase's abbreviation (BAHD) is made by combine the first abbreviation letter (**B**EAT, **A**HCTs, **H**CBT, and **D**AT) of the enzymes that encompasses (**B**EAT: benzylalcohol *O*-acetyltransferase, **A**HCTs: anthocyanin *O*-hydroxycinnamoyltransferases, **H**CBT: anthranilate *N*-hydroxycinnamoyl/benzoyltransferase, and **D**AT: deacetylvindoline 4-*O*-acetyltransferase). The enzymes of the BAHD family acyltransferase are responsible for acyl-CoA dependent acylation of secondary metabolites, typically resulting in the formation of esters and amides. The enzyme HCT catalyzes the synthesis of the *p*-coumaroyl shikimate, the substrate for 3-hydroxylation mediated by C3H (ii of the seven enzymes' list). Alternatively, *p*-coumaroyl CoA can serve as a substrate for chalcone synthase, diverting it to the flavonoid pathway, or CCR (vi of the seven enzymes' list).

The pathway leading to *p*-coumaryl alcohol (the first monolignol, which by polymerization produces lignin, Figure 6) requires only a subset of the above-mentioned enzymes [PAL, C4H, CCR, CAD (i, vi, and vii of the seven enzymes' list, respectively) and 4CL]. The synthesis of coniferyl alcohol (the second monolignol, which by polymerization produces lignin, Figure 6) requires these five enzymes plus three more [C3H, CCoAMT (ii and iv of the seven enzymes' list, respectively), and HCT], making them eight in total. The sinapyl alcohol synthesis (the third monolignol, which by polymerization produces lignin, Figure 6) requires all 10 enzymes, namely the eight required for the coniferyl alcohol synthesis plus the F5H and COMT (iii and v of the seven enzymes' list, respectively) (Bonawitz and Chapple, 2010). The last major step of lignin synthesis is the dehydrogenation and polymerization of these three monolignols (*p*-coumaryl, coniferyl and sinapyl alcohols), catalyzed by peroxidase (POD) and/or laccase (LAC) (Figure 6).

Lignin content and composition have been shown to vary between the non-flowering (gymnosperm, fern and lycophytes) and flowering (angiosperms, consisting of dicots and monocots) vascular plants, as well as within them. The lignin of dicots (group of angiosperms) consists mostly of G and S subunits, while that of some monocots (the other group of angiosperms) contains relatively more H subunits. In contrast, the lignin of non-flowering plants is composed mostly of G subunits, lacking the S subunits (Whetten, 1998; Bonawitz and Chapple, 2010; Li and Chapple, 2010; Vanholme *et al.*, 2010; Weng and Chapple, 2011). Differences in the amount and composition of lignin have also been observed among species of the same plant group, but also between cell types and individual cell layers of the same species (Bonawitz and Chapple, 2010; Vanholme *et al*., 2010).

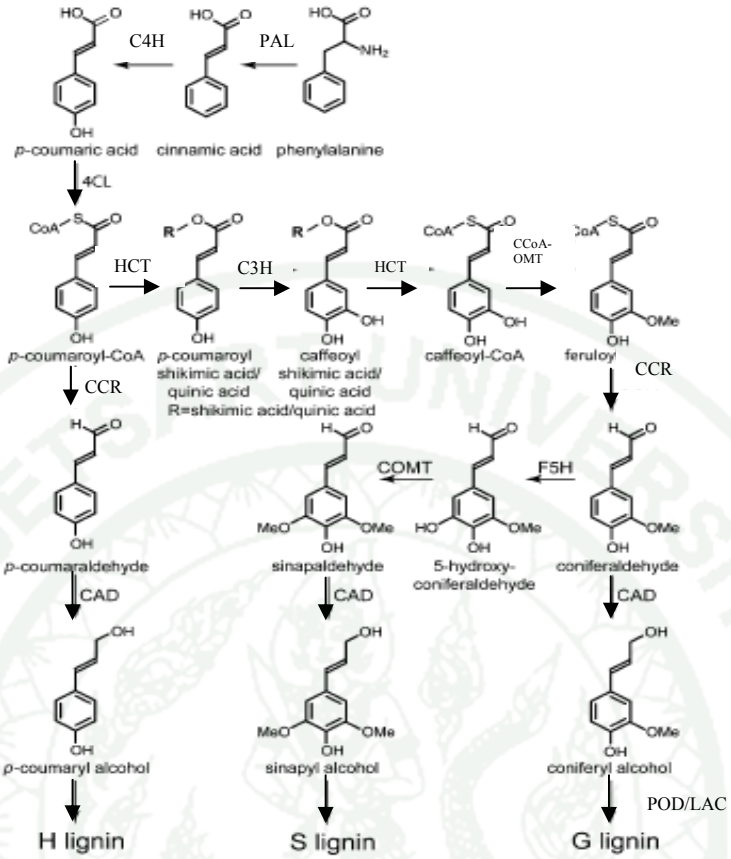

Figure 6 The intermediates and the enzymes catalyzing the various reactions of the monolignol biosynthesis pathway. The pathway starts with phenylalanine, and ends to one of the three monolignols (H-, G-, S- lignin), that by polymerization produce lignin. Enzyme abbreviations are explained in Table 2.

Source: Vanholme *et al*. (2010)

Table 2 The enzymes involved in the monolignol biosynthesis pathway and their abbreviation.

Enzyme	Abbreviation
Phenylalanine ammonia lyase	PAL
Cinnamate 4-hydroxylase	C4H
p-coumaroyl shikimate 3-hydroxylase	C3H
Ferulate (coniferaldehyde) 5-hydroxylase	F5H
Caffeoyl CoA *O*-methyltransferase	CCoAMT
Caffeic acid (5-hydroxyconiferaldehyde) *O*-methyltransferase	COMT
Cinnamoyl-CoA reductase	CCR
Cinnamyl alcohol dehydrogenase	CAD
4-Coumarate: CoA Ligase	4CL
Hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase	HCT
Peroxidases	POD
Laccase	LAC

Monolignol translocation and polymerization

Monolignols, the lignin monomers, are synthesised in the cytoplasm (Golgi or endoplasmic reticulum) and are released into the cell wall, where they undergo oxidation and polymerization to form lignin (Figure 7). The translocation of small molecules across the cell membrane may occur by at least three different mechanisms: exocytosis, transporter-mediated export, and diffusion (Donaldson, 2001; Bonawitz and Chapple, 2010; Li and Chapple, 2010). Monolignols are exported to the extracellular space (i.e. apoplast) by an as yet poorly understood mechanism for their incorporation into the lignin polymer (Bonawitz and Chapple, 2010; Vanholme *et al*., 2010). In one proposed model, the monolignols are translocated over the plasma membrane through their 4-*O*-glucosylated forms, coniferin and syringin, that are deglucosylated upon their arrival by glucosidases located in the cell wall. In another proposed model, monolignols are transported to the plasma membrane by Golgi-derived vesicles. However, Kaneda *et al*. (2008), using [3H]-Phe feeding experiments, neither found evidence for Golgi-derived vesicles involved in monolignol transport, nor did they find any significant label in the monolignol glucosides

(previous model). Thus, at present, there is no convincing support for the role of the two above-mentioned models (monolignol glucosides, and Golgi-derived vesicles) in the transport of monolignols to the cell wall. Other models assume that monolignols are translocated across the plasma membrane by transporters, but tests on several prime candidate transporters (by using reverse genetics) have not as yet revealed any effect (Vanholme *et al*., 2010).

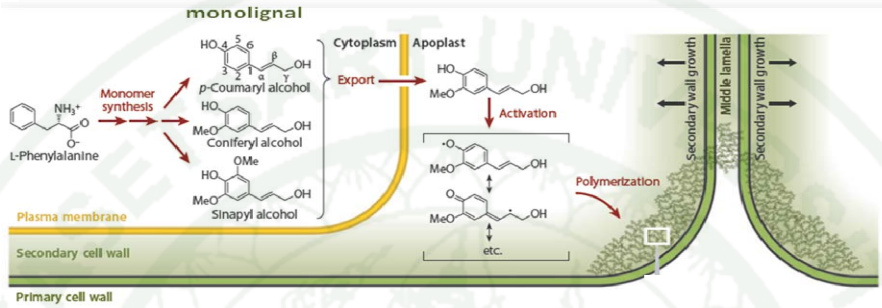

Figure 7 An overview of monolignol biosynthesis, transport and polymerization. Phenylalanine, through a series of enzymatic reactions, is converted to one of the three monolignols (*p*-coumaryl, coniferyl, and sinapyl alcohols). These are subsequently transported to the apoplast (extracellular space), where they undergo oxidation and polymerization to form lignin.

Source: Bonawitz and Chapple (2010)

Lignin polymerization occurs via oxidative radicalization of monolignols, followed by combinatorial radical coupling. After reaching the extracellular space, monolignols firstly undergo a single-electron oxidation, mediated by wall-bound peroxidase and/or laccase. The resulting reactive radical species is relatively stable due to delocalization of the unpaired electron in the conjugated system (Figure 8). Subsequently, two monomer radicals may couple to form a (dehydro) dimer, thereby establishing a covalent bond between both subunits. Monolignol radicals favor coupling at their β positions, resulting essentially in only the β-β, β-5, and β-O-4 dimers (Figure 8). Then, the dimer needs to be dehydrogenated, again to a phenolic radical, before it can couple with another monomer radical. This mode of action, in which a monomer (radical) adds to the growing polymer, is termed endwise coupling: the polymer grows one unit at a time (Vanholme *et al*., 2010).

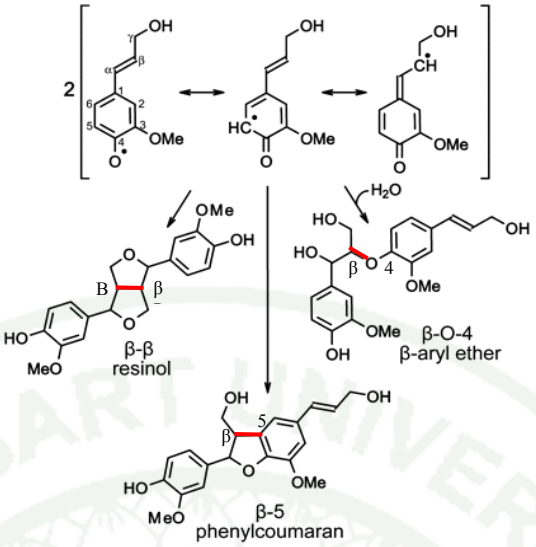

Figure 8 Two dehydrogenated monolignol (coniferyl alcohol) monomer radicals couple to form a dimer. Monolignol radicals favor coupling at their β positions, resulting in the β-β, β-5, and β-O-4 dimers. The red lines indicate the generated bonds.

Source: Vanholme *et al.*(2010)

In plants, lignin synthesis requires the presence of all three above-mentioned processes (monolignol synthesis, translocation and polymerization; summarized in Figure 7) in the lignifying cells, or the transfer of precursors from another cell to the lignifying cell. In addition, the relative amount and activity of key regulatory enzymes affect the rate of flux through each of those three processes.

One of the ways, in which the presence and quantity of lignin biosynthetic enzymes could be modulated, is at the level of transcription. Several lines of evidence suggest that control of lignification at the transcriptional level is important. Many of the enzymes involved in lignification are regulated at the transcriptional level. These enzymes are found as multiple isoforms encoded by different genes varying in their kinetic properties and their distribution throughout the plant. Furthermore, the timing or localization of transcriptional regulation of several of these genes is consistent with the regulation of where and when lignification occurs (Rogers *et al.,* 2004; Bonawitz and Chapple, 2010; Li *et al*., 2010; Singh *et al*., 2010).

Enzymes and reactions of lignin biosynthesis

Phenylalanine ammonia lyase (PAL)

Deamination of phenylalanine to cinnamate is catalyzed by the PAL enzyme. PAL is one of the most intensively studied enzymes in secondary metabolism of plants, because of the key role it plays in phenylpropanoid biosynthesis pathway leading to lignin synthesis. Therefore, PAL has a direct influence on lignin accumulation. PAL is also associated with plant defense mechanisms in response to a wide range of biotic or abiotic stressors, resulting in tissue lignification. For example, low temperature storage in mangosteen induced increased pericarp firmness, as a result of lignin synthesis, and this has been associated with an increase in *PAL* and *POD* genes' expression and their respective enzyme activities (Dangcham *et al*., 2008). Modification of PAL can result in changes in lignin content (Korth *et al*., 2001) or composition, and thus to abnormal phenotypes. For instance, severe inhibition of PAL in *Nicotiana tabacum* led not only to decreased lignin content, but also to stunting, decreased pollen viability and abnormal leaf and flower morphology (Elkind *et al*., 1990).

In *A. thaliana*, PAL is encoded by four genes of which *AtPAL2* has been suggested to be mostly involved in lignin biosynthesis (Costa *et al*., 2003). In *E. japonica*, two PAL genes have been identified (*EjPAL1* and *EjPAL2*) with different expression patterns during fruit development. The former gene (*EjPAL1*) was strongly expressed in mature fruit, whereas the latter (*EjPAL2*) was only expressed in early stages of development. This expression pattern suggests that the *EjPAL2* gene might be more related to phenylpropanoid synthesis (including lignin), since considerable increases in vascular tissue take place during early stages of fruit development (Shan *et al*., 2008).

Cinnamate 4-hydroxylase (C4H)

C4H catalyzes the conversion of cinnamate into 4-hydroxy-cinnamate, a key reaction of the phenylpropanoid pathway. Molecular oxygen is cleaved during this reaction, with one oxygen atom added to the aromatic ring and the other reduced to water. C4H has been purified and characterized to different degrees from several plant

species. In *Populus tremuloides*, the C4H gene was also associated with *cis*-acting regulatory elements that are involved in the biosynthesis of guaiacyl and syringyl monolignols (Lu *et al*., 2006). Instead, down-regulation of C4H, by using RNAi to silence its gene expression, resulted in reduced (30%) lignin content in *Populus tremula* × *tremuloides*, but had no significant effect on the guaiacyl to syringyl ratio (Bjurhager *et al.*, 2010).

4-Coumarate: coenzyme A Ligase (4CL)

The enzyme 4CL catalyzes the conversion of various cinnamic acid derivatives to the activated thioester form (i.e. *p*-coumaroyl CoA), which can then be channeled either (i) further downstream into monolignol biosynthesis or (ii) into flavonoid biosynthesis (Figure 4; see also Figure 6). In the latter case, the chalcone synthase is involved. The 4CL is homologous to the acyl-CoA synthetase, which is the first committed enzyme in the β-oxidation pathway for the breaking down of the long-chain fatty acids. In the catalytic mechanism of both enzymes, the formation of an acyl-adenylate intermediate is involved (Weng and Chapple, 2010).

Four *At4CL*s have been identified in *A. thaliana*, two of which (*At4CL1* and *At4CL2*) have also been associated with lignin biosynthesis (Soltani *et al.*, 2006). Antisense suppression of 4CL activity in both *N. tabacum* and *A. thaliana* led to altered lignin subunit composition as well as to reduced total lignin content (Lee *et al.*, 1997). A 4CL encoding gene, *Ej4CL*, cloned from *E. japonica* has relatively high amino acid homologies with *At4CL*1 and *At4CL*2 (78 and 76%, respectively). However, its relatively low expression observed in lignified fruit of cv. LYQ and its relatively high expression in non-lignified fruit of cv. BS suggest that further efforts must be made to identify members of the lignification-specific 4CL gene family in this species (Shan *et al.*, 2008).

Hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT)

HCT catalyzes two reactions, the one preceding, as well as the one following the insertion of the 3-hydroxyl group into the *p*-coumaroyl shikimate (a monolignol precursor). The former reaction, catalyzed by HCT, is the convertion of *p*-coumaroyl CoA to 4-coumaroyl shikimate. The produced 4-coumaroyl shikimate is hydroxylated by the enzyme 4-coumaroyl shikimate 3-hydroxylase [previously known as coumarate 3-hydroxylase (C3H)], yielding to caffeoyl shikimate (ester). During the latter reaction, catalyzed by HCT, the caffeoyl shikimate is converted to its corresponding CoA (caffeoyl CoA).

Transgenic *Medicago sativa* plants, in which the HCT levels were reduced (by using antisense expression), showed strongly reduced lignin content and striking changes in lignin monomer composition (Shadle *et al*., 2007). HCT is functionally analogous to C3H with regards to its overall role in monolignol biosynthesis. As a consequence, strong down-regulation of HCT is expected to result in similar phenotype as seen in a C3H mutant, described below (Hoffmann *et al*., 2004).

Coumarate 3-hydroxylase (C3H)

C3H is a cytochrome P450 enzyme catalyzing the 3'-hydroxylation of either coumaroyl quinate or shikimate, and yielding to caffeoyl CoA. The produced caffeoyl CoA is subsequently converted to lignin monomers. An interesting study on *A. thaliana* showed that a C3H mutation resulted in dwarf phenotype, while the lignin of the mutant was primarily formed by a monomer (*p*-coumaryl alcohol), which was only a minor component of the lignin in wild type (Franke *et al*., 2002). However, most probably this C3H mutation was not null, since another C3H mutation (performed by using T-DNA insertion) was lethal in the homozygous condition.

Caffeoyl CoA *O*-methyltransferase (CCoAMT)

CCoAMT catalyzes methylation steps in two reaction groups. The first reaction, catalyzed by CCoAMT, is the meta-*o* methylation of caffeoyl CoA, forming the feruloyl CoA. As a consequence, CCoAMT plays an important role in the formation of the coniferyl alcohol moieties (i.e. distinct groups of their molecule), with coniferyl alcohols being lignification precursors (Dwivedi and Campbell 1995; Vander *et al*., 2000).

Although initially thought to be involved only in pathogen defense, CCoAMT was later shown to be expressed in lignifying tissues in a number of species. An *in vivo* study in *Zinnia elegans* showed that the up-regulation of CCoAMT in cultured mesophyll cells induced their differentiation into tracheary elements, suggesting its involvement in lignification. Further evidence on this role comes from the reduced (20–45%) lignin contents, when CCoAMT was suppressed, in different species including *N. tabacum*, *A. thaliana*, *M. sativa* and *Populus tremula* × *alba* (Meyermans *et al*., 2000; Zhong *et al.*, 2000; Marita *et al.*, 2003; Do *et al.*, 2007). These studies also reported that the suppression of CCoAMT reduced the formation of both G - and S - types of lignin, consistent with the notion that caffeoyl-CoA is a precursor of both lignin types in angiosperms.

Caffeate O - methyltransferase (COMT)

COMT catalyzes the *O*-methylation of aromatic diols of monolignol precursors, by using S-adenosyl methionine as the methyl group donor. This methylation takes place in the 3- and 5- positions of those precursors and at the aldehyde level (Bonawitz and Chapple, 2010). This reaction is very important, since it limits the reactivity of the 3-hydroxyl group, and thus reducing the number of sites (on the aromatic ring) that can form bonds to other monolignol molecules during polymerization. COMT is also believed to catalyze the methylation of 5-hydroxyl ferulate to sinapate.

The involvement of COMT in the synthesis of monolignols has also been supported by genetic evidence on both monocots and dicots. This enzyme was found to

be involved in the *in vivo* synthesis of the two lignin units [guaiacyl (G) and syringyl (S), respectively]. A number of COMT isoforms have been identified. For example, in *N. tabacum* COMT isoforms were assigned to lignin synthesis (COMT1) or to defense mechanisms (COMT2), since the latter isoform was highly induced by infection (Collendavelloo *et al*., 1981). Moreover, transgenic plants or mutants downregulated for COMT expression not only possessed a decreased S to G ratio but also a new 5-hydroxyguaiacyl unit in their lignin. These observations strongly suggest that, *in vivo*, COMT is only operating at the level of the methylation of 5-hydroxyferulic acid into sinapic acid (Boudet, 2000).

Cinnamoyl-Coenzyme A reductase (CCR)

CCR catalyzes the reduction of hydroxycinnamoyl-COA thioesters to their corresponding aldehydes (Whetten and Sederoff, 1995). It is the entry point for the lignin-specific branch of the phenylpropanoid pathway and is considered to be a key enzyme controlling the quantity and quality of lignin.

Antisense suppression of CCR not only led to decreased total lignin and dwarfing, but was also associated with collapse of the xylem (Bonawitz and Chapple, 2010). In *N. tabacum*, transgenic lines with downregulated CCR activity (obtained by using an antisense strategy) had lower (50%) lignin content, compared to the controls (wild type). This decrease in lignin content provoked deleterious effects on plant development, including stunting and collapsed xylem vessels (Piquemal *et al.,* 1998). Similarly to *N. tabacum*, down-regulation of CCR in transgenic *Populus tremula* × *Populus alba* plants was associated with reduced lignin content (Leple *et al*., 2007). In the same study, an abnormal coloration (orange-brown, often patchy) of the outer xylem was observed.

At molecular level, plants with CCR deficiency have been shown to produce lignin that contains increased ferulic acid levels. This higher level of ferulic acid shows that intermediates in monolignol biosynthesis can be incorporated into the lignin polymer in the presence of metabolic block (Bonawitz and Chapple, 2010).

Ferulate 5-hydroxylase (F5H)

Hydroxylation of ferulate to 5-hydroxyferulate is catalyzed by F5H, which is a cytochrome P-450-linked monooxygenase. This enzyme has been proven to be extremely difficult to work with, and consequently appears only in a limited number of studies (Chapple *et al.*, 1992; Whetten and Sederoff, 1995; Bonawitz and Chapple, 2010). The employed biochemical strategy was not successful in cloning the F5H encoding gene, as its activity proved either difficult to be detected or was lost upon its purification from plant extracts (Whetten and Sederoff, 1995). A mutation in the F5H encoding gene (*fah-1*) has been identified in *A. thaliana*, where mutant plants lacked sinapate-derived residues in their lignin (Chapple *et al*., 1992). Further analysis of the mutant revealed that it produced lignin that was completely devoid of the S-subunit. Taken together these results indicate that the *FAH1* gene is required for 5-hydroxylation of both monolignol and sinpoylmalate precursors (Bonawitz and Chapple, 2010).

Cinnamyl alcohol dehydrogenase (CAD)

CAD is an NADP (H)-specific oxidoreductase, which catalyzes the reversible conversion of cinnamyl aldehydes to their corresponding alcohols (Whetten and Sederof, 1995; Boerjan *et al.*, 2003; Li *et al*., 2010). This reaction is the last step in the biosynthesis of monolignols. CAD's specific role at the end of the monolignols' biosynthesis pathway might be taken to explain why this enzyme has been considered as an indicator of lignin biosynthesis.

In *N. tabacum*, antisense and knockdown strategies of the CAD genes did not affect lignin content in these plants, though, unlike the controls, their lignin contained substantial levels of coniferaldehyde (Halpin *et al.,* 1994). Similar results were taken in a CAD mutant of *P. taeda* (Ralph *et al.,* 1997). Altered structure of the lignin polymer deposited in the cell wall, as a result of CAD deficiency, has also been reported (Sederoff *et al*., 1999). In *A. thaliana*, nine genes (*AtCAD*s) have been identified, among which two (*AtCAD-C* and *AtCAD-D*) were suggested to be involved in lignin biosynthesis (Sibout *et al*., 2003). Null mutants of those two genes showed significant changes in lignin composition, while the mutant of the latter gene also underwent a

slight change in lignin content. Consequently, the loss of CAD activity results in changes in lignin composition rather than lignin content (Baucher, 1999).

Moreover, CAD has also been shown to be expressed in response to both abiotic (e.g. wounding and mechanical injury, MacLean *et al*., 2007) and biotic (e.g. pathogen elicitors, Campbell and Ellis, 1992; Galliano *et al.*, 1993) stresses. Modulation of the CAD transcripts' levels (*ejcad1*), by either ethylene treatment or low-temperature conditioning, was particularly associated with changes in lignification during ripening in *E. japonica* fruits. The expression of *ejcad1* markedly increased 4 days following exposure to low temperature (0 ^{O}C), while this expression levels' increase preceded the enhancement in fruit firmness, as a result of lignification. These results support the view that lignification is stimulated by low temperature, and this stimulation is possibly mediated by a higher CAD activity (Shan *et al*., 2008).

In mangosteen, CAD activity was highest at 15 minute after impact and then decreased. The increase activity of this enzyme might be among the cause of the increased lignin level (Bunsiri *et al.*, 2003).

Peroxidases (PODs) and oxidases

The last major step in lignin synthesis involves monolignol dehydrogenation prior to its polymerization. This dehydrogenation step has been shown to be catalyzed by different classes of oxidative enzymes, such as peroxidases (PODs) and laccases (a multi-copper oxidase**)** (Boerjan *et al*., 2003). Peroxidases use hydrogen peroxide as a substrate, whereas laccases use oxygen to oxidize their metal centers for enabling oxidation. Both peroxidases and laccases have been considered likely candidates for the activation of monolignol in the apoplast, though the process this takes place is poorly understood (Bonawitz and Chapple, 2010).

Both these two oxidative enzymes belong to large gene families, of which the individual members have overlapping activities. These overlapping activities of the involved genes settle difficulties in studying the underlying processes, since a knockout

of one or few of those genes may have little to no effect on lignification, due to gene redundancy (Vanholme *et al*., 2010).

Antisense POD plants provided direct evidence for their involvement in polymerization of monolignol (i.e. lignin monomer) (Li *et al*., 2010). In *E. japonica*, it was found that the activities as well as the transcript levels of the involved PODs (guaiacyl and syringyl) increased in the cultivar where the fruit firmness increased (through lignification), while both remained at low levels in the cultivar where the fruit firmness did not change during ripening (i.e. no lignification took place) (Cai *et al.*, 2006c; Shan *et al.*, 2008).

POD activity in impacted mangosteen fruit is rapidly increased in 15 min as well as CAD activity (Bunsiri *et al*., 2003). Dangcham (2008) found that POD activity in mangosteen fruit during storage at low temperature (6 ^{O}C) also increased and related to an increase in lignin content.

MYB transcription factor

Transcription factors play a role in regulating plant development and response to environment stimuli. There are composed of the mainly four discrete domains; DNA binding domain, nuclear localization signal, transcription activation domain and oligomerization. In plant, MYB proteins are one of the largest transcription factor families. MYB protein were classified into three major groups based on the number of adjacent repeat in the binding domain. R1R2R3-MYB, R2R3-MYB and R1-MYB (Figure 9). The subfamily contains the two repeat R2R3 DNA binding domain is the largest in higher plants. MYB repeats typically contain regularly spaced tryptophan residues, which build a central tryptophan cluster in the three dimensional helix- turn - helix fold (Du *et al*., 2009).

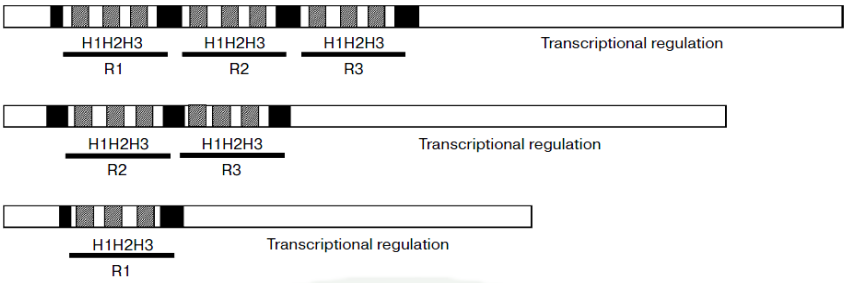

Figure 9 Schematic of functional domains of MYB protein. Shaded boxes represent the most conserved DNA binding domain in MYB proteins; R1, R2, and R3 are repeats of DNA binding domains; crosshatched/hatched bars indicate three a-helices in MYB R1, R2, and R3, where the first, second, and third helices form a helix–turn–helix structure when bound to DNA.

Source: (Du *et al*., 2009)

The R2R3-MYB gene have been extensively studied and members of MYB family have been found to be involved in diverse regulation of the secondary metabolism, control of cell floral and seed development , and control of the cell cycle. Some were also involved in various defense and stress responses and in light and hormone signaling pathways. The regulation of some genes of lignin biosynthesis pathway has begun to elucidate by isolation and characterization of R2R3-MYB (Du *et al*., 2009). The *Pinus taeda* R2R3-MYB transcription factors *PtMYB*1 and *PtMYB*4 that can bind to DNA motifs known as AC elements, which are ubiquitous in the promoter encoding lignin biosynthesis enzymes, can alter the accumulation of transcripts corresponding to genes encoding lignin biosynthesis enzymes in transgenic plants (Morse *et al*., 2009; Patzlaff *et al*., 2003). The study of two MYB proteins, *AmMYB*308 and *AmMYB*330, overexpression of *Antirrhinum* MYBs proteins in transgenic tobacco plants caused a reduction in expression of several lignin biosynthetic genes and decreased in lignin content, suggest that the *Antirrhinum* MYBs are able to regulate the expression of lignin biosynthetic genes and thereby affect lignin biosynthesis (Tamagnone *et al*., 1998). The *Eucalyptus EgMYB*2 bind to the AC elements and their genes are expressed in developing wood that undergoes secondary wall thickening and lignin biosynthesis (Goicoechea *et al*., 2005). In *Arabidopsis*, MYB58 and MYB63 are

transcriptional activators of lignin biosynthesis was supported by the findings that they bind to the AC elements and directly activate the expression of lignin biosynthetic genes. Another MYB transcription factor, MYB85, was also found to activate lignin biosynthetic (Zhou *et al*. 2009).

Plant MYB genes that are also involved in plant responses to diverse abiotic stresses have been identified and functionally characterized. Jin *et al*. (2000) found that *AtMYB*4 responds to wounding and UV. The *AtMYB*2 protein was shown to act as an activator to regulate the expression of abscisic acid (ABA) inducible genes under drought stress conditions in *Arabidopsis* (Urao *et al*., 1996 and Abe *et al*., 2003). The overexpression of *AtMYB*15 results in enhanced drought tolerance and sensitivity to ABA (Ding *et al*., 2009). The *AtMYB*44/*AtMYB*R1 protein regulates ABA mediated stomatal closure under abiotic stresses (Jung *et al*., 2008) and the *AtMYB*60 protein controls stomatal closure and root growth in response to drought stress (Oh *et al*., 2011).The *TaMYB*1 gene encodes a R2R3-MYB protein that is involved in responses to abiotic and ABA stresses (Lee *et al*., 2007). Another R2R3-MYB protein, *TaMYB*2A, improves tolerance to multiple abiotic stresses in transgenic *Arabidopsis* plants (Mao *et al*., 2011). The overexpression of *OsMYB*4 improves the cold and freezing tolerances of transgenic plants (Vannini *et al*., 2004; Pasquali *et al*., 2008; Park *et al*., 2010 and Soltesz *et al*., 2012). The overexpression of *TaMYB33* increases salt and drought tolerance in *Arabidopsis* plants (Qin *et al*., 2012), and the ectopic expression of *TaMYB73* improves the tolerance of transgenic *Arabidopsis* plants to salinity stress (He *et al*., 2012). *AtMYB*60 was specifically expressed in guard cell, and its expression was negatively modulated during drought, which indicated that it was a transcriptional modulator of physiological responses in guard cells and opened new possibilities to engineering stomatal activity to help plants survive desiccation. The R2R3-MYB genes are not only involved in the signal transduction pathways of drought, low temperature and light but also in the signal transduction pathways of nutrientional deficiency, UV-B and low temperature, etc. (Du *et al*., 2009).

Stress, biotic or abiotic, induces lignification

Different types of abiotic stress (e.g. low temperature, gamma radiation, and mechanical injury) have been shown to result in an increase of lignin content in several species (Moura *et al*., 2010). In a similar manner, lignification frequently takes place in response to biotic stress, such as pathogen infection. The lignin, induced by stress imposition, often mentioned as defense lignin, is structurally different than the lignin that is normally produced during development (the so-called developmental lignin). In case of pathogen attack, the defense lignin has been shown to provide a physical barrier, by enhancing the cell wall mechanical strength. This physical barrier deters pathogen invasion in the level of both penetration and diffusion (Moura *et al*., 2010).

Lignification, leading to pericarp firmness increased, has been shown to be induced by physical impact in mangosteen (Ketsa and Atantee, 1998; Busiri *et al.*, 2003). In *E. japonica*, lignification occurred in response to low storage temperature (0 ^{O}C) (Cai *et al*., 2006c; Shan *et al*., 2008). Wounding of *Persea americana* or *Solanum lycopersicum*, by using a radiation beam emitted from a laser, resulted in increased phenolic and lignin deposits in the walls of the cells underlying the wound (Etxeberria *et al*., 2006). Another study showed that wounding, as a result of gamma irradiation, decreased (40%) lignin biosynthesis during tissue healing (Ramamurthy *et al*., 2000). In *Populus tremula* × *alba* leaves, ozone-induced stress increased lignification (Cabane *et al*., 2004). In that study, it was also shown that the defense lignin, produced in response to ozone stress, had a different structure from the developmental lignin. In *M. domestica* fruits, *Penicillium expansum* inoculation increased lignin content (Valentines *et al*., 2005). Interestingly, the defense lignin, induced by *P. expansum*, acted as a physical barrier, decreasing the incidence of the decay. In *Cucurbita maxima* fruit and *B. rapa* vegetable, disease incidents induced lignin synthesis. In both cases, the defense lignin was significantly different in composition from the developmental lignin (Stange *et al.,* 2001; Zhang *et al.*, 2007).

The structural differences between developmental and defense lignin, are regulated by changes in gene expression of the lignin biosynthesis pathway. For example,

in *Oryza sativa* a gene that was involved in the synthesis of defense lignin, was not associated with developmental lignin synthesis (Kawasaki *et al.*, 2006).

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MATERIALS AND METHODS

1. Fruit treatments

Mangosteen fruit (*Garcinia mangostana* L.) were purchased from a commercial grower, located in Chanthaburi, Central East Thailand.The fruit were carefully collected and packed into 10 kg plastic boxes to avoid physical damage and then transported to the laboratory within 6 h. Upon arrival, the fruit were selected based on their size (75-90 g) and color (dark purple). This color corresponds to stage 5 according to the scale defined by Palapol *et al*. (2009). Bunsiri *et al*. (2003) have reported that mangosteen stage with dark purple (stage 5) showed greater firmness after impact compared to mangosteen fruit with reddish brown (stage 3). Magosteen fruit with dark purple may show clearly gene expression related pericarp hardening after impact. Therefore, mangosteen fruit with dark purple were chosen for the experimentation.

For impact treatments, the fruit were dropped from a height of 100 cm onto a concrete floor. Before impact, the fruit were orientated in such a way that the calyx was in the horizontal direction (i.e. parallel to the floor). White powder (talc) was placed on the floor, to clearly indicate the impact area on the fruit. After impact, the fruit were held at 25^{o}C (78-80 % RH). The fruit were then sampled at 0, 5, 10, 15, 20, 25, 30, 60,120, 180 min and 1 day after impact.

In the nitrogen atmosphere experiment, the fruit were dropped from 100 cm and then held in the following two atmosphere compositions: (1) ambient air (21% O_2; control), and (2) nitrogen (very low oxygen atmosphere (0.01% O_2). For this experiment, the above-mentioned measurements were conducted at 0, 1, 2, 3, 4, 5, 6, 12, 18, and 24 h after treatment.Ten fruit (constituting one replication) were placed in an 11 L plastic jar under the ambient air and nitrogen atmospheres. Atmospheric conditions were set up using a flow system with air and N_2 in compressed tanks (OFN grade, 99.99%N_2).

Following the impact treatment, fruit were measured for fruit firmness, total free phenolic content, lignin content, lignin monomer composition and gene expression.

Fruit pericarp was cut into small pieces and immediately frozen in liquid nitrogen. The samples were stored at -80°C for biochemical and molecular analyses. During the experiments, 30 fruit per treatment were sampled, 10 for each replication.

2. Physiological and biochemical measurements

2.1 Pericarp firmness

Fruit firmness, non-impacted and impacted pericarp of the same fruit without peeling (Figure 10) was determined using an Effegi firmness meter (FT011, Italy). A cylinder plunger head with a diameter of 0.2 cm, pressed 0.5 cm deep into the pericarp, was used. The force was recorded in newtons (N).

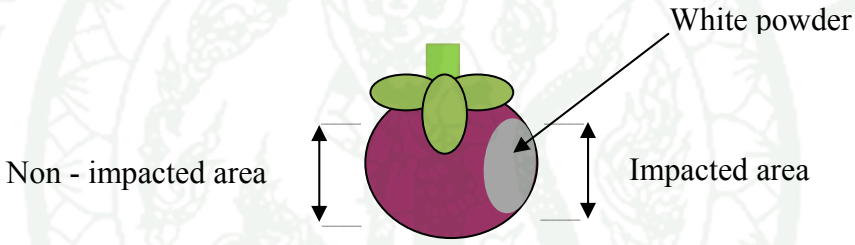

Figure 10 The area of non - impacted and impacted mangosteen pericarp after impact were determined for postharvest changes of physiology, biochemistry and molecular biology.

2.2 Lignin analysis

Lignin was assayed by derivatization with thioglycolic acid (modified from the methods of Bruce and West (1989) and Lange *et al*. (1995). Five grams of pericarp was homogenized with 100 mL methanol for 1 min, and vacuum-filtered using Whatman No. 1 filter paper. The powder was transferred to aluminium cups, and dried at 60 °C for 24 h. Twenty milligrams of dried powder was mixed with 1 mL of 2 N HCl and 0.2 mL of 98% thioglycolic acid. The solutions were heated at 100°C for 4 h in a boiling water bath with gentle agitation (GFL 3017 shaker, Germany). After cooling to room temperature, the mixture was centrifuged at 15700 × *g* for 20 min. The pellets were washed three times with H_2O. The pellet was suspended in 1 mL of 0.5 N NaOH,

agitated gently at 25° C for 18 h to extract the lignin thioglycolate. Subsequently, the samples were centrifuged again at 15700 × *g* for 20 min. The supernatants were transferred to tubes, where 1 mL of concentrated HCl was added and lignin thioglycolic acid was allowed to precipitate at 4°C for 4 h. After centrifugation at15700 × *g* for 20 min, the pellet was dissolved in 1mL of 0.5 N NaOH and diluted to 40 times (100 µL: 3900 µL). The absorbance was subsequently measured at 280 nm.

