A MOLECULAR STUDY OF FRUIT DEVELOPMENT IN BLACK PEPPER (*PIPER NIGRUM* L.)

By

KHEW CHOY YUEN

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ABSTRACT

Black pepper is the most popular spice crops, but the production is hindered by the uneven flowering and fruit ripening that leads to low productivity and quality of the black pepper products. The mechanisms of flowering and fruit development are associated with the agronomic traits in the variety. However, the breeding programme that combined desired agronomic traits to induce synchronize flowering and fruit ripening in black pepper remain a considerable challenge. Therefore, a better understanding of the molecular mechanisms underlying the flower and fruit development will help to provide a strategy for crop improvement. In this study, different molecular tools and experiments were deployed to compare the flower and fruit development in three different commonly planted black pepper varieties in Malaysia with distinct fruit characteristics. The transcriptomic sequencing analysis demonstrated that the differentially expressed genes were expressed more intensely at the flower and early stage of fruit development. Gene ontology (GO) enrichment analysis further support functions of differentially expressed genes between flower and fruit in the categories of carbohydrate metabolic processes, embryo development and DNA metabolic processes while the differentially expressed genes in fruit are the biosynthetic process, secondary metabolic process and catabolic process. Transcriptional analysis of sugar-transporter and carbohydrate metabolism genes in different fruit varieties suggested that the carbohydrate metabolism in black pepper fruit is developmentally regulated and some genes might serve as potential genes for future crop quality improvement. Study on the piperine related genes expression analysis suggested that lysine-derived products might present in all stages of fruit development with transportation active throughout the development stages. The roles of plant hormones during the flower and fruit development were inferred based on the probe-based gene expression analysis and the quantification of the multiple plant hormones. Jasmonic acid and salicylic acid were found to play roles in flowering and fruit set, whereas auxin, gibberellin and cytokinins are important for fruit growth. Abscisic acid has a positive role in fruit maturation and ripening in the development process. A distinct pattern of plant hormones related gene expression profile with the hormones accumulation profiles suggested a complex network of regulation is involved in the signalling process and crosstalk between plant hormones was another level of regulation in the mechanisms. The current study provides

the first insight into the flower and fruit development mechanisms in black pepper and fills the substantial gap in the area for future crop improvement.

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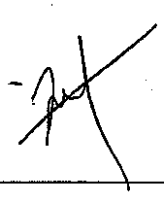
DECLARATION BY CANDIDATE

I, Khew Choy Yuen, higher degree research student of Doctor of Philosophy by Research, from Faculty of Engineering, Computing and Science, Swinburne University of Technology Sarawak Campus hereby declare that this dissertation is original and contains no material which has been accepted for award to the candidate of any other degree or diploma, except where due reference is made in the text of the examinable outcome. To the best of candidate's knowledge, this thesis contains no material previously published or written by another person except where due reference is made in the text of the examinable outcome; and where the work is based on joint research or publications, the relative contributions of the respective workers or authors has been disclosed.

(KHEW CHOY YUEN)

As the principal coordinating supervisor, I hereby acknowledge and certify that the above mentioned statements are legitimate to the best of my knowledge.



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(B) Papers in Conference

1. Khew Choy Yuen, Izumi Mori, Takakazu Matsuura, Takashi Hirayama, Jennifer Ann Harikrishna, Lau Ee Tiing, Zehnder Jarroop & Hwang Siaw San. Role of Plant Growth Factors in Fruit Development and Ripening of Black Pepper (Piper nigrum L.). Proceeding of International Conference on Biochemistry, Molecular Biology and Biotechnology, YSN-ASM & ICBMBB Young Investigator Session. 16th August 2018, P.32.

2. Khew Choy Yuen, Lau Ee Tiing, Hwang Siaw San (2017). De novo Assembly of Fruit Transcriptome in Black Pepper (Piper nigrum L.). Proceeding of 12th Malaysia International Genetics Congress. 25-27th September 2017, P.59.

3. Choy-Yuen Khew, Ee-Tiing Lau and Siaw-San Hwang (2016). High throughput sequencing of Black Pepper (Piper nigrum L.) flower and fruit transcriptome. Proceeding of Monash Science Symposium, Monash University Malaysia, Selangor, 21-23 November 2016, P. 145.

4. Khew, C.Y., Lau, E.T. and Voon, S.C. (2016). Morphological Characterization of Flower and Fruit Development in Black Pepper (*Piper nigrum* L.). Proceeding of 26 th Malaysian Society of Plant Physiology Conference, The Waterfront Hotel, Kuching, 9-11 August 2016, P. 33.

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LIST OF ABBREVIATIONS

NGS	Next-generation sequencing
HPLC-MS/MS	high-performance liquid chromatography coupled to tandem mass spectrometry
KC	Kuching
SA	Semengok Aman
S1	Semengok 1
EST	expressed sequence tag
DAA	day after anthesis
RNA	Ribonucleic acid
CTAB	Cetyl trimethylammonium bromide
cDNA	Complementary DNA
bp	Basepairs
EDTA	Ethylenediaminetetraacetic acid
^{o}C	Degree Celcius
BLAST	Basic Local Alignment Search Tool
LiCl	Lithium chloride
µg	Microgram
µL	Microliter
rpm	revolution per minute
dNTPs	deoxynucleotides
GA	Gibberellic acid
IAA	Indole acetic acid
ABA	Abscisic acid
JA	Jasmonic acid
JA-Ile	jasmonoyl isoleucine
tZ	*trans*-zeatin
iP	isopentenyladenine
DHZ	Dihydrozeatin

CHAPTER 1: INTRODUCTION

1.1 Research Background

Black pepper (*Piper nigrum* L.) cultivation has a long history, and black pepper (often called the King of Spices) remains one of the most popular spice crops. Pepper is utilized for a wide variety of purposes. Its use extends from a humble culinary spice and condiment to providing considerable therapeutic benefits as an antioxidant and an anti-colon toxin, as well as being an antibacterial, antifungal, antidepressant, antidiarrheal, anti-inflammatory, antimutagenic, anticancer, antispasmodic, and insecticidal agent (Ahmad et al., 2012, Khan et al., 2017). However, the non-synchronous aspect of its floral development, as well as its non-uniform fruit maturation within a spike, hinders the production of black pepper. The consequent irregular timing of pepper berry ripening requires a high input of labor for selective harvesting, leads to low productivity and reduces the quality of the pepper products. Nonetheless, pepper remains the most coveted spice in the world in spite of several issues affecting pepper plantations. In 2017, the value of the trade in black pepper amounted to USD 1.5 billion from an output of 4.6 x 10^5 tons (International Pepper Community Statistics), and 60% of global production was from South-East Asia.

The optimum harvesting stage for black pepper (the whole berries) and white pepper (the inner seeds without the outer layer) is at the mature-green or late hard-dough and breaker stage respectively (Paulus, 2011). Usually, only berries that are at these suitable stages of maturity are harvested manually for processing into black or white peppercorns respectively. The characteristics of the fruit differ extensively among different varieties of *P. nigrum* as there is wide genetic diversity of the germplasm in black pepper (Joy et al., 2007). For example, in the three varieties used for this study, besides their useful traits, each has some shortcomings. The 'Semongok Aman' *P. nigrum* variety exhibits more standardized maturation than other varieties recommended by industry; however, it has shorter fruit spikes and a thick fruit pericarp. The pepper variety 'Kuching' has a thinner pericarp, which is important in the production of premium white pepper, but has poor fruit setting with comparatively lower yield. The pepper variety 'Semongok I' has potentially the

highest yield as it has the longest fruit spikes and largest berry size among the three varieties; however, the fruit ripening is not as even as that of 'Semongok Aman' (Paulus, 2011).

