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# Expression Profiling of Candidate Genes for Insight to Pericarp Browning in Litchi (*Litchi chinensis* Sonn.)

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***Authors' contributions***

*This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

Litchi (*Litchi chinensis* Sonn.), a subtropical fruit crop has high commercial value and consumer acceptance owing to its rich juicy aril and attractive bright red pericarp. Anthocyanin, the major pigment present in litchi pericarp reaches its maximum content in fully ripen fruit contributing to its bright red colour. Anthocyanin content in plants depends on the rate of biosynthesis, stability in the vacuoles and the rate at which it is degraded. The biosynthesis of anthocyanin occurs via an intricate phenyl propanoid pathway controlled by plethora of structural and regulatory genes. Several genes encoding enzymes responsible for anthocyanin synthesis have been isolated and characterised in different plants. Litchi fruit being highly perishable, exhibit relatively shorter

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postharvest shelf-life of 2–3 days at ambient conditions which in part can be attributed to the enzymatic and non-enzymatic degradation of anthocyanin. In contrast to the comprehensive understanding of molecular basis of anthocyanin synthesis, the studies on its catabolism or degradation are meagre. Polyphenols oxidases and peroxidases are the major enzymes responsible for anthocyanin degradation leading to the problem of pericarp browning. Laccase, an anthocyanin degradation enzyme expresses about thousand fold higher than the polyphenols oxidase in the pericarp with epicatechin as favourable substrate. A detailed study of the anthocyanin degradation pathway in litchi may be helpful in managing the problem of pericarp browning to preserve its bright red colour as well as to enhance the shelf life and marketability of this valuable fruit crop.

*Keywords: Candidate genes; browning; Litchi; pericarp; anthocyanin.*

## 1. INTRODUCTION

Litchi (*Litchi chinensis* Sonn.), a subtropical fruit crop has a high commercial value and consumer acceptance owing to its rich juicy aril and attractive bright red pericarp. It is a member of family Sapindaceae, or soap berries which also includes longan and rambutan. Colour of the litchi pericarp is an important quality attribute that determines its consumer acceptance [1]. The red colour of litchi fruit pericarp is the expression of anthocyanins. Lee et al. [2,3]. However, litchi fruit exhibit rapid browning within 2-3 days of harvest [4]. This greatly restricts its transportation and commodity value due to rapid deterioration and browning of pericarp during room storage and post-cold storage under ambient conditions [5,6]. The browning or discoloration of litchi pericarp is thought to be due to leaking of enzymes and substrates and subsequent degradation of the red pigments (anthocyanins) and phenols by polyphenol oxidase (PPO), peroxidase (POD) [7,8,9,10,11] and/or phenyalanine ammonia lyase (PAL) [12] and anthocyanase [13].

Various exogenous treatments i.e. low pH cellulose coatings [14], sulphur dioxide ($SO_2$) fumigation [15], hot water and oxalic acid dips [16], ascorbic acid [17], methyl jasmonate [18], 1-methylcyclopropene [19], hydrochloric acid [20], oxalic acid [21], irradiation [20], salicylic acid [22,23], pyrogallol [24], potassium metabisulfite [22], nitric oxide [25], apple polyphenols [26], L-cysteine [27], kojic acid [28], tea seed oil [29], biocontrol bacteria [30], methionine [31] and novel chitosan formulation [32] have been used to delay litchi pericarp browning and improve the shelf life of litchi fruit. However, molecular approaches at genetic level to reduce the expression of the genes responsible for pericarp browning is still going on. Genes responsible for tissue browning are now been identified which will further help us in elucidation of the mechanism behind litchi pericarp browning and applying in improvement of litchi cultivars with longer shelf life through various bio-engineering techniques. Inhibition of gene expression of genes involved in browning in plant tissues might reduce the enzyme activity and hence reduce the tissue browning. e.g. expression of PPO antisense RNA in ‘‘Yali’’ pear can cause them decreases of the PPO activity in pear leaves [33].

## 2. ANTHOCYANIN: THE PIGMENT RESPONSIBLE FOR FRUIT COLOUR

Anthocyanins are a class of flavonoids responsible for red/pink colouration of litchi pericarp [34]. The pericarp contains a considerable amount of anthocyanin type pigments, either in the form of monomers or polymers with Cyanidin-3-rutinoside as the major anthocyanin pigment along with Cyanidin-3-glucoside and malvidin-3-ace-tylglucoside [35,3]. Compared to other fruit crops, the pigment primarily responsible for red colour in apple skin is cyanidin in the form of cyanidin 3-o-galactoside [36,37], while in mangosteen pericarp mainly consist of cyanidin-3-sophoroside, cyanidin-3-glucoside and several other cyaniding derivatives [38]. *Vitis vinifera* varieties usually produce 3-monoglucoside, 3-acetylglucoside, and 3-p-coumarylglucoside derivatives of the aglycones delphinidin, cyanidin, peonidin, petunidin, and malvidin, with malvidin derivatives often being the major forms present. In cultivated strawberry, the glucosylated anthocyanin pelargonidin (pelargonidin 3-glucoside) is the main anthocyanin present in ripe fruit (approximately 88%), along with other pelargonidin-glycosides and cyanidin 3-glucoside [39]. Anthocyanin pigments accumulation in fruit is an important determinant of ripeness and quality as most of the fruits accumulate it only in their ripening phase [40]. It belongs to a diverse group of secondary metabolites, the flavonoid group which plays a variety of functional roles in plants as in petals is intended to attract pollinators, in