Monolignol content was analysed by GC-MS using a modification of the previous methods (Meyer *et al*. 1998; Zhang *et al*. 2007). The pericarp of mangosteen fruit were ground to a powder in liquid nitrogen and extracted with 20 mL of 0.1 M sodium phosphate buffer (pH 7.2) for 30 min at 37°C followed by three extractions with 80% ethanol at 80°C. The tissue was then extracted once with acetone and dried at 70°C in an oven. Samples of lignocellulosic material (20 mg each) were mixed with 4 mL of 2 M NaOH and 40 µL of nitrobenzene. This mixture was incubated on a heat-block in a sealed glass tube at 160°C for 3 h. The reaction products were cooled to room temperature and 10 µL of 1,4-dioxane (containing 100 µg acetovanillone) was added as an internal standard before the mixture was extracted twice with 2 mL of dichloromethane. The aqueous phase was acidified with 6 M HCl to pH 2 and extracted twice with 8 mL of ether. The combined ether phases were dried with anhydrous sodium sulfate and the ether was evaporated in a stream of nitrogen. The dried residue was re-suspended in 50 µL of pyridine, with 10 µL of BSA [*N*,*O*-bis-(trimethylsilyl)-trifluoracetamide)] added and 2 µL aliquots of the silylated products were analysed using an GC-MS QP 2010 Shimadzu at spilt ratio 1:70 equipped with DB -1 column (30 x 0.32 mm). The operation conditions were as follows: initial temperature 40°C for 2 min, 40-200 °C for 4 min ramped at 40°C/min, 200-230°C for 3 min ramped at 10°C/min. Lignin monomer composition was calculated from the integrated areas of the peaks representing the trimethylsilylated derivatives of vanillin, syringaldehyde,vanillic acid and syringic acid. Total nitrobenzene oxidation susceptible guaiacyl units or total G lignin (vanillin and vanillic acid) and syringyl units or total S lignin (syringaldehyde and syringic acid) were determined after correction for recovery efficiencies for each of the products during the extraction procedure relative to the internal standard. The identity of each of the peaks used for quantification of lignin monomer composition was confirmed

using GC-electron impact MS by comparison to authentic compounds. Authentic compounds and all the organic solvents were of GC purity.

2.3 Total free phenolics content

Total free phenolic content in the pericarp were determined using the Folin–Ciocalteu method, where the results are expressed as milligrams gallic acid equivalents (GAE) per mg of fresh weight (Waterhouse, 2002). Frozen pericarp (3 g) tissue was extracted with 20 mL of methanol. The extraction sample was homogenized using a Polytron PT 2100 (Kinematica, Luzeen, Switzerland) then centrifuged at 18000 ×*g* for 20 min. The supernatant was used for analyzing total free phenolic content. The reaction mixture were prepared by mixing 40 μL of methanol solution of extract, with 3.16 mL of distilled water , 0.2 mL of Folin-Ciocalteau's reagent, and 0.6 mL Na_2CO_3 (20:80, w/v). The mixture was incubated in a water bath at 40 °C for 30 min, the reduction of the Folin–Ciocalteau reagent by phenolic compounds measured as the development of a blue color. Absorbance was assessed by a spectrophotometer (1700 UV–visible, Shimadzu, Japan) at 765 nm. The absorbance was measured against a blank that had been prepared in a similar manner, replacing the extract with distilled water. The measured absorbance was compared to a gallic acid calibration curve.

3. Gene expression of the lignified biosynthetic pathway involved in pericarp hardening

3.1 Isolation and cloning of lignin biosynthesis genes and a R2R3 MYB transcription factor

3.1.1 Total RNA extraction

The protocol is modified from Lopez-Gomez and Gomez-Lim (1992). Five gram of frozen pericarp tissue were ground in a Retsch MM301 mixing mill (MM 301, Retsch, Düsseldorf, Germany) and were added to 15 mL of extraction buffer containing 150 mM Tris buffer (pH 7.5), 2% SDS, 2% β-mercaptoethanol, 50 mM EDTA and 0.5 g of polyvinylpolypyrrolidone (PVPP). The mixture was shaken

vigorously for 1 min by vortex, and 1.5 mL of 5M potassium acetate and 4.0 mL of chilled absolute ethanol were added to the mixture. The mixture was shaken vigorously for 1 min, then 15 mL mixture of chloroform and isoamyl alcohol (24:1, v/v) was added. The solution was shaken vigorously for 5 min and centrifuged at 7,000 × *g* for 20 min. The supernatant was collected and extracted by a solution of 10 mL phenol, chloroform and isoamyl alcohol (25:24:1, v/v). The mixture was shaken vigorously for 5 min by vortex and centrifuged at 7,000 × *g* for 20 min. The top aqueous phase was transferred to a new RNAse free Falcon tubes, and re-extracted with 15 mL of mixture of chloroform and isoamyl alcohol (24:1, v/v). The supernatant was collected and precipitated with 8M LiCl to obtain a 3M final concentration at 4 ^{O}C over night. After centrifugation at 12,000 × *g* for 20 min at 4 ^{O}C, the supernatant was carefully poured off and the pellet was washed with 3 mL of 3M LiCl. The RNA pellet re-suspended in 350 µL DEPC water, and transferred to an autoclaved microtube and then 35 µL 3M of potassium acetate and 962.5 µL of chilled absolute alcohol were added. The RNA was precipitated at –80 ^{O}C for 1 h. The mixture was centrifuged in a microcentrifuge at 12,000 × *g* for 30 min at 4 ^{O}C, washed once with 500 µL of chilled 70% ethanol and re-suspended in 50 µL of chilled DEPC water. The RNA was quantified by measuring the absorbance at 230, 260, and 280 nm (abbreviated as A_{230}, A_{260} and A_{280}, respectively) by a spectrophotometer (NanoDrop 1000 spectrophotometer, USA). One absorbance unit at 260 nm corresponded to approximately 40 µg/mL. The RNA concentration of each sample was estimated in µg/mL by the following equation: RNA concentration = A260 x dilution factor x 40 µg/mL. The quality of the purified total RNA was determined by calculating the A_{260}/A_{280} ratio. This ratio gives a quantitative indication of the quality of purified RNA, being good when it ranges between 1.8 and 2.0. A qualitative indication was taken using gel electrophoresis on agarose gel (1%, w/v).

3.1.2 cDNA synthesis (Reverse Transcription Reaction)

Five milligrams of total RNA was treated with deoxyribonuclease I (Turbo DNAfree™ Kit, Ambion, TX, USA) with the aim of removing contaminating genomic DNA. The first stand cDNA was synthesised from 4 µg of total RNA following the protocol of the SuperScript (SuperScript^{TM} III First-Strand Synthesis System for RT-PCR, Invitrogen, USA) as a template to amplify the target genes by PCR

and a template for quantitative real-time PCR. The first strand cDNA was stored at -20 ^{O}C until use.

3.1.3 PCR amplification of cDNA fragments

Lignin biosynthesis genes and a MYB transcription factor were isolated from dark purple pericarp mangosteen tissue (stage 5), using forward and reverse degenerate primers (Table 3), which were designed based on conserved regions of similar genes sequences available from the public database NCBI (National Center of Biotechnology Information http://www.ncbi.nlm.nih.gov/). The reaction mixture consisted of 10× PCR buffer, 50 mM $MgCl_2$, 10 mM dNTPs, 10 μM of each primer, cDNA, 1 U Platinum *Taq* (Invitrogen, USA) and distill water. The reaction conditions were displayed in Table 4.

3.1.4 Rapid amplification of cDNA ends (RACE)

To amplify mangosteen lignin biosynthesis and MYB full length cDNAs, both 5'and 3' RACE were performed to generate full length cDNAs following the protocol of GeneRacer^{TM} kit and SMART™ RACE cDNA amplification Kit SMART respectively.

The cDNA fragments of *GmPAL*, *GmC4H*, *Gm4CL*, *GmHCT*, *GmC3H*, *GmCCR*, *GmCCoAMT*, *GmF5H*, *GmCOMT*, *GmCAD*, *GmPOD* and *GmMYB*30 were obtained using the degenerate primer and 3'RACE primer as 3'RACE strategy. The 3' RACE of these gene fragments were amplified following the protocol of GeneRacer^{TM} kit (Invitrogen, CA, USA) using specific primers based on initial sequence information (Table 5). All specific primers were designed with Tm 60±1^{O}C. The reaction conditions were displayed in Table 6 and 8.
GeneRacer™ 3Primer 5′-GCTGTCAACGATACGCTACGTAACG-3′GeneRacer™ 3Nested Primer 5′-CGCTACGTAACGGCATGACAGTG-3′
The cDNA fragments of *GmC4H*, *GmF5H*, and *GmMYB*30 were obtained using the degenerate primer and 5'RACE primer as 5'RACE strategy. The 5' RACE of these genes were obtained by using SMART™ RACE cDNA Amplification Kit SMART

(Clontech, CA, USA). Specific primers based on initial sequence information (Table 10). The reaction conditions were displayed in Table 11 and 13.

Universal Primer _long

CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT

Universal Primer Short CTAATACGACTCACTATAGGGC

Nested Universal Primer A (NUP) AAGCAGTGGTATCAACGCAGAGT

10X Universal Primer A Mix (UPM) compose of 0.4 µM universal primer long and 2 µM universal primer short.

Table 3 Degenerate primers for fragment amplification of lignin biosynthesis genes and MYB transcription factor.

Gene name	Sequence	Annealing temperature (^{o}C)	Expected size (bp)
Gm4CL	F-TGM TVA TGM MVA AGT TCG AGA R-TYC TTC CKC ARG ATY TTG CC	55	1000
GmCCR	F- AAG AAY TGG TAY TGY TAY GG R- AKD GGR TAY TCM GGG AAK A	55	350
GmC3H	F-CAY TAY GTY AAG GTD AGR AA R-TGW GGV AGC ATH ARD GGN GT	48	740
GmHCT	F-GTV CCD TTY TAY CCY ATG GC R-GTR AAD ATN ACR TTS CCR AA	45	714
GmCCoAMT	F- ATG ACM ACV TCW GCD GAT GA R- DCC RTT CCA BAG RGT GTT GAC	58	400
GmF5H	F- ATG GGB TWC YTB CAY ATG GT R- GTG VAG KCY KAR GGT YTC YT	50	328
GmCAD	F- TAY CCH ATG GTY CCT GGR CA R-CCC ATG TGB CCM ACN CCD CC	60	900
GmMYB30	F-AKT GAA GAA RGG WCC ATG GAS R-ATT CTT GAT CTC RTT RTC WGT TCT	50	400

Table 4 PCR conditions for fragment amplification of lignin biosynthesis genes and MYB transcription factor.

Temperature (°C)	Time	No. of cycles
94	5 min	1
94	30 s	
45-60	30 s	35
(temperature ranges depending on the primer)		
72	1 min	
72	10 min	1

Table 5 Specific primers for3' RACE.

Gene name	Sequence	Expected size (bp)
GmPAL	F_1-AAC TCT GTG AAT GAC AAT CCT TTG ATT and GR3′ Primer F_2- TTC AGT GAG CTT GTT AAT GAC TAC TAC AAC and GR3′N Primer	1,600
GmC4H	F_1-CGC GAT AGA CCA TAT CTT GGA C and GR3′ Primer F_2-GAG ATC AAC ATT ATG GTC AAT CGA GT and GR3′N Primer	970
Gm4CL	F_1-TTG ATC AAA TAC AAG GGA TTC CAA G and GR3′ Primer F_2-GTT AGG TCA AAT GGT TCA AAC ATC A and GR3′N Primer	600
GmC3H	F_1-ATT ATG GAA GAA CAC ACA ATT GCT C and GR3′ Primer F_2-GCA ATA ACA GTG GAA TGG GCA ATG G and GR3′N Primer	1,000
GmHCT	F_1-TTC ACA AAC CTT AAA ACC AGA TAG C and GR3′ Primer F_2-GCT GAT GAC CAA GAA ACC AAA CTG TAT ATC G and GR3′N Primer	1,000
GmCCoAMT	F_1-AAA TTG GTG TCT ACA CTG GCT ACT C and GR3′ Primer F_2-CCT GTT CTT GAC CAA CTA ATT GAA G and GR3′N Primer	750

Table 5 (Continued)

Gene name	Sequence	Expected size (bp)
GmCCR	F_1-CTT TAA ATG CAA GTA CTA TTC ACA TCC TCA A and GR3′ Primer F_2-CCG TGG TGA TGT TGT GGA GAT TCT and GR3′N Primer	600
GmCOMT	F_1-AYC ARG AYA ARR TYY TYR TSG ARA GCT GGT and GR3′ Primer F_2-GAT TCA ACA AGR TYK TYA ACM RKG GAA TG and GR3′N Primer	1,000
GmF5H	F_1-GAA AAC TAA TAT CAA GGC CAT CAT CAT G and GR3′ Primer F_2-GTG CAC TTA AAG AAA CCT TCA GCC and GR3′N Primer	1,000
GmCAD	F_1-CAA GTT CAA GGT AGG GGA CAT AGT and GR3′ Primer F_2-TTG GAT CAT GCA GAA ATT GTA ATC and GR3′N Primer	950
GmPOD	F_1-CAA TCA CAA TGA TAG CAT TTC TTT GGT ACT and GR3′ Primer F_2- TCC AAT CCA CTG GCA TTG ATG TTG and GR3′N Primer	750
GmMYB30	F_1-AGA ACA GAC AAC GAG ATC AAG AAT C and GR3′ Primer F_2-GAG TTG TAT TGA AGA AAG GTC CAT G and GR3′N Primer	1,000

Table 6 PCR reactions for primary condition of 3' RACE.

Reagent	Volume (µL)	Volume (µL)
10x PCR Buffer	2.5	2.5
10 mM dNTPs	0.5	0.5
50 mM MgCl2	1	1
GR3' primer	3	3
Degenerate primer	-	5
Specific primer	2	-
cDNA (GeneRacer)	1	1
Platinum Taq	0.2	0.2
Water	14.8	11.8

Table 7 PCR conditions for primary condition of 3' RACE.

Temperature (^{o}C)	Time	No. of cycles
94	2 min	1
94	30 s	5
72	1 min/kb	
94	30 s	5
72	1 min/kb	
94	30	
55, 60	30 s	32
72	1 min/kb	
72	10 min	1

Table 8 PCR reactions for secondary reaction of 3' RACE.

Reagent	Volume (µL)	Volume (µL)
10x PCR Buffer	2.5	2.5
10 mM dNTPs	0.5	0.5
50 mM MgCl2	1	1
GR3'N primer	0.5	0.5
Degenerate primer	-	5
Specific primer	0.5	-
Initial PCR product	0.5	1
Platinum Taq	0.2	0.2
Water	19.3	11.8

Table 9 PCR conditions for secondary reaction of 3' RACE.

Temperature (^{o}C)	Time	No. of cycles
94	2 min	1
94	30 s	
55,60,65	30	32
68	1 min/kb	
68	10	1

Table 10 Specific primers for 5' RACE.

Gene name	Sequence	Expected size (bp)
GmF5H	GSP_2-GAC AGA TTC CCA AGA CTC AGC TCT GSP_1-CAG TAT TCA CCT CCT GAT CAC AGC	820
GmC4H	GSP_2-ACT CTG AGC CAA CCT ACT TCT CTC GSP_1-GGC GTC CAA GAT ATG GTC TAT CG	830
*GmMYB*30	GSP_2-TCT TCT TCT GGT CTC CAT GGA C GSP_1- GGG CGG AGA TAA TTA GTC CAT CTG	150

Table 11 PCR reactions for primary condition of 5' RACE.

Reagent	Volume (μ L)
10x PCR Buffer	2.5
10 mM dNTPs	0.5
50 mM MgCl2	1
GSP_1	2.5
10x UPM primer	2.5
cDNA 5'RACE (5:50)	0.5
Platinum Taq	0.2
Water	15.3

Table 12 PCR conditions for primary condition of 5' RACE.

Temperature (^{o}C)	Time	No. of cycles
94	30 s	5
72	1 min	
94	30 s	5
70	30 s	
72	1 min	
94	30 s	27
68	30 s	
72	1 min	

Table 13 PCR reactions for secondary condition of 5' RACE.

Reagent	Volume (μ L)
10x PCR Buffer	2.5
10 mM dNTPs	0.5
50 mM MgCl2	1
GSP_2	1
10 µM NUP	1
cDNA	2.5
Platinum Taq	0.2
Water	16.3

* The cDNA in this reaction mean the dilution of 5 µl of the primary PCR product into 245 µl of water.

Table 14 PCR conditions for secondary condition of 5' RACE.

Temperature (^{o}C)	Time	No. of cycles
94	30 s	25
68	30 s	
72	1 min	

3.1.5 PCR products cloning and DNA sequencing.

After visualizing the PCR product by gel electrophoresis on 1% agarose gel, the amplified PCR fragments from each gene were purified by using a gel extraction (QIAprep Gel Extraction, QIAGEN, Germany) and cloned into pGEM-T Vector (pGEM®-T Easy Vector System, Promega, USA). The gene cloning and DNA sequencing was take place by the following five steps:

(A) Ligations using the pGEM®-T Easy vectors.

The ligation reactions were set up using 5 µL 2 × Rapid Ligation Buffer, 1 µL pGEM-T Vector (50 ng μL^{-1}), 3 µL PCR product and 1 µL T4 DNA Ligase till a final volume of 10 µL. The solution components were mixed gently by pipetting, and the reaction was incubated at 4 ^{O}C overnight.

(B) Bacterial transformation by heat shock method.

The one hundred microliters frozen competent cells of *Escherichia coli* strain DH5α were placed in an ice bath until thawed. The ligation reaction (10 µL) was carefully added into each competent cell tube. The tube were gently flicked to mix and then placed on ice for 30 min. The cells were heat-shocked for 45 sec in a water bath at 42 ^{O}C without shaking and immediately place on ice for 5 min. One-milliliter 2XYT medium will be added into the tube and incubated for 1.5 h at 37 ^{O}C with shaking at 200 rpm. For each transformation, 100 µL of reaction mix were plated onto duplicate 2XYT/ampicillin/X-Gal plates and incubated at 37 ^{O}C for a period of 16 to 24 h. White colonies were selected for the next step.

(C) Purification of plasmid DNA using QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany)**.**

Single white colonies containing the inserted genes were cultured in 3 ml 2XYT medium at 37 ^{O}C overnight. One and a half milliliter of cultured bacteria cells were centrifuged at 7,000 ×*g* for 1 min. The pellet cells were re-suspended in 200 µL PD1 buffer (re-suspension buffer). Two hundred microliters of PD2 buffer (lysis buffer) was added and the tube was gently inverted for mixing. The mix was incubated for 1 min at room temperature (25 ^{O}C) until the lysate was homologous. Subsequently, 300 µL of PD3 buffer (neutralization buffer) was added and the tube was inverted immediately and centrifuged at 12,000 × *g* for 10 min. The supernatant was transferred to the spin column. The tube was then centrifuged for 1 min and the flow-through was discarded. The spin column was washed by adding 400 µL of W1 buffer and centrifuged for 1 min, while the flow-through was discarded. The spin column was washed by adding 600 µL washing buffer, centrifuged for 30 sec, and the flow-through was discarded. The tube was centrifuged for 3 min to dry the column matrix. The spin column was placed in the new microcentrifuge tube for DNA elution. DNA will be eluted by adding 50 µL elution buffer, incubated 2 min at room temperature (25 ^{O}C) and centrifuged for 2 min. DNA was kept at -20 ^{O}C until use.

(D) Restriction enzyme digestion.

The presence of size of insert in recombinant plasmid was determined by *Eco* RI restriction digestion (New England Biolabs, Inc., USA). The insert size was checked by gel electrophoresis before sequencing

(E) DNA sequencing and analysis**.**

The sequence analysis of the clone was conducted by automatic sequencing using the ABI PRISM® 377 DNA sequencer (Applied Biosystems, California, USA). The sequences of amplified all fragments were compared with the genes in the Genbank database using the BLAST program from NCBI. The DNA and protein sequences were analysed by the Genious program (Biomatters, NewZealand) and submitted to GenBank (NCBI). Specific primers were tested for specificity using plasmid amplification and the product analysed on a 1.0% (w/v) agarose gel stained with ethidium bromide. The primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/).

3.2 Expression analysis of lignin biosynthesis genes and a MYB transcription factor.

The oligonucelotide primer sets used for qPCR analysis were designed on the basis of 3'-untranslated regions (UTR) of individual genes. The primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). The length of all PCR products ranged from 100 to 200 bp. The gene-specificity of these primer sets were tested using the following: (i) individual PCR products were separated on 1% agarose gels stained with SYBR-safe to examine the size; (ii) the PCR products were cloned into pGEM-T Vector and sequence analysis confirmed. The primer sets were finally used for qPCR as given in Table 15.

Fifty-fold diluted cDNA samples were used for qPCR. qPCR was performed on the Light Cycle 480 system (384-well plates) and the Light Cycler® 480 SYBR Green I Master kit (Roche Diagnostics, Germany) following the manufacturer's instructions. All reactions were performed in four replications using 3 µL of the dilute

template (50x), 2 µL of each primer (2.5 µM) and 5 µL of 2 × Mastermix to final volume of 10 uL. PCR was initiated by 5 min at 95 °C, followed by 50 cycles of 95 °C for 5 s, 60°C for 5 s, 72°C for 10 s and completed by melt curve analysis. No-template controls for each primer pair were included in each run. The mangosteen elongation factor 1 alpha (*GmELF*, EU274578) was used as an internal control to normalize small differences in template amounts with the forward primer 5' - GCC CAA AAG ACC ATC AGA CAA GC -3' and reverse primer 5' - CGG AAG GAC CAA AAG TGA CAA CC -3'. *GmELF* was selected for normalization because of its consistent transcript level throughout the fruit samples with crossing threshold (Ct) values changing by < 2. The standard curve was generated for each gene using cDNA serial dilution (at least 5 dilutions) and the resulting PCR efficiency calculations were imported into relative expression data analysis.

Table 15 Specific primers for real-time PCR (qPCR).

Gene name	Sequence	Annealing temperature (^{o}C)	Expected size (bp)
GmPAL	F-CAA AAG CTG AGG CAA GTT CC R-TGC TGT GCT TCC ACT CTC AT	60	167
GmC4H	F-GAA TAG CGG AGC TCG TGA AC R-GAT GGC CAT TCT GAG ACG TAG	60	161
Gm4CL	F-GCA GGG GAA GTT CCG GTT GCA T R-GGA ACA CCA GCC GCA AGC CT	60	200
GmC3H	F- TTC GGT GCT GGA AGA AGA GT R- GGC CAC TGC TTG TAA TGG AG	60	186
GmHCT	F- GAT CAC AAG CTG GTT AGG C R- TTC ATG TGC TCG GAT TGC	59	171
GmCCoAMT	F- TCG TGG ATG CTG ACA AAG AC R-CCT AGG GTC CAC AGC AAG AG	60	200
GmCCR	F-CTG GGT ATC CCA TTC CTA CC R-CTA ATG GGG TCT TCT GGT TG	58	193
GmCOMT	F-GGG CAA AGA AAG GAC ACA GA R-ATC CAT ACT GCC CCA CAT TG	60	166
GmF5H	F-CTT GGT CTT TAT GGG CTG GA R-CCT TGG AAT GGC AAT GAG TC	60	153
GmCAD	F-CTG ACT CCC ACT ATT GAA GTG R-CAA TTT GGG GTT GCT CTC CTT	60	156
GmPOD	F-TGA TCG AGA GAC ACC CAT G R-GCC CGG AAG TAG GCA TTA TC	60	153
GmMYB30	F-GTG CCT GAA GTG AAA CGT GA R-GAA ATC CAT GCC TTT GTG GT	59	168
GmELF	F-GCC CAA AAG ACC ATC AGA CAA GC R-CGG AAG GAC CAA AAG TGACAA CC	58	140

3.3 Promoter isolation and analysis of *GmF5H* genes

The 5’ flanking region of *GmF5H* were isolated using Genome Walker Kit (Clontech, USA). The genomic DNA (gDNA) was isolated from young mangosteen leaves using the method describe by Dayle and Doyle (1990). The libraries were prepared by separate digestion of 2.5 µg of genomic DNA with 80 units of *Dra*I, *Eco*RV, *Hpa*I, *Sca*I, *Ssp*I, *Ecl* 136 II and *Stu*I (New England Biolabs, Inc., USA). The DNA mixtures were incubated at 37ºC overnight to create blunt-end fragments. All DNA sample were purified using QIAquick Gel Extraction Kit (Qiagen, Germany) following the manu facturer's instructions, then the DNA libraries were ligated with Gonome Walker adapter using Rapid DNA Ligation Kit (Roche, USA) and incubated overnight (16–24 h) at 4°C. All ligated mixtures were purified using QIAquick Gel Extraction Kit (Qiagen, Germany). The 5′ flanking region of F5H was amplified following the protocol of Genome Walker Kit (Clontech, USA). The genespecific primers were designed in the 5′ end of *GmF5H* and used for each genomic-walking PCR. The adaptor primers are shown as below.

AP1 5′-GTAATACGACTCACTATAGGGC-3′ (primary PCR)
AP2 5′-ACTATAGGGCACGCGTGGT-3′ (nested PCR).

The gene-specific primers
5′- AGGGAGACTAGGCCCAAGAGGAAGAGGA -3′ (primary PCR) and 5′-GGAGATATTCCTCCATGGTTTGGGGCAT -3′ (nested PCR) were used for the mangosteen F5H promoter. The first amplification reactions were carried out in 50 µL volumes as the reaction conditions displayed in Table 14. After the first PCR, the second amplification reactions, same as under condition for the full-length cDNA amplification, were carried out in 50 µL volumes using the first PCR product as DNA template, and the first, second reaction conditions displayed in Table 15. PCR products were analysed by gel electrophoresis on a 1% agarose gel, the longest of amplified PCR fragments from each gene were purified using QIAquick Gel Extraction Kit (Qiagen, Germany) and cloned into pGEM®-T Easy Vector (Promega, USA) as described as above. Promoter fragments were analysed for *cis*-elements using PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html).

Table 16 PCR reaction for promoter.

Reagent	Volume (μL)
10x PCR Buffer	5
10 mM dNTPs	1
50 mM $MgCl_2$	1.5
10 μM AP_1, AP_2	1.5
10 μM GSP_1, GSP_2	1.5
DNA	2
Platinum Taq	0.2
Water	38.3

Table 17 PCR conditions for primary condition of promoter.

Temperature (^oC)	Time	No. of cycles
94	3 min	1
94	30 s	7
72	3 min	
94	30 s	32
67	3 min	
67	10 min	1

Table 18 PCR conditions for secondary condition of promoter.

Temperature (^oC)	Time	No. of cycles
94	3 min	1
94	30 s	7
72	3 min	
94	30 s	30
67	3 min	
67	10 min	1

4. Statistics

Ten mangosteen fruit comprised of one replicate, with three replicates being used in each treatment. Five mangosteen fruit were randomly sampled for determination of firmness. The remaining fruit were pooled together for determination of total free phenolics, total lignin content, monolignol content and gene expression. Data were compared in *t*-test. Differences at $P<0.05$ were considered as significant.

RESULTS AND DISCUSSION

Results

1. Changes in fruit firmness and phenolic/lignin content after impact

Mangosteen fruit which had been dropped from a height of 100 cm, showed a color change in the damaged pericarp within 10 min. This developed to almost dark in color 60 min after impact (Figure 11). The color change within the impacted pericarp was confined to the damaged area. Non-impacted and impacted pericarp differed in firmness; the firmness of the impacted pericarp rapidly increased over the period of 30 min to 1 day while firmness of non-impacted pericarp remained stable (Figure 12A).

The impacted pericarp had lower total free phenolic contents than non-impacted pericarp. Total free phenolic content of the impacted pericarp gradually decreased throughout the study period, whereas that of the non-impacted pericarp remained stable (Figure 12B). Lignin content of impacted pericarp increased continuously throughout the period after impact, whereas that of the non-impacted pericarp did not change to any great extent (Figure 12C).

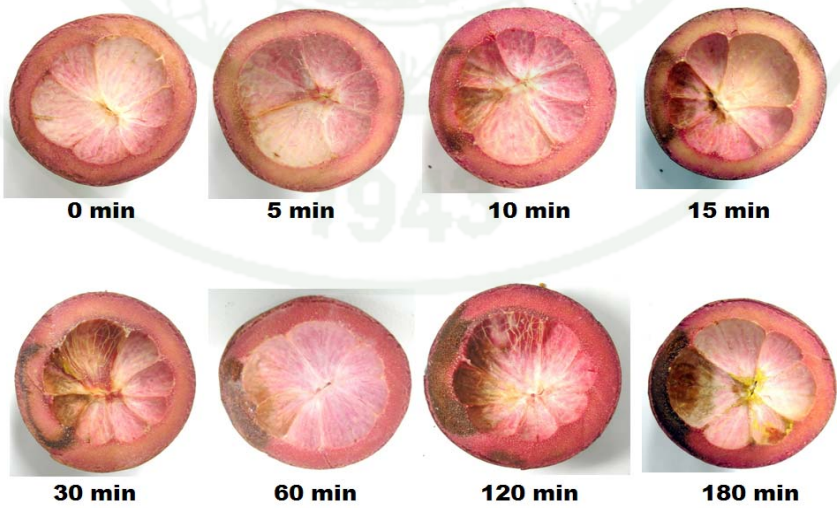

Figure 11 Changes in color of mangosteen pericarp after impact at a height of 100 cm and measured at 0, 5, 10, 15, 30, 60, 120 and 180 min after impact.

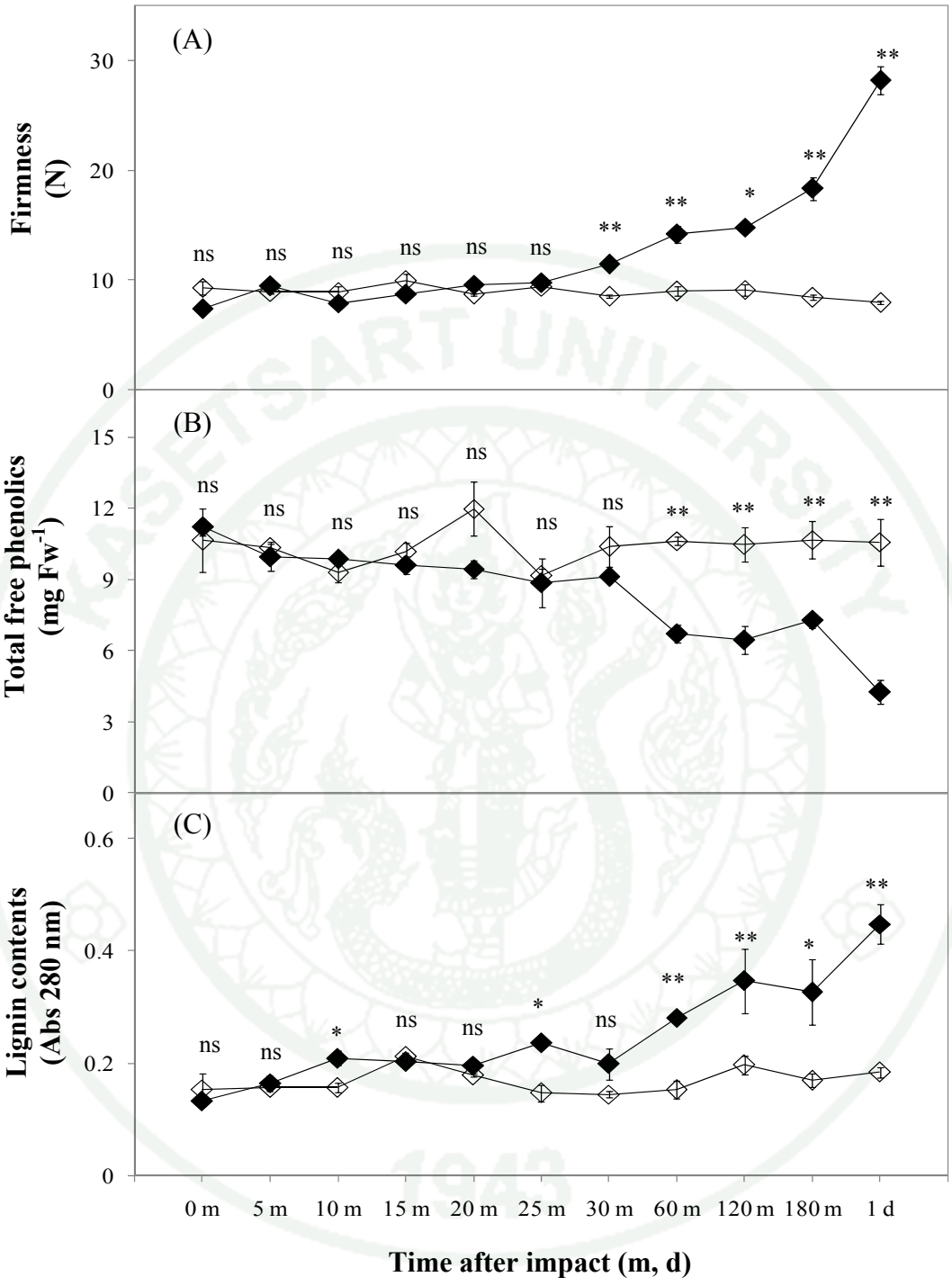

Figure 12 Changes in firmness (A), total free phenolic (B) and lignin contents (C) of mangosteen pericarp with and without impact after impact from a height of 0 (◊) and 100 cm (◆) and measured at 0, 5, 10, 15, 20, 25, 30, 60, 120 and 180 min and 1 day after impact. Data are means ± SE of three replications.

2. Changes in monolignol content after impact

GC-MS was used to measure the nitrobenzene oxidation products of cell walls after extraction from non-impacted and impacted pericarp. Nitrobenzene oxidation degrades the phenylpropane structure from a C6-C3 to a C6-C1 unit. The degraded products were *p*-hydroxybenzaldehyde, vanillin, syringaldehyde and the corresponding acids *p*-hydroxybenzoic acid, vanillic acid, and syringic acid. This was confirmed the formation of nitrobenzene oxidation products which showed the retention times of authentic standards *p*-hydroxybenzaldehyde, vanillin, acetovanillone (internal standard), *p*-hydroxybenzoic acid, syringaldehyde, vanillic acid, syringic acid without impact (Figure 13A) and after impact (Figure 13B). The products were identified by comparison of their retention times and mass spectra with standards (Table 19).

GC-MS result showed higher G (vanillin and vanillic acid) and S (syringaldehyde and syringic acid) lignin in the impacted pericarp than the non-impacted pericarp. The vanillin levels were 5-fold higher than vanillic acid (Figure 14A and B). Similarly the syringaldehyde levels were higher than syringic acid in both non-impacted and impacted pericarp (Figure 15A and B). Interestingly, G lignin concentrations were twice as high as S lignin in the impacted pericarp (Figure 16A and B).

Table 19 Chromatographic and spectroscopic characteristics of the lignin monomers in mangosteen pericarp after impact from a height of 100 cm, using nitrobenzene oxidation, GC-MS.

Peak no.	Retention time (min)	M^+ (M/Z)	Lignin monomer
1	5.96	267, 223,193	*p*- hydroxybenzaldehyde
2	6.47	194, 209, 224	Vanillin
3	6.70	193, 223, 238	Acetovanillone (Internal standard)
4	6.88	267, 223, 193	*p*- hydroxybenzoic acid
5	7.05	224, 239, 254	Syringaldehyde
6	7.44	223, 267, 297, 312	Vanillic acid
7	8.00	253, 313, 327, 342	Syringic acid

Note : Total H lignin (*p*- hydroxybenzaldehyde + *p*- hydroxybenzoic acid)
Total G lignin (vanillin + vanillic acid)
Total S lignin (syringaldehyde + and syringic acid)

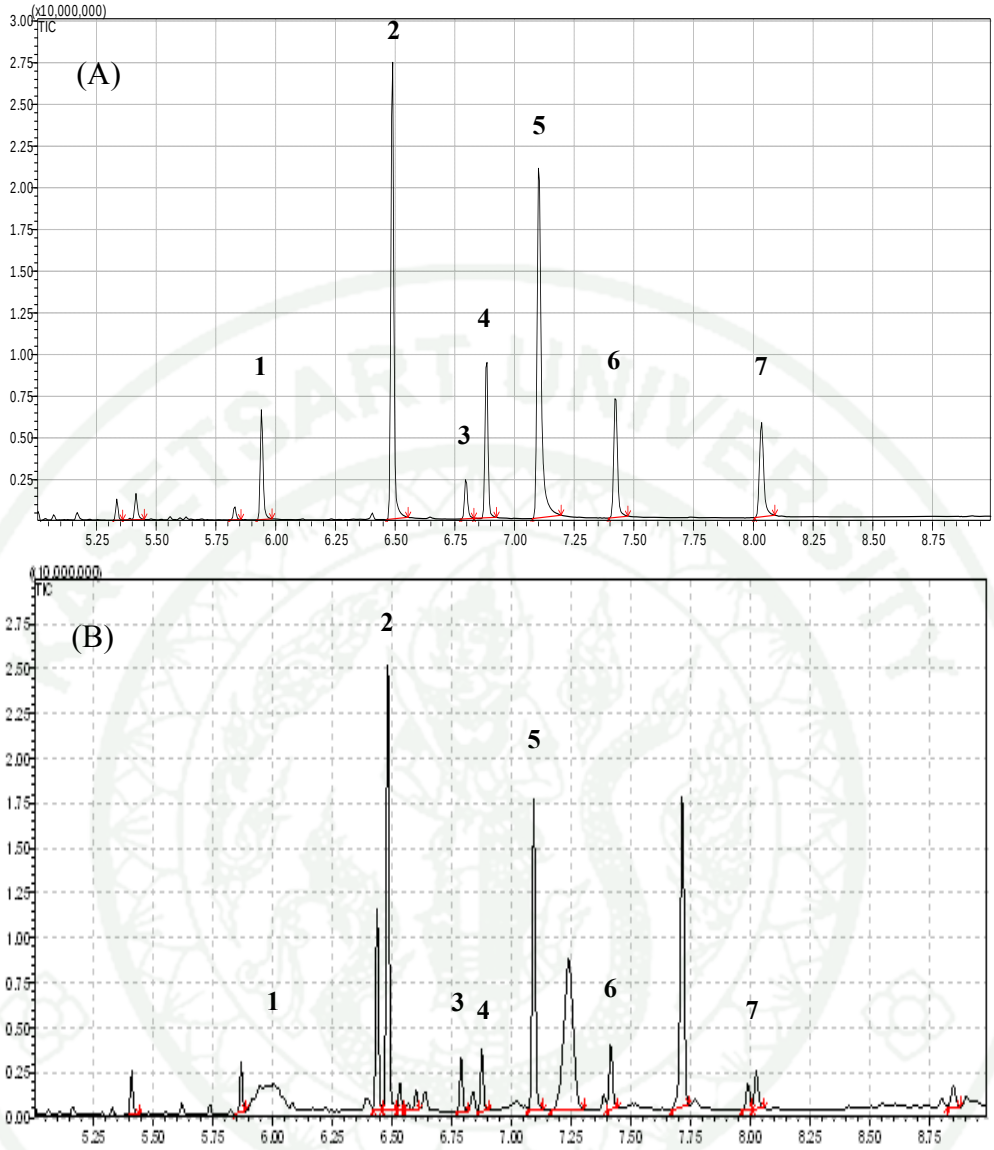

Figure 13 GC-MS analysis of trimethylsilyl of reaction products obtained by nitrobenzene oxidation. (A) Total ion chromatograms of trimethylsilyl of benzaldehyde and benzoic acid from authentic chemicals 1) authentic *p*- hydroxybenzaldehyde, 2) authentic vanillin, 3) acetovanillone (internal standard), 4) authentic *p*-hydroxybenzoic acid, 5) authentic syringaldehyde, 6) authentic vanillic acid and 7) authentic syringic acid. (B) Total ion chromatograms of trimethylsilyl of benzaldehyde and benzoic acid from mangosteen pericarp after impact from a height of 100 cm.