The development and ripening of black pepper fruit have received little scientific attention. To date, only one study has been available on the black pepper fruit transcriptome, concentrating solely on the biosynthesis of piperine (Hu et al., 2015). The integrated process of black pepper development, including the mechanism and molecular bases, remains elusive, which restricts the possibilities of refining black pepper fruit quality using crop improvement methodology. Since the critical factor that determines the productivity of a plant, especially in perennial fruit crops like black pepper, is the floral transition. The crucial first step for the reproductive phase is the differentiation of vegetative primordia into reproductive primordia, which will determine the blossom intensity and yield capacity. Over the last 20 years, research on flower development has seen great progress in model plants and has provided a better understanding of how a flower develops. The most important landmark in the understanding of flower development was the development of the ABC model that states that three different functions act in concert to specify organ identity in the four whorls of the flower. However, a recent revision of the model has proposed that the development of each organ requires the creation of large protein complexes.

In angiosperms, the fundamental destiny of the flower is to transition into a fruit in order to guarantee the survival through seed dispersal of the next generation. The fruit is the plant's most vital organ in order to maximise the efficiency of the dispersal process (Pesaresi et al., 2014). The flower and fruit development process is a genetically programmed event, and this process is influenced by environmental factors and endogenous signals which ultimately lead to floral transition and transformation of the fruit (Ravindran, 2003). *Arabidopsis* is the most studied model system in flower development (Seymour et al., 2013), while research on fleshy fruit development and ripening is particularly emphasised in tomato as a model system (Giovannoni, 2004). Research conducted on dry and fleshy fruits and other fleshy-fruited species has found that they have strong similarities in the molecular circuits

to control fruit development. This indicates the conservation of regulatory networks in fruit morphologies among wide range of angiosperm (Seymour et al., 2013). Nonetheless, even with the broad range of studies on the regulatory mechanism in model plants, information on the functional conservation of the regulatory genes between fruit types is still minimal.

A recent advance in high-throughput sequencing technologies has provided new tools to gather large amounts of genetic data from non-model crops (Unamba et al., 2015). This development has presented the prospect of powerful and accurate analysis of transcriptomes, which will be extremely helpful in studying the development of flowers and fruit in other types of fruits. As in the latest model platform of illumina Hiseq is capable to generate hundreds of millions of reads which up to 150 bp with 99.5% accuracy. The technology enable the direct use of RNA samples for sequencing without laborious work in cloning and plasmid extraction. The sequencing technology help in reduce the cost and time to generate enormous data in single run. In spite of these advantages, the sequence reads generated from the next-generation sequencing (NGS) technology like illumina is generally short that required the sequence assembly tools to reconstruct the transcriptome based on reference genome. For non-model plant like black pepper that without reference genome, *de novo* assembly strategy was used to tie pieces of sequences to form a transcripmoe. The applications of NGS technology included with transcriptomic characterization, target genes or isoforms identification, gene expression quantification as well as genetic variation identification.

Other than the genetically influence, plant hormones are recognised to be closely related to the development of flowers and fruit by translating the gene functions into changes at the cellular level in growth and cellular differentiation (Chandler, 2011, Dong et al., 2014). As with many developmental processes in plants, plant hormones play an important role in pollination and in synchronising the signals between the developing seed and its surrounding fruit tissues, as well as in regulating fruit ripening (McAtee et al., 2013). The functions related to plant hormones do not operate on their own in a certain phase; the regulation of different facets of flower and fruit development encompasses a complex network of several hormones (Kumar et al., 2014). Information on the interaction between plant

hormones at various phases of fruit development, however, remains limited, especially in plants of non-model origin like black pepper. Nevertheless, substantial gains have been achieved in characterising hormone responses with the recent progress in mass spectrometry techniques (Rohrmann et al., 2011, Osorio et al., 2011). These initiatives have resulted in the detection of discrete and intersecting patterns in the biosynthesis activity of plant hormones among plant species (Liu et al., 2005, Gao et al., 2013, Sun et al., 2012, Zhang et al., 2009). These findings thus allow better control of flower and fruit development for the improvement of crop quality.

1.2 Research Aims and Objectives

This study overall aim is to obtain insights into the genetic mechanisms and the variations in the endogenous levels of plant hormones in the flower and fruit development of black pepper through a comparative study of three different black pepper varieties in Malaysia. Therefore, the study is focus on two main objectives with the first objective on study the genetic mechanisms of flower and fruit development in black pepper with the specific objectives as following:

(1) To morphologically characterize the fruit spikes in three different varieties of black pepper.

(2) To perform next-generation sequencing and *de novo* assembly of the flower and fruit transcriptome in black pepper

(3) To genetically characterize the transcriptome and analyse the transcriptome gene expression

(4) To study probe-based gene expression profiling of selected target genes in different stages of flower and fruit development.

Meanwhile, the second objective of the study is the quantitative analysis of hormonal changes during flower and fruit development in black pepper with the specific objectives as following:

(1) To extract plant hormones from different stages of flower and fruit development in three different black pepper varieties.

(2) To quantify of endogenous levels of diverse plant hormones at different stages of flower and fruit development through high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS).

(3) To elucidate the relationship of the plant hormone profiles to the transcription profiles of the genes related to plant hormone metabolisms.

1.3 Research Contributions and Impact to Society

Research in model organisms has produced a great deal of significant data on various molecular variables that contribute to plant growth and development, but it has some constraints. The study of additional plant models like black pepper will provide unique insights into flower and fruit development processes as many duplication events happen at the genome level that may lead to different unique gene functions. Another reason for studying particular molecular-genetic characteristics in specific plants is the existence of interesting characters in such plants that are lacking in model plants. For black pepper, the unique pungency and other secondary metabolites that make up the flavor will serve as a source of interesting characters for genetic study.

Research on the genetic mechanism that governs flower and fruit development remains scarce, although pepper is an important spice crop with typical climacteric fruit characteristics. Lately, however, with the availability of high-throughput sequencing technologies has provided advanced tools to reveal the complex networks of genetic regulatory pathways in flower and fruit development in black pepper. This will lead to the genetic characterisation of several important traits and open up new frontiers for crop improvement. The outcome of this study is the first report describing the molecular study of black pepper fruit development. Thus it will make a contribution to the gap in the molecular understanding of the fruit development in black pepper for future crop improvement.

The current study enables the identification of key gene activity responsible to the development of fruit in black pepper. In the past, plant breeders produced new varieties with changes in the phases of development and modifications of plant architecture by identifying the required phenotypes in a few plants among the large numbers of plants in a breeding population. Now with the increased knowledge and powerful gene sequence-based diagnostics provide plant breeders with more precise selection objectives and assays to operate in rationally planned crop improvement programmes. We can expect yield potential to increase and harvested product quality portfolios to better fit an increasing diversity of market requirements in the black pepper industry.