seeds and fruits as seed dispersal, as feeding deterrents and as protection against damage from U.V. radiation Holton et al. 1995.

## 3. ANTHOCYANIN BIOSYNTHESIS

Flavonoids are widely distributed among land plants. They may be classified into about a dozen groups, such as chalcones, flavones, flavonols and anthocyanins based upon their structure. Anthocyanin biosynthesis has been extensively studied in petunia, snapdragon and maize, resulting in the elucidation of the biosynthetic pathway. Anthocyanins are most frequently o-glycosylated (usually glucosylated) at the C3-position, followed by the C5 position. Glycosylation of anthocyanins results in slight reddening. Genes of anthocyanin biosynthetic pathway have been isolated using various methodologies, like protein purification, trans-poson tagging, differential screening, and polymerase chain reaction (PCR) amplification.

The anthocyanin biosynthetic pathway is well established [41,42]. Two classes of genes are required for anthocyanin biosynthesis, the structural genes encoding the enzymes that directly participate in the formation of anthocyanins and other flavonoids, and the regulatory genes that control the transcription of structural genes. The pathway is also controlled in response to different developmental and environmental cues [43,44,55,46,47].

According to Deroles [48], anthocyanin biosynthetic pathway can be divided into two sections, the early and late sections. In the early section are the formation of the dihydroflavanols, comprising phenylalanine ammonialyase (PAL), Cinnimate 4-hydroxylase (C4H), 4-Coumarate: CoA ligase (4CL), Chalcone Synthase (CHS), Chalcone isomerase (CHI) and flavanone 3-hydroxylase (F3H). Genes of these sections are called early genes. The late sections leads to the formation of dihydroflavanol reductase (DFR), anthocyanidins synthase (ANS) and UDP Glucose: Flavonoid 3-O-glucosyltransferase (UFGT) and genes forming these enzymes are called late genes. The key regulatory genes in biosynthesis of anthocyanin vary with fruit species. Zhao et al. 2012 suggested that UFGT plays an important role in anthocyanin biosynthesis in the pericarp of litchi and its expression strongly influences fruit coloration in litchi. The color of red and black grapes results from the accumulation of anthocyanins that are usually only located in the skin of the berry. Also in grape berry, expression of the UDP-glucose: flavonoid3-O glucosyltransferase (UFGT) was critical for anthocyanin biosynthesis [49] with white-skinned grape cultivar lacking in anthocyanins because of absence of UFGT gene [50]. In other fruits like grapes [51], apples [52], red pear [53], MYB transcription factors regulates the biosynthetic genes of anthocyanin pathway. While in Strawberries (*Fragaria* × *ananassa* Duch.), the putative DFR gene plays a main role during colour development [54,55].

Tsuda et al. [56] found that chalcone synthase gene and dihydroflavanol 4- reductase gene are the key regulatory genes in the anthocyanin biosynthesis in mature red peach and nectarine. The enzymes involved in the flavonoid biosynthesis pathway are localized in the cytosol. After biosynthesis, flavonoids are transported to vacuoles or cell walls [57]. The precursors for the synthesis of all flavonoids, including anthocyanins, are malonyl-COA and p-coumaroyl-COA. Chalcone synthase (CHS) catalyzes the stepwise condensation of three acetate units from malonyl-COA with p-coumaroyl-COA to yield tetrahydroxychalcone (THC). Chalcone isomerase (CHI) then catalyzes the stereospecific isornerization of the yellow-colored tetrahydroxychalcone to the colorless naringenin. (2S)-Naringenin is hydroxylated at the 3-position by flavanone 3-hydroxylase (F3H) to yield (2R, 3R)-dihydrokaempferol, a hydroflavonol. Flavonoid 3´-hydroxylase (F3´H) and flavonoid 3´, 5´-hydroxylase (F3´5´H), which are P450 enzymes, catalyze the hydroxylation of dihydrokaempferol (DHK) to form (2R, 3R)-dihydroquercetin and dihydromyricetin, respectively. For converting the colorless dihydroflavonols (DHK, DHQ, and DHM) to anthocyanins, at least three enzymes are needed. The first is reduction of dihydroflavonols to flavan9, 4-cis-diols (leucoanthocyanidins) by dihydroflavonol4-reductase (DFR). Further oxidation, dehydration, and glycosylation of the different leucoanthocyanidins produce the corresponding brick-red pelargonidin, red cyanidin, and blue delphinidin pigments. Anthocyanidin synthase (ANS, also called leucoanthocyanidin dioxygenase), which belongs to the OGD family, catalyzes the synthesis of corresponding colored anthocyanidins. After synthesis, anthocyanin is transported to vacuolar lumen where they are stored. Transport mechanisms of anthocyanins may be redundant or depend on plant species and organs. The first and most established mechanism involves transport of anthocyanins via a glutathione S-