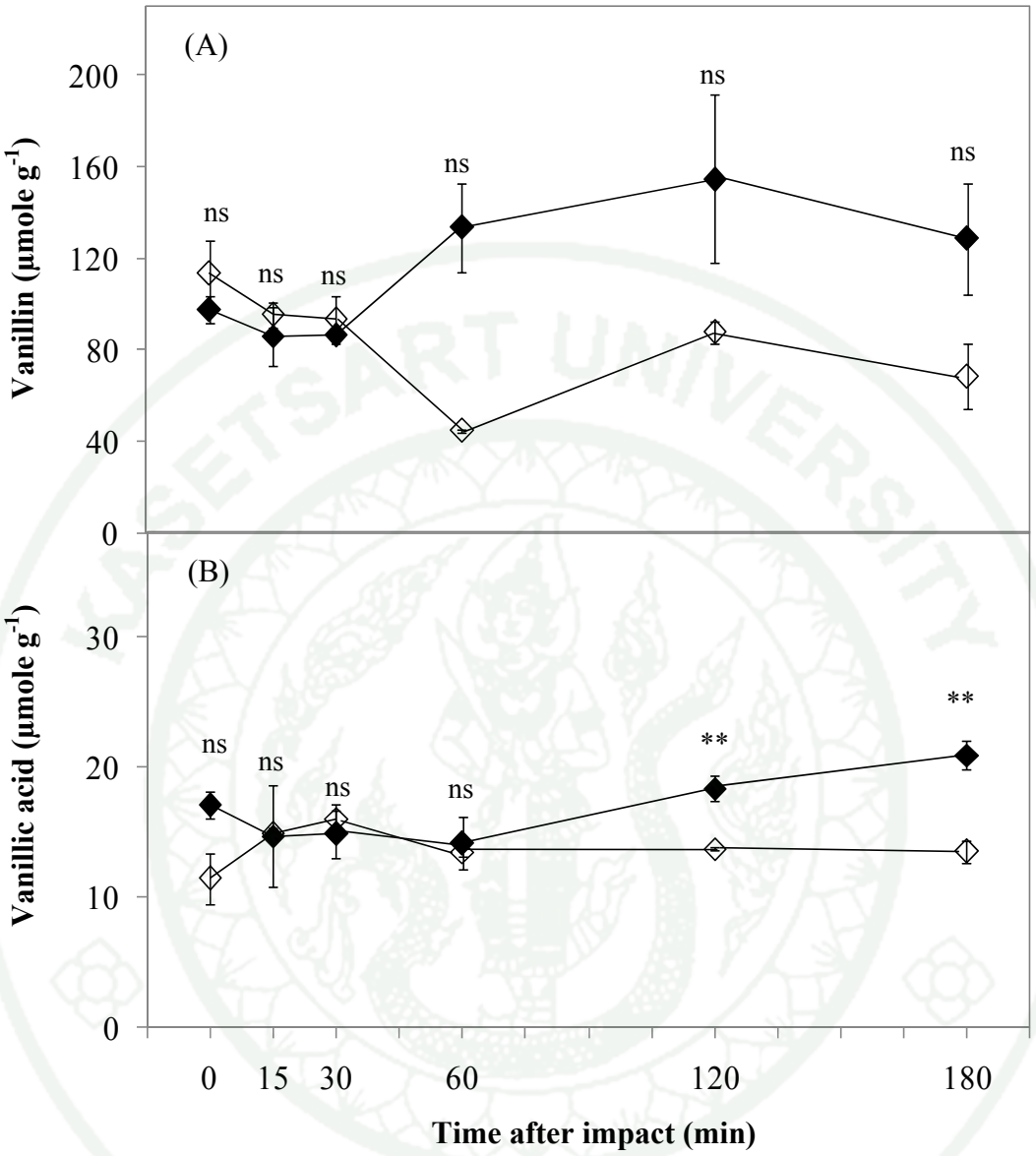

Figure 14 GC-MS analysis of trimethylsilyl of reaction products obtained by nitrobenzene oxidation. Lignin monomer composition, vanillin (A) and vanillic acid (B) in mangosteen pericarp after impact from a height of 0 (◊) and 100 cm (◆) and measured at 0, 30, 60, 120 and 180 min after impact. Data are means ± SE of three replications.

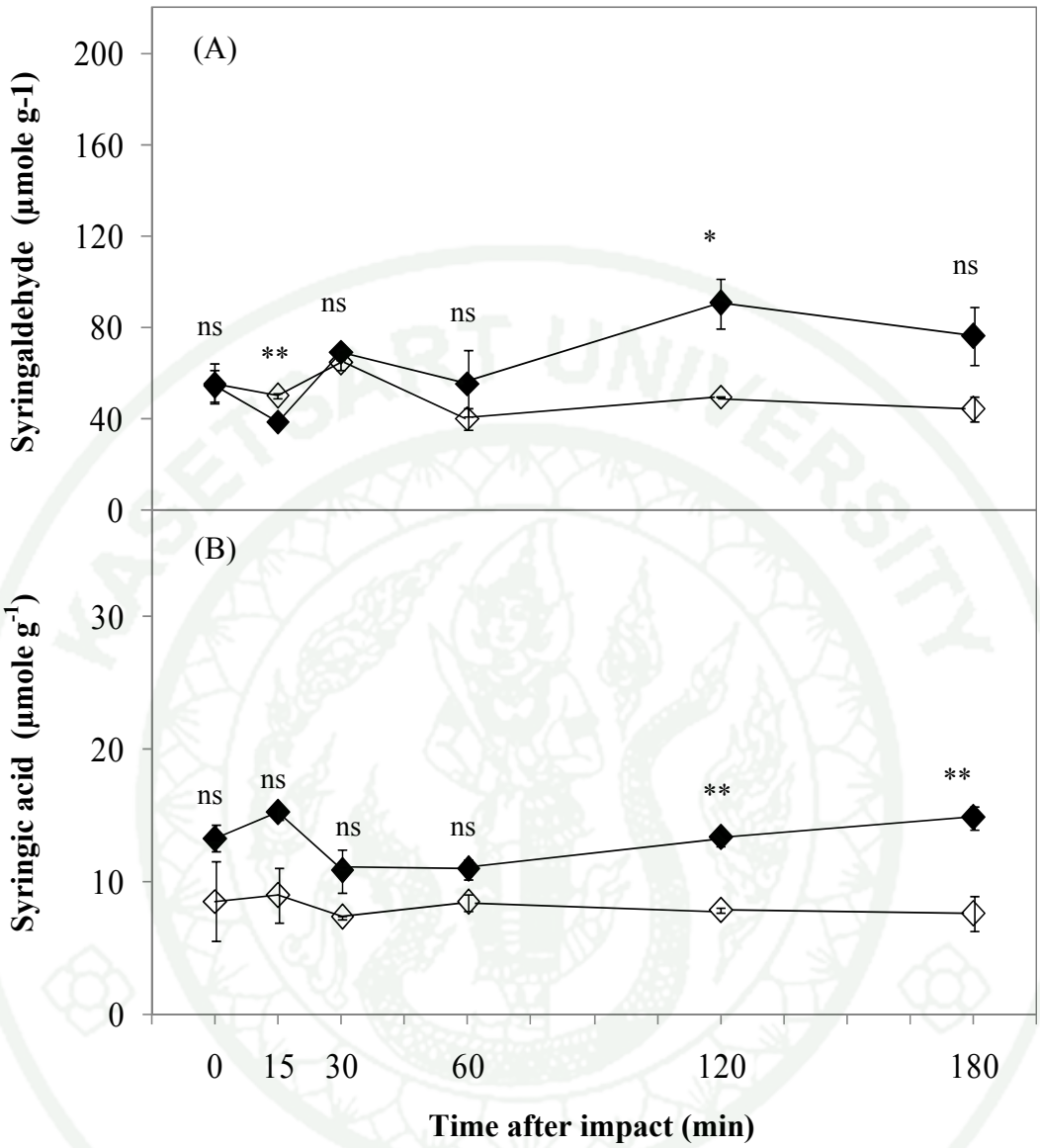

Figure 15 GC-MS analysis of trimethylsilyl of reaction products obtained by nitrobenzene oxidation. Lignin monomer composition, syringaldehyde (A) and syringic acid (B) in mangosteen pericarp after impact from a height of 0 (◊) and 100 cm (◆) and measured at 0, 30, 60, 120 and 180 min after impact. Data are means ± SE of three replications.

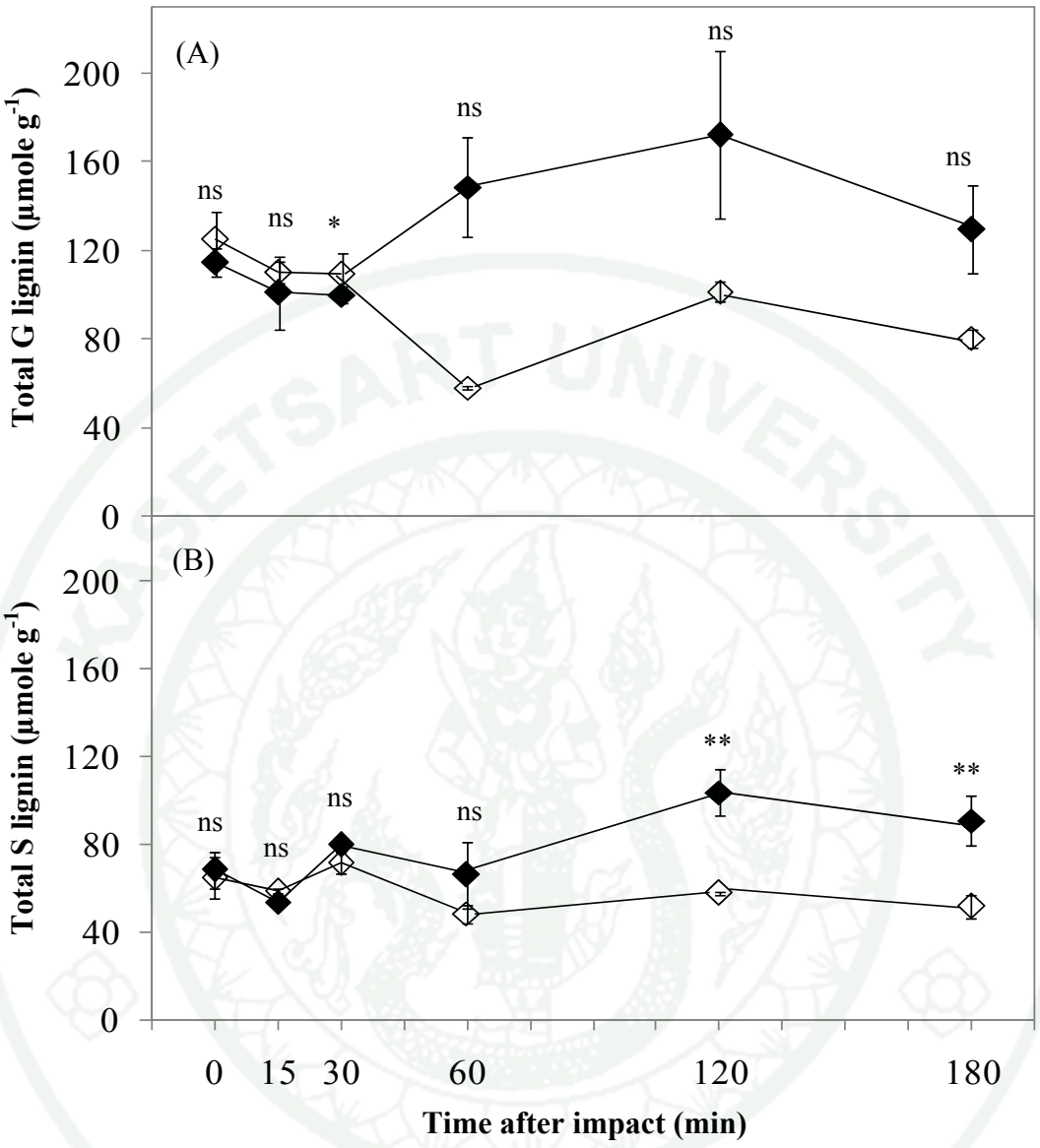

Figure 16 Changes in lignin monomer composition as total G (vanillin + vanillic acid) (A) and S (syringaldehyde + syringic acid) (B) lignin in mangosteen pericarp after impact from a height of 0 (◊) and 100 cm (◆) and measured at 0, 30, 60, 120 and 180 min after impact. Data are means ± SE of three replications.

3. Changes in fruit firmness, total free phenolic contents and lignin of impacted pericarp under enhanced nitrogen atmosphere

The firmness of impacted pericarp kept under nitrogen atmosphere was less than that of impacted pericarp kept in air, and remained relatively stable from 0 to 6 h, then increased to almost the same extent as that of fruit kept in air (Figure 17A). The levels of total free phenolic contents in impacted pericarp kept in ambient air and nitrogen atmosphere declined in similar patterns (Figure 17B). However, lignin content of the impacted pericarp kept in nitrogen atmosphere showed only a slight increase. There was a large difference in lignin content between pericarp in air and nitrogen atmosphere by the end of the experiment (Figure 17C).

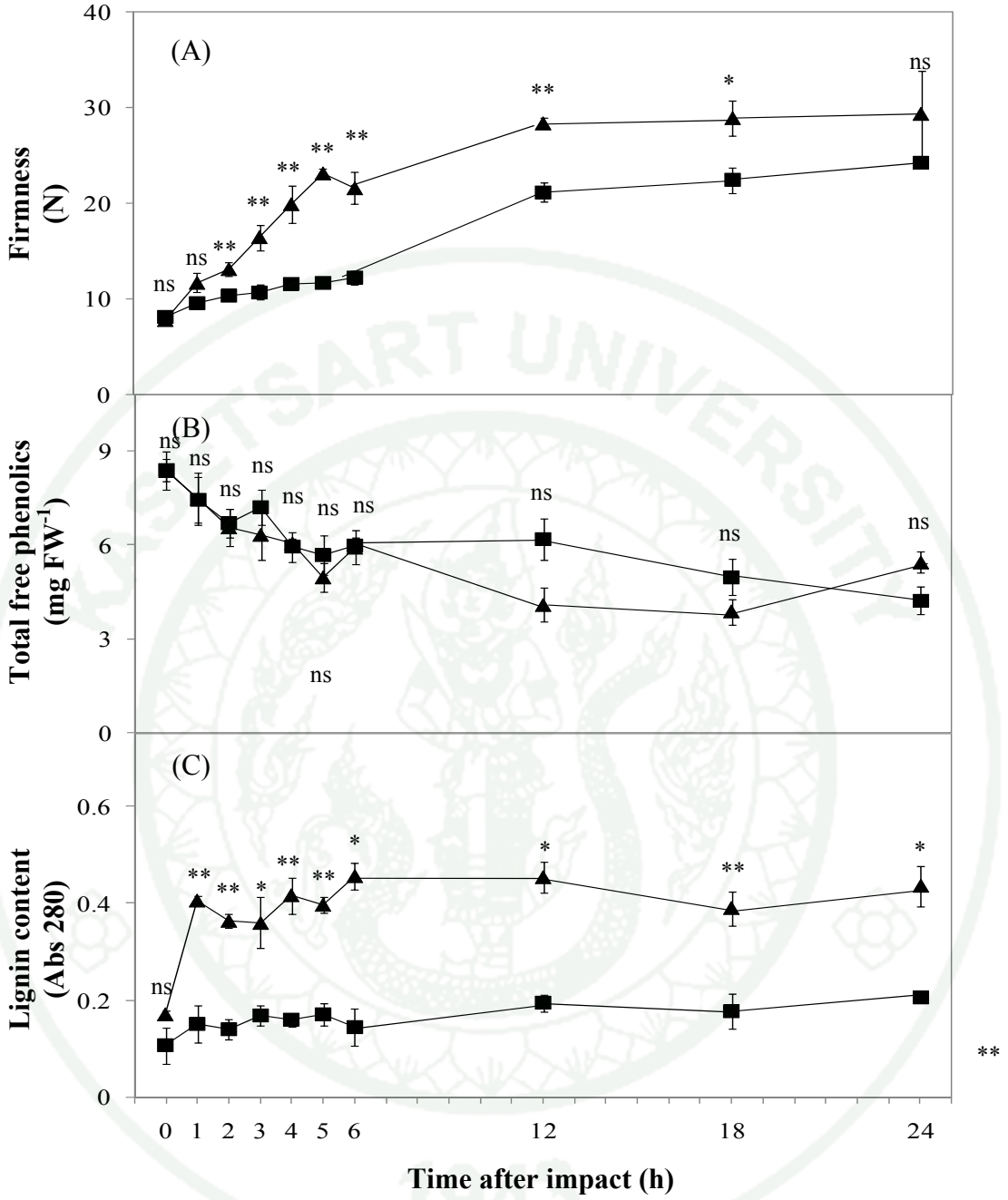

Figure 17 Changes in firmness (A), total free phenolic (B) and total lignin contents (C) in mangosteen pericarp after impact from a height of 100 cm and then kept in ambient air (▲) and nitrogen atmosphere (■) and measured at 0, 1, 2, 3, 4, 5, 6, 12, 18 and 24 h after impact. Data are means ± SE of three replications.

4. Changes in monolignol content of impacted pericarp under enhanced nitrogen atmosphere

Analysis of lignin monomer composition by nitrobenzene oxidation indicated that the vanillin and vallinic acid levels in impacted pericarp kept in nitrogen atmosphere was lower than those kept in ambient air (Figure 18A and B). As the same as the level of syringaldehyde and syringic acid that the impacted pericarp were kept in nitrogen atmosphere showed lower level than the pericarp were keep in ambient air (Figure 19A and B).

G and S lignin showed higher level in impacted pericarp kept in ambient air than those kept in nitrogen atmosphere. Interestingly, G lignin concentrations were twice as high as S lignin in the impacted pericarp (Figure 20A and B).

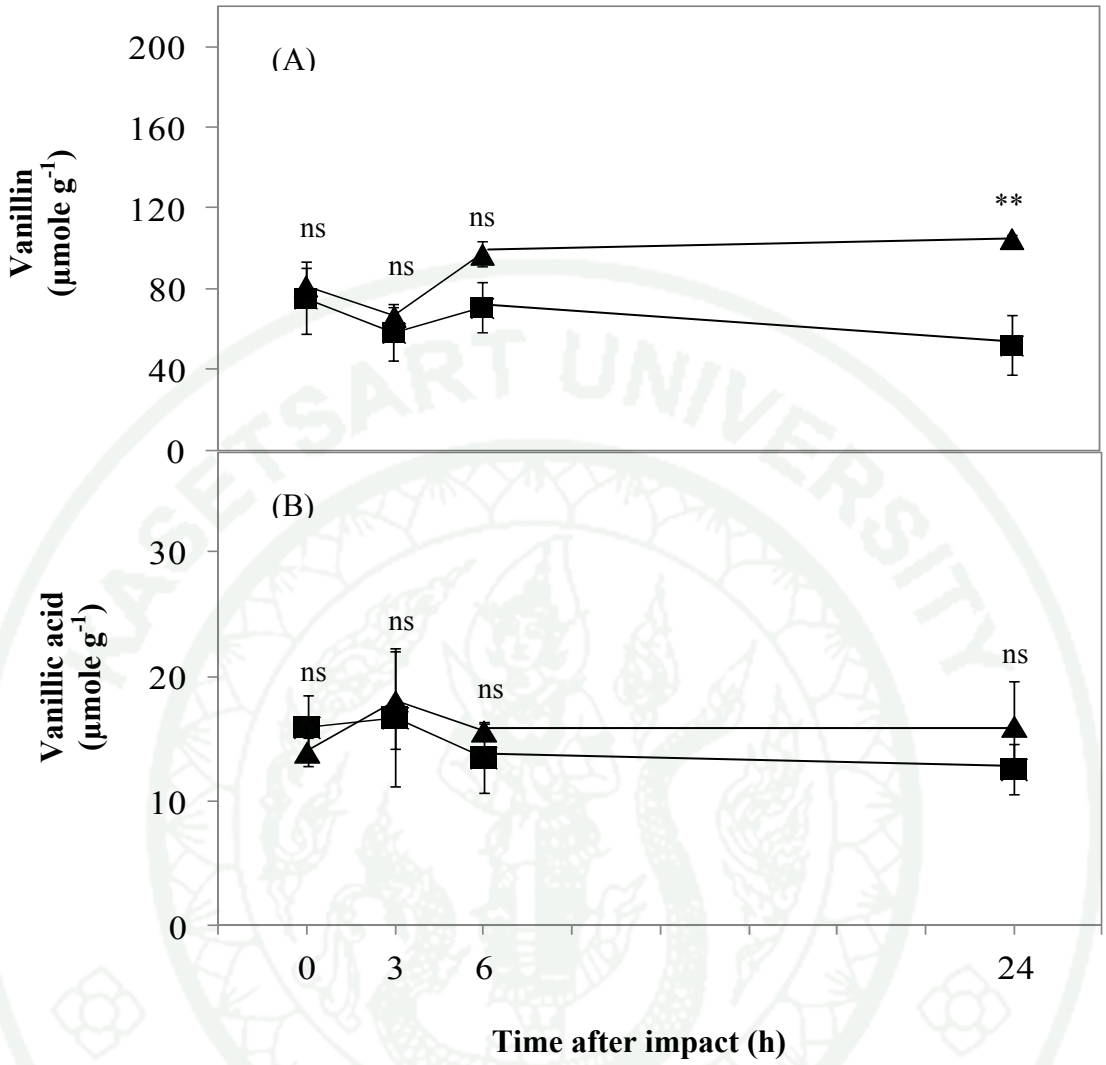

Figure 18 GC-MS analysis of trimethylsilyl of reaction products obtained by nitrobenzene oxidation. Lignin monomer composition, vanillin (A) and vanillic acid (B) in mangosteen pericarp after impact from a height of 100 cm then kept them in ambient air (▲) and nitrogen atmosphere (■) for 0, 3, 6 and 24 h.after impact. Data are means ± SE of three replications.

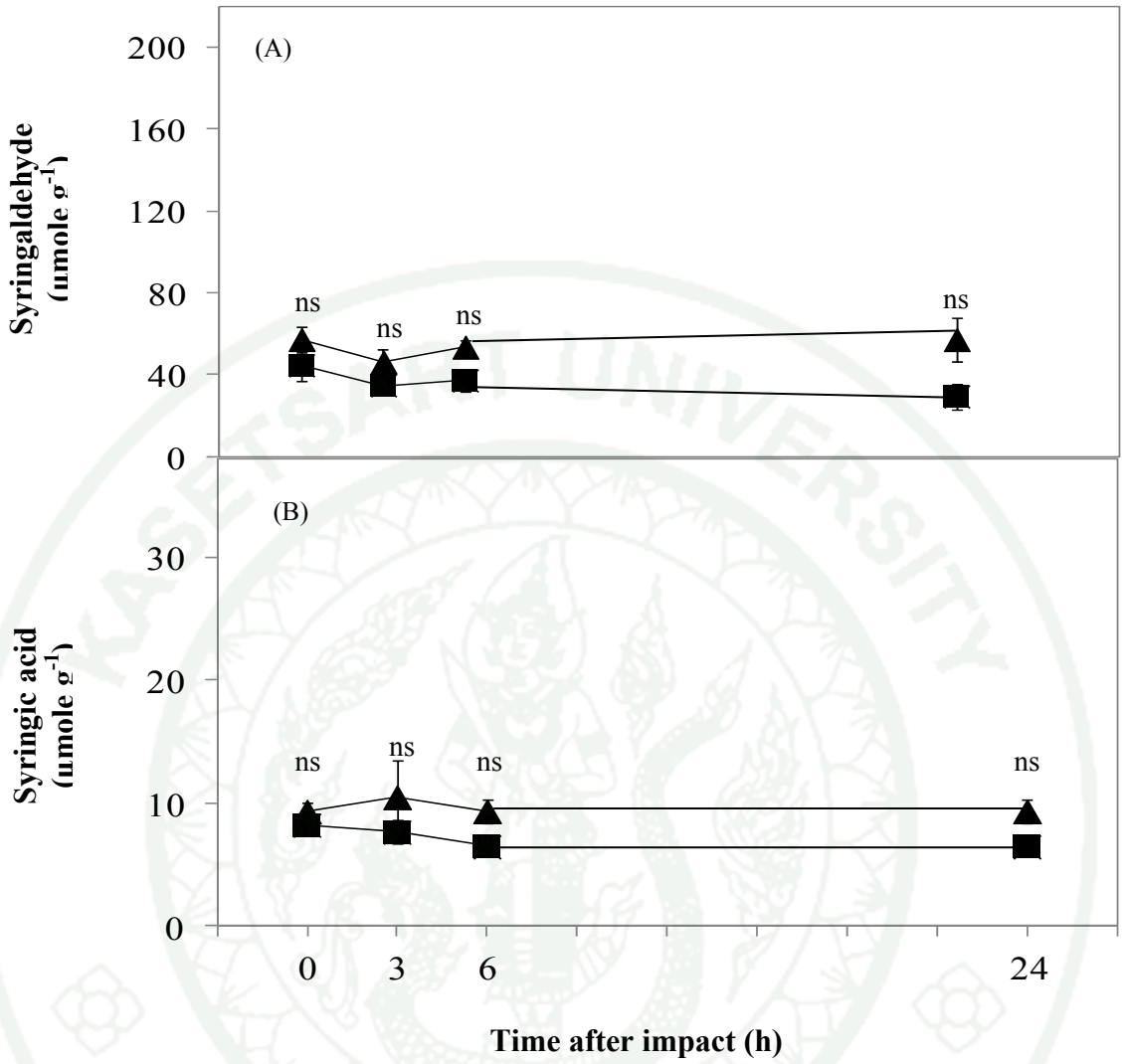

Figure 19 GC-MS analysis of trimethylsilyl of reaction products obtained by nitrobenzene oxidation. Lignin monomer composition, syringaldehyde (A) and syringic acid (B) in mangosteen pericarp after impact from a height of 100 cm then kept them in ambient air (▲) and nitrogen atmosphere (■) for 0, 3, 6 and 24 h after impact. Data are means ± SE of three replications.

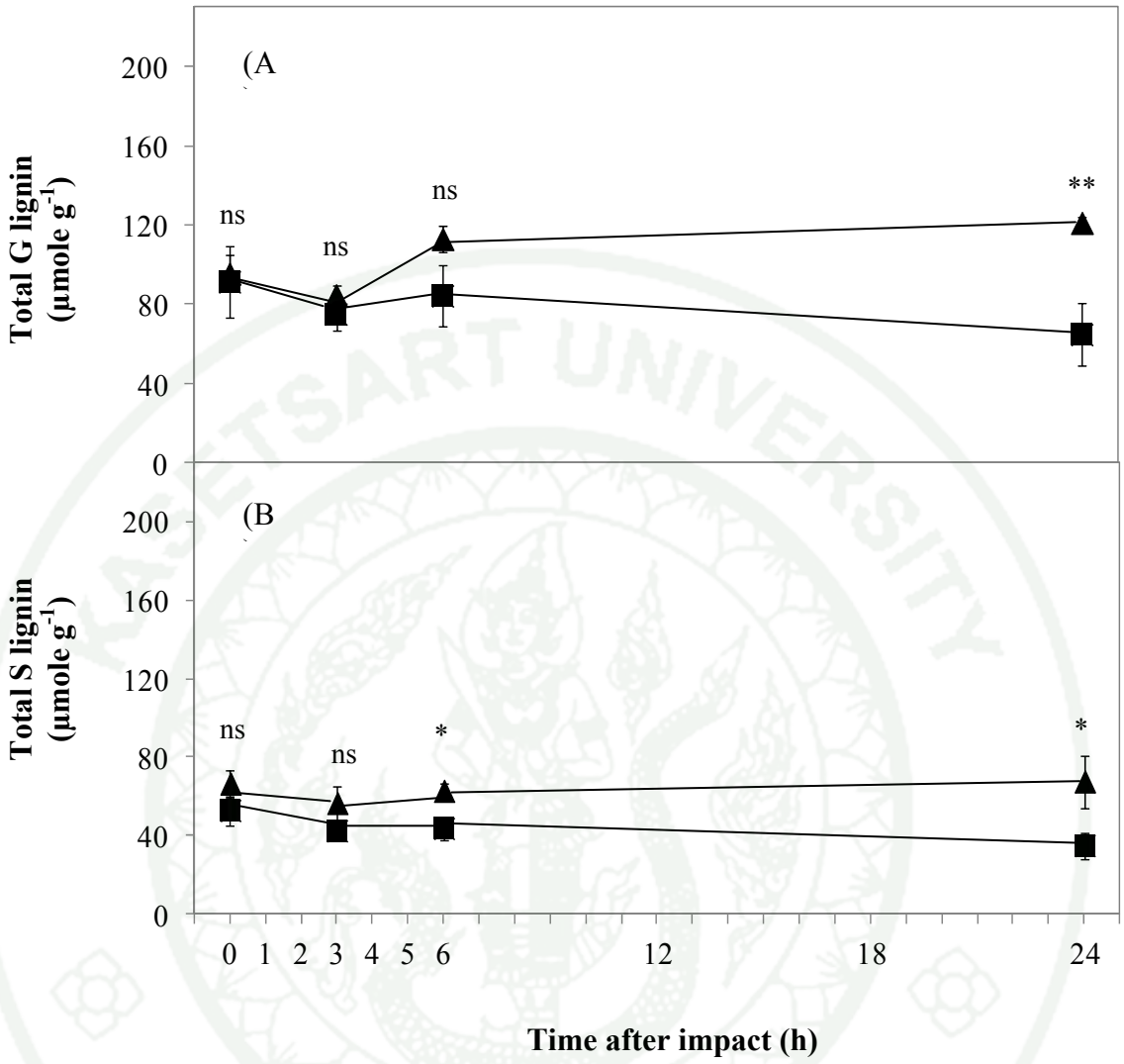

Figure 20 Changes in firmness lignin monomer composition as total G (vanillin + vanillic acid) (A) and S (syringaldehyde + syringic acid) (B) lignin in mangosteen pericarp after impact from a height of 100 cm and then kept in ambient air (▲) and nitrogen atmosphere (■) and measured at 0, 1, 2, 3, 4, 5, 6, 12, 18 and 24 h after impact. Data are means ± SE of three replications.

5. Isolation and cloning of lignin biosynthesis genes and a R2R3 MYB transcription factor

cDNA from mangosteen pericarp was isolated and characterized all the lignin biosynthesis genes and a MYB transcription factor, using forward and reverse degenerate primers (Table 3) and specific primers (Table 5 and 10).

5.1 *p*-coumaroyl shikimate 3-hydroxylase (C3H)

The partial-length *GmC3H* (accession no. KJ671469) had 1176 bp transcript encoding a predicted protein of 392 amino acid (Appendix Figure 1 and 2). *GmC3H* shared 87-79% homology with *Populus tomentosa*, *Trifolium pretense*, *Eucalyptus globules*, *Hibiscus cannabinus* and *Pinus taeda* (Table 20). Highly conserved of *GmC3H* was shown in Appendix Figure 3.

5.2 Hydroxycinnamoyl coenzyme A shikimate hydroxycinnamoyl transferase (HCT)

The partial-length *GmHCT* (accession no. KJ671470) had 1104 bp transcript encoding a predicted protein of 368 amino acid (Appendix Figure 4 and 5). *Gm HCT* shared 84-77% homology with *Populus tomentosa*, *Populus tomentosa*, *Cucumis sativus*, *Coffea Arabica* and *Pinus radiate* (Table 20). Highly conserved of *GmHCT* was shown in Appendix Figure 6.

5.3 Cinnamyl alcohol dehydrogenase (CAD)

The partial-length *GmCAD* (accession no. KJ671471) had 885 bp transcript encoding a predicted protein of 295 amino acid (Appendix Figure 7 and 8). *GmCAD* shared 85-78% homology with *Gossypium hirsutum*, *Nicotiana attenuate*, *Eucalyptus saligna*, *Hibiscus cannabinus* and *Populus nigra* (Table 20). Highly conserved of *GmCAD* was shown in Appendix Figure 9.

5.4 Peroxidase (POD)

The partial-length *GmPOD* (accession no. KJ671472) and 624 bp transcript encoding a predicted protein of 208 amino acid (Appendix Figure 10 and 11). *GmPOD* shared 78-60% homology with *Populus tomentosa*, *Brassica rapa*, *Arabidopsis thaliana*, *Camellia oleifera* and *Theobroma cacao* (Table 20). Highly conserved of *GmPOD* was shown in Appendix Figure 12.

5.5 Caffeoyl CoA O-methyltransferase (CCoAMT)

The partial-length *GmCCoAMT* (accession no. KJ671473) had 537 bp transcript encoding a predicted protein of 179 amino acid (Appendix Figure 13 and 14). *GmCCoAMT* shared 91-85% homology with *Gossypium hirsutum, Populus trichocarpa, Eucalyptus camaldulensis, Brassica rapa* and *Betula luminifera* (Table 20). Highly conserved of *GmCCoAMT* was shown in Appendix Figure 15.

5.6 4-coumarate coenzyme A ligase (4CL)

The partial-length *Gm4CL* (accession no. KJ671474) had 1044 bp transcript encoding a predicted protein of 348 amino acid (Appendix Figure 16 and 17). *Gm4CL* shared 88-80% homology with *Populus tomentosa*, *Pyrus pyrifolia*, *Eucalyptus camaldulensis*, *Hibiscus cannabinus* and *Eriobotrya japonica* (Table 20). Highly conserved of *Gm4CL* was shown in Appendix Figure 18.

5.7 Caffeic acid O-methyltransferase (COMT)

The partial-length *GmCOMT* (accession no. KJ671475) had 585 bp transcript encoding a predicted protein of 195 amino acid (Appendix Figure 19 and 20). *GmCOMT* shared 84-80% homology with *Eucalyptus camaldulensis, Populus tomentosa, Pyrus x bretschneideri, Theobroma cacao* and *Gossypium hirsutum* (Table 20). Highly conserved of *GmCOMT* was shown in Appendix Figure 21.

5.8 Ferulate-5-hydroxylase (F5H)

The full-length *GmF5H* (accession no. KJ671476) had 1568 bp transcript encoding a predicted protein of 523 amino acid (Appendix Figure 22 and 23). *GmF5H* shared 78-76% homology with *Populus trichocarpa, Eucalyptus globules, Hibiscus cannabinus, Pyrus x bretschneider* and *Brassica napus* (Table 20). Highly conserved of *GmF5H* was shown in Appendix Figure 24.

5.9 Cinnamoyl CoA reductase (CCR)

The partial-length *GmCCR* (accession no. KJ671477) had 540 bp transcript encoding a predicted protein of 180 amino acid (Appendix Figure 25 and 26). *GmCCR* shared 89-62% homology with *Eucalyptus amygdalina*, *Hibiscus cannabinus*, *Populus trichocarpa, Hevea brasiliensis* and *Pinus radiata* (Table 20). Highly conserved of *GmCCR* was shown in Appendix Figure 27.

5.10 Cinnamate 4-hydroxylase (C4H)

The full-length *GmC4H* (accession no. KJ671478) had 1518 bp transcript encoding a predicted protein of 506 amino acid (Appendix Figure 28 and 29). *GmC4H* shared 95-90% homology with *Citrus sinensis, Populus tomentosa, Gossypium arboretum, Cucumis sativus* and *Eucalyptus urophylla* (Table 20). Highly conserved of *GmC4H* was shown in Appendix Figure 30.

5.11 MYB transcription factor

In order to isolated and characterize the mangosteen MYB transcription factor that play a role in lignin biosynthesis, two degenerate primers were designed in the R2R3 MYB domain. MYB gene was isolated by 3’ race and full length sequence obtained by 5’ race. MYB repeats typically containing regularly spaced tryptophan residues, which builds a central tryptophan cluster in the three-dimensional helix–turn–

helix fold. The tryptophan residues form a cluster in a hydrophobic core in each repeat and stabilize the structureof the DNA binding domain. R2R3 MYB gene is the gene that encoded proteins characterized by two 50 to 52 residue-long imperfect repeats. Each of these MYB repeats contains three helices, with the second and third helices forming a helix-turn-helix structure when bound to DNA (Figure 21 and 22).

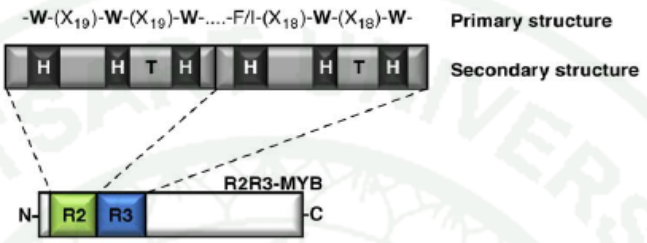

Figure 21 The primary and secondary structures of a typical R2R3-MYB are indicated. H, helix; T, turn; W, tryptophan; X, amino acid (X).

Source : (Dubos *et al*., 2010)

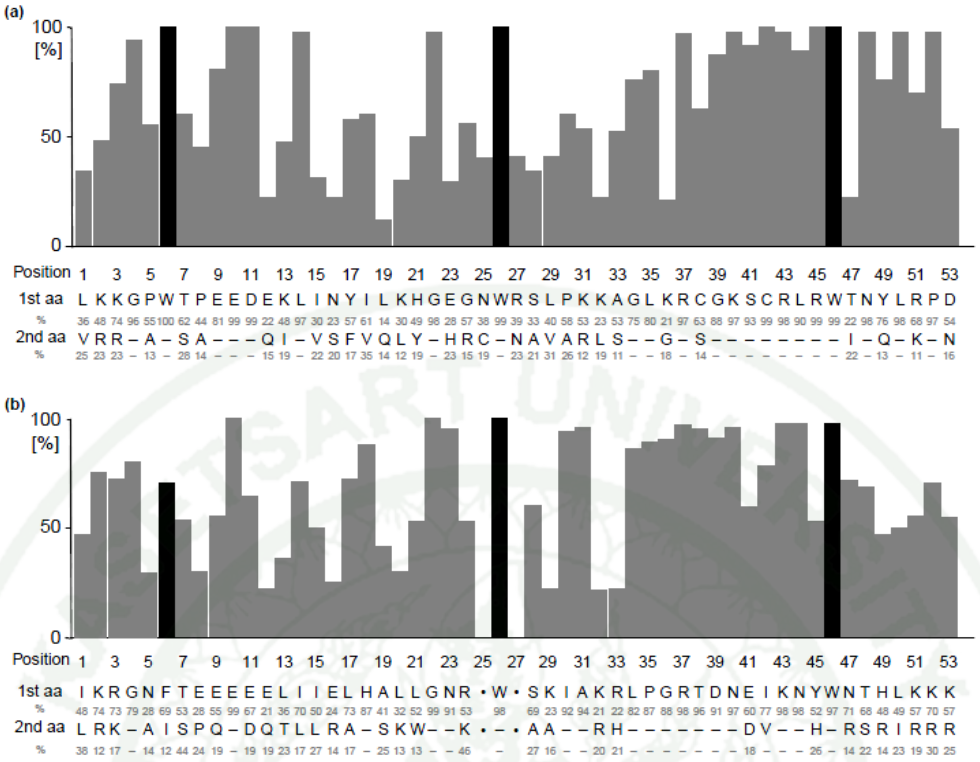

Figure 22 Consensus sequence and the level of conservation of R2R3-type MYB domains from *Arabidopsis thaliana*. (a) R2 and (b) R3 were aligned to show maximal identity, which required the introduction of two gaps amino- and carboxy-terminal to the second tryptophan (W) residue in the R3 MYB-repeat sequence.