CHAPTER 2: LITERATURE REVIEW

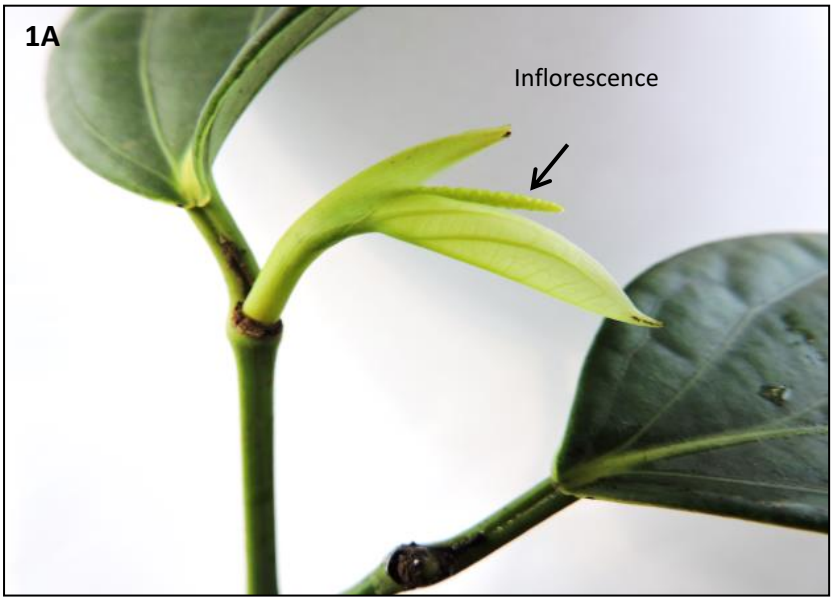
2.1 Black Pepper (*Piper nigrum* L.)

Black pepper (*Piper nigrum*) (hereinafter referred to as 'pepper') belongs to the Piperaceae family and is a perennial climbing plant of the Magnoliid subclass, known as basal angiosperms (Gordo et al., 2012). It originates from the Western Ghats of Kerala in India and grows well in hot tropical climates with high humidity. Pepper is extensively used as a spice and seasoning. The piperine alkaloid and essential oil in its oleoresin determine the pepper quality. Piperine causes the pungency whereas the essential oil contributes to the distinctive flavour (Bagchi and Srivastava, 2003). Pepper is commercially traded in the form of dried whole pepper fruit. The ripe fruit seed is white pepper (whole or ground) and the unripe fruit is green pepper. Pepper is largely cultivated in Asian countries, and Malaysia is among the top five pepper producing countries, with a production of 31,057 metric tons in the year 2018 (Malaysian Pepper Board Statistics). The state of Sarawak alone accounts for 95% of pepper production in the country.

Cytological studies (Jose, 1984; Mathew, 1958) suggest that the basic chromosome number of *Piper* is x = 13, whereas *Piper nigrum* is tetraploid (2n = 52). Results of chromosome counts conducted by Sim (1985) reported that the four local Malaysia varieties of black pepper (Kuching, Bangka, Bangkayang and Lampung) have 2n=52 and thus are tetraploids. Chance cross-pollination between different species of *Piper* might have occurred when more than one species climbed up the same support trees. Due to the absence of a pollen transfer mechanism, subsequent gene flow is restricted in these progenies. High successful vegetative propagation ensures further survival and spread of these progenies. The present day *Piper nigrum* cultivars are the descendants of such segregating populations which are vegetative propagated by farmers through cuttings (Ravindran, 2000). Breeding and conservation programs by humans based on good fruit set, pungency and so on contribute to the diversity of cultivars.

There are about a hundred varieties of pepper under cultivation (Bagchi and Srivastava, 2003), there are significant differences between the varieties in terms of

several morphological and floral characteristics. These characteristics include the shape of the leaf, the size, the spike length, the density, the distribution of male, female and bisexual inflorescence along the spike, fruit setting, fruit weight as well as the maturation time (Bagchi and Srivastava, 2003, Chen et al., 2018). Pepper fruit takes from six to nine months to reach the fully ripened stage from flowering, depending on the time of flowering and the variety (Sim and Rosmah, 2011). The pepper inflorescence is a catkin-like fleshy spike. It arises at the node opposite the leaf on a plagiotropic branch (Figure 1A). Depending on the colour of the bracts in different varieties, the inflorescence can be green, light green or golden yellow. The inflorescences at maturity are from 5 to 20 cm long and bear 50 to 120 flowers, depending on the variety. The flowers are minute, sessile and without perianths (Figure 1B). They may be bisexual (hermaphroditic) or unisexual, with staminate and pistillate flowers borne on the same or different plants (Hasan-Iljas, 1960). Pepper can bloom throughout the year, and the flowering is unsynchronised, which requires intensive labour in flower- picking in order to control the flowering (Zu et al., 2016).



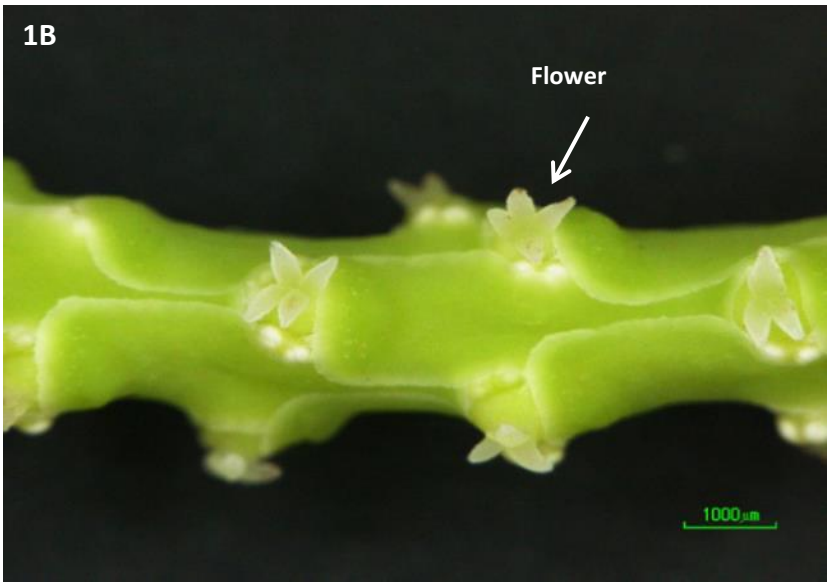


Figure 1: Inflorescence and flowers of pepper. (1A) An inflorescence arises opposite a leaf at the node. (1B) The sessile, small white flowers.

Pepper is valued for its fruit, and botanically, the fruit is a sessile globose drupe, contains a single seed and is commonly known as a pepper berry. The berry like fruits or peppercorns are generally around 4 to 6 mm in diameter, but the size and shape vary in different cultivars. The fruits contain a single seed and when mature they become reddish-yellow or orange-yellow, while the young berries are green in colour. The length of the spike varies within varieties (Zachariah and Parthasarathy, 2008). The seed is off-white and hollow, adhering to the pericarp (Figure 2). The exocarp of the fruit is thin with a fleshy mesocarp and a hard endocarp (Figure 2). As the fruit develops, the inner layer of the endocarp merges with the testa which is the outer layer of the seed (Mourão and Beltrati, 2000). The cells of the inner seed coat become thick-walled and are filled with tannin. The seed is about 3 to 4 mm in diameter and has a minute embryo with little endosperm and copious perisperm (Figure 2). The perisperm is made up of large cells containing starch and piperine (Bagchi and Srivastava, 2003). The embryo has a hexagonal appearance when viewed from the top while the endosperm is top-shaped.