transferase (GST)-like protein and a multi-drug resistance-like protein (a type of ABC transporter).

## 4. TRANSCRIPTIONAL REGULATION OF THE ANTHOCYANIN BIOSYNTHESIS

The spatial and temporal expression of structural genes in anthocyanin biosynthesis is determined by a combination of R2R3 Myb, basic helix–loop–helix (bHLH) and WD40-type transcriptional factors and their interaction. This has been well established in maize, Arabidopsis, petunia and some other plants [57], and in Japanese morning glory [58]. The WD40 and bHLH proteins are pleiotropic and are involved in multiple processes in addition to anthocyanin synthesis, such as the control of vacuolar pH in petunia flowers and the formation of trichomes and root hairs in Arabidopsis. It is believed that they affect these processes via their interactions with specific MYB proteins, such as PH4 in petunia and GL1/Wer in Arabidopsis [57]. Genes of the flavonoid pathway are known to be co-ordinately induced and transcription factors that directly regulate the expression of the structural genes of the pathway have been identified in several species. The pathway is regulated by the interaction of the DNA-binding R2 R3 MYB transcription factors and MYC-like basic helix–loop–helix (bHLH) and WD40-repeat proteins [59,60].

The R2R3 MYB genes associated with the flavonoid pathway represent the most abundant class of MYB genes in plants. Mostly MYBs in the control of flavonoid biosynthesis are positive regulators that enhance the expression of the structural flavonoid pathway genes. But repressors have also been characterized, such as *FaMYB1* in strawberry (*Fragaria* x *ananassa* Duch.) and *VvMYB4* in the berries of grapevine [60,61]. Strawberry *FaMYB1* was reported to suppress anthocyanin and flavanol accumulation in transgenic tobacco lines and over-expression of this gene inhibited the biosynthesis of proanthocyanidins in the leaves of Lotus corniculatus [62]. In fruits, particularly in grapevine, the regulation of flavonoid biosynthesis, 14 flavonoid biosynthesis related R2R3 MYB family members have been described [60,63]. Lai et al. [64] described a litchi R2R3-MYB transcription factor gene, *LcMYB1*, which demonstrates a similar sequence as other known anthocyanin regulators. These results suggest that *LcMYB1* controls anthocyanin biosynthesis in litchi and LcUFGT might be the structural gene that is targeted and regulated by *LcMYB1*. Furthermore, the overexpression of *LcMYB1* induced anthocyanin accumulation in all tissues in tobacco, confirming the function of *LcMYB1* in the regulation of anthocyanin biosynthesis. Also in his other work on transcriptomic study of litchi pericarp, 53 litchi R2R3-MYB TFs were identified as being expressed in the litchi fruit pericarp [65].

Anthocyanins biosynthesis in grapevine berries is regulated by *VvMYBA1* and *VvMYBA2*, that are homologs of Arabidopsis *AtMYB75*, *AtMYB90*, *AtMYB113*, and *AtMYB144* [66]. *MdMYB110a*, a paralog of *MdMYB10*, regulates anthocyanin accumulation in the red-flesh apple phenotype [67]. bHLH proteins involved in flavonoid biosynthesis have been characterized in grapevine, apple, and strawberry [68,69,70,71]. WD40 proteins role in the regulatory complex of anthocyanin biosynthesis was reported for the first time in Arabidopsis TRANSPARENT TESTA GLABRA 1 (*TTG1*) locus [72] since which homologs have been characterized from fruit species including apple [73], grapevine [74], pomegranate [75], and strawberry [71]. Lai et al. [65] identified genes encoding enzymes in the flavonoid biosynthesis pathway in litchi pericarp. Transcripts corresponding to seven PAL genes, five 4CLs and two C4Hs with differential expression patterns were identified and higher expression of CHS and CHI gene were observed in the red stage of litchi pericarp. Furthermore, other than UFGT gene, *unigene 0016938* and *unigene 001639* showed the highest expression levels in the pericarp with highest anthocyanin concentration. Also, a GST gene (Glutathione s-transferase) gene (*Unigene 0021409*) was found significantly up-regulated during litchi pericarp colouration.