Source : (Stracke *et al*., 2001)

The full length of *GmMYB*30 were aligned with other MYBs. The pattern of gene alignment showed the structure composing of helix-turn-helix structure as the R2R3 MYB in *Arabidopsis*. The sequence of *GmMYB*30 conserved with R2R3-type MYB domains from *Arabidopsis thaliana* (Figure 23).

The sequence of *GmMYB* were translate to deduce amino acid and alignment using Genious program. *GmMYB30* (accession no. KJ671479) had 1377 bp transcript encoding a predicted protein of 381 amino acid. After blast the full-length of amino acid

sequence *GmMYB30* with TAIR (The Arabidopsis Information Resource) This database maintains of genetic and molecular biology data for the model higher plant(*Arabidopsis thaliana*.) *GmMYB30* gene of mangosteen fruit shared high homology to *Arabidopsis* MYB30 and MYB94 (TAIR, http://www.arabidopsis.org/cgi-bin/wublast/wublast access 11 April 2014) (Figure 24).

Figure 23 Protein sequence alignment of *GmMYB30* with other MYB regulators from other plants. Identical residual are shown in dark grey (W), there are mean tryptophan.

Sequences producing significant alignments:	Score (bits)	E Value
AT3G28910.1 | Symbols: ATMYB30, MYB30 | myb domain protein ...	209	2e-54
AT3G47600.1 | Symbols: MYB94, ATMYBCP70, ATMYB94 | myb doma...	205	4e-53
AT1G74650.1 | Symbols: ATY13, ATMYB31, MYB31 | myb domain p...	205	5e-53
AT5G62470.2 | Symbols: MYB96 | myb domain protein 96 | chr5...	204	9e-53
AT1G08810.1 | Symbols: MYB60, AtMYB60 | myb domain protein ...	204	1e-52
AT5G62470.1 | Symbols: MYB96, MYBCOV1, ATMYB96 | myb domain...	200	1e-51

Figure 24 Blast the full-length of *GmMYB30* gene in TAIR data base. The *GmMYB30* gene showed high homology to *Arabidopsis* MYB30 and MYB94.

Table 20 Alignment of partial amino acid sequences of lignin biosynthesis genes from mangosteen (*Garcinia mangostana* L.) pericarp with similar genes in fruit of other plants.

Gene name	Species	Indentities %	Accession number
PAL	*Garcinia mangostana*	98	ACM62741
	Medicago sativa	88	AEW25950.1
	Ulmus pumila	88	AAY82486.1
	Populus trichocarpa	89	ACC63887.1
	Robinia pseudoacacia	89	ACF94716
C4H	*Citrus sinensis*	95	NP_001275824.1
	Populus tomentosa	93	AFZ78542
	Gossypium arboreum	91	AAG10196
	Cucumis sativus	91	CAK95273
	Eucalyptus urophylla	90	AGJ71350.1
4CL	*Populus tomentosa*	88	AFC89540
	Pyrus pyrifolia	88	AFY97682
	Eucalyptus camaldulensis	87	ACX68559
	Hibiscus cannabinus	82	AGJ84134
	Eriobotrya japonica	80	ABV44809
CCR	*Eucalyptus amygdalina*	87	AAT74893
	Hibiscus cannabinus	89	ABK30883
	Populus trichocarpa	88	CAC07424
	Hevea brasiliensis	62	ADU64758

Table 20 (Continued)

Gene name	Species	Indentities %	Accession Number
HCT	*Populus tomentosa*	84	AFZ78609
	Hibiscus cannabinus	84	AFN85668
	Cucumis sativus	80	AEJ88365
	Coffea arabica	82	ABO40491
	Pinus radiata	77	ABO52899
C3H	*Populus tomentosa*	87	AFZ78540
	Trifolium pratense	84	ACX48910
	Eucalyptus globulus	86	ADG08112
	Hibiscus cannabinus	85	AGA60530
	Pinus taeda	79	AAV36205
CCoAMT	*Gossypium hirsutum*	90	ACF48821
	Populus trichocarpa	91	CAA10217
	Eucalyptus camaldulensis	85	ACY66929
	Brassica rapa	86	ABE41833
	Betula luminifera	88	ACJ38669
COMT	*Populus tomentosa*	81	AFZ78575
	Pyrus x bretschneideri	81	AGS44640
	Eucalyptus camaldulensis	81	ACY66932
	Theobroma cacao	84	EOY23716
	Gossypium hirsutum	80	ACT32029

Table 20 (Continued)

Gene name	Species	Indentities %	Accession Number
F5H	*Populus trichocarpa*	78	CAB65335
	Eucalyptus globulus	78	ACU45738
	Hibiscus cannabinus	77	AGR85825
	Pyrus x bretschneideri	78	AGR44939
	Brassica napus	76	ABG73616
CAD	*Nicotiana attenuata*	81	AFP43764
	Eucalyptus saligna	78	AAG15553
	Hibiscus cannabinus	80	ADK24218
	Populus nigra	85	ADN96445
POD	*Populus tomentosa*	74	AGX27515
	Brassica rapa	74	ACT35471
	Arabidopsis thaliana	74	CAA66961
	Camellia oleifera	60	ACT21094
	Theobroma cacao	78	EOY07788
MYB	*Glycine max*	87	AGN96216
	Cucumis sativus	92	XP_004157466
	Citrus macrophylla	90	ABK59039
	Arabidopsis thaliana	89	AAC83617
	Paeonia suffruticosa	89	AGG69481

6. Differential gene expression in fruit pericarp after impact

Expression of the genes encoding enzymes of the lignin biosynthesis pathway as well as an R2R3 MYB transcription factor were examined using quantitative real-time RT-PCR. The transcript levels all genes were detectable in the pericarp of both impacted and non- impacted pericarp. The expression patterns of *GmPAL*, *GmHCT*, *GmCCR, GmC4H* and *GmC3H* (Figure 25) after impact showed lower transcript levels than in the non–impacted pericarp during the same time course. The expression of these genes decreased between 60 min to 1 day after impact. This subset of genes showed a negative correlation between the transcript level and lignin content (r = 0.76, 0.70, 0.53, 0.46 and 0.73, respectively) (Appendix Figure 35) and firmness (r = 0.77, 0.80, 0.64, 0.60 and 0.84, respectively) (Appendix Figure 34). The transcript levels of *GmCCR*, *GmCCoAMT*, *GmPOD* and *GmCAD* (Appendix Figure 34 and 35) showed little correlation with lignin content and firmness, and showed variable differences between impacted and control pericarps (Appendix Figure 34, 35, 38 and 39).

In contrast, the expression of *GmF5H* (Figure 25H) in the non- impacted pericarp remained at a relatively low level throughout the time course, while in impacted pericarp transcription levels increased from 30 min to 1 day after impact. This correlated well with increasing firmness and lignin content. The expression of the R2R3 MYB *GmMYB30* (Figure 25L) in impacted pericarp showed a significant increase in transcript level after 25 min. This increase in MYB30 expression was not seen in non-impacted pericarp.

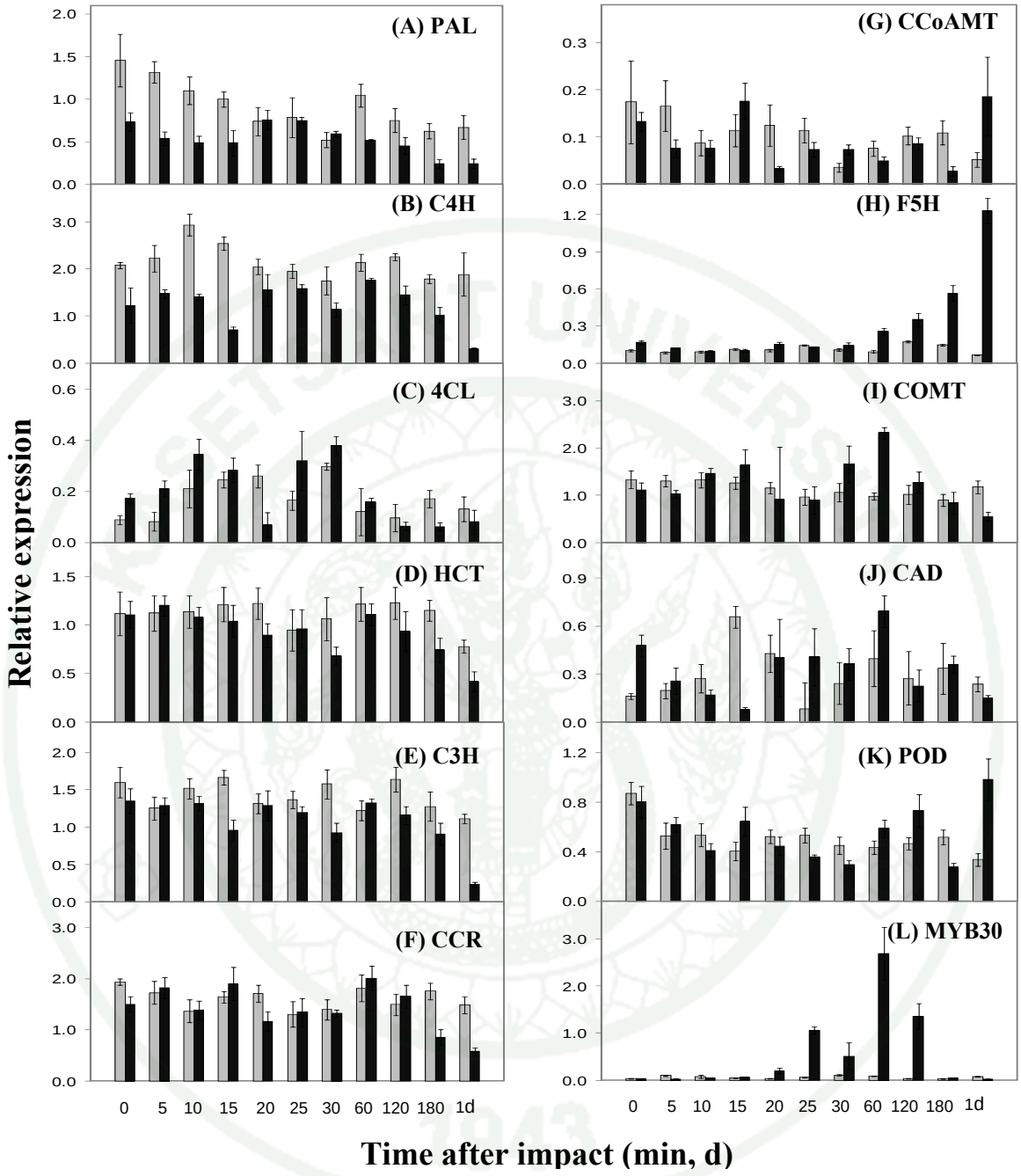

Figure 25 Expression profiling of lignin biosynthesis and MYB transcription factors genes in mangosteen pericarp after impact from a height of 0 (□) and 100 cm (■) and analysed at 0, 5, 10, 15, 20, 25, 30, 60, 120, 180 min and 1 day after impact. Real - time PCR was used to analyse *GmPAL* (A), *GmC4H* (B), *Gm4CL* (C), *GmHCT* (D), *GmC3H* (E), *GmCCR* (F), *GmCCoAMT* (G), *GmF5H* (H), *GmCOMT* (I), *GmCAD* (J), *GmPOD* (K) and *GmMYB*30 (L) express patterns. The transcription level was calculated with respect to ELF level.

7. Differential gene expression in impacted fruit under elevated nitrogen atmosphere

The expression of *GmC4H*, *GmC3H* and *GmCCR* (Figure 26) in fruit held under nitrogen atmosphere showed a negative correlation with firmness and lignin content. *GmF5H* (Figure 26H) expression gradually increased in both atmosphere treatments. However, fruit held in ambient air showed higher *GmF5H* transcript levels at 5, 18 and 24 h after impact. This expression showed a positive correlation with the firmness and lignin content (Appendix Figure 36, 37, 40 and 41).

The expression of *GmMYB30* (Figure 26L) in the fruit held in air showed higher expression earlier in air than under elevated nitrogen atmosphere. The increase in transcript levels of this MYB was delayed by the nitrogen atmosphere.

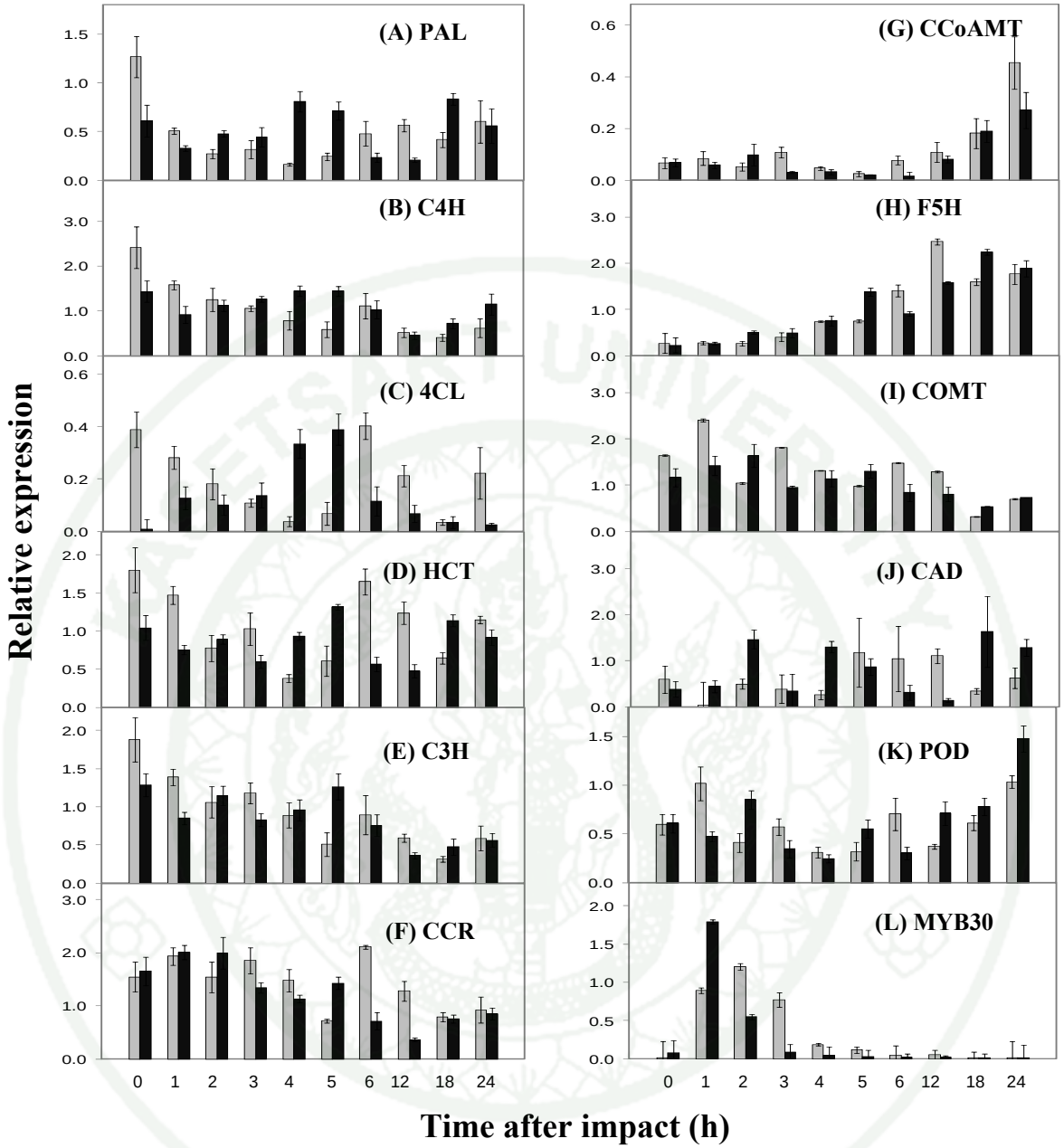

Figure 26 Expression profiling of lignin biosynthesis and MYB transcription factors genes in mangosteen pericarp after impact from a height of 100 cm and then kept in ambient air (■) and nitrogen atmosphere (□)and analysed at 0, 1, 2, 3, 4, 5, 6, 12, 18 and 24 h after impact. Real - time PCR was used to analyse *GmPAL* (A), *GmC4H* (B), *Gm4CL* (C), *GmHCT* (D), *GmC3H* (E), *GmCCR* (F), *GmCCoAMT* (G), *GmF5H* (H), *GmCOMT* (I), *GmCAD* (J), *GmPOD* (K) and *GmMYB*30 (L) express patterns. The transcription level was calculated with respect to ELF level.

8. Promoter isolation and analysis of GmF5H gene

PCR – based DNA walking of seven libraries *Dra*I, *Ecl*136 II, *Eco*RV, *Hpa*I, *Sca*I, *Ssp*I and *Stu*I was used to isolated 5' flanking regions of *GmF5H* of size 939 bp. The regulatory region of the sequence was analysed by PLACE (http://www.dna.affrc.go.jp/PLACE/). This showed several putative cis-elements for regulatory motifs involved in plant development and stress. The combinatorial interaction of cis-acting element by R2R3-MYB and tissue – specific activation of phenylpropaniod biosynthesis gene (CANNTG) were found in the *GmF5H* promoter (±41, ±138, +514). The wound –responsive element or WUN-motif (TCATTACGAA) was found in the *GmF5H* promoter (+381) (Figure 27 and 28).

```
  1    ACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGTAGCACAGATGAACACTGCTTGACG      60
 61    GTCCTACATTGTTTACTTGTTTCAGGGCGTAGAAATACGATTTCAATGAGTAAGGTCTTC     120
121    TTTTCCGGTATAACCAACATTTGGCCTACACTTTATTTACGTAACTTTATAGATATTTAT     180
181    AATTGTTAAAACTTTTTAGCTTTTTTATTGTCAAAAAAAAAAGATTACTATTTTTGAAAT     240
241    ATGCTTTATATCTTTTGTGAAATTTATAATTTTTCTATTTTTAAAATTTCTTATAATTAC     300
301    GTTTATGTTTTTTAGATTTGTTAAAAAAAATTTATCTTTTCCAATTAATATTCAATTTTT     360
361    TAAAAAAATTAGTTTTTTATTTCATTAAGAAAAAGATAGGTTTTCAATTTTGTTTTGGAG     420
421    ATTTTGTTTTTTTAATTAATTTTTAAAAATTTTCGAAATGCAAGAGGAGAAGTTGGAAGC     480
481    AACGAATATAAGATGTTCATCATAAGATTCAAACATTTGTTAAGGAGTTGAAATTATAAT     540
541    TTATCCTTAATTAAAATAATTAAAATTTAAGGATTTGTATGAAATTATTAATATCCGAAA     600
601    GTGGTTGTTTATAATAGTATAGATAAATTATATTACACAAACGAAGACCAATTCAAGCGA     660
661    TGCAAACAAAACACAATTCAAATTTGGCGTGACGAAGTGAAAGCAAAGAAAACATCACAC     720
721    AAAATGGACAGGAAAAAGAATTATAAAACACCCACCAGCTTCAGTAGTTATTGTGCTTGA     780
781    CCAACATTTCTATATAACTGAAGAACCCCCATGGTATATCTTCCTCAAAACATATAAGCT     840
841    CAAACCAAATGCCAAGCGCACCACTATCTTTCCTCCAATAAAAAAAAAAAAAGACCACCAT     900
901    CTTTCCTATTTCCTTATTTCAGACATTCATACACTATG      939
```

Figure 27 Nucleotide sequence of *GmF5H* 5' flanking regions. The basal promoter elements (TATA and CAAT boxes) are highlighted in grey. The single underline letters indicate the combinatorial interaction of cis-acting element by R2R3-MYB and tissue – specific activation of phenylpropaniod biosynthesis gene (CANNTG). The double underline letters indicate the wound – responsive element (TCATTACGAA). The bold letters indicate the translation start site (ATG).

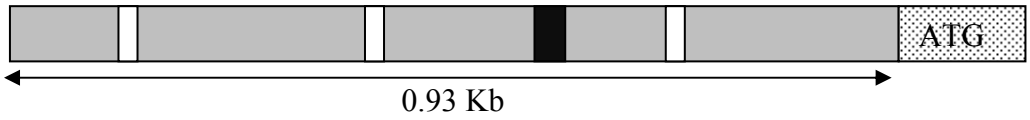

Figure 28 The picture of the sequence of F5H promoter . The white box indicate the MYB binding site and tissue – specific activation of phenylpropaniod biosynthesis gene. The black box indicate wound – responsive element. The dot box of ATG indicates the translation start site.

Discussion

In this study the effect of impact on mangosteen pericarp tissue was examined. The physiological and biochemical changes including fruit firmness, lignin content, lignin monomers, total free phenolics and gene expression in impacted pericarp were determined and analysed compared to non-impacted pericarp.

Wounding is one of many abiotic stresses that produce signals that propagating from injured tissue into adjacent non-injured tissue and induces *de novo* synthesis of specific wound-induced proteins. Pericarp hardening from the mangosteen after impact is one of the abiotic stresses. When physical wounding occurred, the physiological signals induce many mechanisms in plants such as respiration, ethylene biosynthesis, phenolic metabolism and wound healing (Saltveit 2000).

The mechanical stress in fruits results in variations in metabolism of phenolic compounds. The phenolic compounds are reported to be involved in disease resistance in two ways; first by contributing to healing of wound by lignification of cell wall around wounded zones and second, through the antimicrobial properties. The most immediate response to wounding is oxidation of pre-existing phenolic compounds resulting in their degradation. Total phenolics of many commodities such as mangosteen, mango, citrus and tomato have also been found to decrease during stresses (Bunrisi *et al*., 2003; Chidtragool *et al*., 2011; XU *et al.,* 2007). In mangosteen after impact, the first physiological change was browning of the pericarp at 10 min (Figure 11), with firmness and lignin content being altered 30 min and 25 min after impact, respectively (Figure12 A and C). The total free phenolics decreased 5 min after impact and gradually decreased during the time after impact. The main phenolic acids in mangosteen pericarp were identified as *p*-coumaric and sinapic acids, both of them declined in response to impact (Bunsiri *et al.*, 2003). The decline of *p*-coumaric and sinapic acids in response to impact was also associated with an increase in total lignin content. These changes may be linked to the reduction of soluble phenolics pool as lignin is synthesised (Vanholme *et al*., 2010). Similar result was reported in tomato fruit pericarp (Lagrimini *et al*., 1993). After wounding, lesion of membranes results in brutal decompartmentalization which brings into contact between enzymes such as polyphenol

oxidase (PPO) and peroxidae (POD) and substrates particularly phenolic compounds which normally accumulated in the vacuole. The brown discoloration or necrosis which appeared in impacted mangosteen pericarp after impact showed clearly 10 min after impact and the color turned more dark brown thereafter. Dark brown of impacted pericarp can be explained with a decrease in L value after impact (data not shown). The brown color changed concomitantly with increased firmness and the lignin content similar to jicama (Aquino-Bolaños *et al*., 2004).

The phenolic metabolism not only causes tissue browning but also involves in lignin biosynthesis. Phenolics are the main substrate for lignin synthesis, when the lignin biosynthesis begins, the substrate is changed enzymatically to lignin (Vermerris and Nicholson, 2006). In mangosteen, after impact, total free phenolics deceased concomittantly with an increase in total lignin content. The PAL gene expression in impacted pericarp showed a good correlation with total free phenolics decreased. This change pattern may be due to more turnover than synthesis resulting in decreased total free phenolics.

Phenolic compounds are associated with health-promoting activities. Reyes and Cisneros-Zevallos (2003) found that wounding in purple flesh potato increased the accumulation of phenolic compounds by 60% with a parallel of 85% increase in antioxidant capacity. Total soluble phenolics also increased in wounded iceberg lettuce (Ke and Saltveit, 1989a, 1989b; Saltveit, 2004), salicylic acid-treated grape berries (Chen *et al.*, 2006) and high O_2-treated blueberry fruit (Zheng *et al*., 2003). Ju and Bramlage (2000) reported that during cold storage, free phenolics in 'Delicious' apple fruit cuticle increased in early storage, and then remained constant. These suggested that 1) the turnover of phenolic metabolism is less rapid than their synthesis, resulting in increased phenolics or 2) the phenolics increase due to the protection of oxidative damage.

Wounding is a physical stress that damages plant tissues, often increases many enzymatic activities in phenylpropaniod pathway and phenolic metabolism (Saltveit *et.al.,* 2005).

Wounding also increases peroxidase activity and lignin content of tissues, with cell wall lignification localized in wounded and adjacent cells (Ke and Saltveit, 1989). Wounding, UV, light, irradiation, chilling injury and pathogen attack are stresses can induce lignin biosynthesis. Less is known about the transcriptional activation of stress-induced lignin biosynthesis (Zhong and Ye, 2009). Lignin can be induced by pathogen infection or wound provides a physical barrier by enhancing the cell wall to block the penetration and diffusion of pathogen (Zhang *et al*., 2007).

Lignin content of the impacted pericarp increased gradually during after impact. The lignin content of impacted pericarp increased significantly higher than non-impacted pericarp 25 min after impact. The lignins are synthesized via the oxidative coupling of three monolignols, *p* -hydroxyphenyl (H), guaiacyl (G), and syringyl (S). The decline of total free phenolics and also the main phenolic compounds (*p*-coumaric and sinapic acids) in response to impact was associated with the increase in total lignin content. *p*-Coumaric acid decreased more rapidly in impacted pericarp fruit than sinapic acid (Bunsiri *et al*., 2003).This might be due to *p*-coumaric acid generating the monolignols; *p*-courmaryl alcohol (H lignin), coniferyl alcohol (G lignin) and sinapyl alcohol (S lignin).

The lignin composition varies among plant species and tissues. In general, lignins from gymnosperms and related species are rich in G units and contains low amounts of H units, whereas dicot lignins are mainly composed of G and S units (Weng and Chapple, 2010). It is important to note that differences in monolignol content are controlled by key enzymes that are often limiting, e.g. F5H is limiting for S unit production (Weng and Chapple, 2010). Compression wood in coniferous trees also contains significant amounts of *p*-coumaryl alcohol. Wound-induced lignin in wheat leaves (*Triticum aestivum*) revealed polymers with higher proportions of H and S, while leaves inoculated with *B. cinerea* caused an induction of sinapyl alcohol dehydrogenase but no significant increase in *p*-courmaryl alcohol (Mitchell *et al*., 1994).

Data analysis of mangosteen monolignols revealed that the impacted pericarp had higher levels of G (vanillin and vanillic acid) and S (syringaldehyde and syringic

acid) monolignols than the non-impacted pericarp which accounted significantly for the increase in lignin content. This change was similar to Chinese cabbage leaf stalk that was infected by *Erwinia carotovora subsp. carotovora* (Ecc) showed G and S lignin increased significantly compared with H lignin (Zhang *et al*., 2007).

Monolignols are synthesized in the cytoplasm and translocated to the cell wall for subsequent polymerization. Lignin monomers are differentially targeted to discrete regions of various lignifying cell walls; for example, *p*-coumaryl alcohol is mainly targeted to the middle lamella and coniferyl alcohol to the secondary wall of the xylem elements. Sinapyl alcohol, by contrast, is targeted to discrete regions in fiber-forming cell walls. The physiological significance of this is apparently straightforward: differential targeting permits the construction of lignified cell walls with overall quite distinct biophysical properties (Davin and Lewis, 2005). Recently researchers have found that monolignol are transported into cell wall across the plasma membrane process by ABC-transporter. The specific ABC transporters regulate variation of lignin composition to cell specific e.g., vessel element walls are rich in G lignin whereas fiber walls contain S-G lignin (Wang *et al*., 2013).

Wounding in *Populus* induced cell wall to synthesize the thick-walled fibers and secondary cell wall to make cell wall like extremely thick walls. This wound-induced reaction resulted in three modes of wall-thickening 1) xylem fibers showed the presence of a supplementary S2 layer, 2) group of fibers developed a continuously thickened secondary cell wall and 3) the fibers walls developed a sclereid-like sublayering. These changes showed the proportion of G lignin increased, whereas a reduction of H lignin (Frankenstein *et al*., 2006). The increased level of G and S lignin in impacted pericarp may involve in the fiber synthesis and ABC-transporter would regulate lignin composition to cells, resulting in pericarp hardening with higher G and S lignin after impact (Frankenstein *et al*., 2006; Wang *et al*., 2013). However, this needs further study for elucidation.

The firmness of impacted pericarp at 30 m after impact increased significantly compared to the non-impacted pericarp. An increase in fruit firmness is an uncommon

postharvest textural change, which has been reported only relatively few fruit crops. This trait is caused by cell wall secondary lignification, induced as a response to stress (Ketsa and Atantee, 1998; Boerjan *et al*., 2003; Boudet *et al*., 2003; Bunsiri *et al*., 2003; Moura *et al*., 2010).

The data confirm previous studies, where pericarp hardening is clearly associated with an increase in firmness and lignin content and is concomitant with a decrease in total free phenolics (Ketsa and Koolpluksee, 1993; Ketsa and Atantee, 1998; Bunsiri *et al*., 2003; Dangcham *et al.*, 2008). The prelimnary study of impacted fruit showed higher respiration rate than non-impacted fruit, while ethylene production of impacted fruit was lower than that of non-impacted fruit (data not shown). The results of increased respiration and decreased ethylene production in impacted fruit were similar to Ketsa and Koolpuksee (1993) and Ketsa and Meenaphan (1995), respectively. The ethylene production decreased in the impacted fruit may be due to impacted pericarp lost of capability to convert ACC to ethylene (Ketsa and Meenaphan, 1995).

Impacted mangosteen kept under elevated nitrogen had lowered lignification and hence a lower firmness, compared to impacted fruit in ambient air. Similarly the whole fresh button mushrooms (*Agaricus bisporus*) stored in low oxygen atmosphere reduced the accumulation of lignin (Jiang *et al*., 2014). The peeled bamboo shoots (*Phyllostachys praecox f. prevelnalis*) kept under low oxygen atmosphere also reduced the cellulose and lignin contents (Sheng *et al*., 2004).

It is understood that lignification is an oxidative process. The last major step in lignin synthesis involves monolignol polymerization, which uses many oxidative enzymes, e.g. peroxidase, laccases or phenol oxidases. After the activation of monolignols by these enzymes, oxidized monolignol radicals couple to form three dimensionally cross-linked structures. This polymerization constitutes the final step of lignin biosynthesis. These enzymes use oxygen and reactive oxygen species for the reaction, and therefore lowering O_2 levels affect monolignol polymerization, thus reducing lignification (Imberty *et al*., 1985).

Wounding induces genes related to lignin biosynthesis, such as PAL, C4H, F5H, CAD, CCR and 4CL (Delessert *et al*., 2004; Soltani *et al*., 2006; Moura *et al*., 2010), resulting in accumulation of lignin surrounding the wound site. In previous research on fruit lignification, loquat provides the most comprehensive set of observations on lignin and monolignol precursors, and their associated enzymes and genes (Shan *et al*., 2008). The expression of genes encoding cinnamyl alcohol dehydrogenase (CAD) and peroxidase (POD), *EjCAD1* and *EjPOD*, were most closely associated with loquat flesh lignification. *EjCAD1* expression was stimulated by low temperature, which may contribute to low temperature injury in fruit (Shan *et al*., 2008). To test the hypothesis that mangosteen pericarp hardening results from transcriptional changes in the genes encoding the lignin biosynthetic pathway, gene expression was examined during a time course after impact. The pattern of gene expression of F5H, COMT, CAD and POD in impacted fruit showed some increases over non-impacted fruit. However, the expression of genes encoding early steps of lignin biosynthesis such as PAL, C4H, 4CL, HCT, C3H and CCR decreased following impact. F5H showed a strong increase in expression in impacted fruit from 30 min to 1 day after impact while in the pericarp of the non-impacted fruit remained at a relatively low level throughout the time course. F5H is a cytochome P450 dependent monooxygenase that catalyses the hydroxylation of ferulic acid, coniferaldehyde and coniferyl alcohol, leading to sinapic acid and syringyl lignin biosynthesis (Zhao *et al*., 2010; Bonawitz and Chapple, 2010).

Up-regulation of F5H in poplar tree was found to increase the S/G ratio that subsequently led to increased pulping efficiency. Conversely, down-regulation of F5H in alfalfa caused a decrease in the S/G ratio. *Arabidopsis* transgenic lines overexpressing F5H showed that it contained S lignin in a larger proportion (92%). Therefore, F5H is a key enzyme involved in synthesizing monolignol sinapyl alcohol (Zhao *et al*., 2010).

Among the different lignin monomers, S lignin seems to be particularly involved in plant defense, and some plants selectively accumulated S lignin in response to pathogen attack (Menden *et al.,* 2007). *Arabidopsis* plants overexpressing F5H under the C4H promoter accumulated high levels of S lignin and showed an increase in

resistance to nematode infection (Wuyts *et al*., 2006). It is possible that S monolignol biosynthesis initially evolved as a defense response following the appearance of F5H (Zhao *et al*., 2010).

In impacted pericarp, the firmness level, lignin content and the level of S lignin increased during the time after impact. S lignin show significantly increased at 120 -180 min after impact. These changes conformed to the pattern of F5H expression level. Hence, the result of F5H may be a key enzyme involved in synthesizing monolignol sinapyl alcohol and involved in the pericarp hardening in mangosteen.

Wounding also induced the synthesis of lignin precursors and ferulate esters in *Solanum tuberosum* (Bernards and Lewis, 1992). Ruegger *et al*., (1999) found that in *Arabidopsis* leaves, F5H has been shown to be induced by senescence. In Kenaf (*Hibiscus cannabinus* L.), qPCR analysis suggests that *Hc*F5H is highly induced by wounding. These results point to the fact that plants enhance lignin production in order to protect them from wounding through the regulation of the genes involved in lignin biosynthesis such as F5H (Kim *et al*., 2013).

The expression of stress-induced genes in plants is largely regulated by specific transcription factors. Transcription factors belonging to NAC, MYB and WRKY gene families, have been shown to regulate the lignin biosynthetic pathway in various plant species (Vanholme *et al*., 2010). MYB proteins have been shown to be involved in many significant physiological and biochemical processes, including regulation of primary and secondary metabolism, cell development and cell cycle, participation in defense and response to various biotic and abiotic stresses (Du *et al.*, 2009). Some of these MYB transcription factors have been shown to regulate the entire phenylpropanoid metabolism, and the others were proposed to specifically regulate the lignin biosynthesis (Zhong and Ye, 2009; Zhao and Dixon, 2011).

The phenylpropanoid metabolism also appears to be regulated at the transcriptional level. The stresses induce significant differences in accumulation of different phenylpropanoid gene transcription level. It is well known that lignin is

induced in response to environmental cues, such as wounding, mechanical stress and plant pathogens, and some monolignol pathway genes are specifically induced by stress. In this study *ZmMYB*31 and *ZmMYB*42 are both induced by wounding while Jin *et al.* (2004) reported *AtMYB*4, responded to wounding and UV in *Arabodopsis*.

Full length coding sequence of a MYB transcription factor, *GmMYB*30 was isolated. This is homologous to two genes in *Arabidopsis* (*AtMYB*30 and *AtMYB*94) that are involved in stress response (Vailleau *et al*., 2002; Nikiforova *et al*., 2003). The expression of *GmMYB*30 in impacted fruit showed a significant increase after 20-120 min. *GmMYB*30 and *GmF5H* showed low expression in non-impacted fruit, whereas *GmMYB*30 increased in 20 min, while the expression of *GmF5H* gradually increased 25 min after impact.

The proteins of the R2R3-MYB class are plant-specific and are involved in the processes such as primary and secondary metabolism, cell destination and identity, development and responses to abiotic and biotic stress. Previous studies have verified that *Arabidopsis AtMYB*30 overexpression accelerated and intensified the hypersensitivity response (HR) after an attack from avirulent strains of *Pseudomonas syringae*. This suggested that MYB acted as a positive regulator of cell death in response to the attack of pathogenic bacteria (Murilo *et al*., 2014). Chen *et al*. (2014) reported mRNA of *AhMYB*30 increased under salt stress.

In loquat, MYB genes were involved in stress condition such as chilling injury and flesh lignification. The result indicated that the *Ej*MYB1 interacted with *Ej*4CL1 promoter and AC element acted as an activator. *Ej*MYB1 activated promoter of *Arabidopsis* and loquat lignin biosynthetic genes (Xu *et al.,* 2014).

In the *Arabidopsis* no MYB site was found in F5H promoter. The transient expression system showed that the SND1 bound to the promoter region of F5H and activated F5H expression without *de novo* protein synthesis. This indicated that the regulation of F5H by SND1 is through direct binding to F5H promoter rather than through downstream transcription factors such as *At*MYB46 and /or *At*MYB58 (Zhao *et*

al., 2010). In mangosteen, isolated the *Gm*F5H promoter found the motif that similar to the MYB binding site and tissue-specific activation of phenylpropaniod biosynthesis gene (CANNTG). Moreover, the motif of wound-responsive element gene was found (TCATTACGAA). *GmMYB*30 showed homologous to two genes in *Arabidopsis* (*AtMYB*30 and *AtMYB*94) that are involved in stress response (Vailleau *et al*., 2002; Nikiforova *et al*., 2003). This information may indicate that *GmMYB*30 and *GmF5H* gene co-ordinate regulated lignin biosynthesis in mangosteen fruit after impact. The function of promoters of *GmMYB*30 and *GmF5H* in mangosteen is currently under investigation via transient expression system.

Wound induced expression of the *F5H* gene in connection with its signal transduction molecules in plants. In wounded *Camptotheca. acuminate,* MJ (methyl jasmonic acid) and H_2O_2 (wound-signaling molecules) induced expression of *Ca*F5H1. H_2O_2 is known as a downstream regulator in the JA-associated wound-signaling pathway in tomato (Orozco-Cardenas *et al*., 2001). H_2O_2 stimulates the expression of late genes containing proteinase inhibitors and polyphenol oxidase genes related to plant defense. *CaF5H1* gene is up-regulated by H_2O_2 implies that its products might be involved in defense processes. In mangosteen, these signal molecules may involve in regulation of genes in lignin biosynthesis pathway (Kim *et al*., 2006). This should be further studied.

Additionally, it was hypothesized that N_2 atmosphere would reduce pericarp hardening and lignin content. The levels of F5H expression increased gradually in both ambient air and N_2 atmosphere. It is interesting that the impacted fruit kept in the ambient air showed high expression levels of F5H and POD. These results suggest monolignols may be oxidized by peroxidases (Vanholme *et al*., 2010). This supports the finding that the impacted fruit kept in the nitrogen atmosphere still had a little rising of firmness and lignin content during the time after storage suggesting some oxygen activity of peroxidase enzyme not likely still existing inside the fruit.