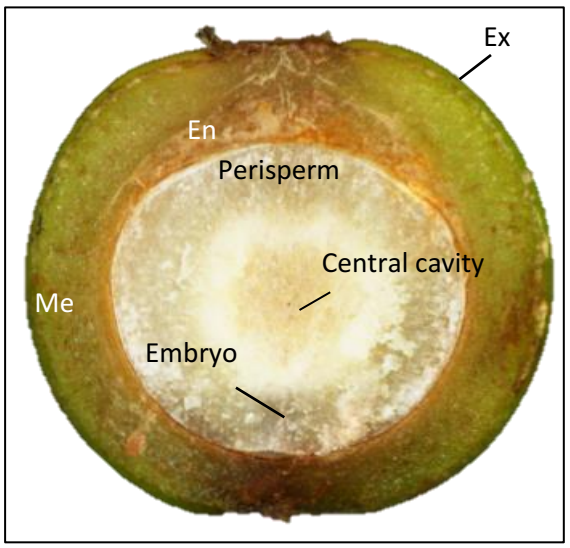


Figure 2: The pepper fruit. (Ex: Exocarp, Me: Mesocarp, En: Endocarp)

In Malaysia, there are three popularly cultivated pepper varieties; 'Kuching' (KC), 'Semengok Aman' (SA) and 'Semengok 1' (S1), each with various distinct morphological differences (Chen et al., 2018). A study by Paulus (2011) indicated that the SA and S1 varieties have a thicker pericarp and are more suitable for the production of premium pepper and green pepper products, while the KC variety is the preferred variety for the production of creamy white pepper. The flower of the KC is pale yellow, compared to the green color in the SA and S1, and the fruit set is not as good as that of the SA and S1. The SA variety has abundant fruit spikes with a good fruit setting. It has bigger berries and longer fruit spikes than the KC. Furthermore, the SA has more uniform fruit ripening, which allows the crop to be harvested over a shorter period. The S1 variety, also known as 'Panniyur 1' and originally introduced into Malaysia from India in 1993, has the most extended fruit spikes with a bigger berry size compared to the other varieties cultivated in Malaysia (Paulus, 2011). Apart from morphological analysis based on careful observations of these varieties, flower and fruit development mechanism information in pepper is very limited, other than two preliminary studies carried out in Malaysia (Sim, 1979a) and India (Sasikumar B, 1992).

2.2 Genetic regulation on flower and fruit development

2.2.1 Research on flower genetic mechanisms

In angiosperms, flowers are crucial to evolutionary success being an integral aspect of the synchronized transition from the vegetative stage to the reproductive stage. This floral transition is prompted by environmental signals (for e.g., photoperiod, light quality, moisture stress, temperature and nutrient conditions) and endogenous signals (Parvathi M. Sreekumar, 2014). The homeotic genes of the flower, which encode transcription factors (TFs), specify the organization of floral organ identities (Schwarz-Sommer et al., 1990, Yanofsky et al., 1990, Coen and Meyerowitz, 1991). According to the classical ABC model (Coen and Meyerowitz, 1991) that is based on *Arabidopsis thaliana* L. and *Antirrhinum majus* L., three functional gene classes specify the identity of floral organs that interact genetically to regulate flower development (Coen and Meyerowitz, 1991). In the ABC model, a eudicot flower is composed of four concentric whorls bearing a series of floral organs that sequentially arranged from the outer whorl (sepals) to the inner whorls (petals, stamens and carpels) (Dreni and Zhang, 2016). In the first whorl, A-class genes define sepal identity, and in conjunction with B-class function genes, are responsible to determine the second whorl petal identity. Class B and C genes are accountable to specify the third whorl stamen identity, after which the C function genes carry out the termination and specialization of the floral meristem (FM) into the carpel. Subsequent studies on petunia (*Petunia* x *hybrid s*ort. ex E. Vilm.) discovered more genes that govern ovule identity and development within the carpel, and a D-class gene was added to the original model (Angenent et al., 1995, Colombo et al., 1995). Lastly, an E-class gene was revealed to be crucial, in combination with the A, B, C, and D genes, in the identification of all floral organs (Pelaz et al., 2000, Theißen, 2001, Ditta et al., 2004).

Further genetic research on *A. thaliana* uncovered five separate genes that facilitate floral homeotic functions in the plant. The A-class function is expedited by *APETALA1* (AP1) and *APETALA2* (AP2) in dictating the development of petals and sepals; the B-class function by *APETALA3* (AP3) and *PISTILLATA* (PI) to influence development of petals and stamens, and the C-class function by *AGAMOUS* (AG) to

stipulate development of stamens and carpels (Krizek et al., 2000). These genes all encode recognized TFs (Yanofsky et al., 1990, Jack et al., 1992, Mandel et al., 1992, Goto and Meyerowitz, 1994, Jofuku et al., 1994), implying that the A, B and C genes may regulate the transcription of certain specific ('target genes') whose products are directly or indirectly related with floral organ formation or function. Homeotic genes consisting of the A, B, C and E classes detail in part the genetic networks regulating elements of the initiation, development and architecture of the floral organs. However, little information is available on how these gene functions influence cellular level changes involving the formation of various floral organs with specific shapes and sizes.

2.2.2 Research on fruit genetic mechanisms

The ultimate fate of the flower is to become a fruit to ensure seed dispersal for the survival of the next generation. Depending on the traits of a burst in respiration and a sharp rise of ethylene production during the commencement of maturation, fruits can be classified into climacteric and non-climacteric. The tomato is considered a typical climacteric fruit model with extensive studies carried out on analysis of its molecular genetic structure in fruit development and ripening. The formation of a fruit requires close information sharing on development among ovules and carpels. It has been speculated that the signals that begin fruit development originate from the grains of pollen (O'Neill, 1997, O'Neill and Nadeau, 1996) as well as in ovules after successful fertilisation (Gillaspy et al., 1993a), resulting in a change in the process of pistils development from senescence to fruit setting (Vercher et al., 1984, van Doorn and Woltering, 2008). The maturation of gynoecia becomes fruits, so the patterning of carpel takes place before the architecture of the fruit. In turn, the identity of the carpel is regulated by the floral homeotic C function genes, including all the AG sub-clade members (Dreni et al., 2011). These members were first identified and named in *Arabidopsis thaliana* (Yanofsky et al., 1990, Becker and Theißen, 2003). Comparative studies have also shown the function of AG sub-clade members were conserved from monocots to the basal angiosperms (Dreni et al., 2011, Bradley et al., 1993, Davies et al., 1999, Yellina et al., 2010).

In addition to AG genes, fruit formation and maturation involve various other MADS-box transcription factors. Vrebalov et al. (2002) revealed that the RIN-MADS function is disordered by the classical mutation *rin*. MACROCALYX (MC), another MADS-box gene which is also silenced in *rin* plants, is also closely related to RIN-MADS. Antisense suppresion of *RIN-MADS* and *MC* has discovered that RIN-MADS alone is needed for the maturation of tomato fruit (Vrebalov et al., 2002). On the other hand, all *miR156*-targeted SBP-Box transcription factors seem to control the patterning, determinacy, and early development of tomato fruit, as the inhibition of their expression by *Arabidopsis miR156b* overexpression caused additional new floral organs to grow from the fruit's stylar end. The class I KNOTTED1-like homeobox (KNOX) gene LeT6/TKN2 and the NO Apical Meristem/Cup-shaped Cotyledon (NAM/NAC) transcription factor Goblet gene were upregulated in transgenic plant ovaries and linked to meristem maintenance (Silva et al., 2014). The mutation of Mouse-ear (Me) gene causes an aberrant TKN2 mRNA to lose expression and also leads to additional carpel growth. This growth indicates that the determinacy and the number of carpel in tomato is regulated by SPLs through down-regulation of TKN2 (Parnis et al., 1997). However, in *Arabidopsis*, the down-regulation of the miR156-targeted SPL genes had no noticeable effect on the determinacy of gynoecium or the number of carpel, implying that tomato has a unique control factor compared to *Arabidopsis* (Xing et al., 2013).