## 5. ANTHOCYANIN DEGRADATION IN FRUIT

As much is known about the anthocyanin biosynthesis, but very less information is available about their in planta degradation mechanisms [45,76,77]. Anthocyanin degradation may be due to enzymatic or non-enzymatic reaction [13]. The non enzymatic degradation of anthocyanin may be due to: the hydrolysis of the 3-glycosidic linkages producing the more labile aglucone, and hydrolytic opening of the pyrylium ring to form a substituted chalcone. [78]. According to Huang et al. [79], anthocyanase (anthocyanin-b-glucosidase) may be involved in removing the sugar groups, leading to the anthocyanin decolorization. Zhang

et al. [13] reported that the product from the anthocyanidin degradation had a similar structure to catechol (a good substrate for polyphenol oxidase), which, in turn, could accelerate enzymatic browning reaction by the enzyme polyphenol oxidase. He also found, an anthocyanase, catalyzing anthocyanin hydrolysis and producing anthocyanidin from litchi fruit pericarp showing high activity suggesting that anthocyanase might contribute to the browning of litchi pericarp involved in the anthocyanase-anthocyanin-PPO reaction.

Anthocyanin degradation occurs in different plant organs in response to a variety of environmental and developmental conditions. In post-harvest cases, anthocyanin degradation occurs due to changes in the vacuoles that decrease the stability of the pigments and cause either chemical degradation or increased vulnerability to degrading enzymes (e.g.b-glucosidases, peroxidases) present in the vacuoles. Changes in the vacuolar pH, such as increased pH in senescing tissue, may decrease the stability of the anthocyanins and cause chemical degradation [80]. Anthocyanins in litchi fruit are degraded after harvest, accompanied by fruit browning [81,34,82,83,84]. Peroxidase activity initially increases in the exocarp and during long-term storage in the endocarp, while PPO activity increases during long-term storage in the exocarp [81,34,82,83,84]. PPO enzymes in higher plants are located in the plastids of both photosynthetic and non-photosynthetic tissues [85]. It was proposed that anthocyanins are first hydrolyzed by an anthocyanase (b-glucosidase), forming anthocyanidins [70]. The compounds thus formed can then be oxidized by PPO and/or peroxidise. Wang et al. [86] cloned the litchi PPO gene (*LcPPO*) and described its expression patterns. He found an up-regulation of *LcPPO* expression at early stage of post harvest storage that accelerates PPO protein synthesis and PPO activity increases further accelerating litchi pericarp browning problem. Later Fang et al. [87] compared expression levels of PPO and ADE/LAC in the pericarp tissue during fruit browning and found that ADE/LAC expression levels were about 1,000-fold higher than those of the PPO.

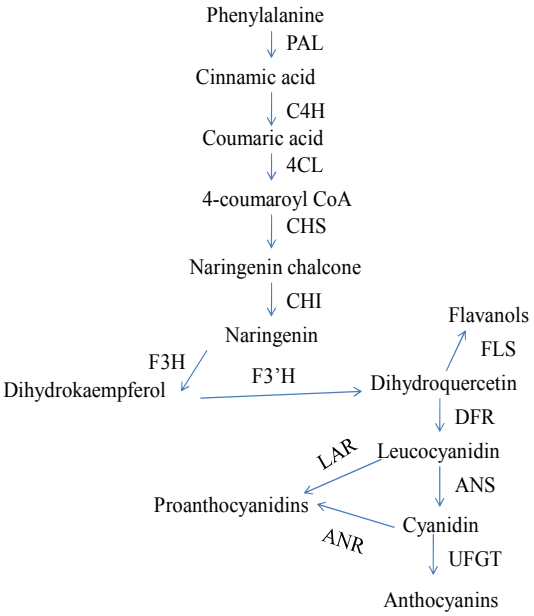


**Fig. 1.**

Figure: Simplified scheme of the flavonoid biosynthesis. Enzymes names are abbreviated as follows; PAL, phenylalanine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4 coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavanone 3'-hydroxylase; DFR, dihydroflavonol reductase; FLS, flavonol synthase; ANS, anthocyanidin synthase; UFGT, UDP-flavonoid glucosyltransferase; ANR, anthocyanidin reductase and LAR, leucoanthocyanidin reductase.

## 6. CONCLUSIONS

Litchi, an important subtropical fruit, is of high commercial value. Pericarp browning is a major post-harvest problem in litchi which renders the fruit unmarketable. There is much information available related to the genes involved in anthocyanin biosynthesis. But very little information is available at the genetic level pertaining to anthocyanin degradation mechanisms. Hence more research should be focussed towards revealing the anthocyanin degradation pathway during browning which may be helpful for us in preserving the red colour of litchi pericarp and extending the shelf-life and hence the marketability of this lustrous fruit.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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