The process of hardening in mangosteen after impact was summaried in figure 29. The impacted pericarp sent a signal (s) to increased respiration rate, decreased

ethylene synthesis and phenolics metabolism. The impacted pericarp of mangosteen also showed brown color after impact. This may cause the damage cell leading to the contact between PPO enzyme and phenolics forming brown pigments. The phenolic compounds of impacted pericarp decreased, whereas the lignin content, lignin composition increased. The reduction of total free phenolics may be due to either the turnover of phenolics to lignin occurred more rapidly than their synthesis or total free phenolics were used as the substrate of PPO for browning reaction. The reduction of ethylene production may be due to impairing ACC oxidase activity.

F5H showed a strong increase in expression in impacted pericarp, while the expression of the non-impacted pericarp remained stable after impact. The expression of F5H showed a high correlation with an increase in lignin content, firmness, G and S lignin. This indicated that F5H may be a key enzyme in lignin involved in synthesizing monolignol sinapyl alcohol and also involved in plant defense. The isolation of F5H promoter found two elements similar to the MYB binding site and tissue-specific activation of phenylpropaniod biosynthesis gene and the motif of wound-responsive element gene. Isolation MYB transcription, the sequence of *GmMYB*30 conserved with R2R3-type MYB domains. This indicated that *GmMYB*30 and *GmF5H* gene co-ordinate regulated for lignin biosynthesis in mangosteen fruit after impact.

N_2 atmosphere decreased firmness and lignin content. The last major step in lignin synthesis involves monolignol polymerization, which uses many oxidative enzymes. These enzymes use oxygen and reactive oxygen species for the reaction, and therefore lowering O_2 levels (N_2 atmosphere) affected of the monolignol polymerization and then reducing lignifications.

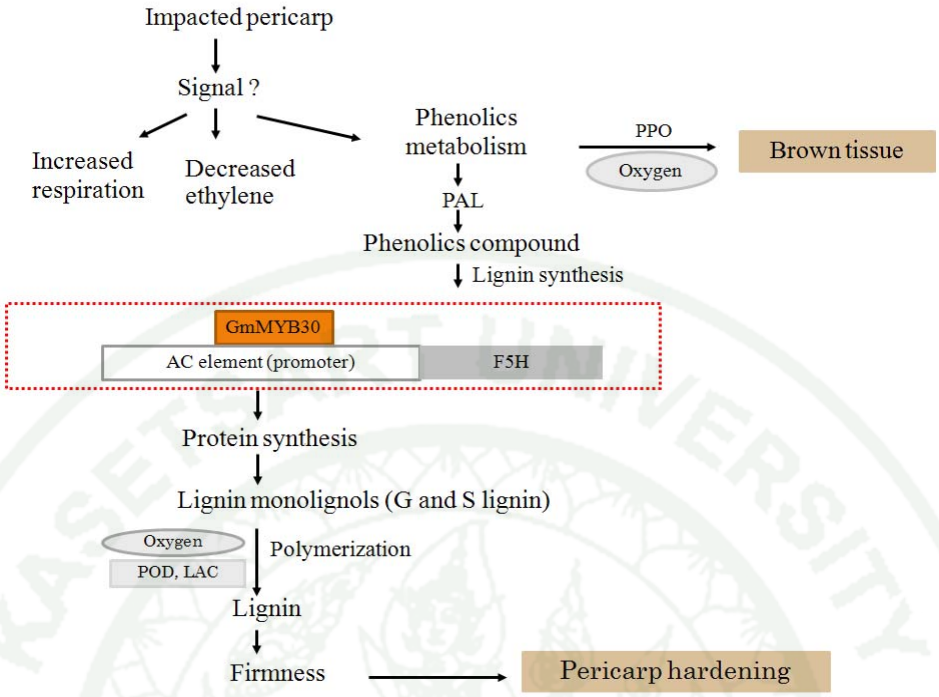

Figure 29 The interrelationships of impacted pericarp that induced many changes in metabolism processes.

CONCLUSIONS

Study on lignin biosynthesis and gene expression of mangosteen fruit after impact can be concluded as following:

1. Pericarp hardening in mangosteen fruit after impact was associated with an increase in lignin biosynthesis.

2. Firmness, lignin content and G and S lignins in pericarp increased rapidly after impact.

3. Total free phenolic content decreased rapidly in damaged pericarp following impact.

4. N_2 delayed an increase in firmness and reduced lignin contentin impacted pericarp.

5. *GmF5H* expression increased after impact correlating with fruit firmness and lignin content.

6. Transcription factor R2R3*GmMYB*30 involved in impact response that is one of potential regulators of the pericarp hardening in mangosteen after impact.

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APPENDIX

Appendix Table 1 Pericarp firmness of mangosteen fruit after impact from a height of 0 and 100 cm at 0, 5, 10, 15, 20, 25, and 30 min as well as 1, 2, 3, and 24 h after impact.

Height	Time after impact (min)										
	0	5	10	15	20	25	30	60	120	180	1 day
0 cm	9.31	8.95	8.95	9.93	8.69	9.41	8.53	8.98	9.12	8.43	7.97
100 cm	7.38	9.48	7.91	8.72	9.61	9.77	11.46	14.18	14.80	18.35	28.22
t-test	ns	ns	ns	ns	ns	ns	**	**	*	**	**

ns = non-significantly different

* = significantly different at $P \leq 0.05$

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

Appendix Table 2 Pericarp firmness of mangosteen fruit after impact from a height of 100 cm at 0, 1, 2, 3, 4, 5, 6, 12, 18 and 24 h after impact.

Atmosphere	Time after impact (h)									
	0	1	2	3	4	5	6	12	18	24
Air	7.78	11.70	13.10	16.37	19.90	23.12	21.57	28.33	28.84	29.25
Nitrogen	9.20	9.57	10.33	10.69	11.53	11.70	12.19	21.14	22.39	24.23
t-test	ns	ns	*	*	**	**	**	**	*	ns

ns = non-significantly different

* = significantly different at $P \leq 0.05$

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

Appendix Table 3 Lignin contents of mangosteen fruit after impact from a height of 0 and 100 cm at 0, 5, 10, 15, 20, 25, and 30 min as well as 1, 2, 3 and 24 h after impact.

Height	Time after impact (min)										
	0	5	10	15	20	25	30	60	120	180	1 day
0 cm	0.15	0.15	0.15	0.21	0.17	0.14	0.14	0.15	0.19	0.17	0.18
100 cm	0.13	0.16	0.20	0.20	0.19	0.23	0.19	0.28	0.34	0.32	0.44
t-test	ns	ns	*	ns	ns	*	ns	*	**	*	**

ns = non-significantly different

* = significantly different at $P \leq 0.05$

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

Appendix Table 4 Lignin contents of mangosteen fruit after impact from a height of 100 cm at 0, 1, 2, 3, 4, 5, 6, 12, 18 and 24 h after impact.

Atmosphere	Time after impact (h)									
	0	1	2	3	4	5	6	12	18	24
Air	0.17	0.40	0.36	0.36	0.41	0.39	0.45	0.45	0.38	0.43
Nitrogen	0.10	0.15	0.14	0.16	0.15	0.17	0.14	0.19	0.17	0.20
t-test	ns	**	**	*	**	**	*	*	**	*

ns = non-significantly different

* = significantly different at $P \leq 0.05$

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

Appendix Table 5 Total free phenolic contents of mangosteen fruit after impact from a height of 0 and 100 cm at 0, 5, 10, 15, 20, 25, and 30 min as well as 1, 2, 3, and 24 h after impact.

Height	Time after impact (min)										
	0	5	10	15	20	25	30	60	120	180	1 day
0 cm	10.64	10.32	9.27	10.16	11.97	9.24	10.37	10.62	10.47	10.67	10.57
100 cm	11.20	9.93	10.69	9.58	9.44	8.85	9.54	6.71	6.45	7.27	4.25
t-test	ns	ns	ns	ns	ns	ns	ns	**	**	**	**

ns = non-significantly different

* = significantly different at $P \leq 0.05$

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

Appendix Table 6 Total free phenolic contents of mangosteen fruit after impact from a height 100 cm at 0, 1, 2, 3, 4, 5, 6,12, 18 and 24 h after impact.

Atmosphere	Time after impact (h)									
	0	1	2	3	4	5	6	12	18	24
Air	8.36	7.46	6.55	6.30	6.03	4.95	5.91	4.08	3.86	5.44
Nitrogen	8.4	7.42	6.69	7.20	5.93	5.66	5.99	6.184	4.97	4.23
t-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

ns = non-significantly different

* = significantly different at $P \leq 0.05$

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

Appendix Table 7 Changes in lignin monomer composition vanillin in mangosteen fruit after impact from a height of 0 and 100 cm at 0, 15, 30, 60, 120 and 180 min after impact.

Height	Time after impact (min)					
	0	15	30	60	120	180
0 cm	113.52	95.25	93.161	44.82	87.68	68.59
100 cm	97.53	86.03	133.45	86.59	154.64	128.83
t-test	ns	ns	ns	ns	ns	ns

ns = non-significantly different

Appendix Table 8 Changes in lignin monomer composition vanillic acid in mangosteen fruit after impact from a height of 0 and 100 cm at 0, 15, 30, 60, 120 and 180 min after impact.

Height	Time after impact (min)					
	0	15	30	60	120	180
0 cm	11.464	14.86	16.053	13.5	13.72	13.522
100 cm	17.072	14.706	14.883	14.144	18.378	20.929
t-test	ns	ns	ns	ns	**	**

ns = non-significantly different

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

Appendix Table 9 Changes in lignin monomer composition G lignin in mangosteen fruit after impact from a height of 0 and 100 cm at 0, 15, 30, 60, 120 and 180 min after impact.

Height	Time after impact (min)					
	0	15	30	60	120	180
0 cm	124.98	110.11	109.21	58.001	101.4	81.348
100 cm	114.61	100.74	148.64	99.956	172.34	160.27
t-test	ns	ns	*	ns	ns	ns

ns = non-significantly different

* = significantly different at $P \leq 0.05$

Appendix Table 10 Changes in lignin monomer composition syringaldehyde lignin in mangosteen fruit after impact from a height of 0 and 100 cm at 0, 15, 30, 60, 120 and 180 min after impact.

Height	Time after impact (min)					
	0	15	30	60	120	180
0 cm	55.354	49.945	64.279	39.917	49.379	44.144
100 cm	54.395	38.474	68.989	55.19	90.323	76.041
t-test	ns	**	ns	ns	*	ns

ns = non-significantly different

* = significantly different at $P \leq 0.05$

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

Appendix Table 11 Changes in lignin monomer composition syringic acid lignin in mangosteen fruit after impact from a height of 0 and 100 cm at 0, 15, 30, 60, 120 and 180 min after impact.

Height	Time after impact (min)					
	0	15	30	60	120	180
0 cm	8.52	8.9373	7.291	8.421	7.8323	7.5483
100 cm	13.234	15.198	10.805	10.91	13.316	14.794
t-test	ns	ns	ns	ns	**	**

ns = non-significantly different

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

Appendix Table 12 Changes in lignin monomer composition S lignin in mangosteen fruit after impact from a height of 0 and 100 cm at 0, 15, 30, 60, 120 and 180 min after impact

Height	Time after impact (min)					
	0	15	30	60	120	180
0 cm	64.823	58.882	71.57	48.338	57.653	51.692
100 cm	68.268	53.75	79.793	66.099	103.64	90.834
t-test	ns	ns	ns	ns	**	**

ns = non-significantly different

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

Appendix Table 13 Changes in lignin monomer composition G+S lignin in mangosteen fruit after impact from a height of 0 and 100 cm at 0, 15, 30, 60, 120 and 180 min after impact

Height	Time after impact (min)					
	0	15	30	60	120	180
0 cm	189.81	170.48	180.78	109.59	159.05	128.22
100 cm	181.76	154.49	231.95	166.06	282.96	251.1
t-test	ns	ns	ns	**	ns	**

ns = non-significantly different

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

Appendix Table 14 Changes in lignin monomer composition vanillin in mangosteen fruit after impact from a height of 100 cm at 0, 3, 6 and 24 h after impact.

Atmosphere	Time after impact (h)			
	0	3	6	24
Air	81.533	66.489	97.301	105.03
Nitrogen	75.395	58.338	70.696	52.123
t-test	ns	ns	ns	**

ns = non-significantly different

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

Appendix Table 15 Changes in lignin monomer composition vanillic acid in mangosteen fruit after impact from a height of 100 cm at 0, 3, 6 and 24 h after impact.

Atmosphere	Time after impact (h)			
	0	3	6	24
Air	13.963	18.065	15.66	16.034
Nitrogen	15.952	16.725	13.526	12.583
t-test	ns	ns	ns	ns

ns = non-significantly different

Appendix Table 16 Changes in lignin monomer composition G lignin in mangosteen fruit after impact from a height of 100 cm at 0, 3, 6 and 24 h after impact.

Atmosphere	Time after impact (h)			
	0	3	6	24
Air	95.495	84.553	112.96	121.07
Nitrogen	91.347	75.063	84.222	64.707
t-test	ns	ns	ns	**

ns = non-significantly different

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

Appendix Table 17 Changes in lignin monomer composition syringaldehyde in mangosteen fruit after impact from a height of 100 cm at 0, 3, 6 and 24 h after impact.

Atmosphere	Time after impact (h)			
	0	3	6	24
Air	56.902	45.87	53.194	56.865
Nitrogen	44.336	34.548	36.919	29.08
t-test	ns	ns	ns	ns

ns = non-significantly different

Appendix Table 18 Changes in lignin monomer composition syringic acid in mangosteen fruit after impact from a height of 100 cm at 0, 3, 6 and 24 h after imapct.

Atmosphere	Time after impact (h)			
	0	3	6	24
Air	9.346	10.509	9.3717	10.497
Nitrogen	8.25	7.6763	6.482	5.6583
t-test	ns	ns	ns	ns

ns = non-significantly different

Appendix Table 19 Changes in lignin monomer composition S lignin in mangosteen fruit after impact from a height of 100 cm at 0, 3, 6 and 24 h after impact.

Atmosphere	Time after impact (h)			
	0	3	6	24
Air	66.248	56.379	62.566	67.362
Nitrogen	52.586	42.224	43.402	34.738
t-test	ns	ns	*	*

ns = non-significantly different

* = significantly different at $P \leq 0.05$

Appendix Table 20 Changes in lignin monomer composition G+S lignin in mangosteen fruit after impact from a height of 100 cm at 0, 3, 6 and 24 h after impact.

Atmosphere	Time after impact (h)			
	0	3	6	24
Air	161.74	140.93	175.53	188.43
Nitrogen	143.93	117.29	127.62	99.445
t-test	ns	ns	ns	**

ns = non-significantly different

* = significantly different at $P \leq 0.05$

```
   1 cactatgtta aggttagaaa agtttgcact cttgagcttt tcactgccaa gaggcttgaa
  61 gggttaagac ccattagaga agatgaggtc gctgccatgg ttgaatctat cttcaaggat
 121 tgcaatactc ctgaaggcca tggaaagagc ctgcaagtga ggaagtactt gggagtagta
 181 gccttgaaca acataacaag gatagcattt ggaaagcgat tcatgaatga tgaaggaata
 241 atggatgagc aagggttaga gttcagggcc attggagcca atgggcttaa gctcggtgcg
 301 acacttgcca tggcagagca catcccatgg ctacgctgga tgttcccact tgaagaagag
 361 gcatttgcaa aacatggggc tcgtagagat cgtctcacta gagctattat ggaagaacac
 421 acaattgctc gccaacaaag tgggggtgcc aaacaacact ttgtggatgc attgcttact
 481 ttgaaagata agtatgatct tagtgaggac actatcattg gacttctttg ggacatgata
 541 actgcaggca tggacactgt tgcaataaca gtggaatggg caatggctga actaatcaag
 601 aatccaagag tgcaacaaaa ggctcaagag gagctagacc gcgtagttgg cttagagcgt
 661 gtcatgaccg aggctgattt cttgagcctc ccatacttac aatgcgtagc caaagaatca
 721 ctaagattcc accctccaac ccctctgatg cttccccacc gcgccaatgc taatgtcaaa
 781 gtcgctggtt atgacatacc caaaggatca aatgtgcatg tcaatgtgtg ggctattgct
 841 cgcgatcctg ctgtctggaa aaacccatta gagtttcgac cagagaggtt ccttgaggaa
 901 gatgttgaca tgaaaggtca cgatttcagg ctactccctt tcggtgctgg aagaagagtg
 961 tgccctggtg cgcaacttgg tatcaacttg gtcacatcca tgcttggaca tttgttgcac
1021 cgtttttgct ggacacctcc tgacggcgtg aagccagaag agattgacat ggccgaaaat
1081 ccggggttag tcacttacat gagggctcca ttacaagcag tggccactcc taggatgcct
1141 tctcacttgt acaaacgcgt tcctgcagat ttgtaatttt atatttggta agaccagctt
1201 taagcttgct gcttcgcaat ttgatgtttg gtttatggaa aagttgaaaa ctgctgaaaa
1261 tcagaccata ttatgttttg ccaatgtttg agtcattttt ttttgtttga tgtccattta
1321 acatgctgat gctattagca actccaagtt gctattgtaa taaccttatt cacacctcta
1381 acggacgtac aaaaatgctc tcgtattctc tttaaaaaaa aaaaaa
```

Appendix Figure 1 The partial nucleotide sequences of *GmC3H* cDNA fragment (Accession no. KJ671469).

```
YVKVRKVCTLELFTAKRLEGLRPIREDEVAAMVESIFKDCNTPEGHGKSLQVRKYLGV
VALNNITRIAFGKRFMNDEGIMDEQGLEFRAIGANGLKLGATLAMAEHIPWLRWMFPL
EEEAFAKHGARRDRLTRAIMEEHTIARQQSGGAKQHFVDALLTLKDKYDLSEDTIIGL
LWDMITAGMDTVAITVEWAMAELIKNPRVQQKAQEELDRVVGLERVMTEADFLSLPYL
QCVAKESLRFHPPTPLMLPHRANANVKVAGYDIPKGSNVHVNVWAIARDPAVWKNPLE
FRPERFLEEDVDMKGHDFRLLPFGAGRRVCPGAQLGINLVTSMLGHLLHRFCWTPPDG
VKPEEIDMAENPGLVTYMRAPLQAVATPRMPSHLYKRVPADL
```

Appendix Figure 2 The partial deduced amino acid sequences of *GmC3H* cDNA fragment.

```
Mangosteen          ------------------------------------------------------------
Eucalyptus          MALPLILLSIPLLFLLLAHQLYQRLRFKLPPGPRAWPVVGNLYDIKPVRFRCFAEWSQAY 60
Poplar              --MNLLLIPISFITILLTYKIYQRLRFKLPPGPRPWPIVGNLYDVKPVRFRCFAEWAQAY 58
Red_clover          -MALFLTIPLSLITIFLFYTLFQRLRFKLPPGPRPWPVVGNLYDIKPVRFRCFAEWAQSY 59
Kenaf               -MGP-LVITISIFALFLAFKLYQRLRFKLPPGPRAWPVVGNLYDVKPVRFRCYAEWAQAY 58
Loblolly_pine       ------------------------------------------------------------

Mangosteen          ------------------------------------------------------------
Eucalyptus          GPIISVWFGSTLNVVVSSSELAKEVLKENDQQLADRHRSRSAAKFSRDGQDLIWADYGPH 120
Poplar              GPIISVWFGSTLNVIVSNTELAKEVLKENDQQLADRHRSRSAAKFSRDGKDLIWADYGPH 118
Red_clover          GPIISVWFGSTLNVIVSNSELAKEVLKEKDQQLADRHRSRSAAKFSRDGQDLIWADYGPH 119
Kenaf               GPVISVWFGSTLNVIVSNTELAREVLKERDQQLADRHRTRSAAKFSRDGQDLIWADYGPH 118
Loblolly_pine       ------------------------------------------------------------

Mangosteen          YVKVRKVCTLELFTAKRLEGLRPIREDEVAAMVESIFKDCNTPEGHGKS--LQVRKYLGV 58
Eucalyptus          YVKVRKVCTLELFTPKRLEALRPIREDEVTAMVESIFKDCTNPDNSGKT--LLVKKYLGA 178
Poplar              YVKVRKVCTLELFSPKRLEALRPIREDEVTAMVESIFNDCTHPENNGKT--LMVKKYLGA 176
Red_clover          YVKVRKVCTLELFSPKRIEALRPIREDEVTAMVESIFNDSTNSENLGKG--ILMRKYIGA 177
Kenaf               YVKVRKVCTLELFSPKRLEALRPIREDEVTAMVESIFIDSTNPDAGSKGTSLLVRKYLGA 178
Loblolly_pine       ------------------------------------------------------------

Mangosteen          VALNNITRIAFGKRFMNDEGIMDEQGLEFRAIGANGLKLGATLAMAEHIPWLRWMFPLEE 118
Eucalyptus          VAFNNITRLAFGKRFMNAEGVIDEQGLEFKAIVSNGLKLGASLAMAEHIPWLRWMFPLEE 238
Poplar              VAFNNITRLAFGKRFQNAEGVMDEQGLEFKAIVSTGLKLGASLAMAEHIPWLRWMFPLEE 236
Red_clover          VAFNNITRLAFGKRFVNSEGVMDEQGVEFKAIVANGLKLGASLAMAEHIPWLRWMFPLEE 237
Kenaf               VAFNNITRLAFGKRFVNSEGIMDEQGHEFKAIVANGLKLGASLAMAEHIPWLRWMFPLEE 238
Loblolly_pine       VAFNNITRLAFGKRFVNEEGKMDPQGVEFKEIVATGLKLGASLTMAEHIPYLRWMFPLEE 60
                    **:*****:****** * ** :* ** **: * :.******:*:******:*********

Mangosteen          EAFAKHGARRDRLTRAIMEEHTIARQQSGGAKQHFVDALLTLKDKYDLSEDTIIGLLWDM 178
Eucalyptus          EAFAKHSARRDRLTRAIMEEHTVARQKSG-AKQHFVDALLTLKDKYDLSEDTIIGLLWDM 297
Poplar              DAFAKHGARRDRLTRAIMDEHTLARQTSGGAKQHFVDALLTLQEKYDLSEDTIIGLLWDM 296
Red_clover          EAFAKHGARRDRLTRAIMDEHTQARQKSGGAKQHFVDALLTLQDKYDLSEDTIIGLLWDM 297
Kenaf               EAFAKHGARRDRLTRAIMDEHTLARQKSGGAKQHFVDALLTLQEKYDLSEDTIIGLLWDM 298
Loblolly_pine       GAFAKHGARRDNVTKAIMEEHTLARQTSG-AKQHFVDALLTLQEKYDLSEDTIIGLLWDM 119
                     *****.****.:*:***:*** *** ** ************::****************

Mangosteen          ITAGMDTVAITVEWAMAELIKNPRVQQKAQEELDRVVGLERVMTEADFLSLPYLQCVAKE 238
Eucalyptus          ITAGMDTTAISVEWAMAELIKNPRVQQKAQEELDRVVGFERVVTEPDFSNLPYLQCIAKE 357
Poplar              ITAGMDTTAISVEWAMAELIKNPRVQQKAQEEVDSVVGFERVMTEADFSGLPYLQCVAKE 356
Red_clover          ITAGMDTTAISVEWAMAELIKNPRVQKKAQEELDKVIGFERVMTETDFSSLPYLQSVAKE 357
Kenaf               ITAGMDTTAISVEWAMAELIRNPRVQQKAQEELDRVIGFERVMSETDFSSLPYLQSVAKE 358
Loblolly_pine       ITAGMDTTAITVEWAMAELVRNPRIQQKAQEEIDRVVGRDRVMNETDFPHLPYLQCITKE 179
                    *******.**:********::***:*:*****:* *:* :**:.*.**  *****.::**
```

Appendix Figure 3 Alignment of partial deduced amino acid sequence of *GmC3H* cDNA fragment with other plants, poplar (accession no AFZ78540), red clover (accession no ACX48910), eucalyptus (accession no ADG08112), kenaf (accession no AGA60530) and loblolly pine (accession no AAV36205). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been conserved. The dot (.) means that the semi-conserved substitutions are observed.

```
Mangosteen          SLRFHPPTPLMLPHRANANVKVAGYDIPKGSNVHVNVWAIARDPAVWKNPLEFRPERFLE 298
Eucalyptus          ALRLHPPTPLMLPHRSNSHVKIGGYDIPKGSNVHVNVWAIARDPAVWNSPLEFRPERFLE 417
Poplar              ALRLHPPTPLMLPHRANANVKVGGYDIPKGSNVHVNVWAVARDPAAWKNPLEFRPERFLE 416
Red_clover          ALRLHPPTPLMLPHRANANVKIGGYDIPKGSNVHVNVWAVARDPAVWKNATEFRPERFLE 417
Kenaf               ALRLHPPTPLMLPHRANANVKIGGYDIPKGSNVHVNVWAVARDPAVWKEPEEFRPERFLE 418
Loblolly_pine       ALRLHPPTPLMLPHKATQNVKIGGYDIPKGSNVHVNVWAIARDPAVWKDPLTFRPERFLE 239
                    :**:**********::. :**:.***************:*****.*:..  ********

Mangosteen          EDVDMKGHDFRLLPFGAGRRVCPGAQLGINLVTSMLGHLLHRFCWTPPDGVKPEEIDMAE 358
Eucalyptus          EDVDMKGHDFRLLPFGAGRRVCPGAQLGINLVTSMLGHLLHHFVWTPPQGTKPEEIDMSE 477
Poplar              EDVDMKGHDFRLLPFGAGRRVCPGAQLGINLVTSMLGHLLHHFCWTPPEGMKPEEIDMSE 476
Red_clover          EDVDMKGHDFRLLPFGAGRRVCPGAQLGINMVTSMLGHLLHHFCWAAPEGVNPEDIDMTE 477
Kenaf               EDVDMKGHDFRLLPFGAGRRVCPGAQLGINLVTSMLGHLLHHFCWTPAEGVKAEEIDMLE 478
Loblolly_pine       EDVDIKGHDYRLLPFGAGRRICPGAQLGINLVQSMLGHLLHHFVWAPPEGMQAEDIDLTE 299
                    ****:****:**********:*********:* ********:* *:..:* :.*:**: *

Mangosteen          NPGLVTYMRAPLQAVATPRMPSHLYKRVPADL 390
Eucalyptus          NPGLVTYMSTPVQAVATPRLPSELYKRVPYEM 509
Poplar              NPGLVTYMRTPLQAVATPRLPSHLYKRVAVDI 508
Red_clover          NPGMVTYMRTPLQVVASPRLPSELYKRVPADI 509
Kenaf               NPGLVAYMRTPLQAMATPRLPSHLYKRVAVDI 510
Loblolly_pine       NPGLVTFMAKPVQAIAIPRLPDHLYKRQPLN- 330
                    ***:*::*  *:*.:* **:*..**** . :
```

Appendix Figure 3 (Continued).

```
   1 gtaccgtttt atcctatggc cggcaggctt cggagggacg acgacggtcg tattgagata
  61 tactgtaacg gagaaggggt gctatttgtc gaggccgaaa ccacgtcggt tattgatgat
 121 ttcggtgact tcgcgcctac tccggacctc aagcagctta ttccctccat cgattactcc
 181 ggcgacatcg ggagctttcc tctcctcgtt ttgcaggtga cgtacttcaa atgtggtggt
 241 gtctcacttg gtgttggtat gcagcaccac gtggcagatg ggtattcagg cctgcacttt
 301 gtgaatacct ggtccgacat ggctcgtggt cttgacatta ccattccacc attcatcgac
 361 cggactctcc ttcgtgccag agaccctcct caaccagcat tccagcacat tgagtatcag
 421 ccacctccac cattgaaatc cccttcacaa accttaaaac cagatagcac aaaagttgcc
 481 attttcaagt tttccaaggg ccaaattagt gccctcaaag ccaaggcaaa ggaagctgga
 541 aacacagtaa gctacagctc gtatgagatg ttggctggtc atgtgtggag atgcacgtgc
 601 aaggcgcgag gacttgctga tgaccaagaa accaaactgt atatcgcaac cgatggtcgt
 661 tcgaggttgc gccctccgct tccgccaggt tacttcggca atgtcatatt tacagctaca
 721 ccaattgcag tagcgggcga tcttcaatcg aagcccactt ggtatgctgc gagtaggatt
 781 catggtgctt tgtctcgtat ggacaatgag tacttaaggt cagcccttga ttaccttgaa
 841 cttcagcctg atttgtctgc tcttgttcgg ggagctcata cattcaagtg tccaaatctt
 901 gggatcacaa gctgggttag gctgccaatt cacgatgcgg atttcggttg ggggaggcct
 961 atattcatgg ggcctggcgg gattgcgtac gagggcttgt catttatcct gcctagtgct
1021 actattgatg ggagtatgtc agtggcaatt gccttgcaat ccgagcacat gaaactgttc
1081 gagaagctct tatatgacat ttaagaaacc aatgttctct ctgtttttat ttgtcccttc
1141 tgttaaatct gtttatgttt gttcagtgtt cacatcctta aaggaagaag ctctgcgtaa
1201 aaatgtaacc tgtagagcac ccatatgtta ctttctgtct gcatactaat gaactttgga
1261 aactcaaatt atagatctaa ttggatacaa gtacagctgt tattaagaaa aaaaaaaaaa
1321 aaaaa
```

Appendix Figure 4 The partial nucleotide sequences of *GmHCT* cDNA fragment (Accession no. KJ671470).

```
PFYPMAGRLRRDDDGRIEIYCNGEGVLFVEAETTSVIDDFGDFAPTPDLKQLIPSIDY
SGDIGSFPLLVLQVTYFKCGGVSLGVGMQHHVADGYSGLHFVNTWSDMARGLDITIPP
FIDRTLLRARDPPQPAFQHIEYQPPPPLKSPSQTLKPDSTKVAIFKFSKGQISALKAK
AKEAGNTVSYSSYEMLAGHVWRCTCKARGLADDQETKLYIATDGRSRLRPPLPPGYFG
NVIFTATPIAVAGDLQSKPTWYAASRIHGALSRMDNEYLRSALDYLELQPDLSALVRG
AHTFKCPNLGITSWVRLPIHDADFGWGRPIFMGPGGIAYEGLSFILPSATIDGSMSVA
IALQSEHMKLFEKLLYDI
```

Appendix Figure 5 The partial deduced amino acid sequences of *GmHCT* cDNA fragment.

```
Kenaf           MIINVKESTMVQPAGETPRRSLWNANVDLVVPRFHTPSVYFYRPTGAANFFDPQVMKEAL 60
Cucumber        MIITVKDSTMVTPAEETPHKSLWNSNVDLVVPSMHTPSVYFYRPTGDSNFFDAEVLKEGL 60
Poplar          MIINVKESTMVQPAEETPRRGLWNSNVDLVVPRFHTPSVYFYRPTGAPNFFDAKVLKGAL 60
Coffee          MKIEVKESTMVRPAHETPRRNLWNSNVDLVVPNFHTPSVYFYRPTGSSNFFDAKVLKDAL 60
Mangosteen      ------------------------------------------------------------
Pine            MLITVKNSQMVRPAAPTPQRDLWNSNVDLVVPRIHTASVYFYRPTGSPDFFSMNILRDAL 60


Kenaf           SKALVPFYPMAGRLKRDEDGRIEIDCNAEGVLFVEAETTSVIDDFGDFAPTLELRQLIPT 120
Cucumber        SKALVPFYPMAGRLRRDEDGRIEIYCNAEGVLLVEAETTAVIDDFGDFAPTLQLRQLIPA 120
Poplar          SKALVPFYPMAGRLRRDEDGRIEINCNAEGVLFVEAETTSVIDDFADFAPTLELKQLIPT 120
Coffee          SRALVPFYPMAGRLKRDEDGRIEIECNGEGVLFVEAESDGVVDDFGDFAPTLELRRLIPA 120
Mangosteen      -----PFYPMAGRLRRDDDGRIEIYCNGEGVLFVEAETTSVIDDFGDFAPTPDLKQLIPS 55
Pine            SKLLVPFYPMAGRLKRDPDGRIEINCNGEGVLLVEAITDSVIDDFGDFAPTMELKQLIPK 120
                     *********:** ****** **.****:*** : .*:***.***** :*::***


Kenaf           VDYSGGISTYPLLVLQVTYFKCGGASLGVGMQHHAADGYSGLHFINTWSDMARGLDLTIP 180
Cucumber        VDYSGGIESYPLLVLQVTYFKCGGVSLGVGMQHHVADGYSGLHFVNTWSDMSRGLDLMLQ 180
Poplar          VDYSGGISTYPLLVLQVTYFKCGGVSLGVGMQHHAADGFSGLHFVNTWSDMARGLDLTIP 180
Coffee          VDYSQGISSYALLVLQVTYFKCGGVSLGVGMQHHAADGFSGLHFINSWSDMARGLDVTLP 180
Mangosteen      IDYSGDIGSFPLLVLQVTYFKCGGVSLGVGMQHHVADGYSGLHFVNTWSDMARGLDITIP 115
Pine            VNYSEDISSYPLLVLQVTFFKCGGVSLGVGMQHHVADGYAGIHFINTWSDVARGLDITLP 180
                ::** .* ::.*******:*****.*********.***::*:**:*:***::****: :


Kenaf           PFIDRTLLRARDPPQPAFHHIEYQPPPALNTPPQSTG---PESTAVSIFKLTREQLNALK 237
Cucumber        PYIDRTLLRARDPPQPAFRHVEYQPAPPMKNPVQAD----PEGTTVSIFKFTREQLNLLK 236
Poplar          PFIDRTLLRARDPPQPVFHHVEYQPPPSMKTVLETSK---PESTAVSIFKLSRDQLSTLK 237
Coffee          PFIDRTLLRARDPPQPQFQHIEYQPPPTLKVSPQTAKSDSVPETAVSIFKLTREQISALK 240
Mangosteen      PFIDRTLLRARDPPQPAFQHIEYQPPPPLKSPSQTLK---PDSTKVAIFKFSKGQISALK 172
Pine            PFIDRTLLRARNPPTPKFQHIEYQQPPPLKDTSGIMNGE-KTDISVAIFKLTKEQLEILK 239
                *:*********:** * *:*:*** .*.::              *:***::: *:. **


Kenaf           AKSKEDGNSVNYSSYEMLSGHVWRSVCKARGLTDDQGTKLYIATDGRSRLRPPLPPGYFG 297
Cucumber        AKSKENGNTINYSSYEMLSGHVWRSTCKARELPEDQDTKLYIATDGRARLRPPLPNGYFG 296
Poplar          AKAKEDGNNISYSSYEMLAAHVWRSTCKARELPDDQETKLYIATDGRSRLRPQLPPGYFG 297
Coffee          AKSKEDGNTISYSSYEMLAGHVWRCACKARGLEVDQGTKLYIATDGRARLRPSLPPGYFG 300
Mangosteen      AKAKEAGNTVSYSSYEMLAGHVWRCTCKARGLADDQETKLYIATDGRSRLRPPLPPGYFG 232
Pine            GKARENGNNIAYSSYEMLSGHIWRCACKARNLAEDQETKLYIATDGRNRLRPSIPPGYFG 299
                .*::* **.: *******:.*:**..**** *  ** ********** **** :* ****


Kenaf           NVIFTATPIAVAGDLMLKPTWYAASRIHDALVRMDDEYLRSALDFLELQPDLSALVRGAH 357
Cucumber        NVIFTTTPLAVAGELMSNPTWFAASKIHDALTRMDNDYLRSALDYLEIQPNISALVRGAH 356
Poplar          NVIFTATPIAVAGEMQSKPTWYAAGKIHDALVRMDNDYLKSALDYLELQPDLSALVRGAH 357
Coffee          NVIFTATLIAIAGDLEFKPVWYAASKIHDALARMDNDYLRSALDYLELQPDLKALVRGAH 360
Mangosteen      NVIFTATPIAVAGDLQSKPTWYAASRIHGALSRMDNEYLRSALDYLELQPDLSALVRGAH 292
Pine            NVIFTTTPMAVTGDIISKPTYYAASVIHEALGRMDDEYLRSALDYLELQPDLTALVRGAH 359
                *****:* :*::*::  :*.::**. ** ** ***::**:****:**:**::.*******
```

Appendix Figure 6 Alignment of partial deduced amino acid sequence of *GmHCT* cDNA fragment with other plants, poplar (accession no AFZ78609), kenaf (accession no AFN85668), cucumber (accession no AEJ88365), coffee (accession no ABO40491) and pine (accession no ABO52899). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been conserved. The dot (.) means that the semi-conserved substitutions are observed.

```
Kenaf           TFKCPNLGITSWARLPIHDADFGWGRPIFMGPGGIPYEGLSFVLPSPNNDGSLSVAISLQ 417
Cucumber        TFRCPNLGITSWVRLPIHDADFGWGRPIFMGPGGIAYEGLSFIIPSASDDGSLSVAISLQ 416
Poplar          SFRCPNLGITSWVRLPIHDADFGWGRPIFMGPGGIAYEGLSFILPSPTNDGSMSVAISLQ 417
Coffee          TFKCPNLGITSWVRLPIHDADFGWGRPIFMGPGGIAYEGLSFILPSPTNDGSMSVAISLQ 420
Mangosteen      TFKCPNLGITSWVRLPIHDADFGWGRPIFMGPGGIAYEGLSFILPSATIDGSMSVAIALQ 352
Pine            TFRCPNIGITSWSRLPIHDADFGWGRPIFMGPGGIAYEGLAFVLPSSVNDGSLSVALGLQ 419
                :*:***:***** **********************.****:*::**.  ***:***:.**

Kenaf           TEHMKVFEKLFYDI 431
Cucumber        NRHMKVFEKLFFDI 430
Poplar          AQHMKLFEKFIYDI 431
Coffee          GEHMKLFQSFLYDI 434
Mangosteen      SEHMKLFEKLLYDI 366
Pine            PDHMVRFAKMLYEI 433
                  **  * .::::*
```

Appendix Figure 6 (Continued.)

```
  1 tatcctatgg ttcctgggca tgaggtcgtt gctgaggtga tagaggtggg atccagtgtg
 61 agcaagttca aggtagggga catagttggg gttggtttga tcgttggatc atgcagaaat
121 tgtaatcctt gcaatacaga cattgaacaa tattgtaaga acaaaatatg gtcctacaac
181 gacgtttaca ccgatggtac accaactcaa ggtggattcg ctcaatctat ggtcgtagac
241 caaaaatttg caatgaaaat cccagatgga atgtcaccag aacaagttgc accactttta
301 tgtgccgggg tgacagtgta tagcccatta agccacttcg gactgaagca gagtggacta
361 agcggaggaa ttttaggcct cggaggggta ggccgtatgg gggtcaagat agctaaggca
421 atgagtcacc atgtaacggt aattagctct tctgataaga agagagatga ggcattggac
481 cacctcggag ctgacgacta cttggttagc tccgacgagg ctcagatgca agctgcggct
541 gattcattag attatatcat tgatactgtg cctgtgttcc acccccttga gccatacctt
601 tcattgttga agcttgatgg gaagttgatc ttaatgggtg ttattaataa gcctctccaa
661 tttttgaccc cagttgttat gcaagggagg aaaacaataa ctgggacctt cattggcagc
721 atcaaggaaa cagaagagat gcttgaattt tgcaaagaaa agggactgac tcccactatt
781 gaagtgatta aaatggacta tattaacaca gcatttgaaa ggcttgcaaa gaacgatgtt
841 agataccggt ttgttgtgga tgtcgcagga agcaagtttg aataataaat aattaaggag
901 agcaacccca aattggactt caatatactg ataattctaa tggacttcaa tttactattt
961 gagtgataaa aagaaaaaaa aaaaaaaaaa aaaaaaaaaa
```