The majority of molecular genetic studies on flower and fruit development have been performed on model plant species. In the relatively large Magnollid clade to which pepper belongs (where stem lineage seems to have deviated before the division between eudicots and monocots), no molecular-genetic models have yet been expounded (Bremer et al., 2009). Thus, only by expanding the range of molecular-genetic plants available for study will give a clearer picture of flower and fruit development. The study of additional plant models like pepper will provide unique insights into flower and fruit development processes as many duplication events happen at the genome level that may lead to different unique gene functions (Rijpkema et al., 2006). Another reason for studying particular molecular-genetic characteristics in specific plants is the existence of interesting characters in such plants that are lacking in model plants.

2.2.3 Next-generation sequencing technology

The availability of next-generation sequencing (NGS) technology allows in-depth sequencing to reveal the molecular-genetic characteristics of flower and fruit development in different plants and to measure the expression of the detected transcripts. An Illumina Hiseq 2500 platform can generate hundreds to thousands of millions of paired reads per run (Holm et al., 2019) and compute the gene expression through calculating the number of reads per kilobase of transcript per million mapped reads (RPKM) (Kim et al., 2018). For model organisms such as *Arabidopsis* and tomato, comprehensive genomic and transcript sequence data are present as a reference for reads mapping but for non-model organisms with no reference sequence, short reads sequencing will be a potential problem. Black pepper is non-model organism with no available genome data and limited expressed sequence tags (ESTs) have to employ the *de novo* sequencing technologies which is typically used in non-model plants to assemble the reads without mapping to the reference sequences in order to identify the novel genes (Zhou et al., 2012).

The results of investigations on the transcriptome of pepper flower and fruit development have been reported earlier but mainly focused on the piperine synthesis mechanism (Hu et al., 2015, Park et al., 2012). Research on the genetic mechanism that governs flower and fruit development remains scarce, although pepper is an important spice crop with typical climacteric fruit characteristics. Lately, however with the availability of high-throughput sequencing technologies has provided advanced tools to reveal the complex networks of genetic regulatory pathways in flower and fruit development in pepper. This will lead to the genetic characterisation of several important traits and open up new frontiers for crop improvement. Some of the fruit crop genomes that have been sequenced earlier include tomato (*Solanum lycopersium*), grapevine (*Vitis vinifera*), apple (*Malus* x *domestica*), diploid strawberry (*Fragaria vesca*) and banana (*Musa acuminate*) (Seymour et al., 2013). The availability of these resources underpins advances in the breeding of fruit crops by harnessing variations in crops, and in combination with the alteration of the target proteins or transcripts, provides a powerful thrust for a massive step change in crop improvement.

2.3 Role of plant hormones in flower and fruit development

Plant hormones are known as the first transducers of genetic information to synchronise the signals from floral organ initiation to fruit development and maturation (Chandler, 2011). Extensive studies on the action of plant hormones in regulating flower and fruit development have been carried out on *Arabidopsis* as dry fruit and tomato as fleshy fruit (Kumar et al., 2014, Chandler, 2011). Both plants are model systems with well-characterised genetic information in flower and fruit development. However, other systems like pepper need to explored further as the development mechanisms might be different, as demonstrated in strawberry and grape (Symons et al., 2012). Prior studies have shown that treating unpollinated flowers with plant hormones can stimulate tomato fruit growth as well as in other horticultural plants, indicating that plant hormones may substitute the fertilization signal to induce the growth of fruit (Gillaspy et al., 1993a, Vivian-Smith and Koltunow, 1999). Therefore, further research to understand the dynamic actions of plant hormones in pepper will allow fine regulation of the flower and fruit development process for future crop improvement.

2.3.1 The research on ethylene and gibberellic acid

Pepper berries have the characteristics of a climacteric fruit, in which ethylene controls ripening (Latifah et al., 1998, Collings et al., 2018). Extensive studies on the role of ethylene in regulating fruit ripening in other climacteric fruits like tomato have been well established and have shown potential in manipulating the ripening process (Alexander and Grierson, 2002, García-Salinas et al., 2016). Therefore, in order to induce greater uniformity in pepper fruit ripening, antisense technology using ACC oxidase genes for ethylene was used by Sim et al. (2001). However, no significant improvement in uniformity was found in the pepper plants. Research suggests that the associated functions of plant hormones are not limited to a specific stage; instead, an intricate network of several plant hormones is engaged in controlling various aspects of fruit development (Kumar et al., 2014). Moreover, it has been claimed that reliance on a particular hormone depends on plant species, which ultimately leads to core cell cycle genes being activated (McAtee et al., 2013). Studies on gibberellic acid have been found to trigger fruit development in various

plant species and treatment with combination of plant hormones resulted in normal fruit growth even without fertilization (Gillaspy et al., 1993b, Vivian-Smith and Koltunow, 1999). This demonstrated the need for interactions between diverse plant hormones to induce fruit development and ripening. Hence, it is crucial to establish the pattern of hormone accumulation for individual crops and even within varieties in order to be able to apply this information towards yield and quality improvement of a crop.

2.3.2 The research on abscisic acid, auxin and cytokinins

The use of various plant hormones, such as abscisic acid, auxin, cytokinins, salicylic acid and jasmonic acid, for improving uniform ripening and fruit quality has been explored for a number of crops: In climacteric fruits such as tomato (Srivastava and Handa, 2005a), mango (Zaharah et al., 2012), and apple (Schaffer et al., 2013) and non-climacteric fruits such as strawberries (Symons et al., 2012) and grapes (Fortes et al., 2015). Abscisic acid (ABA) has been shown to trigger the ripening process in mango and it has been suggested that the accretion of ABA before the climacteric stage initiate ethylene production (Zaharah et al., 2012). Also, auxin (IAA) level has been demonstrated to influence the production of ethylene by inducing 1-aminocyclopropane-1-carboxylic acid synthase (ACS) activity during strawberry fruit ripening (Vendrell and Palomer, 1997). Similarly in peach, a climacteric ethylene burst resulted in accelerated degradation of endogenous IAA on the third day of ripening (Wu et al., 2003).

Cytokinins, particularly trans-zeatin (tZ), their most active form, are responsible for regulating cell division and sink strength in tomato fruit (Srivastava and Handa, 2005b). Elevated cytokinins concentration are associated with fruit set and early growth stages in tomato (Matsuo et al., 2012). In orange and grape fruit, a reduction in cytokinin levels before ripening has been recorded, suggesting that cytokinins can inhibit maturation (Davies and Böttcher, 2014). Similarly in diverse fruits like grapes, kiwifruit, tomato and strawberry, 2-isopentenyladenine (iP) levels have been known to escalate during ripening, suggesting that cytokinin is involved in the ripening process (Böttcher et al., 2015b). It is also significant that jasmonic acid

(JA) has recently been identified as involved in the ripening of strawberry fruit as a repressor in ABA biosynthesis (Garrido-Bigotes et al., 2018b).

2.3.3 The utilization of mass spectrometry on plant hormones study

Treatment with exogenous auxin on pepper fruit spikes has been reported earlier to increase the fruit size and overall yield of the fruit (Pillai et al., 1977). However, the role of other plant hormones during the pepper fruit developmental process has not been well documented, and the interplay of plant hormones in regulating flower and fruit development remains unknown. Therefore, comprehensive profiling of the different plant hormones in the growth of the flower and fruit in pepper is crucial to gain information on the functional roles of the developmental process. Due to its extreme sensitivity and selectivity, mass spectrometry (MS) has become a useful tool for multi-targeted profiling of various major plant hormones (Pan and Wang, 2009). Gas chromatography mass spectrometry (GC-MS) is a method broadly used to examine plant hormones. However, the sample preparation is laborious especially in separation and purification which require extensive works. Furthermore, chemical derivatization is another step needed in sample preparation to increase volatility and sensitivity for GC-MS analysis (Marcos and Pozo, 2015). Hence, liquid chromatography tandem mass spectrometry (LC-MS/MS) has been found as the efficient way to simultaneously quantify and profile multiple plant hormones in single run without the necessary of derivatization (Chiwocha et al., 2003). In order to rectify the hormone loss during sample preparation and the separation of chromatographic, good internal standards such as stable isotope-labelled compounds with the same chemical structure as target analytes are playing an important role (Pan and Wang, 2009). These internal standards reduce the quantification problems that may result from potential ion yield variability owing to ion suppression. Therefore, the application of the right tools to investigate the changes in the endogenous levels of plant growth regulators is essential to give a clear picture of the different plant hormones regulation in pepper flower and fruit development.

2.4 Relevance of flower and fruit development genes in hormone pathways

Plant flower and fruit development are well regulated by the genetic mechanism, and the process is stimulated with the aid of exogenous and endogenous indicators including plant hormones. Intensive studies on the role of plant hormones in flower and fruit development in the model plants of *Arabidopsis*, rice and tomato have been accumulated. Nevertheless, the current concern is to synthesize the information to understand the way plant hormones integrate into the genetic mechanism to manipulate flower and fruit development, especially in pepper.

2.4.1 Gibberellins homeotic genes regulation

The correlation between homeotic genes and hormone pathways has been the subject of contemporary studies APETALA1 (AP1; B-class) (Yamaguchi et al., 2014); SQUAMOSA Promoter Binding-Like (SPL/SBP) (Yu et al., 2012), AGAMOUS (AG; C class) (Weigel et al., 1992) and LEAFY (LFY) (Blázquez et al., 1998). Several connections between ABCE model genes and gibberellins (GAs) may be inferred from lines of research evidence. It has been long established that GA governs the transcriptional activity of the flower meristem identity gene LFY (Blázquez et al., 1998), which induces the expression of a gibberellin catabolism gene and causes changes in the GA level (Yamaguchi et al., 2014). Reduced GA levels allow accumulation of DELLA proteins to promote flowering with SPL/SBP transcription factors by activating the APETALA1 gene (Silva et al., 2018). AG expression is facilitated by the GA-DELLA signalling pathway (Yu et al., 2004) which influences the GA biosynthesis enzymes of the gibberellin 20-Oxidase (GA20OX) and gibberellin 3-Oxidase (GA3OX) families to promote bioactive GA_1 and GA_4 development (Rizza and Jones, 2019). In *Arabidopsis*, GA3OX1 expression ensues in stamen filaments, while GA3OX2-4 expression occurs in anthers. GA3OX1 and GA3OX3 mutations cause silique fertility defects (Mitchum et al., 2006, Hu et al., 2008). Studies on GA integration patterns by incorporating homeotic genes indicate a cell division and expansion system controlled by GA underlying flower organogenesis. The DEFECTIVE IN ANTHER DEHISCENCE 1 (DAD1)

expression, which deactivates the stamen filament defect in plants with GA insufficiency, was found to be regained by loss-of-function of DELLA REPRESSOR of GA1-3 (RGA) (Plackett et al., 2011). DAD1 is engaged in the initial stage of biosynthesis of jasmonate (JA) (Yuan and Zhang, 2015) and the discovery implies an interconnection of GA and JA in the control of the growth of stamen.

2.4.2 Jasmonate homeotic genes regulation

Prior studies have shown that the JA ZIM-domain 1 (JAZ1) protein, a crucial JA signalling repressor, interrelates with DELLA proteins *in vivo*. DELLAs prevent JAZ1 inhibitory connection with a vital transcriptional activator of JA responses (MYC2), thereby improving MYC2's capacity to govern its destination genes. In contrast, GA also prompts DELLA degeneration, which enables JAZ1 to bind MYC2 and suppress MYC2-dependent JA-signaling outputs (Hou et al., 2010). JAZs interrelate with MYB21 and MYB24 in order to reduce their transcription role. When the JA signal occurs, the F-box protein CORONATINE INSENSITIVE1 (COI1) accrues JAZs to the Skp-Cullin-F-box-type E3 ubiquitin ligase complex (SCF^{COI1}) for ubiquitination and degradation via the 26S proteasome. The expression of the genes integral to JA-regulated anther development and filament elongation is then triggered by the MYB21 and MYB24 (Song et al., 2011). In rice plants, overexpression of mJAZ3, mJAZ4, mJAZ6, mJAZ7 and mJAZ11 demonstrated low fertility and abnormal growth of the spikelet, comparable to that of *Arabidopsis* eg1 mutants (Hori et al., 2014). Further, research on strawberry fruit revealed that JA signalling-related genes like FaMYC2 and FaJAZ1 were rapidly stimulated by JA treatment at the early fruit production stage and that their expression showed a substantial decline from the fruit development to ripening stages (Garrido-Bigotes et al., 2018b). In addition, there was a major reduction in the expression of gene encoding for JA metabolism enzymes, coexistent with a decrease in JAs and an increase in ABA levels from flowering to fruit ripening (Garrido-Bigotes et al., 2018a). This outcome indicates that the pathways of JA and ABA have an antagonistic interaction in the fruit development and ripening processes.

2.4.3 Abscisic acid homeotic genes regulation

There have been two types of ABA signal transduction pathways reported. The first siganaling pathway constituted with the ABA receptor, PYR/PYL/RCARs (PYLs) together with the ABA negative regulators, Protein phosphatase typre 2C (PP2C) and ABA positive regulator, SNF1 related kinases subfamily 2 (SnRK2). These three key elements are subjected to phosphorylation or dephosphorylation to mediate the transduction of the signal (Nakashima & Yamaguchi-Shinozaki, 2013; Nishimura et al., 2010; Santiago et al., 2009). Upon ABA binding, PYLs interact with their binding pockets ligand that belong to the superfamily of the START protein. Once ABA is bound, PYL shuts down the entrace of the ligand-binding pocket that constituted with two highly conserved β-loops and established a 'cap-lock' configuration, which then binding to the PP2C phosphatase activity site and inhibiting PP2C activity in particular. Subsequently, SnRK2s are produced from PP2C dephosphorylating that phosphorylate transcription factors or downstream proteins, which induce ABA-responsive gene expression (Shen et al., 2006).