Appendix Figure 7 The partial nucleotide sequences of *GmCAD* cDNA fragment (Accession no. KJ671471).

```
PMVPGHEVVAEVIEVGSSVSKFKVGDIVGVGLIVGSCRNCNPCNTDIEQYCKNKIWSY
NDVYTDGTPTQGGFAQSMVVDQKFAMKIPDGMSPEQVAPLLCAGVTVYSPLSHFGLKQ
SGLSGGILGLGGVGRMGVKIAKAMSHHVTVISSSDKKRDEALDHLGADDYLVSSDEAQ
MQAAADSLDYIIDTVPVFHPLEPYLSLLKLDGKLILMGVINKPLQFLTPVVMQGRKTI
TGTFIGSIKETEEMLEFCKEKGLTPTIEVIKMDYINTAFERLAKNDVRYRFVVDVAGS
KFE
```

Appendix Figure 8 The partial deduced amino acid sequences of *GmCAD* cDNA fragment.

```
Eucalyptus      ---------MGSLEKERTTTGWAARDPSGVLSPYTYSLRNTGPEDLYIKVLSCGICHSDI 51
Cotton          ---------MGGLETERTTTGWAARDPSGVLSPYTYTLRSTGPEDVFVKVMCCGICHTDL 51
Kenaf           MQFFDQRPKMGSLETERKTTGWAARDPSGILSPYTYSLRNTGPEDVFIKVICCGICHTDI 60
Mangosteen      ------------------------------------------------------------
Poplar          ---------MGSLETERKIVGWAATDSTGHLAPYTYSLRDTGPEDVFIKVISCGVCHTDI 51


Tobacco         HQVKNDLGMSNYPMVPGHEVVGEVVEVGSNVTKFNVGDTVGVGVLVGCCRSCRPCNQDIE 111
Eucalyptus      HQIKNDLGMSHYPMVPGHEVVGEVLEVGSEVTKYRVGDRVGTGIVVGCCRSCGPCNSDQE 111
Cotton          HQAKNDLGMSNYPMVPGHEVVGEVLEVGSDVSKFRVGDIVGVGCIVGCCRNCRPCDSDNE 111
Kenaf           HQAKNDLGMSNYPMVPGHEVVGEVVEVGSDVAKFAAGEIVGVGLLVGCCRNCRPCDTDRE 120
Mangosteen      ------------PMVPGHEVVAEVIEVGSSVSKFKVGDIVGVGLIVGSCRNCNPCNTDIE 48
Poplar          HQIKNDLGMSXYPMVPGHEVVGEVVEVGSDVTKFKVGDVVGVGVIVGSCKNCNPCKSEIE 111
                            ********* ** **** * *   *  ** *  ** *  * **    *

Tobacco         QYCNKRIWSYNDVYSDGKPTQGGFATSMVVDQKFVVKIPDGMSPEQAAPLLCAGLTVYSP 171
Eucalyptus      QYCNKKIWNYNDVYTDGKPTQGGFAGEIVVGQRFVVKIPDGLESEQAAPLMCAGVTVYSP 171
Cotton          QYCLKKIWSYNDVYTDGKPTQGGFAGSMVVDQKFVVNIPEGMAPEQVAPLLCAGVTVYSP 171
Kenaf           QYCAKKIYSYNDVYTDGNPTRGGFAGSMVVDQKFVVKIPDGMAPEQVAPLLCAGITVYSP 180
Mangosteen      QYCKNKIWSYNDVYTDGTPTQGGFAQSMVVDQKFAMKIPDGMSPEQVAPLLCAGVTVYSP 108
Poplar          QYCNKKIWSYNDVYTDGKPTQGGFAESMVVDQKFVVKIPDGMSPEQAAPLLCAGLTVYSP 171
                ***   *  ***** ** ** ****   ** * *   ** *   ** *** *** *****

Tobacco         LNHFGLKQSGLRGGILGLGGVGHMGVKIAKAMGHHITVISSSDRKKEEALEHLGADEYLV 231
Eucalyptus      LVRFGLKQSGLRGGILGLGGVGHMGVKIAKAMGHHVTVISSSDKKRTEALEHLGRDAYLV 231
Cotton          LNHFGLMGSGLRGGILGLGGVGHMGVKIAKAMGHHVTVISSSDKKKVEALEHLGADDYLV 231
Kenaf           LNHFGLTASGLSGGVLGLGGVGHMGVKIAKAMGHHVTVISSSDKKKLEALEYLGADEYLV 240
Mangosteen      LSHFGLKQSGLSGGILGLGGVGRMGVKIAKAMSHHVTVISSSDKKRDEALDHLGADDYLV 168
Poplar          LKHFGLKQSGLRGGILGLGGVGHMGVKIAKAMGHHVTVISSSDKKREEAMEHLGADEYLV 231
                *  ***  *** ** ******* ********* ** ******* *  **   ** * ***

Tobacco         SSDTTRMQEAADSLDYIIDTIPVFHPLEPYISLLKLDGKLILMGVINTPMQFITPMVMLG 291
Eucalyptus      SSDENGMKEATDSLDYIFDTIPVVHPLEPYLALLKLDGKLILTGVINAPLQFISPMVMLG 291
Cotton          SSDAEGMQKAADSLDYIIDTVPVFHPLEPYLSLLKLDGKLILTGVINTPLQFVTPMVMLG 291
Kenaf           STDAEGMQKAPKSLDYIIDTVPVFHPLEPYLSLLRHDGKLILMGVINTPLQFLTPVVILG 300
Mangosteen      SSDEAQMQAAADSLDYIIDTVPVFHPLEPYLSLLKLDGKLILMGVINKPLQFLTPVVMQG 228
Poplar          SSDVEGMQKAADQLDYIIDTVPVVHPLEPYLSLLKLDGKLILMGVINAPLQFVTPMVMLG 291
                * *   *  *   **** ** ** ******  **  ****** **** * **  * *  *

Tobacco         RKTITGSFIGSMKETEEVLEFCKEKGLTSQIEVVKMDYVNTAMERLEKNDVRYRFVIDVA 351
Eucalyptus      RKSITGSFIGSMKETEEMLEFCKEKGLTSQIEVIKMDYVNTALERLEKNDVRYRFVVDVA 351
Cotton          RKSITGSFIGSMKETEEMLNFCKEKNLTSMIEVVKMDYINTAMERLEKNDVRYRFVVDVA 351
Kenaf           RKEITGSFIGSMKETEELLAFCKEKDLRSTIEVVKMDYINTAMARVAKNDVRYRFVVDVA 360
Mangosteen      RKTITGTFIGSIKETEEMLEFCKEKGLTPTIEVIKMDYINTAFERLAKNDVRYRFVVDVA 288
Poplar          RKSITGSFIGSMKETEEMLEFCKEKGLASMIEVIKMDYINTAFERLEKNDVRYRFVVDVA 351
                ** *** **** ***** * ***** *   *** **** ***  *  ********* ***

Tobacco         GSKLDQ-- 357
Eucalyptus      GSKLD--- 356
Cotton          GSKLDQ-- 357
Kenaf           GSKIDQIN 368
Mangosteen      GSKFE--- 293
Poplar          GSKLIP-- 357
                ***
```

Appendix Figure 9 Alignment of partial deduced amino acid sequence of *GmCAD* cDNA fragment with other plants, cotton (accession no ACF71455), tobacco (accession no AFP43764), eucalyptus(accession no AAG15553), kenaf (accession no ADK24218) and poplar (accession no ADN96445). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment.

```
  1 gtttcttgtg ctgatattct cgctctttct gctagggatg gcattgtctt gttgggaggg
 61 ccaaaagtgg aaatgaagac aggtaggaag gatagtaggg aaagctacta caaagtggtg
121 gaagactata ttcccaatca caatgatagc atttctttgg tactttccct cttccaatcc
181 actggcattg atgttgaagc ggcagttgct cttctaggag cccattcagt gggccgagtc
241 cactgtgtca atttagttca aagactttac cctacaatcg atccaacttt ggacccatca
301 tatgccgaat accttaaaac tcgttgtccc agcccaaacc cagacccaaa ggcagtcgaa
361 tacgctagaa atgatcgaga gacacccatg attattgaca acaattacta caagaacttg
421 ctcaacaaaa agggtttgct ctccattgat caacaattaa cttcggatcc aattacatcg
481 ctttacgtca cgaaaatggc cgccgataat gcctacttcc gggcccaatt tgcccgggcc
541 gtgcttttgt tgtccgagaa caatccactt acagaaatcc aaggggagat aagggaagat
601 tgtcgctatg tgaatagcaa ttagctctaa ctaattgaac ttagttcaac tagtaagaac
661 tttagttcct ttctttgagg tctcaaattc gagtcccaaa aatataatgt tgctcaaaaa
721 aaataataat aatagcaatt agctctacta aataacgtgg caaaaaaagt tgccgacgat
781 ttttcaaata cctaaaataa agataatcat ataaaatgta aaatatttga taaaaaaaaa
841 aaaaaaaaaa aaaaaaaaaa aaaaaaaa
```

Appendix Figure 10 The partial nucleotide sequences of *GmPOD* cDNA fragment (Accession no. KJ671472).

```
SCADILALSARDGIVLLGGPKVEMKTGRKDSRESYYKVVEDYIPNHNDSISLVLSLFQ
STGIDVEAAVALLGAHSVGRVHCVNLVQRLYPTIDPTLDPSYAEYLKTRCPSPNPDPK
AVEYARNDRETPMIIDNNYYKNLLNKKGLLSIDQQLTSDPITSLYVTKMAADNAYFRA
QFARAVLLLSENNPLTEIQGEIREDCRYVNSN
```

Appendix Figure 11 The partial deduced amino acid sequences of *GmPOD* cDNA fragment.

```
Canola          ------------------------------------------------------------
Arabidopsis     ----MANAKPFCLLGFFCLLLQLFSIFHIG-----NGELEMNYYKESCPKAEEIIRQQVE 51
Poplar          -MASSNIWTHFCSSTLIVLLLPLLLQFHSG-----KSELQFNYYAQGCPRAEEIIKEQVI 54
Cocoa           MLGKMATDRHHCCSSFIFLLLPLLLQFHSG-----KSDLQLNYYAESCPNAEEIIKQEVI 55
Mangosteen      ------------------------------------------------------------
Camellia        -MGSKVLFFFAILSLSVLFSLNLNLAFAENEEIEEQVGLVMNFYKDTCPQAEEVIREQVK 59


Canola          ------------WLRNLFHDCVVKSCDASLLLETARGVESEQKSTRSFGMRNFKYVKVIK 48
Arabidopsis     TLYYKHGNTAVSWLRNLFHDCVVKSCDASLLLETARGVESEQKSKRSFGMRNFKYVKIIK 111
Poplar          KLYNKHRNTAVSWVRNLFHVCIVKSCDASLLLETVNGIESEKASQRSLGMRNFKYVNTIK 114
Cocoa           KLYDKHGNTAVSWVRNLFHDCMVKSCDASLLLETVNGIQSEQLSDRSFGMRNFKYVKTIK 115
Mangosteen      ------------------------------------------------------------
Camellia        LLYKRHKNTAFSWLRNIFHDCAVTSCDASLLLDSTRRSLSEKETDRSFGLRNFRYLDTIK 119


Canola          DALEKECPSTVSCADIVALSARDGIVMLKGPKIDMIKTGRRDSRGSYLSDVETLVPNHND 108
Arabidopsis     DALEKECPSTVSCADIVALSARDGIVMLKGPKIEMIKTGRRDSRGSYLGDVETLIPNHND 171
Poplar          AAVESECPVTVSCADIVALSARDGIVMLGGPRVEM-KTGRRDSTVSYGAVVEDFIPNHND 173
Cocoa           QALEKECPMTVSCADIVSLSARDGIVLLGGPRIEM-KTGRKDSKESYLTEVENTIPNHND 174
Mangosteen      -----------SCADILALSARDGIVLLGGPKVEM-KTGRKDSRESYYKVVEDYIPNHND 48
camellia        EAVERECPGVVSCSDILVLSARDGIVALGGPYIPL-KTGRRDGRKSRAEVLEQYLPDHNE 178
                          **:**: ******** * ** : : ****:*.  *    :*  :*:**:


Canola          SLSSVLSNFNSMGIDVEATVALLG--------AHSVGRVHCVNLVHRLYPTIDPTLDPDY 160
Arabidopsis     SLSSVLSTFNSIGIDVEATVALLG--------AHSVGRVHCVNLVHRLYPTIDPTLDPSY 223
Poplar          SISLVLSRFQSIGVDVEGTVALLASFVLFLPGSHSVGRVHCVNLVHRIYPTVDPSMDPDY 233
Cocoa           TMELVLSRFQSIGIDTEGTVALLG--------AHSVGRVHCVNLVHRLYPTVDPTLDPHY 226
Mangosteen      SISLVLSLFQSTGIDVEAAVALLG--------AHSVGRVHCVNLVQRLYPTIDPTLDPSY 100
Camellia        SMSVVLERFASIGIDTPGVVALLG--------AHSVGRTHCVKLVHRLYPEVDPVLNPDH 230
                ::. **. * * *:*. ..****.        :*****.***:**:*:** :** ::* :


Canola          ALYLKNRCPSPNPDPNAVLYSRNDRETPMVVDNMYYKNIMAHKGLLVIDDELASDPRTAP 220
Arabidopsis     ALYLKKRCPSPTPDPNAVLYSRNDRETPMVVDNMYYKNIMAHKGLLVIDDELATDSRTAP 283
Poplar          AEYLKGRCPPPDPDPQAVLYARNDRETPMILDSYYYKNLLGHKGLLMVDQQLTSDPLTSP 293
Cocoa           AEYLKGRCPTPDPNPKAVLYARNDRKTPMILDNMYYKNLLEHKGLLLVDQQLTSDPTTSP 286
Mangosteen      AEYLKTRCPSPNPDPKAVEYARNDRETPMIIDNNYYKNLLNKKGLLSIDQQLTSDPITSL 160
Camellia        VEHMLHKCPDPIPDPKAVQYVRNDRGTPMKLDNNYYRNILDNKGLLIVDHQLATDKRTKP 290
                . ::  :** * *:*:** * **** *** :*. **:*:: :**** :*.:*::*  *


Canola          FVAKMAADNGYFHEQFSRGVRLLSETNPLTGDQGEIRKDCRYVNSK- 266
Arabidopsis     FVAKMAADNGYFHEQFSRGVRLLSETNPLTGDQGEIRKDCRYVN--- 327
Poplar          YVEKMAADNGFFHDQVSRAVVLWSENNPLTGNQGEIRKDCRYVNSN- 339
Cocoa           FVEKMAADNGYFHDQFARAVLLLAENNPLTGDQGEVRKDCRYVNSD- 332
Mangosteen      YVTKMAADNAYFRAQFARAVLLLSENNPLTEIQGEIREDCRYVNSN- 206
Camellia        FVKKMAKSQDYFFKEFARAITILSENNPLTGTKGEIRKQCNVANKLH 337
                :* *** .: :*  :.:*.: : :*.****  :**:*::*. .*
```

Appendix Figure 12 Alignment of partial deduced amino acid sequence of *GmPOD* cDNA fragment with other plants, poplar (accession no AGX27515), canola (accession no ACT35471), *Arabidopsis* (accession no CAA66961), camellia (accession no ACT21094) and cocoa (accession no EOY07788). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been conserved. The dot (.) means that the semi-conserved substitutions are observed.

```
  1 agggcaattc ttgaacatgt tgttgaagct aatcaatgcc aaaaacacca tggtgtctac
 61 actggctact ctctcttggc cactgccctt gcccttcctg atgatgccaa gatcctggcc
121 atggacataa acagagagaa ctatgagttg ggactgccca taatccaaaa ggctggtgtc
181 gcccataaga ttgatttcaa agaaggccct gctttgcctg ttcttgacca actaatcgaa
241 gatggcaaaa gtcatggatc atttgacttc atattcgtgg atgctgacaa agacaattac
301 ctaaactacc acaagaggct tatagagctt gttaaagttg gagggctgat tggctacgac
361 aacaccctat ggaatggaac tgtggcggca ccagatgatg ctccaatgag gaagtacatc
421 aaatattata gggactttgt tttggagctt aataagactc ttgctgtgga ccctaggatt
481 gagatttgca tgcttcctgt tggagatggg atcactctct gccgtcggat caagtgattg
541 attaaatgaa atgaagggat gttatattat gtattaatat atatccttac ttttctttat
601 tgttaattgt tcttcattgg gatgtactta atttccatgg taagttggtc agaaactccc
661 aacatgtaat cgaacttttc gtttacttat tgtttttatt catcaaaaaa aaaaaaaaaa
721 naaaaaaaaa aaaaaaaa
```

Appendix Figure 13 The partial nucleotide sequences of *GmCCoAMT* cDNA fragment (Accession no. KJ671473).

```
AILEHVVEANQCQKHHGVYTGYSLLATALALPDDAKILAMDINRENYELGLPIIQKAG
VAHKIDFKEGPALPVLDQLIEDGKSHGSFDFIFVDADKDNYLNYHKRLIELVKVGGLI
GYDNTLWNGTVAAPDDAPMRKYIKYYRDFVLELNKTLAVDPRIEICMLPVGDGITLCR
RIK
```

Appendix Figure 14 The partial deduced amino acid sequences of *GmCCoAMT* cDNA fragment.

```
Eucalyptus      ----------MAANAEPQQTQPAKHSEVGHKSLLQSDALYQYILETSVYPREPESMKEL 49
Birch           ----------MATNGEDNQNQASRHQEVGHKSLLQSDALYQYILETSVYPKEPESMKEL 49
Brassica        MATTTAEATKTSSTNVEDKQSQNLRHQEVGHKSLLQSDDLYQYILETSVYPREPESMKEL 60
Poplar          ------------------------------------------------------------
Cotton          ----------MATNTQEQQSQAGRHQEVGHKSLLQSDALYQYILETSVYPREPEPMKEL 49
Mangosteen      ------------------------------------------------------------

Eucalyptus      REITAKHPWNLMTTSADEGQFLNMLLKLINAKN-TMEIGVYTGYSLLATALALPDDGKIL 108
Birch           REVTAKHPWNIMTTSADEGQFLNMLLKLINAKN-TMEIGVYTGYSLLATALALPDDGKIL 108
Brassica        REVTAKHPWNIMTTSADEGQFLNMLIKLVNAKN-TMEIGVYTGYSLLATALALPEDGKIL 119
Poplar          --------------ADEGQFLNMLLKLVNAKN-TMEIGVYTGYSLLATALAIPEDGKIL 44
Cotton          RELTAKHPWNLMTTSADEGQFLNMLLKLINAKN-TMEIGVYTGYSLLATALAIPDDGKIL 108
Mangosteen      ----------------------AILEHVVEANQCQKHHGVYTGYSLLATALALPDDAKIL 38
                                      :* ::::*::   . ***************:*:*.***

Eucalyptus      AMDINRENFEIGLPVIQKAGLAHKIDFREGPALPLLDQLVQDEKNHGTYDFIFVDADKDN 168
Birch           AMDINRENYELGLPVIEKAGVAHKIDFREGPALPLLDHLIADEKNHGSYDFIFVDADKDN 168
Brassica        AMDVNRENYELGLPIIEKAGVAHKIDFREGPALPVLDQLVADEKNHGTYDFIFVDADKDN 179
Poplar          AMDINRENYELGLPVIQKAGVAHKIDFKEGPALPVLDQMIEDGKCHGSFDFIFVDADKDN 104
Cotton          AMDVNRENYELGLPVIQKAGVAHKIDFKEGPALPVLDQLVEDEKNHGSFDFIFVDADKDN 168
Mangosteen      AMDINRENYELGLPIIQKAGVAHKIDFKEGPALPVLDQLIEDGKSHGSFDFIFVDADKDN 98
                ***:****:*:***:*:***:******:******:**::: * * **::***********

Eucalyptus      YINYHKRLIDLVKVGGLIGYDNTLWNGSVVAPADAPLRKYVRYYRDFVLELNKALAVDPR 228
Birch           YINYHKRLIDLVKVGGLIGYDNTLWNGSVVAPPDAPLRKYVRYYRDFVLELNKALAADPR 228
Brassica        YINYHKRLIDLVKVGGVIGYDNTLWNGSVVAPPDAPMRKYVRYYRDFVLELNKALAADPR 239
Poplar          YINYHKRLIELVKVGGLIGYDNTLWNGSVVAPPDAPMRKYVRYYRDFVLELNKALAADPR 164
Cotton          YLNYHKRLIELVKVGGLIGYDNTLWNGSVVAPPDAPLRKYVRYYRDFVMELNKALAVDPR 228
Mangosteen      YLNYHKRLIELVKVGGLIGYDNTLWNGTVAAPDDAPMRKYIKYYRDFVLELNKTLAVDPR 158
                *:*******:******:**********:*.** ***:***::******:****:**.***

Eucalyptus      IEICMLPVGDGITLCRRVS 247
Birch           IEICMLPVGDGITLCRRIK 247
Brassica        IEICMLPVGDGITICRRIS 258
Poplar          IEICMLPVGDGITLCRRIQ 183
Cotton          IEICMLPVGDGITLCRRLK 247
Mangosteen      IEICMLPVGDGITLCRRIK 177
                *************:***:.
```

Appendix Figure 15 Alignment of partial deduced amino acid sequence of *GmCCoAMT* cDNA fragment with other plants, eucalyptus (accession no ACY66929), birch (accession no ACJ38669), brassica (accession no ABE41833), poplar (accession no CAA10217) and cotton (accession no ACF48821). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been conserved. The dot (.) means that the semi-conserved substitutions are observed.

```
   1 aagggtgtga tgttgacgca caaaggacta gtaacaagcg tggcccaaca agttgatggc
  61 gaaaacccta atctctatat gcacgagaag gatgtgatct tatgtgtgtt gccattgttt
 121 catatttact cattaaattc tgtgctcctt tgtgggttac gagttggggc aactgttttg
 181 ttgatgcaaa aatttgagat tgtttcttta atggaaaatg tgcaaaaata caaggtgacc
 241 attgcacctt ttgttcctcc tattgttttg gctatggcaa agagtccgga ggtggacaag
 301 tatgatttgt cgtcgattag aacggtgatg tccggagcgg cgcccatggg aaaggagttg
 361 gaggacactc tcagagctaa gctgcctatt gctaaacttg gtcaggggta tgggatgact
 421 gaggcagggc cggtgctagc aatgtgtttg gcatttgcaa aagagccatt cgagataaaa
 481 tcaggtgctt gtggaacagt ggtcaggaat gctgaaatga agattgtaga cccagagact
 541 ggcttctccc tacccaggaa tcaacctgga gagatttgca ttaggggcaa ccaaatcatg
 601 aaaggctatg tgaatgaccc agaggctaca gagaggacta tagacaaaca aggatggttg
 661 cacacaggag atataggata catcgacgat gatgatgaac tttttattgt tgataggttg
 721 aaagaattga tcaaatacaa gggattccaa gttgctcctg ctgaattaga agctatgttg
 781 attgcacatc cccatatctc agatgctgca gttataccaa tgaaagatga ggctgcaggg
 841 gaagttccgg ttgcattcgt agttaggtca aatggttcaa acatcaccga agatgaaatt
 901 aaacaatata tctctaaaca ggttattttc tataagagga tcaacagggt gtttttacg
 961 gacacaattc caaaagctcc atctggcaaa atcttgagaa aagatttgag agctaggctt
1021 gcggctggtg ttcccaatca gtgacaatta taacattgtg acattttgat gttattatac
1081 cactcagtta ttttaagacc agagacaaac acgtccttaa ttccaaattc ccttctctga
1141 atagtccttt atacgaggac ggaaaaaggg acaaaaattt tcagggttgt gaattttagt
1201 gccttctgtc cttcccgtga agggaagtta ctaaaatgtg tctgtatcat ttgcaaattg
1261 tattaatttt ctttcttttc ttctatataa tggtgtcatg tactatagat aacacgttgg
1321 acaacttcgg aactctaaaa ttgtgtttgt actatgtgaa aatctataaa ttttaagttg
1381 attatttctt aaaaaaaaaa aaaaaaaa
```

Appendix Figure 16 The partial nucleotide sequences of *Gm4CL* cDNA fragment (Accession no. KJ671474).

```
GVMLTHKGLVTSVAQQVDGENPNLYMHEKDVILCVLPLFHIYSLNSVLLCGLRVGATV
LLMQKFEIVSLMENVQKYKVTIAPFVPPIVLAMAKSPEVDKYDLSSIRTVMSGAAPMG
KELEDTLRAKLPIAKLGQGYGMTEAGPVLAMCLAFAKEPFEIKSGACGTVVRNAEMKI
VDPETGFSLPRNQPGEICIRGNQIMKGYVNDPEATERTIDKQGWLHTGDIGYIDDDDE
LFIVDRLKELIKYKGFQVAPAELEAMLIAHPHISDAAVIPMKDEAAGEVPVAFVVRSN
GSNITEDEIKQYISKQVIFYKRINRVFFTDTIPKAPSGKILRKDLRARLAAGVPNQ
```

Appendix Figure 17 The partial deduced amino acid sequences of *Gm4CL* cDNA fragment.

```
Mangosteen      ------------------------------------------------------------
Eucalyptus      MEAKPSEQPREFIFRSKLPDIYIPDNLSLHAYCFENISEFADRPCVINGATGRTYTYAEV 60
Poplar          MEANKDQV-KEFIFRSKLPDIYIANHLPLHTYCFEKLSQFKDNPCLINGPTGDIYTYADV 59
Kenaf           MEANQDG--HEFIFRSSLPDINIPNHLPLHTYCFENLSNFKDGPCLINAPTGRVYTYAQV 58
Pear            --MEHHHKDDEFIFRSKLPDIYIPNHLPLHTYCFENISQFMDRPCLINGNNGDTFTYADV 58
Loquat          ------------------------------------------------------------

Mangosteen      ------------------------------------------------------------
Eucalyptus      ELISRRVSAGLNGLGVGQGDMIMLLLQNCPEFVFAFLGASYRGAISTTANPFYTPGEIAK 120
Poplar          ELTSRKVASGLYKFGLQQGDVILLLLQNSPEFVFAFLGASFIGAISSTANPFYTSAEIAK 119
Kenaf           HLTCRKVAAGLNKLGIQQGDVIMLLLHNSPEFVFAFLGASFRGAITTTANPFFTPAEIAK 118
Pear            ELTSRKVASGLHKIGIHQTDVIMLLLQNSPEFVFAFLGASNIGAVVTTANPFYTPAEMAK 118
Loquat          ------------------------------------------------------------

Mangosteen      ------------------------------------------------------------
Eucalyptus      QASAAQAKIVITQAAYADKVRPFAEENGLSV----VCIDT-APEGCLHFSELMQADENAA 175
Poplar          QATASKAKLIITQAAFAEKVQQFAQENDHVKIMTIDSLTD-N---CLHFSELTSSDENEI 175
Kenaf           QASASKTRLFITQAVYAEKVKNFALDKDIKI----ITIDT-TPEGCLHFSELTRVHEDEI 173
Pear            QAKASNAKLIITQSAYVEKVNDFALKNDVEIMVVDSAETEEDGNTYRHFSELTSADENDI 178
Loquat          ------------------------------------------------------------

Mangosteen      ------------------------GVMLTHKGLVTSVAQQVDGENPNLYMHEKDVILCVL 36
Eucalyptus      PAADVKPDDVLALPYSSGTTGLPKGVMLTHRGQVTSVAQQVDGDNPNLYFHKEDVILCTL 235
Poplar          PAVKIKPDDVVALPYSSGTTGLPKGVMLTHKGLVTSVAQQVDGENPNLYFHERDVILCVL 235
Kenaf           PAVKINPDDVVALPFSSGTTGLPKGVMLTHKSLVTSVAQHVGGDNPNIYFHERDVILCLL 233
Pear            PAVKIYPEDVVALPYSSGTTGLPKGVMLTHKGLVTSVAQQVDGENPNLYFHSEDVILCVL 238
Loquat          ------------------------KGVMLTHKSLVTSVAQQVDGENPNLYYSTNDVVLCVL 37
                                        ******   ****** * * *** *    ** ** *

Mangosteen      PLFHIYSLNSVLLCGLRVGATVLLMQKFEIVSLMENVQKYKVTIAPFVPPIVLAMAKSPE 96
Eucalyptus      PLFHIYSLNSVMFCALRVGAAILIMQKFEIMALMELVQRYRVTILPIVPPIVLAIAKSAE 295
Poplar          PLFHIYSLNSVFLCGLRAGSAILLMQKFETVALMDLVQKYKVTIAPLVPPIFLAIAKSPV 295
Kenaf           PLFHIYSLNCILLCSLRAGAAILIMQKFEILPLMELVEKYSVTIAPFVPPIILAIAKTPD 293
Pear            PLFHIYSLNSVFLCGLRVGAAILIMQKFEITKLLELVEKYKVTIAPFVPPIVLSIAKSPD 298
Loquat          PLFHIYSLNSVLLCGLRAGAAILMMNKFEIVSLLGLIDKYKVSIAPIVPPIVLAIAKFPD 97
                *********    * ** *   * * ***   *      * * * * **** *  **

Mangosteen      VDKYDLSSIRTVMSGAAPMGKELEDTLRAKLPIAKLGQGYGMTEAGPVLAMCLAFAKEPF 156
Eucalyptus      VDRYDLSSIRTIMSGAAPMGKELEDTVRAKLPNAKLGQGYGMTEAGPVLAMCLAFAKEPF 355
Poplar          VDQYDLSSIRTVLSGAAPMGKELEDTVRAKLPNAKLGQGYGMTEAGPVIAMCLAFAKEPF 355
Kenaf           IQKYDLSSIRMVISGAAPMGKKLEDAVRDRLPNAKLGQGYGMTETVLALNL--AFAKEPW 351
Pear            LDRYDLSSIRMVMSGAAPMGKELEDTVRAKLPNAKLGQGYGMTEAGPVLSMCLAFAKEPF 358
Loquat          LDKYDLSSIRVLKCGGAPLGKELEDAVRAKFPNVTLGQGYGMTEAGPVLTMSLAFAKQPF 157
                   *******    * ** ** ***  *   *   *********         **** *
```

Appendix Figure 18 Alignment of partial deduced amino acid sequence of *Gm4CL* cDNA fragment with other plants, eucalyptus (accession no ACX68559), poplar (accession no AFC89540), kenaf (accession no AGJ84134), pear (accession no AFY97682) and loquat (accession no ABV44809). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been conserved. The dot (.) means that the semi-conserved substitutions are observed.

```
Mangosteen      EIKSGACGTVVRNAEMKIVDPETGFSLPRNQPGEICIRGNQIMKGYVNDPEATERTIDKQ 216
Eucalyptus      EIKSGACGTVVRNAEMKIVDPETGASLPRNQAGEICIRGHQIMKGYLNDPEATANTIDKE 415
Poplar          EIKSGACGTVVRNAEMKIVDPETGDSQPRNKAGEICIRGSQIMKGYLNDPEATERTIDKD 415
Kenaf           ETKSGACGTVVRNAEMKIVDPETGTSLPRNQSGEICIRGSQIMKGYLNDPEATKRTIDKE 411
Pear            EIKSGACGTVVRNAEMKIVDPDTGASLTRNQAGEICIRGSQIMKGYLNDPEATERTVDKR 418
Loquat          EVKPGGCGTVVRNAELKIVDPESGASLPRNQPGEICIRGDQIMKGYLNDPESTRTTIDKE 217
                * * * ********* *****  * *  **  ******* ****** **** *  * **

Mangosteen      GWLHTGDIGYIDDDDELFIVDRLKELIKYKGFQVAPAELEAMLIAHPHISDAAVIPMKDE 276
Eucalyptus      GWLHTGDIGYIDDDDELFIVDRLKELIKYKGFQVAPAELEAMLIAHPSISDAAVVPMKDE 475
Poplar          GWLHTGDIGYIDEDELFI-VDRLKELIKYKGFQVAPAELESMLIAHPSISDAAVVPMKDE 474
Kenaf           GWLHTGDIGYIDEDNELFIVDRLKELIKYKGFQVAPAELEAMLISHPNISDAAVVPMKDE 471
Pear            GWLHTGDIGYIDGDDELFIVDRLKELIKYKGFQVAPAELEAMLIAHPNISDAAVVPMKDE 478
Loquat          GWLHTGDIGFIDDDDELFIVDRLKELIKYKGFQVAPAELEALLITHPSVSDAAVVPKKDE 277
                ********* ** *     ********************  ** **  ***** * ***

Mangosteen      AAGEVPVAFVVRSNGSNITEDEIKQYISKQVIFYKRINRVFFTDTIPKAPSGKILRKDLR 336
Eucalyptus      VASEVPVAFVVKSNGSVITEDEIKQYISKQVVFYKRINRVFFTDAIPKAPSGKILRKDLR 535
Poplar          AAGEVPVAFVVRSNGSKITEDEIKQYISKQVIFYKRIGRVFFTEAIPKAPSGKILRKDLR 534
Kenaf           AAGEVPVAFIVRSNHSNITEDEIKQFISKQVVFYKRLARVFFVDTIPKAPSGKILRKDVR 531
Pear            AAGEIPVAFVVRSNGSKISEDDIKQYISKQVVFYKRIGRVFFIDKIPKAPSGKILRKDLR 538
Loquat          AAGEVPVAFVVRSNNSQLTEDEVKQFISKQVVFYKRINRVFFIEAIPKSPSGKILRKDLR 337
                 * * **** * ** *   **  ** ***** ****  ****   *** ********* *

Mangosteen      A-RLAAG------VPNQ----- 346
Eucalyptus      A-KLASG------VYN------ 544
Poplar          AMVSAGDIPHQIPNMTYMQNQH 556
Kenaf           A-KLAAH------VPN------ 540
Pear            A-KLAAGLP----N-------- 547
Loquat          A-KLAAGFP----N-------- 346
                *   *
```

Appendix Figure 18 (Continued)

```
  1 ttcaacaagg ttttcaacaa tggaatgttt gatcactcga ccattactat gaaaaaacta
 61 cttgagacgt acacagggtt tggaggtgtc acaacagtag tggatgttgg gggtggaata
121 ggagcttcac tcaacctaat tatctctaaa tacccgtcaa ttaagggtat taatttcgat
181 ttgcctcatg ttattgagga tgctccagct tatcccggaa tagagcatgt tggtggagac
241 atgtttgtta gcgtcccaaa ggctgatgca atttttatga agtggatatg ccacgactgg
301 agcgatgaac actgcttaaa gttcttgaaa aattcctata acgcgctgcc agaaaacggg
361 aaggtgattg ttgtggaatg cattcttcca gaggctccgg acacaagcct tgcaagcaag
421 ttggtcatcc acattgacac aattatgttg gcccacaatc ccgggggcaa agaaaggaca
481 cagaaggagt ttgaggcctt ggctaaggga gcaggcttcc aaggtttcca agttgcttgc
541 tgtgctgtgg gtacatatgt catggaattc cttaaaaagc cttgaaattg ggtgtcatgt
601 ttgtttgatt caatgtgggg cagtatggat tatgaaatgg tggtttctgt ttgcgattgt
661 gaagagtaat tttctgttaa atgtgagtac tgttagaaaa ggaataaacg tggttgtttt
721 gtgtcagata aacatttcaa tacatgtggg cgatgattgt tggagcactt gagaaatata
781 taggttgcat aacgacggac aattctatat tatcccaaaa aaaaaaaaaa aaaaaa
```

Appendix Figure 19 The partial nucleotide sequences of *GmCOMT* cDNA fragment (Accession no. KJ671475).

```
NKVFNNGMFDHSTITMKKLLETYTGFGGVTTVVDVGGGIGASLNLIISKYPSIKGINF
DLPHVIEDAPAYPGIEHVGGDMFVSVPKADAIFMKWICHDWSDEHCLKFLKNSYNALP
ENGKVIVVECILPEAPDTSLASKLVIHIDTIMLAHNPGGKERTQKEFEALAKGAGFQG
FQVACCAVGTYVMEFLKKP
```