The second pathway is the pathway of magnesium chelatase H subunit (CHLH), where a cluster of WRKY transcription repressors are antagonized by the CHLH as an ABA receptor to alleviate the inhibition of ABA-responsive genes (Shen et al., 2006, Wu et al., 2009). The PYL–PP2C–SnRK2 and CHLH pathways are actively involved in the regulation of tomato and strawberry fruit ripening. The expression of the three key component genes (PYL, PP2C and SnRK2) in tomato corresponded to the fruit development process (Sun et al., 2011), while the virus-induced gene silencing (VIGS) in strawberry fruit repress the PYR1 and CHLH genes and greatly inhibited the process of fruit colouration (Chai et al., 2011, Jia et al., 2011). In sweet cherry, PaPYL, PaPP2C and PaSnRK2 expression was correlated with development and ripening. When fruit ripening was triggered, the expression of these genes was a direct effect from the exogenous application of ABA and IAA. This association was stronger when compared to that of CHLH during the development and ripening of fruit (Wang et al., 2015).

2.4.4 Auxin homeotic genes regulation

Auxins are recognized as improving the expression of different transcripts in the gene families like Aux/IAA, Gretchen Hagen3 (GH3) and Small Auxin Up RNA (SAUR) in the early stage of response to auxin signalling (Abel and Theologis, 1996). Auxin-responsive element (AuxRE) included with the early auxin-responsive gene promoters incorporate with a conserved cis-responsive TGTCTC element (Guilfoyle et al., 1998). In order ot respond with auxin, the auxin response factors (ARFs) were formed through the binding of transcription factors with the TGTCTC motif. Various developmental processes, including flowering and fruit setting are governed by ARFs (Guilfoyle and Hagen, 2007). An amino-terminal B3-type DNA Binding Domain (DBD) and two C-terminal Aux/IAA domains (CTD) with a flexible middle domain form a standard ARF protein which coordinating protein-protein interaction to act either as an activation domain (AD) or a suppression domain (RD) (Korasick et al., 2014). Furthermore, the Aux/IAA domain is known to influence the composition of either homodimers or heterodimers form in between ARF with Aux-IAA or ARF (Guilfoyle and Hagen, 2001, Guilfoyle and Hagen, 2007). The central region segregates the DBD from the CTD, and its amino acid makeup is crucial in defining if an ARF protein acts as an activator or a suppressor (Ouellet et al., 2001, Tiwari et al., 2003). Silencing of the *Solanum lycopersicum* ARF 9 (SIARF9) increased the cell division activity and formed bigger fruit than the wild type (de Jong et al., 2015) while upregulation of SIARF2 in transgenic tomato induced flower organ senescence, which suggests the role of auxins in regulating flower development (Ren et al., 2017, Sakamoto et al., 2006, Takei et al., 2001).

2.4.5 Cytokinins homeotic genes regulation

The role of cytokinins (CK) in fruit and seed development has been studied for several decades. In numerous plant species, adenosine phosphate-isopentenyl transferase (IPT) catalyses the first stage of CK biosynthesis, which produces isopentenyladenine (iP) nucleotides as precursors of CK (Kakimoto, 2001, Takei et al., 2001, Sakamoto et al., 2006). The cytochrome P450 monooxygenases, CYP735A1 and CYP735A2 in *Arabidopsis* transform the iP-nucleotides into the nucleotides of trans-zeatin (tZ) (Takei et al., 2004). CK nucleotides generated by

IPTs and CYP735As have to be transformed in free-base form to become biologically active. A CK-activating enzyme (LOG) was detected in rice and *Arabidopsis* which specifically converts CK nucleotides into active nucleobases (Kurakawa et al., 2007b, Kuroha et al., 2009b).

Degradation or conjugation lead to denature of CKs, and irreversible CK degradation in many species of plants is catalyzed by cytokinin oxidase/dehydrogenase (CKX). CKX is a flavin adenine dinucleotide-containing oxidoreductase that responsible for the deactivation of metabolic CK which react with unsaturated N^6 side chains from tZ and iP as well as their respective ribosides selectively (Matsuo et al., 2012). A study on ckx3 ckx5 double mutant in *Arabidopsis* revealed that they developed bigger inflorescence and floral meristems, suggesting the function of CKX in controlling the activity of reproductive meristems (Bartrina et al., 2011). Overall, substantial studies on different plants have shed further light on the relationship between plant hormone-related genes, and reproductive organ patterning genes and have shown that they constitute a network responsible for flower and fruit formation. Such information enables a better understanding of the complex regulatory interactions that exist in the gene-regulatory and hormonal signalling networks that govern the processes of floral and fruit growth.

CHAPTER 3: MATERIALS AND METHODS

3.1 Morphological characterization on fruit setting of three different black pepper varieties

Fruit spikes were collected from two years old mature pepper plants (*Piper nigrum* L.) of SA, S1 and KC varieties at a pepper farm managed by pepper farmer in Kampong Karu, Borneo Highland Sarawak, Malaysia. The three varieties were planted randomly in the farm and three plants were randomly selected for the collection of samples for each variety. The black pepper varieties were identified based on the morphological difference on leaves and fruit spikes as describes in Book of Pepper Production Technology in Malaysia (Paulus, 2011). Meanwhile, the stages of the fruit spikes were determined by comparison to the description and tables as reported by Sim & Rosmah (2011) in the Book of Pepper Production Technology in Malaysia. The samples were transported to the laboratory for further examination. Longitudinal sections of pepper fruits were examined under the multi-purpose zoom microscope (Nikon AZ100) at a magnification of 10x. The data on length of fruit spike, diameter of a berry, compactness of fruit setting per spike, number of berries per fruit spike, weight per fruit spike and weight of 100 berries were measured from three different black pepper varieties (SA, S1 and KC) and each variety with 10 replicates collected from three plants. The data were subjected to statistical analysis of one-way ANOVA and Tukey posthoc test for the significant analysis.

3.2 Plant materials and RNA preparation

Samples were collected from two years old mature pepper plants (*Piper nigrum* L.) of SA, S1 and KC varieties at a pepper farm in Kampong Karu, Borneo Highland Sarawak, Malaysia. The three varieties were planted randomly in the farm (same area) and two plants were randomly selected for the collection of samples for each variety. Six fresh hermaphrodite flower spike (1 day after anthesis, DAA) and six fruit spikes (14 DAA) (Figure 3) were collected from two biological replicates (two plants) of each pepper variety and snap frozen in liquid nitrogen before storage at -80^{o}C until use. Two grams of frozen plant tissues were used to extract the total RNA

by using a modified CTAB method (Lau et al., 2012). The total RNA was extracted from frozen grinded tissues using 20 mL of extraction buffer (2% CTAB, 1% PVP, 0.1 M Tris-HCl, 25 mM EDTA, 1.4 M NaCl and 1% ß-mercaptoethanol) and incubated at 65^{o}C for 45 mins. The homogenate was centrifuged at 13,000 rpm at 4^{o}C for 15 mins to remove the plant debris. Equal volumes of phenol were added, and the tubes were gently inverted for at least six times. The mixture was then centrifuged at 13,000 rpm at 4^{o}C for another 15 mins. The supernatant was collected in a new tube. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added and mixed by gently inverting the tube six times. The sample was later centrifuged at 13,000 rpm at 4^{o}C for 15 mins, and the supernatant was collected in a new tube with 1/3 volume of 8 M LiCl and precipitated at -20^{o}C for 2 hours. The total RNA was recovered by centrifuging at 13,000 rpm at 4^{o}C for 15 mins and washing with 2 mL of 70% ethanol before being air-dried and dissolved in 500 µl of DEPC-treated water. The RNA was purified and treated with RQ1 RNase-free DNase (Promega, USA) to remove DNA contamination. The RNA purity and concentration were measured at 260/230 nm and 260/280 nm using a spectrophotometer (NanoPhotometer P330, IMPLEN, Germany) while the RNA integrity was measured on an Agilent 2100 Bioanalyzer with RNA 6000 Nano Reagents Kit (Agilent Technologies, 5067-1511, Lithuania). The RNA Integrity Number (RIN) was calculated using an algorithm adapted for plant RNA profiles. All RIN values were between 7.4 and 8.5.