Appendix Figure 20 The partial deduced amino acid sequences of *GmCOMT* cDNA fragment.

```
Cocoa           MGSTG-ETQMTPNQVSDEELNLFAMQLTSASVLPMVLTSAIELDLFEIMAKAGPGAFLSP 59
Cotton          MGSTG-ETQMTPTQVSDEEANLFAMQLTSASVLPMVLKSAIELDLLEIMAKAGPGAFLSP 59
Eucalyptus      MGSTGSETQMTPTQVSDEEANLFAMQLASASVLPMVLKAAIELDLLEIMAKAGPGAFLSP 60
Poplar          MGSTG-ETQMTPTQVSDEEAHLFAMQLASASVLPMILKTAIELDLLEIMAKAGPGAFLST 59
Pear            MGSTP-ETQMTPTQVSDEEANLFAMQLASGSILPMVLKAAIELDLLEVMAKAGPGAFVSP 59
Mangosteen      ------------------------------------------------------------

Cocoa           NELASQLPTKNPDAPVMLDRILRLLASYSVLNCSLRTLPDGKVERLYSLGPVCKFLTKNE 119
Cotton          KELASQLPTSNPDAPVMLDRILRLLATYSILTCSLRTLPDGKVERLYGLGPVCKFLTKNE 119
Eucalyptus      GEVAAQLPTQNPEAPVMLDRIFRLLASYSVLTCTLRDLPDGKVERLYGLAPVCKFLVKNE 120
Poplar          SEIASHLPTKNPDAPVMLDRILRLLASYSILTCSLKDLPDGKVERLYGLAPVCKFLTKNE 119
Pear            ADIASQLPTKNPDAPVMLDRILRLLASYSILTYSLRTLPDGKVQRLYGLGPVCKFLTKNE 119
Mangosteen      ------------------------------------------------------------

Cocoa           DGVTLSSLSLMNQDKVLMESWYYLKDAVLEGGIPFNKAYGMTAFEYHGTDPRFNKVFNRG 179
Cotton          DGVTLSALSLMNQDKVLMESWYYLKDAVLEGGIPFNKVYGMTAFEYHGTDPRFNKVFNRG 179
Eucalyptus      DGVSIAALNLMNQDKILMESWYYLKDAVLEGGIPFNKAYGMTAFEYHGTDPRFNKIFNRG 180
Poplar          DGVSVSPLCLMNQDKVLMESWYYLKDAILDGGIPFNKAYGMTAFEYHGTDPRFNKVFNKG 179
Pear            DGASIGSLCLMNQDKVLMESWYHLKESVLEGGIPFNKAYGMTAFEYHGTDPRFNKVFNKG 179
Mangosteen      ----------------------------------------------------FNKVFNNG 8
                                                                     ***:**.*

Cocoa           MNDHSTTTMRKILETYDGFEGVKTLVDVGGGIGASLSMIISKHPSIKGINFDLPHVIEDA 239
Cotton          MSDHSTITMKKILETYDGFEGLKTLVDVGGGTGATLNMIVTKHPSIKGINFDLPHVIEDA 239
Eucalyptus      MSDHSTITMKKILETYKGFEGLETVVDVGGGTGAVLSMIVAKYPSMKGINFDLPHVIEDA 240
Poplar          MSDHSTITMKKILETYKGFEGLTSLVDVGGGTGAVVNTIVSKYPSIKGINFDLPHVIEDA 239
Pear            MADHSTITMKKLLEIYNGFEGLTSIVDVGGGTGAVLNMIVSKYPSIKDINFDLPHVIEDA 239
Mangosteen      MFDHSTITMKKLLETYTGFGGVTTVVDVGGGIGASLNLIISKYPSIKGINFDLPHVIEDA 68
                * **** **:*:** * ** *: ::****** ** :. *::*:**:*.************

Cocoa           PAYPGVEHVGGDMFVSVPKGDAIFMKWICHDWSDEHCSKFLKNCYQALPDNGKVIVAECN 299
Cotton          PAYPGVEHVGGDMFESVPKGDAIFMKWICHDWSDEHCSKFLKKCYEALPDSGKVIVAECI 299
Eucalyptus      PPLPGVKHVGGDMFVSVPKGDAIFMKWICHDWSDDHCAKFLKNCYDALPNNGKVIVAECV 300
Poplar          PSFPGVEHVGGDMFVSVPKADAVFMKWICHDWSDAHCLKFLKNCYDALPENGKVILVECI 299
Pear            PQYPGVEHVGGDMFVSVPKGDAIFMKWICHDWSDEHCLKFLKNCYVALPDNGKVIVAECI 299
mangosteen      PAYPGIEHVGGDMFVSVPKADAIFMKWICHDWSDEHCLKFLKNSYNALPENGKVIVVECI 128
                *  **::******* ****.**:*********** ** ****:.* ***:.****:.**

Cocoa           LPDYPDPSLATKLVVHIDCIMLAHNPGGKERTAKEFEALAKGAGFQGFQVKCCAFGTYIM 359
Cotton          LPDYPDPSLATKLVVHIDCIMLAHNPGGKERTEKEFEALARSAGFQGFQVKCCAFGTYIM 359
Eucalyptus      LPVYPDTSLATKNVIHIDCIMLAHNPGGKERTQKEFETLAKGAGFQGFQVMCCAFGTHVM 360
Poplar          LPVAPDTSLATKGVVHVDVIMLAHNPGGKERTEKEFEGLAKGAGFQGFEVMCCAFNTHVI 359
Pear            FPVAPDSSLATKGVVHIDAIMLAHNPGGKERTEKEFEALAKGFGFQGFRVVCCAFNTYAI 359
Mangosteen      LPEAPDTSLASKLVIHIDTIMLAHNPGGKERTQKEFEALAKGAGFQGFQVACCAVGTYVM 188
                :*  **.***:* *:*:* ************* **** **:. *****.* ***..*: :
```

Appendix Figure 21 Alignment of partial deduced amino acid sequence of *GmCOMT* cDNA fragment with other plants, cocoa (accession no. EOY23716), cotton (accession no. ACT32029), eucalyptus (accession no.ACY66932), poplar (accession no. AFZ78575) and pear (accession no. AGS44640). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been conserved. The dot (.) means that the semi-conserved substitutions are observed.

```
Cocoa           EFLKTV 365
Cotton          EFVKRV 365
Eucalyptus      EFLKTA 366
Poplar          EFRKKA 365
Pear            EFFKKN 365
Mangosteen      EFLKKP 194
                ** *
```

Appendix Figure 21 (Continued)

```
   1 acatgggggt gcttgaccaa catttctata taactgaaga acccccatgg tatatcttcc
  61 tcaaaacata caagctcaaa ccaaatgcca agcgcaccac catctttcct ccaataaaaa
 121 aaaaaaagac caccatcttt cctatttcct tatttcagac attcatacac tatgccccaa
 181 aatatggagg aatatctcct ccaaacttat aactatctaa caaatccaga acaaccattt
 241 ctcctcctaa taatctcatt cctcttcctc ttgggcctag tctccctcct ccgccggaga
 301 ccaccttacc caccaggccc aaaaggctta ccaatcatag gaaacatgtt aatgatggac
 361 caactaaccc accgtggctt agccaaacta tcccaaaaat atggtggcat attccaccta
 421 aaaatgggtt atctccacat ggtttgcatt tcttccccag aaacagcccg ccaagtcctc
 481 caagtccaag ataatatctt ctccaatagg tcagcaacca tagcgattcg ccacttaacc
 541 tacgaccgtg ctgacatggc ttctgcccac tatggaccct tttggaggca gatgaggaag
 601 atatgtgtta tgaggctttt cagccggaaa agagctgagt cttgggaatc tgtccgccac
 661 gaagttgact ctatggtcaa atccgtcgca gggaacgttg ggcaccaggt caacgtggga
 721 gagttgatat ttcgtttgac tatgaacatt acttataggg ctgcctttgg gtctaagacc
 781 caagggcagg atgagtttat tggaatattg caagagtttt ctaagctgtt tggtgctttt
 841 aatattgcgg attttatacc ttggcttagt tgggtggacc ctcaggggct taatgctagg
 901 ctggaaaaag ctaggaatgc tcttgacaag tttattgact tgattataga tgatcatatg
 961 aagaagagag aaattggtaa tgttaatggc tgtgatcagg aggtgaatac tgatatggtg
1021 gataatttgc tggatttcta taccgaggtg gaggaggcca aagtttccga atctgacgat
1081 ttgcagaatt ctattaaact tacgaaaact aatatcaagg ccatcatcat ggatgtaatg
1141 cttggtggga cggaaacagt ggcatccgca atagagtggg ccctagtgga gctcatgaga
1201 agcccacaag acttgaaacg ggtccaacaa gagctagctg atgtggtggg cctggatcgc
1261 cgggtcgagg aatccgatat cgagaagctt actttcttaa aatgtgcact taaagaaacc
1321 ttaagacttc acccgccgat tcctctcctt ttacatgaaa ctgcagagga cgctgtcgtt
1381 ttgggctttt acgtgccgaa aagatctcgg gtcatgatca acgtttgggc catagggcgc
1441 gacccaaatt cgtgggagga cccagagagt tttaagccct ctaggttttt aaaagaagga
1501 atgcctgatg ttaaggggag taatttcgaa tttattcctt ttgggtcggg tcggaggtcc
1561 tgcccgggaa tgcaacttgg tctttatggg ctggagctgg cagtggccca tttacttcat
1621 tactttaatt gggagttacc agatgggatg aagcctagtg agatggacat gagcgatata
1681 tttggactca ctgcgcctcg ggcgagtcga ctcattgcca ttccaaggac acgttcgatt
1741 tgtccgattt gaagaaatgg gtgtattatt tggaagatgc attgaacatg aggacttttt
1801 tcagttttac cttcttttac tttgtggttt ttttatactg ccaaatattg agtgagtgga
1861 gaaattatgg gaaagaaaaa ataacggccg aagggaagtc aaggaaaatt atttcgagat
1921 tttccattta ctttttcact atgttcataa gaatcctatc ctcattcatg tgctttgtgc
1981 gaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa a
```

Appendix Figure 22 The full length nucleotide sequences of *GmF5H* cDNA fragment (Accession no. KJ671476).

MEEYLLQTYNYLTNPEQPFLLLIISFLFLLGLVSLLRRRPPYPPGPKGLPIIGNMLMM
DQLTHRGLAKLSQKYGGIFHLKMGYLHMVCISSPETARQVLQVQDNIFSNRSATIAIR
HLTYDRADMASAHYGPFWRQMRKICVMRLFSRKRAESWESVRHEVDSMVKSVAGNVGH
QVNVGELIFRLTMNITYRAAFGSKTQGQDEFIGILQEFSKLFGAFNIADFIPWLSWVD
PQGLNARLEKARNALDKFIDLIIDDHMKKREIGNVNGCDQEVNTDMVDNLLDFYTEVE
EAKVSESDDLQNSIKLTKTNIKAIIMDVMLGGTETVASAIEWALVELMRSPQDLKRVQ
QELADVVGLDRRVEESDIEKLTFLKCALKETLRLHPPIPLLLHETAEDAVVLGFYVPK
RSRVMINVWAIGRDPNSWEDPESFKPSRFLKEGMPDVKGSNFEFIPFGSGRRSCPGMQ
LGLYGLELAVAHLLHYFNWELPDGMKPSEMDMSDIFGLTAPRASRLIAIPRTRSICPI

Appendix Figure 23 The full length deduced amino acid sequences of *GmF5H* cDNA fragment.

```
Mangosteen          --------MEEYLLQTYNYLTNPEQPFLLLIISFLFLLGLVSLLRRRPPYPPGPKGLPII 52
Kenaf               ------------------------------------------------------------
Eucalyptus          MDIFYFYSQLQSLVQT--QLQQSPMTLLLSVVPLLLFLGLVARLRRKPPFPPGPRGLPVI 58
Pear                ---------MDSLLQS--LQPLKSMTPLVFIIPLLFLLPLIFRFRRLPPYPPGPKGLPLI 49
Poplar              ---------MDSLLQS--LQTLP-MSFFLIIISSIFFLGLISRLRRRSPYPPGPKGFPLI 48
Rapeseed            ---------MESSISQTLSQVLDPTTGILIVVSPLIFVGLITR-RRRPPYPPGPRGWPII 50

Mangosteen          GNMLMMDQLTHRGLAKLSQKYGGIFHLKMGYLHMVCISSPETARQVLQVQDNIFSNRSAT 112
Kenaf               ------------------------------------------------------------
Eucalyptus          GNMLMMGELTHRGLASLAKKYGGIFHLRMGFLHMVAVSSPDVARQVLQVHDGIFSNRPAT 118
Pear                GNMLMMDQLTHRGLAKLAKQYGGIFHLRMGFLHMVAVSNPDVARQVLQVQDNIFSNRPAT 109
Poplar              GSMHLMDQLTDRGLAKLAKQYGGLFHMRMGYLHMVAGSSPEVARQVLQVQDNMFSNRPAN 108
Rapeseed            GNMSMMDQLTHRGLANLAKKYGGLCHLRMGFLHMYAVSSPDVAKQVLQVQDSVFSNRPAT 110

Mangosteen          IAIRHLTYDRADMASAHYGPFWRQMRKICVMRLFSRKRAESWESVRHEVDSMVKSVAGNV 172
Kenaf               ------------MAFAHYGPFWRQMRKLSVMKLFSRKRAESWESIRDEVDSHVKAVASNA 48
Eucalyptus          IAISYLTYDRADMAFAHYGPFWRQMRKLCVMKLFSRKRAESWESVRDEVDTMVRTVAGSE 178
Pear                IAISYLTYDRADMAFAYYGPFWRQMRKLCVMKLFSRKRAESWESVRDEVDSAVRTVTVHV 169
Poplar              IAISYLTYDRADMAFAHYGPFWRQMRKLCVMKLFSRKRAESWESVRDEVDSMVKTVESNI 168
Rapeseed            IAISYLTYDRADMAFAHYGPFWRQMRKVCVMKVFSRKRAESWASVRDEVDKMIRSVSSNV 170
                              ** *:**********:.**::********* *:*.***. :::*

Mangosteen          GHQVNVGELIFRLTMNITYRAAFGSKTQ-GQDEFIGILQEFSKLFGAFNIADFIPWLSWV 231
Kenaf               GDVVNMGELIFNLTKNIIYRAAFGCISQQGQEEFIRILQEFSKLFGAFNMADFIPWLGWA 108
Eucalyptus          GTAVNIGELVFELTRDIIYRAAFGTSSTEGQDEFISILQEFSKLFGAFNIADFIPYLSWI 238
Pear                GSAVNIGELVFSLTKNIIYRAAFGTSSQEGQDEFIAILQEFSKLFGAFNIADFIPSLGWV 229
Poplar              GKPVNVGELIFTLTMNITYRAAFGAKN-EGQDEFIKILQEFSKLFGAFNISDFIPWLGWI 227
Rapeseed            GKSINVGEQIFALTRNITYRAAFGSACEKGQDEFIRILQEFSKLFGAFNVADFIPYFGWV 230
                    *  :*:** :* ** :* ******     **:*** *************::**** :.*

Mangosteen          DPQGLNARLEKARNALDKFIDLIIDDHMKKREIGN-VNGCDQEVNTDMVDNLLDFYTEVE 290
Kenaf               DPQGLNTRLEKARGALDKFIDTIIDKKMRN-------NGGSDVGDTDMVDDLLTFCT--E 159
Eucalyptus          DPQGLTARLVKARQSLDGFIDHIIDDHMDKKRNKTSSGGGDQEVDTDMVDDLLAFYS--D 296
Pear                DPQGLNNRLAKARESLDRFIDTIIDDHMEKKKNNKGLNDGE----TDMVDELLAFYS--E 283
Poplar              DPQGLTARLVKARKALDKFIDHIIDDHIQKRKQNNYSEEAE----TDMVDDMLTFYS--E 281
Rapeseed            DPQGINKRLVKARNDLDGFIDDIIDEHIKKKENQNSVDAGD-VVDTDMVDDLLAFYS--E 287
                    ****:. ** ***  ** *** ***.:: :        .    *****::* * :  :

Mangosteen          EAKVSESDD-LQNSIKLTKTNIKAIIMDVMLGGTETVASAIEWALVELMRSPQDLKRVQQ 349
Kenaf               EAQVNQSED-LQNSIKLTRDNIKAIIMDVMFGGTETVASAIEWALAELMKSPEDMKRVQQ 218
Eucalyptus          EAKVNESDD-LQNSIRLTRDNIKAIIMDVMFGGTETVASAIEWAMAELMRSPEDLKKVQQ 355
Pear                EAKVNESEDNLQSAIKLTRDNIKAIIMDVMFGGTETVASAIEWAMSELMKSPEDLKRVQQ 343
Poplar              ETKVNESDD-LQNAIKLTRDNIKAIIMDVMFGGTETVASAIEWAMAELLKSPEDIKRVQQ 340
Rapeseed            EAKLVSETADLQNSIKLTRDNIKAIIMDVMFGGTETVASAIEWALTELLRSPEDLKRVQQ 347
                    *::: ..   **.:*:**: **********:*************: **::**:*:*:***
```

Appendix Figure 24 Alignment of full – length of amino acid sequence of *GmF5H* cDNA fragment with other plants, kenaf (accession no. AGR85825), eucalyptus (accession no. ACU45738), pear (accession no. AGR44939), poplar (accession no. CAB65335) and rapeseed (accession no. ABG73616). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been conserved. The dot (.) means that the semi-conserved substitutions are observed.

```
Mangosteen          ELADVVGLDRRVEESDIEKLTFLKCALKETLRLHPPIPLLLHETAEDAVVLGFYVPKRSR 409
Kenaf               ELAEVVGLDRRVEESDMEKLTSLKCTLKETLRLHPPIPLLLHETAEDTVVAGYQIPAKSR 278
Eucalyptus          ELADVVGLDRRVEESDFEKLTYLKCCLKETLRLHPPIPLLLHETAEDAVISGYRIPARSR 415
Pear                ELADVVGLDRRPEETDFEKLTYLKCALKETLRLRPPIPLLLHETSEDAVVAGYRIPKRSR 403
apoplar             ELADVVGLERRVEESDFDKLTFFKCTLKETLRLHPPIPLLLHETSEDAEVAGYYVPKKTR 400
Rapeseed            ELAEVVGLDRRVEESDIEKLTFLKCTLNETLRLHPPIPLLLHETAEDTEIDGYFVPKKSR 407
                    ***:****:** **:*::*** :** *:*****:**********:**: : *: :* ::*

Mangosteen          VMINVWAIGRDPNSWEDPESFKPSRFLKEGMPDVKGSNFEFIPFGSGRRSCPGMQLGLYG 469
Kenaf               VMINAWAIARDKDSWEDPDSFKPSRFLKEGVADYKGSNFEFIPFGSGRRSCPGMTLGLYA 338
Eucalyptus          VMINAWAIGRDPGSWTEPDKFKPSRFLESGMPDYKGSNFEFIPFGSGRRSCPGMQLGLYA 475
Pear                VMINAWAIGRDKDSWEDAESFKPSRFLKEGVPDFKGSNFEFIPFGSGRRSCPGMQLGLYA 463
Poplar              VMINAYAIGRDKNSWEDPDSFKPSRFLEPGVPDFKGNHFEFIPFGSGRRSCPGMQLGLYA 460
Rapeseed            VMINAFAIGRDKNSWVDPETFRPSRFLEPGVPDFKGSNFEFIPFGSGRRSCPGMQLGLYA 467
                    ****.:**.** .** :.:.*:*****: *:.* **.:*************** ****.

Mangosteen          LELAVAHLLHYFNWELPDGMKPSEMDMSDIFGLTAPRASRLIAIPRTRSICPI- 522
Kenaf               LDLAVAHLLHCFTWELPDGMKPSELDMSDVFGLTAPRAARLYAVPKKRLICPLF 392
Eucalyptus          LDMAVAHLLHCFTWELPDGMKPSEMDMGDVFGLTAPRSTRLVAVPTPRLVGALY 529
Pear                LEMAVAHLLHCFTWELPDGMKPSELDMNDVFGLTAPRASRLIAVPSKRVVCPL- 516
Poplar              LDLAVAHLLHCFTWELPDGMKPSELDMTDMFGLTAPRATRLVAVPRKRVVCPL- 513
Rapeseed            LELAVAHILHCFTWKLPDGMKPSELDMSDVFGLTAPKATRLYAVPCTRLICAL- 520
                    *::****:** *.*:*********:** *:******:::** *:*  * : .:
```

Appendix Figure 24 (Continued)

```
  1 aagaattggt attgttacgg aaaagcggtg gcggagatgg cggcttggga ggtggccaag
 61 gacaagggag tggacctagt ggtagtgaac ccagtactag tacttgggcc acttctacaa
121 gccactttaa atgcaagtac tattcacatc ctcaagtacc taactggctc agccaagact
181 tatgccaatt ctgtccaagc ttatgtgcac gttaaggacg ttgctttggc ccacattatt
241 gtgtttgaga ctccctccgc ctctggccgc tacctttgcg ccgagactgt ccttcaccgt
301 ggtgatgttg tggagattct cgtaaagctc ttccctgggt atcccattcc taccaagtgc
361 tcagatgaga agaacccaag agcaaagccc tacaagttca ccaacaagaa gctcaaggat
421 ttgggcttgg aatttacccc ggtgaggcag tgcctatacg taactgtcaa gtgcttgcag
481 gaaaaaggtc acctcccaat tccaaaacaa ccagaagacc ccattagaat ccaaacttag
541 ttctcttatg gatttaagtg ggtgacattt aggtacttgt ggtacatgta attgattaac
601 tagttggggg aacactcgcc aagaaccaat gtaccatgtg ctgtcttttg tatggtttct
661 tttgttgatg ttttactgtt cttaaaccaa atgatgtttt attgttctta agccatataa
721 tacaattcct attctaaaaa aaaaaaaaaa aaaaaaaaaa a
```

Appendix Figure 25 The partial nucleotide sequences of *GmCCR* cDNA fragment (Accession no. KJ671477).

```
NWYCYGKAVAEMAAWEVAKDKGVDLVVVNPVLVLGPLLQATLNASTIHILKYLTGSAK
TYANSVQAYVHVKDVALAHIIVFETPSASGRYLCAETVLHRGDVVEILVKLFPGYPIP
TKCSDEKNPRAKPYKFTNKKLKDLGLEFTPVRQCLYVTVKCLQEKGHLPIPKQPEDPI
RIQT
```

Appendix Figure 26 The partial deduced amino acid sequences of *GmCCR* cDNA fragment.

```
Eucalyptus      ------------------------------------------------------------
Kenaf           ------------------------------------------------------------
Poplar          MPVDASSLSGQGQTICVTGAGGFIASWMVKLLLDKGYTVRGTARNPADPKNSHLRELEGA 60
Pine            --MTAGKQTEEGQTVCVTGAGGFIASWLVKLLLERGYTVRGTVRNPEDQKNAHLKQLEGA 58
Rubber          MPVDTSSLSGHGQTVCVTGAGGFIASWIVKLLLERGYTVKGTVRNPDDPKNSHLRELEGA 60
Mangosteen      ------------------------------------------------------------

Eucalyptus      -------------------------------------EQMVEPAVIGTRNVIVAAAEAK 22
Kenaf           -------------------------------------EQMVEPAVNGTKNVIMAAAEAK 22
Poplar          EERLTLCKADLLDYESLKEAIQGCDGVFHTASPVTDDPEEMVEPAVNGTKNVIIAAAEAK 120
Pine            EERLTLVKADLMDYNSLLNAINGCQGVFHVASPVTDDPEEMVEPAVNGTKNVLDACAVAG 118
Rubber          KERLTLCKADLLDYESLRKAIMGCDGVFHAASPVTDDPEQMVEPAVNGTKNVVIAAAEAK 120
Mangosteen      ------------------------------------------------------------

Eucalyptus      VRRVVFTSSVGAITMDPNRGPDVVVDESCWSDLDFCKSTKNWYCYGKAVAEKSACAEAKE 82
Kenaf           VRRVVFTSSIGAVYMDPNRSPDVVVDESCWSDLEFCKNTKNWYCYGKAVAEQAAWETAKE 82
Poplar          VRRVVFTSSIGAVYMDPNKGPDVVIDESCWSDLEFCKNTKNWYCYGKAVAEQAAWDMAKE 180
Pine            VRRVVFTSSIGAVYMDPSRDYDALVDENCWSNLDYCKETKNWYCYGKTVAEKAAWERAKD 178
Rubber          VRRVVFTSSIGAVYMDPNRNPDVVVDESCWSDLDFCKNTKNWYCYGKAVAEQAAWEVAKE 180
Mangosteen      ----------------------------------------NWYCYGKAVAEMAAWEVAKD 20
                                                        *******:*** :*   **:

Eucalyptus      RGVDLVVINPVLVLGPLLQSTVNASIIHILKYLTGSAKTYANSVQAYVHVKDVALAHILV 142
Kenaf           KGVDLVVVAPVLVLGPLLQSTVNASTVHILKYLTGSAKTYANSVQAYVHVRDVALAHILV 142
Poplar          KGVDLVVVNPVLVLGPLLQPTVNASITHILKYLTGSAKTYANSVQAYVHVRDVALAHILV 240
Pine            KGLDLVVVNPCVVLGPVLQSSINASIIHILKYLTGSAKTYANSVQAYVHVRDVAEAHILV 238
Rubber          KGVDLVAVNPVLVLGPLLQSTVNASIIHILKYLTGSAKTYANSVQAYAHVKDVALAHILV 240
Mangosteen      KGVDLVVVNPVLVLGPLLQATLNASTIHILKYLTGSAKTYANSVQAYVHVKDVALAHIIV 80
                :*:***.:: * :****:**.::***  ********************.**:*** ***:*

Eucalyptus      FETPSASGRYLCAESVLHRGDVVEILAKFFPEYNLPT----------------------- 179
Kenaf           FENPSASGRYLCAESVLHRG---------------------------------------- 162
Poplar          FETPSASGRYLCSESVLHRGEVVEILAKFFPEYPIPTKCSDEKNPRKQPYKFSNQKLRDL 300
Pine            YESPSASGRYLCAESVLHRGDVVDLLASMFPQYPIPTKVKEDGKPRVKPWKVSNQKLKDL 298
Rubber          YEIPSASGRYLCAESVLHRGEVVEILAKSFPEYPIPTRCSDEKNPRAKPYKFSNQKLKDL 300
Mangosteen      FETPSASGRYLCAETVLHRGDVVEILVKLFPGYPIPTKCSDEKNPRAKPYKFTNKKLKDL 140
                :* *********:*:*****

Eucalyptus      ------------------------------------
Kenaf           ------------------------------------
Poplar          GFEFTPVKQCLYETVKSLQEKGHLPIPKQAAEESLKIQ 338
Pine            GLEFTPAKQCLYETVISLQEKGHISK------------ 324
Rubber          GMEFTPVKQCLYETVKSLQERGHLPIPKQPEDSIRIQS 338
Mangosteen      GLEFTPVRQCLYVTVKCLQEKGHLPIPKQPEDPIRIQT 178
```

Appendix Figure 27 Alignment of partial deduced amino acid sequence of *GmCCR* cDNA fragment with other plants, eucalyptus (accession no. AAT74893), kenaf (accession no. ABK30883), poplar (accession no. CAC07424), pine (accession no. AFC38436) and rubber (accession no. ADU64758). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been conserved. The dot (.) means that the semi-conserved substitutions are observed.

```
   1 atggggatgc aaataaaggc atttacaata cttaattgtc caattctctt ccactagaat
  61 atcaaaacca tggatttact cctattggag aagacccttt tggctctttt ctttgcagcc
 121 gtagtggcca tcgtgatttc caccctccgt ggcaagcgtt tcaagctgcc accgggtcct
 181 ctacccattc ccgttttcgg aaactggctc gaagtgggag atgacctaaa ccaccggaac
 241 ctaactgagt tagccaaaaa attcggacaa atcttccttc tccggatggg acaacgcaat
 301 cttgttgtgg tctcctctcc tgagttggct aaagacgttt tgcacaccca aggagttgag
 361 tttgggtccc gaacgcgtaa cgttgtgttc gacatcttca caggagaagg ccaggacatg
 421 gttttcactg tctatggcga gcattggagg aagatgagga ggatcatgac cgtccctttc
 481 ttcaccaaca aggttgtcca acaatacaga tttggttggg agtatgaagc ggcacaagtg
 541 gtggaggatg ttaggaagaa cccggacgct gcaacaaaag ggatagttct aaggaagagg
 601 ttgcagctaa tgatgtacaa caatatgtac aggattatgt tcgacaggag gtttgagagt
 661 gaggatgatc ccttgttcaa taagctcaag gctttgaatg gagagagaag taggttggct
 721 cagagttttg agtataatta cggtgacttt attccaattt tgaggccttt cttgagaggg
 781 tatttaaaga tttgcaagga ggtgaaggaa aggaggttgc agctctttaa ggactatttt
 841 gttgaagaaa ggaagaaatt ggcaagtaca aagagcatga gcaacgagag cttgaaatgc
 901 gcgatagacc atatcttgga cgcccaacag aagggagaaa tcaacgagga caacgttctt
 961 tactcgttga aaatatcaac gttgctgcaa ttgagatcaa cattatggtc aatcgagtgg
1021 ggaatagcgg agctcgtgaa ccatcctgaa atccagaaaa aacttcatca cgaactagac
1081 acaatcctcg gtccaggcaa ccaaatcacc gagccagaca cccaaaagct cccatacctc
1141 caggcagtga tcaaagaaac cctacgtctc agaatggcca tcccactcct cgtcccgcac
1201 atgaacctca acgatgccaa gctcggtggc tatgacatcc ccgcagagag taagatcttg
1261 gtcaatgctt ggtggctagc caacaatcca gccaactgga agaatcctga agaattccgg
1321 cccgaaaggt tcttagaaga ggagtccaaa gttgaggcta atgggaatga ctataagtac
1381 ctcccatttg gagttggtag gaggagttgc cctggaatta tcttggcatt gccaatcctt
1441 gggattacaa tagggagatt ggtgcagaac tttgagcttt tgcctcctcc aggacagtcc
1501 aggattgata cctcggagaa aggtgggcag ttcagcttgc atatattgaa gcattccacc
1561 atagttgcca agcctcggtc gttctagaaa ttgttctctt atagttgatg tgtttcctga
1621 ttccgtcctt ttttgatttg tattgttggt ggtgtaatgt tactcttaaa tgcttacctt
1681 gaattgtgat ggaattgacc gaaaaaaaaa aaaaaaaaa
```

Appendix Figure 28 The full length nucleotide sequences of *GmC4H* cDNA fragment (Accession no. KJ671478).

MDLLLLEKTLLALFFAAVVAIVISTLRGKRFKLPPGPLPIPVFGNWLEVGDDLNHRNL
TELAKKFGQIFLLRMGQRNLVVVSSPELAKDVLHTQGVEFGSRTRNVVFDIFTGEGQD
MVFTVYGEHWRKMRRIMTVPFFTNKVVQQYRFGWEYEAAQVVEDVRKNPDAATKGIVL
RKRLQLMMYNNMYRIMFDRRFESEDDPLFNKLKALNGERSRLAQSFEYNYGDFIPILR
PFLRGYLKICKEVKERRLQLFKDYFVEERKKLASTKSMSNESLKCAIDHILDAQQKGE
INEDNVLYSLKISTLLQLRSTLWSIEWGIAELVNHPEIQKKLHHELDTILGPGNQITE
PDTQKLPYLQAVIKETLRLRMAIPLLVPHMNLNDAKLGGYDIPAESKILVNAWWLANN
PANWKNPEEFRPERFLEEESKVEANGNDYKYLPFGVGRRSCPGIILALPILGITIGRL
VQNFELLPPPGQSRIDTSEKGGQFSLHILKHSTIVAKPRSF

Appendix Figure 29 The full length deduced amino acid sequences of *GmC4H* cDNA fragment.

```
Orange          MDLNGWCNSGNQNMCCCQSYVKRGYDRVLSFNGLITVSKLRGKRFKLPPGPLPVPVFGNW 60
Poplar          MDL--------------LLLEKTLLGSFVAVLVAILVSKLRGKRFKLPPGPLPVPVFGNW 46
Cotton          MDL--------------LFLEKALLGLFVAVVLAITISKLRGKRFKLPPGPLPVPVFGNW 46
Cucumber        MDL--------------LLLEKTLLGLFLSVVLAIAISKLRGKRFKPPPGPLPVPIFGNW 46
Eucalyptus      MDL--------------LLLEKTLLGLFAAAIVAIAVSKLRGKRFRLPPGPLPVPIFGNW 46
Mangosteen      MDL--------------LLLEKTLLALFFAAVVAIVISTLRGKRFKLPPGPLPIPVFGNW 46
                ***                *     . :    * :*.******: ******:*:****

Orange          LQVGDDLNHRNLSDLAKKYGDVLLLRMGQRNLVVVSSPDHAKEVLHTQGVEFGSRTRNVV 120
Poplar          LQVGDDLNHRNLTDLAKKFGDILLLRMGQRNLVVVSSPDLAKEVLHTQGVEFGSRTRNVV 106
Cotton          LQVGDDLNHRNLTDLAKKFGDIFLLRMGQRNLVVVSSPELAKEVLHTQGVEFGSRTRNVV 106
Cucumber        LQVGDDLNHRNLTDLAKKFGDIFLLRMGQRNLVVVSSPELAKEVLHTQGVEFGSRTRNVV 106
Eucalyptus      LQVGDDLNHRNLTDLAKRFGDILLLRMGQRNLVVVSSPDLSKEVLHTQGVEFGSRTRNVV 106
Mangosteen      LEVGDDLNHRNLTELAKKFGQIFLLRMGQRNLVVVSSPELAKDVLHTQGVEFGSRTRNVV 106
                *:**********::***::*:::***************: :*:*****************

Orange          FDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQQQRFNWEDEAARVVEDVKKDPE 180
Poplar          FDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQQYRYGWEEEAAQVVEDVKKNPE 166
Cotton          FDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQQYRFGWEDEAARVVEDVRKNPE 166
Cucumber        FDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQQYRQGWEFEAQSVVDDVKKNPE 166
Eucalyptus      FDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQQYREGWEAEAAAVVEDVKKNPA 166
Mangosteen      FDIFTGEGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQQYRFGWEYEAAQVVEDVRKNPD 166
                ******:****************************** * .** **  **:**:*:*

Orange          AATNGIVLRRRLQLMMYNNMYRIMFDRRFESQDDPLFNRLKALNGERSRLAQSFEYNYGD 240
Poplar          AATHGIVLRRRLQLMMYNNMYRIMFDRRFESEEDPLFNKLKALNGERSRLAQSFDYNYGD 226
Cotton          AATNGIVLRRRLQLMMYNNMYRIMFDTRFESEDDPLFVRLKALNGERSRLAQSFEYNYGD 226
Cucumber        SATTGIVLRRRLQLMMYNNMYRIMFDRRFESEDDPLFHKLRALNGERSRLAQSFEYNYGD 226
Eucalyptus      AAREGIVLRRRLQMMMYNNMYRIMFDRRFDSEDDPLFVKLKALNGERSRLAQSFDYNYGD 226
Mangosteen      AATKGIVLRKRLQLMMYNNMYRIMFDRRFESEDDPLFNKLKALNGERSRLAQSFEYNYGD 226
                :*  *****:***:************ **:*::**** :*:**************:*****

Orange          FIPILRPFLRGYLKICKEVKERRLQLFKDYFVEERKKLASTKSMSNESLKCAIDHILDAQ 300
Poplar          FIPILRPFLRGYLKICKEVKERRLQLFKDYFVEERKKLGSTKSMSNEGLKCAIDHILDAQ 286
Cotton          FIPILRPFLRGYLKICKEVKDRRLQLFKDHFVEERKKLGSTKSMNNDGLKCAIDHILDAQ 286
Cucumber        FIPILRPFLRGYLKICKEVKETRLKLFKDYFVEERKKLANTKSTTNEGLKCAIDHILDAQ 286
Eucalyptus      FIPILRPFLRGYLKICKEVKERRLQLFKDYFVDERKKLASVKRMDNEGLKCAMDHILEAQ 286
Mangosteen      FIPILRPFLRGYLKICKEVKERRLQLFKDYFVEERKKLASTKSMSNESLKCAIDHILDAQ 286
                ********************: **:****:**:*****...*   *:.****:****:**

Orange          TKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNHPEIQKKLRNELDTVLGPGHQI 360
Poplar          KKGEINEDNVLYIVENINVAAIETTLWSIEWEIAELVNHSLIQKKLRDELDTVLGPGHQI 346
Cotton          QKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNHPEIQKKLRHELDTVLGPGNQI 346
Cucumber        QKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNHPEIQRKLRNELDTVLGPGVPI 346
Eucalyptus      QKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNHPQIQRKLREEIDAVLGPGVPV 346
Mangosteen      QKGEINEDNVLYSLKISTLLQLRSTLWSIEWGIAELVNHPEIQKKLHHELDTILGPGNQI 346
                 ********** ::  .:  :.:******* *******. **:**:.*:*::****  :
```

Appendix Figure 30 Alignment of full – length of amino acid sequence of *GmC4H* cDNA fragment with other plants, orange (accession no. NP_001275824.1), poplar (accession no. AFZ78542), eucalyptus (accession no. AGJ71350.1), cotton (accession no. AAG10196) and cucumber (accession no. CAK95273). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been conserved. The dot (.) means that the semi-conserved substitutions are observed.

```
Orange          TEPDTHKLPYLQAVIKETLRLRMAIPLLVPHMNLHDAKLGGYDVPAESKILVNAWWLANN 420
Poplar          TEPDTYKLPYLNAVIKETLRLRMAIPLLVPHMNLHDAKLGGFDIPAESKILVNAWWLANN 406
Cotton          TEPDTHKLPYLQAVIKETLRLRMAIPLLVPHMNLHDAKLGGYDIPAESKILVNAWWLANN 406
Cucumber        TEPDTQKLPYLQAVVKETLRLRMAIPLLVPHMNLHDAKLAGYDIPAESKILVNAWWLANN 406
Eucalyptus      TEPELHKLPYLQAVIKETLRLRMAIPLLVPHMNLHDAKLGGYDIPAESKILVNAWWLANN 406
Mangosteen      TEPDTQKLPYLQAVIKETLRLRMAIPLLVPHMNLNDAKLGGYDIPAESKILVNAWWLANN 406
                ***:  *****:**:******************:****.*:*:***************

Orange          PAQWKKPEEFRPERFLEEESKVEANGNDFRYLPFGVGRRSCPGIILALPILGITIGRLVQ 480
Poplar          PAKWKNPEEFRPERFFEEEAKVEANGNDFRYLPFGVGRRSCPGIILALPILGITLGRLVQ 466
Cotton          PANWKNPEEFRPERFFEEEAKVEANGNDFRYLPFGVGRRSCPGIILALPILGITLGRLVQ 466
Cucumber        PANWKNPEEFRPERFLEEESKVEANGNDFKYLPFGAGRRSCPGIILALPILGITIGRLVQ 466
Eucalyptus      PAHWKKPEEFRPERFLEEEAKVEANGNDFRYLPFGVGRRSCPGIILALPILGVTIGQLVQ 466
Mangosteen      PANWKNPEEFRPERFLEEESKVEANGNDYKYLPFGVGRRSCPGIILALPILGITIGRLVQ 466
                **:**:*********:***:********::*****.***************:*:*:***

Orange          NFELLPPPGQSKIDTAEKGGQFSLHILKHSTIVAKPRSF 519
Poplar          NFELLPPPGQSKIDTSEKGGQFSLHILKHSTIVAKPRSF 505
Cotton          NFELLPPPGQSQIDTTEKGGQFSLHILKHSTIVAKPRQF 505
Cucumber        NFELLPPPGQSKLDTSEKGGQFSLHILKHSTIVVKPRVF 505
Eucalyptus      NFELLPPPGQSKLDTTEKGGQFSLHILKHSTIVLKPRSF 505
Mangosteen      NFELLPPPGQSRIDTSEKGGQFSLHILKHSTIVAKPRSF 505
                ***********:.**:**************** *** *
```

Appendix Figure 30 (Continued)

```
   1 atgcagactc acgataaagg cctgcaccag gtctcttcgc acagaaagtg gggagagccc
  61 atattgaaga aaggtccatg gagaccagaa gaagatatca ttttagtctc ttatatacgg
 121 gaacatggtc ctgggaattg gagatcggtt cccactaata ctggtttgct tagatgcagc
 181 aagagttgca ggctcagatg gactaattat ctccgccctg gaataaagcg tggtggcttc
 241 acagaacatg aagagaagat gattatccat ctccaagctc ttttgggaaa tagatgggca
 301 gccatagcat catatcttcc ccaaagaaca gacaacgaga tcaagaatca ttggcatacc
 361 catctaaaga aaattcatga gcaaaaacaa agaccaccca atcaagaatc aaaagaccaa
 421 acactggaag tattgagcca agatggtgtt actggagagt taaaagcaga aattagtgct
 481 ctacccatta cttgtaacaa cccttgtcac ccagtaatat ttgagagcta ccctttttcc
 541 ccatcagctt cttccagtga gtcatctggc ttgagcacca attcagctat agttaacgaa
 601 gtatatgatg gagatgaagc aaacaagaaa tgtgcttgga atcaagaaca tggcctttct
 661 tataatgggt ggtcagaagt tccaaaaatt gcaggtcata caaggaatag cagaagttgt
 721 ggatggatga aaaattatgt gcctgaagtg aaacgtgaaa gctaccggaa agaagaagaa
 781 gatttgatca tcaacttaca tgagcaactt gggaacagtt ggtctgctaa tgctattcca
 841 ttaccggaga aaaccgacgc cgagataatg aaatatgaga gtatccacca caaaggcatg
 901 gatttcaatg aacagccaat tggcgaaatt tccccaagtg aaaaagacaa cgtaagtttt
 961 attgaccaat tggaaaactt ggcagaaaca ccttccagtg gattctcctt ttttagctct
1021 gaacttgaca tgggaaatac tatgacccca atagggtggt ttatggagga aagttcaaat
1081 ccatttgaga catttgaagc agatttttgg acggagccat tcctgctaga ttctgaccgc
1141 tactaaatat gattgtaatg ttacgtcctc gatggataat tggtaaattt atggtactgg
1201 tgaaattatg ttgtcactat aattgaagtg cctatgattt gactcggatg acgatgatga
1261 tggttagata tttcataatg aatctagagc tgggttttta ataccactta tatctcgtgt
1321 aaatagattc tcaatgaaat aaaatcatcc catttcgaag aaaaaaaaaa aaaaaaa
```

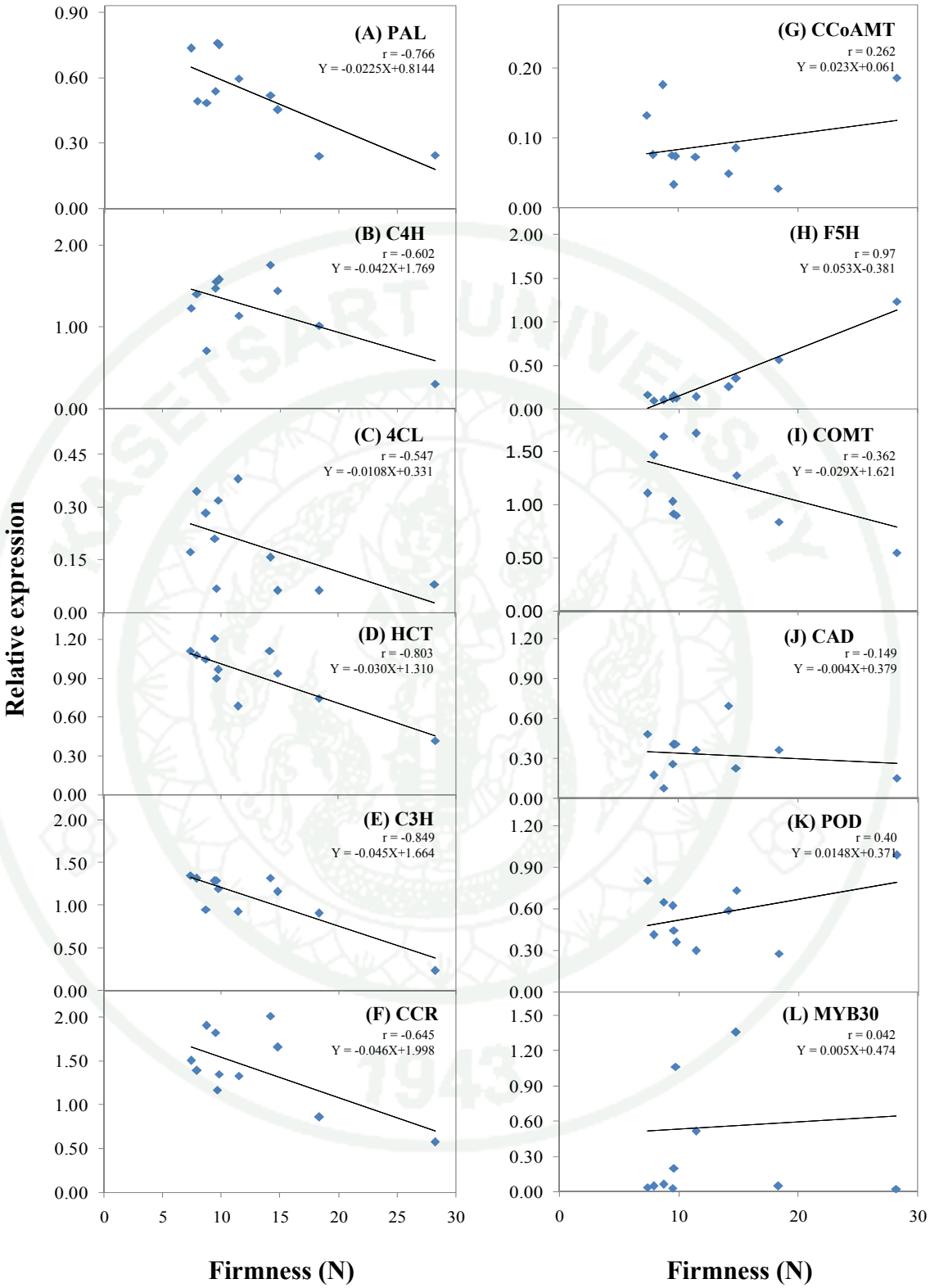
Appendix Figure 31 The full length nucleotide sequences of *GmMYB30* cDNA fragment (Accession no. KJ671479).

```
MQTHDKGLHQVSSHRKWGEPILKKGPWRPEEDIILVSYIREHGPGNWRSVPTNTGLLRCSKSCRLRWTNYL
RPGIKRGGFTEHEEKMIIHLQALLGNRWAAIASYLPQRTDNEIKNHWHTHLKKIHEQKQRPPNQESKDQTL
EVLSQDGVTGELKAEISALPITCNNPCHPVIFESYPFSPSASSSESSGLSTNSAIVNEVYDGDEANKKCAW
NQEHGLSYNGWSEVPKIAGHTRNSRSCGWMKNYVPEVKRESYRKEEEDLIINLHEQLGNSWSANAIPLPEK
TDAEIMKYESIHHKGMDFNEQPIGEISPSEKDNVSFIDQLENLAETPSSGFSFFSSELDMGNTMTPIGWFM
EESSNPFETFEADFWTEPFLLDSDRY
```

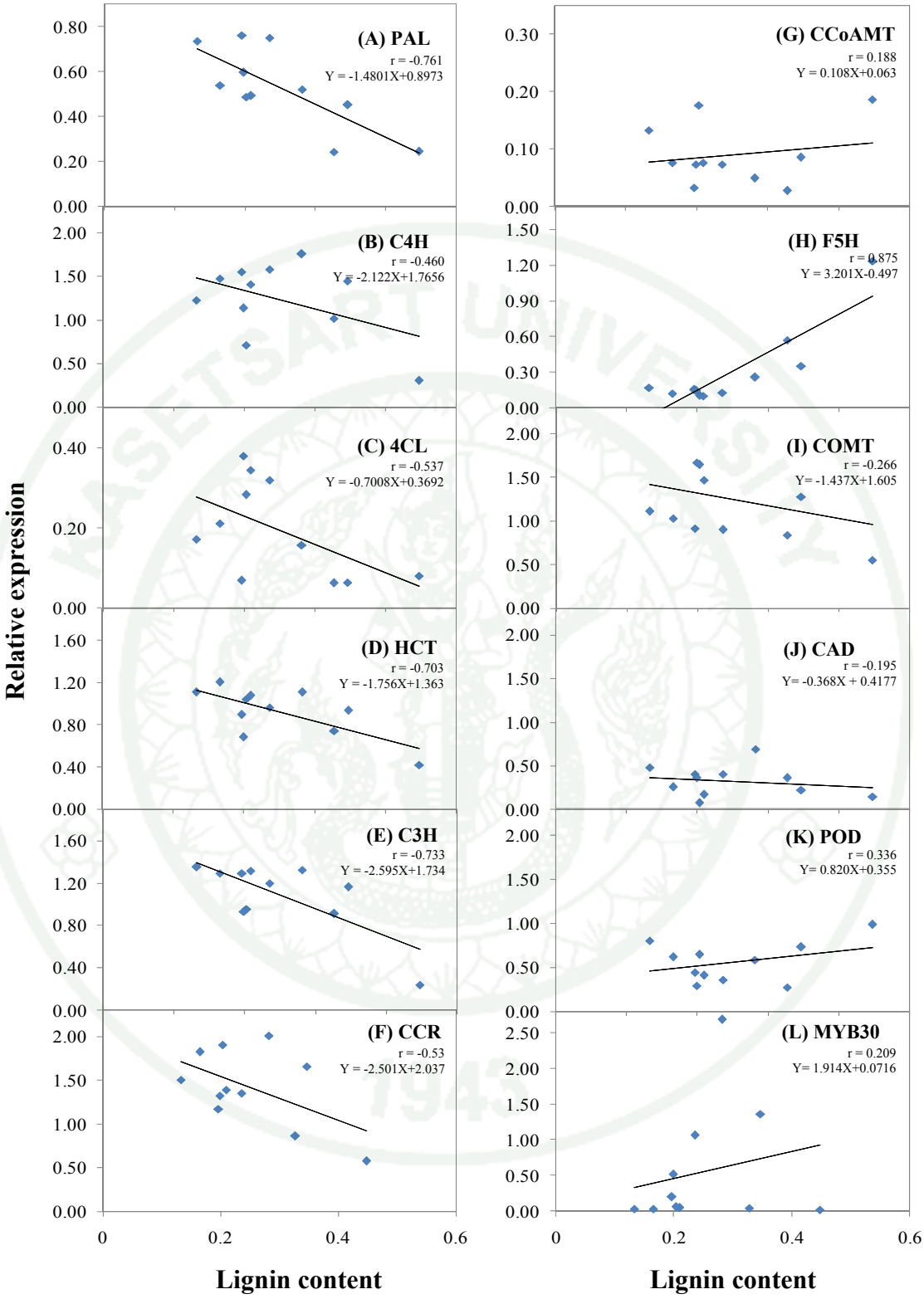
Appendix Figure 32 The full length deduced amino acid sequences of *GmMYB30* cDNA fragment.

```
MYB94_Arabidopsis      ----------MGRPPCCDKIGVKKGPWTPEEDIILVSYIQEHGPGNWRSVPTHTGLRRCS 50
R2R3_MYB_Medicago      ----------MVRPPCCEKIGVKKGPWTPEEDIILVSYIQEHGPGNWRSVPTTTGLMRCS 50
MYB30_Arabidopsis      ----------MVRPPCCDKGGVKKGPWTPEEDIILVTYIQEHGPGNWRAVPTNTGLLRCS 50
R2R3_MYB60_Vitis       ----------MGRPPCCDKVGIKKGPWTPEEDIILVSYIQEHGPGNWRSVPTNTGLLRCS 50
GmMYB30                MQTHDKGLHQVSSHRKWGEPILKKGPWRPEEDIILVSYIREHGPGNWRSVPTNTGLLRCS 60
                                :       :  :***** ********:**:********:*** *** ***

MYB94_Arabidopsis      KSCRLRWTNYLRPGIKRGNFTEHEEKMILHLQALLGNRWAAIASYLPERTDNDIKNYWNT 110
R2R3_MYB_Medicago      KSCRLRWTNYLRPGIKRGNFNDHEEKMIIHLQALLGNRWAAIASYLPERTDNDIKNYWNT 110
MYB30_Arabidopsis      KSCRLRWTNYLRPGIKRGNFTEHEEKMIVHLQALLGNRWAAIASYLPQRTDNDIKNYWNT 110
R2R3_MYB60_Vitis       KSCRLRWTNYLRPGIKRGNFTPHEEGMIIHLQALLGNKWAAIASYLPQRTDNDIKNYWNT 110
GmMYB30                KSCRLRWTNYLRPGIKRGGFTEHEEKMIIHLQALLGNRWAAIASYLPQRTDNEIKNHWHT 120
                       ******************.*. *** **:********:*********:****:***:*:*

MYB94_Arabidopsis      HLKKKLKKMNDSCDSTINNGLDNKDFSISNKNTTSHQSSNS---SKGQWERR-LQTDINM 166
R2R3_MYB_Medicago      HLRKKLKKINQTGD----------------ENEVEENSIPQ---VKGQWERR-LQTDIHM 150
MYB30_Arabidopsis      HLKKKLNKVNQDSHQELDRS----SLSSSPSSSSANSNSNI---SRGQWERR-LQTDIHL 162
R2R3_MYB60_Vitis       HLKKKIKKFQSALS---------------PHMASDSTTSTC---TNHQFVPRSYAGDDHH 152
GmMYB30                HLKKIHEQKQRPPNQESKDQTLEVLSQDGVTGELKAEISALPITCNNPCHPVIFESYPFS 180
                       **:*  :: :                                  .

MYB94_Arabidopsis      AKQALCDALSIDKPQNPTNFSIPDLGYGPSSSSSSTTTTTTTTRNT-----NPYPSGVYA 221
R2R3_MYB_Medicago      AKQALCEALSLDKPTP----ILAENQTSPYASNTENIARLLEKWMK-----KPENSVETT 201
MYB30_Arabidopsis      AKKALSEALSPAVAPI----ITSTVTTTSSSAESRRSTSSASGFLR-----TQETSTTYA 213
R2R3_MYB60_Vitis       RRGSSFEVINGHSSAH----PSLNSPISTYASSTENISRLLEGWMRSSPKATKEKLHQNS 208
GmMYB30                PSASSSESSGLSTNSAIVNEVYDGDEANKKCAWNQEHGLSYNGWSEVP---KIAGHTRNS 237
                          :  :  .                   .: .                .      :

MYB94_Arabidopsis      SSAENIARLLQNFMKDTPKTSVPLPVAATEMAITTAAS---SPSTTEGDGEGIDHSLFSF 278
R2R3_MYB_Medicago      NSGNSIMVVTGSGSREGGQN--TIACKQKDQAFDSLVS---FNSLNSDCSQSVSVEEKNF 256
MYB30_Arabidopsis      SSTENIAKLLKGWVKNSPKT------QNSADQIASTEV---KEVIKSDDGKECAGAFQSF 264
R2R3_MYB60_Vitis       SLEEGSIDMTGNSMAVAAVTSVQCYRPKLEQGGGELVANDEFESILEYENLNDDHHQTTD 268
GmMYB30                RSCGWMKNYVPEVKRESYRKEEEDLIINLHEQLGNSWSANAIPLPEKTDAEIMKYESIHH 297
                                        .                       .

MYB94_Arabidopsis      NSIDEAEEKP------KLIDHDINGLITQGSLSLFEKWLFDEQSHDMIINNMSLEGQE-- 330
R2R3_MYB_Medicago      LAMDSCFFQY------QSKPNQE----TQDPLMFMENWPFDDEAAQCNEDLMNVSMEENT 306
MYB30_Arabidopsis      SEFDHSYQQAGVSPDHETKPDITGCCSNQSQWSLFEKWLFED-SGGQIGDILLDENTN-- 321
R2R3_MYB60_Vitis       ATIPSDDHDHD-----HEMKMDHDQKKHNPPLSFLEKWLLDESAAQGEE--MMDQLSP-- 319
GmMYB30                KGMDFNEQPIG-----EISPSEKDNVSFIDQLENLAETPSSGFSFFSSELDMGNTMTPIG 352
                         :             .                : :   .  :      :

MYB94_Arabidopsis      -VLF------------------------ 333
R2R3_MYB_Medicago      PGLF------------------------ 310
MYB30_Arabidopsis      --FF------------------------ 323
R2R3_MYB60_Vitis       --IF------------------------ 321
GmMYB30                WFMEESSNPFETFEADFWTEPFLLDSDRY 381
                          :
```

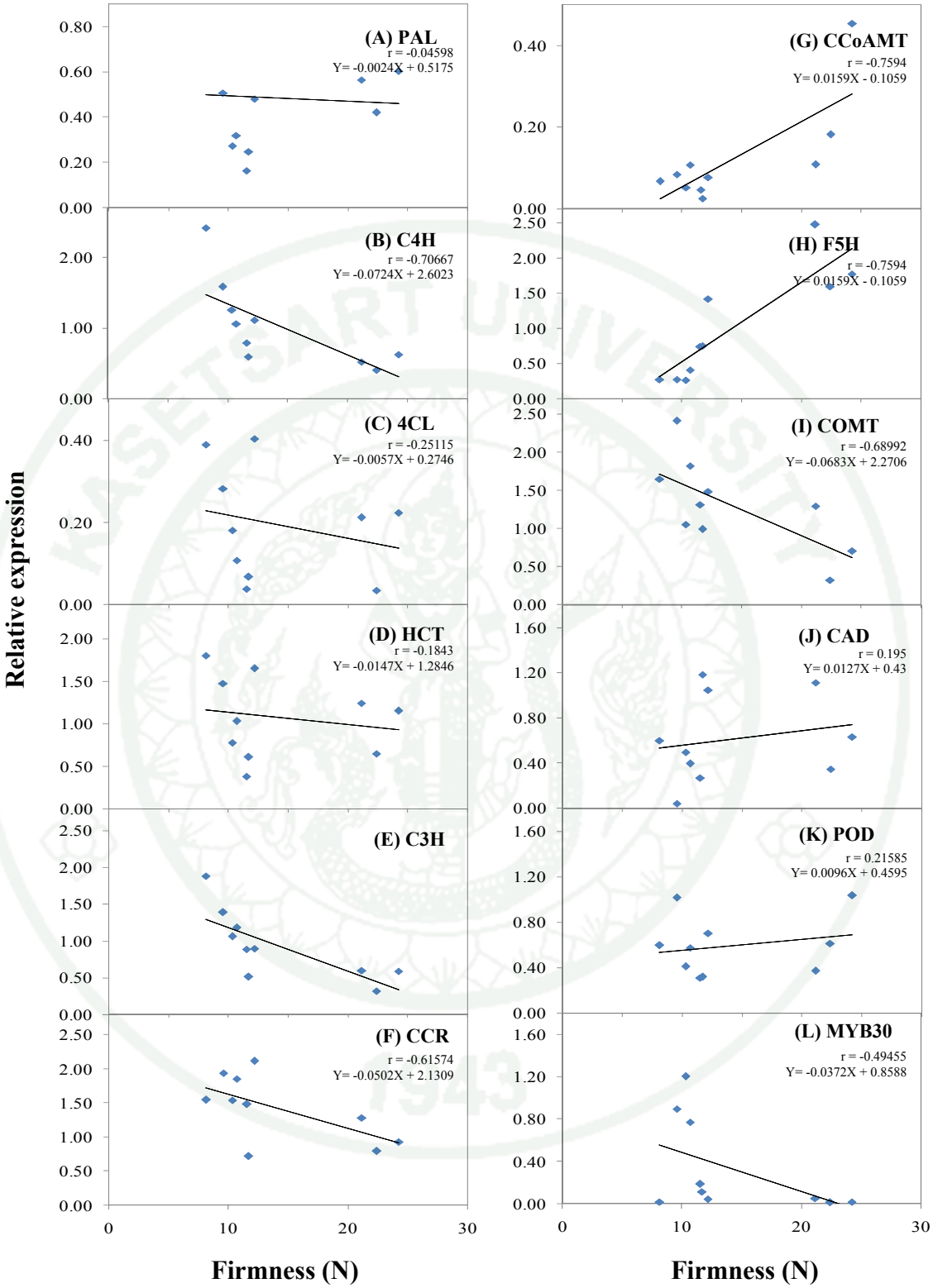
Appendix Figure 33 Alignment of full – length of amino acid sequence of *GmMYB30* cDNA fragment.



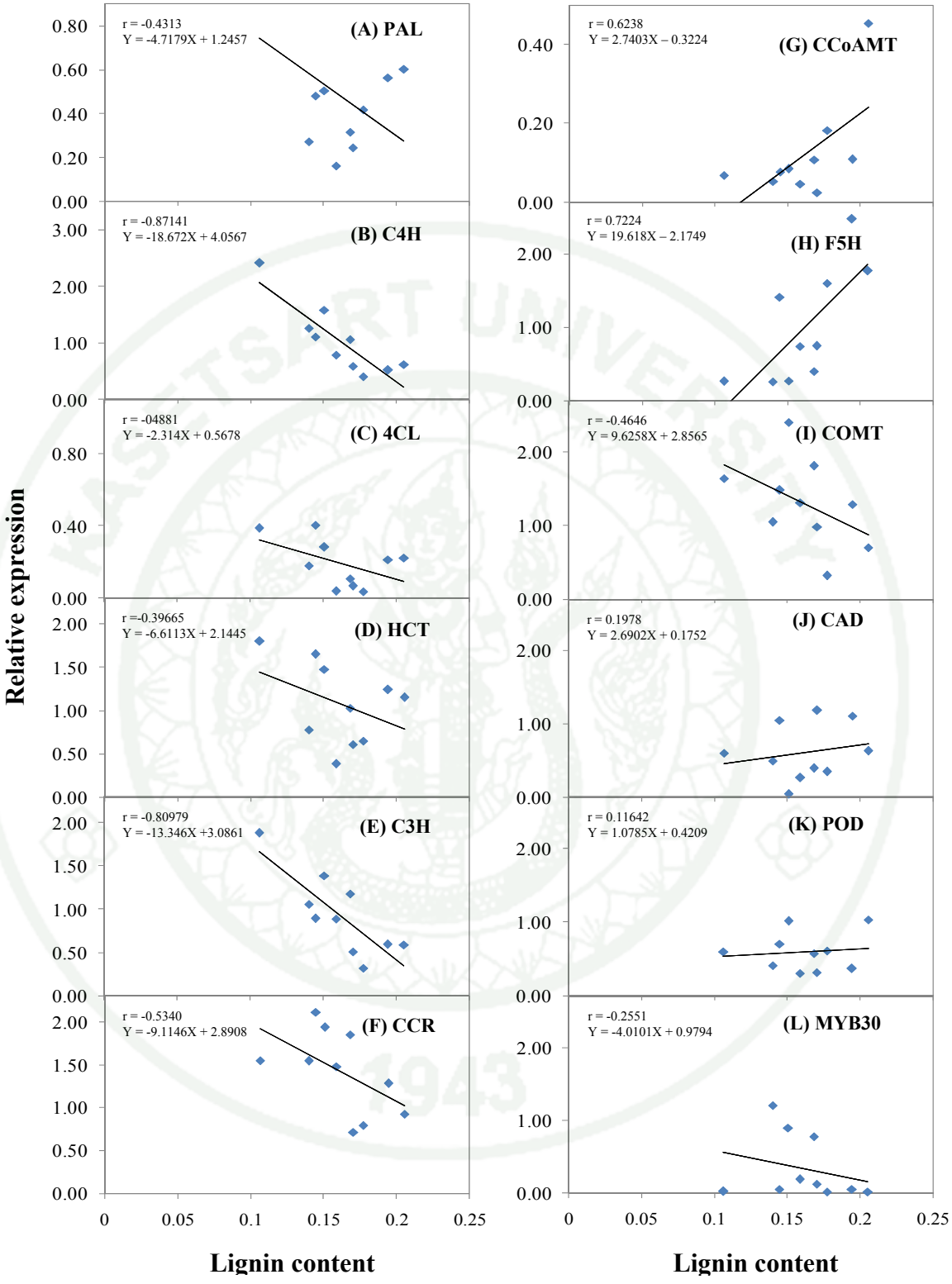
Appendix Figure 34 The correlation of the firmness and the gene expression in lignin biosynthesis of impacted mangosteen pericarp.



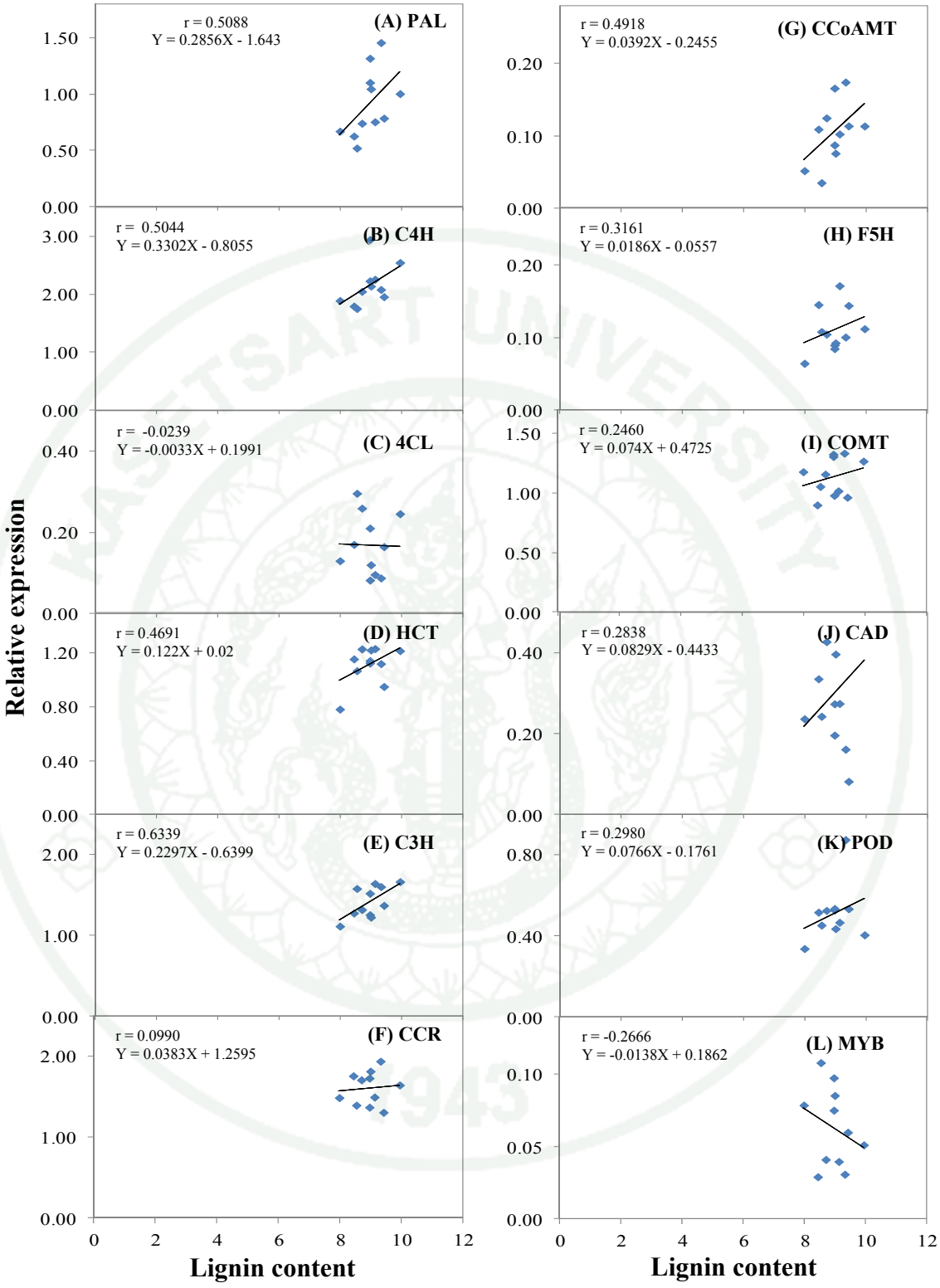
Appendix Figure 35 The correlation of lignin content and the gene expression in lignin biosynthesis of impacted mangosteen pericarp.



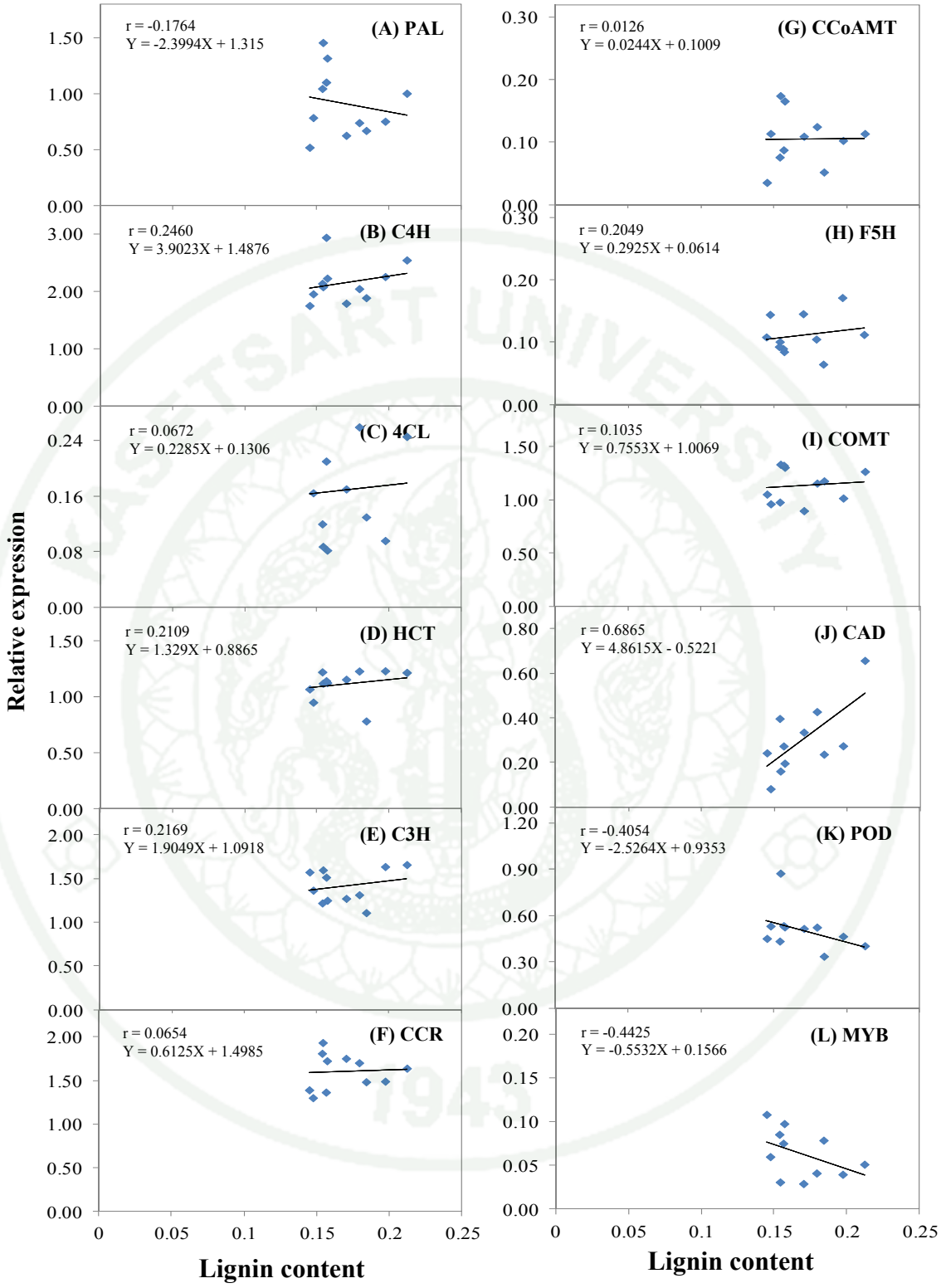
Appendix Figure 36 The correlation of the firmness and the gene expression in lignin biosynthesis of impacted mangosteen pericarp under elevated nitrogen atmosphere.



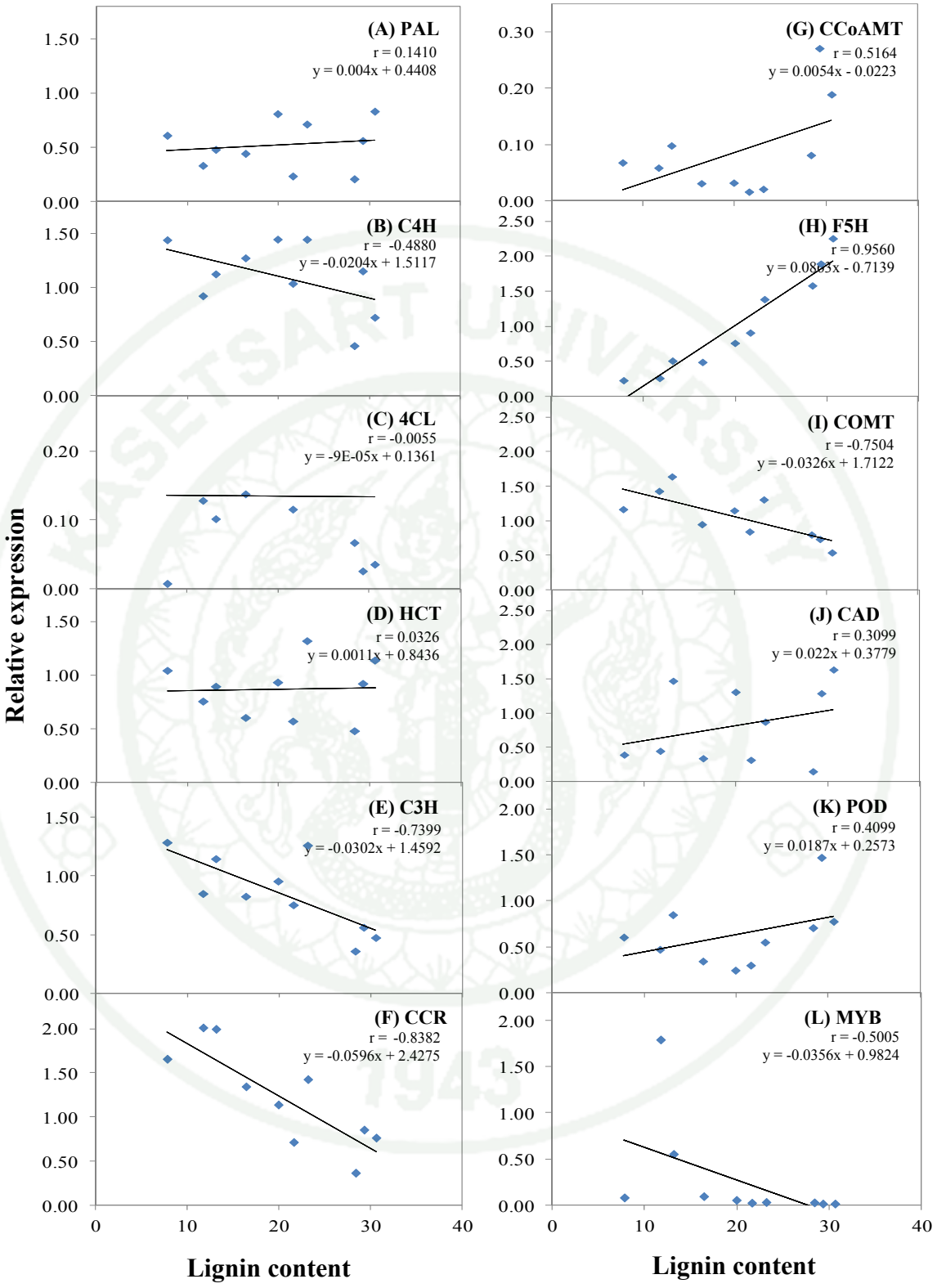
Appendix Figure 37 The correlation of the lignin and the gene expression in lignin biosynthesis of impacted mangosteen pericarp under elevated nitrogen atmosphere.



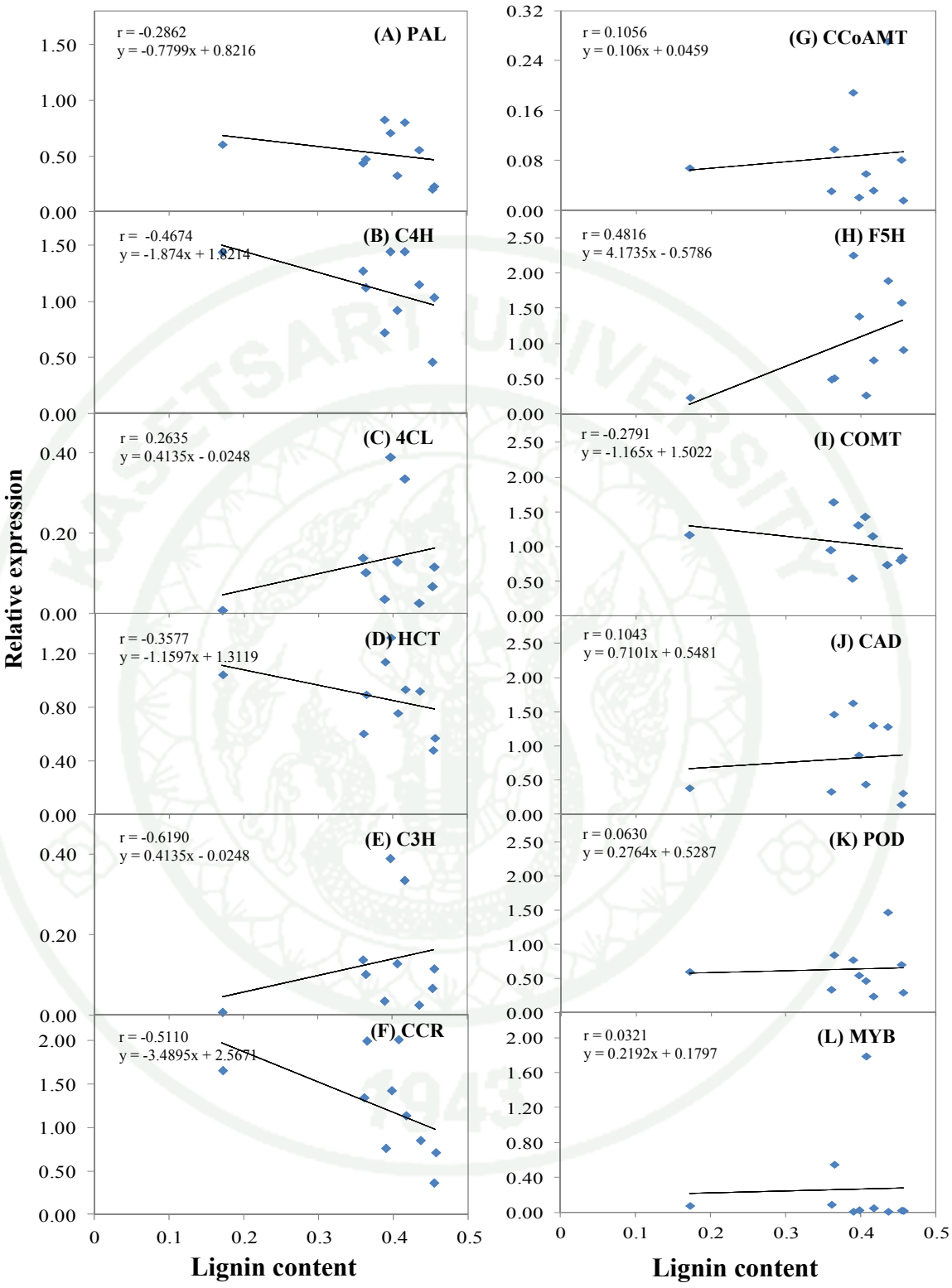
Appendix Figure 38 The correlation of the firmness and the gene expression in lignin biosynthesis of non-impacted mangosteen pericarp.



Appendix Figure 39 The correlation of lignin content and the gene expression in lignin biosynthesis of non- impacted mangosteen pericarp.



Appendix Figure 40 The correlation of the firmness and the gene expression in lignin biosynthesis of impacted mangosteen pericarp under ambient air.



Appendix Figure 41 The correlation of the lignin and the gene expression in lignin biosynthesis of impacted mangosteen pericarp under ambient air.

CURRICULUM VITAE

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Outstanding Oral Presentation Award, The 15th Royal Golden Jubilee Congress, Thailand Research Fund, 28-30 May 2014, Pattaya, Thailand