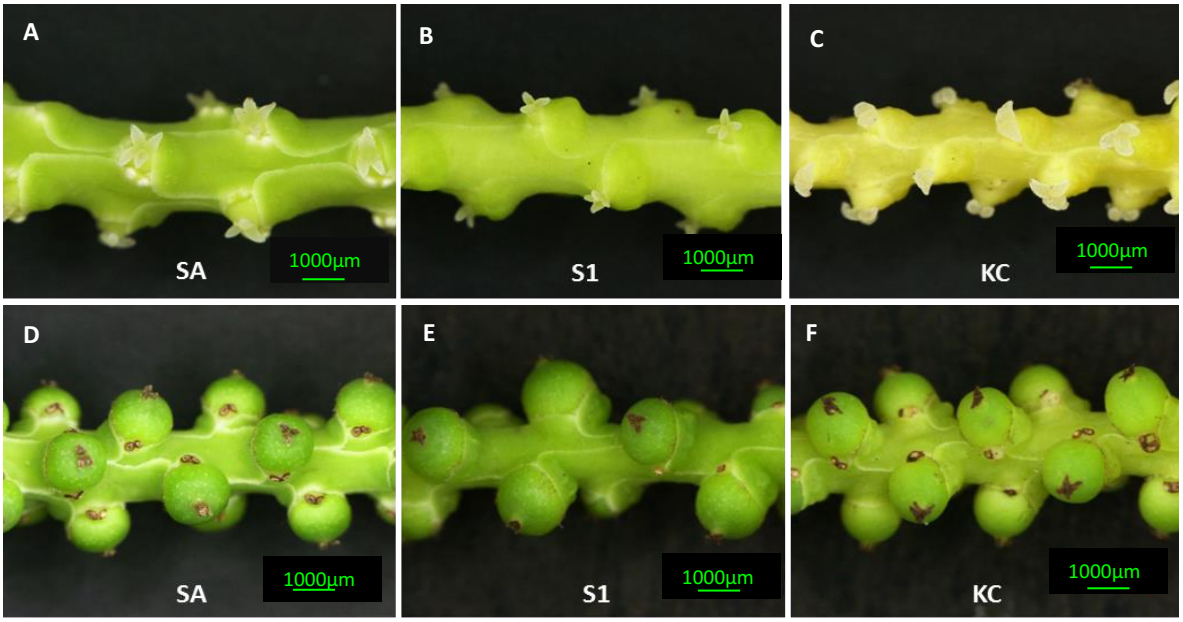


Figure 3: Flower and fruit samples of *P. nigrum*. Complete emergence flower 1 DAA of A: SA; B: S1; C: KC. Fruit half covered by subtracting bracts (14 DAA) in D: SA; E: S1; F: KC.

3.3 Sequencing and *de novo* assembly

The total RNA from twelve samples were converted into cDNA library for subsequent sequencing using the Illumina® TruSeq® RNA Sample Preparation Kit v2 (Illumina, USA). The final cDNA libraries were sequenced as 12 libraries distributed across two lanes of an Illumina HiSeq 2500 (Illumina, USA), with 1% Illumina PhiX control library spiked into each lane at the Institute of Bioscience, University Putra Malaysia. The 100 base-pair paired-end sequencing reads were obtained in FASTQ format text file, and PhiX sequences were excluded from the raw reads. Then the clean reads were subjected to base quality ($Q \geq 30$), ambiguous base call ($N \leq 2$) and sequencing adapter trimming to ensure that only good quality bases derived from the mRNAs were used for the *de novo* assembly. Trimmed reads of 34 base pairs or shorter were discarded. All the assemblies were performed on a workstation with 12 cores and 64 GB random access memory using SOAP denovo-Trans program (version 2.04; http://soap.genomics.org.cn/soapdenovo.html) and CLC Genomics Workbench (version 9.5.2).

3.4 Annotation and gene ontology analysis

The assembled contigs were imported in the bioinformatics tool Blast2GO (version 2.99) and were parsed on the National Center for Biotechnology Information (NCBI) of non-redundant protein database BLASTX (E value 1e-3). The functional annotation included Gene Ontology (GO) terms (http://www.geneontology.org) (Ashburner et al., 2000); Enzyme Commission numbers (EC code) (Schomburg et al., 2004); InterPro terms (Zdobnov and Apweiler, 2001) and metabolic pathways (Kyoto Encyclopedia of Genes and Genomes, KEGG) (Ogata et al., 1999). The "Augment Annotation by ANNEX" function was used to refine the annotations. The GOslim of the plant-specific condensed version of the GO was run for the annotation. The differentially expressed genes further underwent GO enrichment analysis by CLC Genomics Workbench (version 9.5.2) based on hypergeometric distribution (Falcon and Gentleman, 2006).

3.5 Measurement of gene expression

The flower (1 DAA) and fruit tissues (14 DAA) of each variety were separately isolated for comparison purpose. Differentially expressed genes were identified using the CLC Genomics Workbench (version 9.5.2) by screening with a threshold false discovery rate (FDR) < 0.05 and log Fold Change (logFC) > 2 or < -2. The number of total genes reads estimated the gene expression level located in the transcriptome based on the number of mapped reads for each unigene normalised as a Fragments Per Kb per Million reads (FPKM) value. The list of up/down-regulated genes was identified by filtering the differentially expressed genes via excel filter function. Once the list of up/down-regulated transcripts was obtained, they were compared to one another using a free web service at http://bioinformatics.psb.ugent.be/webtools/Venn/. Furthermore, a list of exclusively expressed transcripts was identified by filtering the genes expression file. The transcripts were considered uniquely expressed if they are expressed (fpkm > 0) in all replicates in one group, and not expressed (fpkm $= 0$) in all other groups and their replicates. The GO and KEGG pathways enrichment analysis was carried out using the generated gene set from the previous analysis.

3.6 Probe-based gene expression analysis

Samples were collected from two years old mature pepper plants (*Piper nigrum* L.) of SA, S1 and KC varieties at a pepper farm in Kampong Karu, Borneo Highland Sarawak, Malaysia. The RNA for gene expression studies was prepared from the samples harvested at six developmental stages of three black pepper varieties (KC, SA and S1) in three biological replicates (3 plants) following the CTAB method mentioned earlier. Six fresh flower spikes and six fruit spikes were collected from different fruit development stages as characterized as stage 1, flowering stage with complete emergence of stigma (1 DAA); stage 2, fruit set stage with fruit half covered by subtending bracts; stage 3, fruit grow and expansion stage with the fruit nearly touching each other; stage 4, fruit maturation stage, fruit in mature green color; stage 5, breaker stage, greenish-yellow in colour; and stage 6, ripe stage, red (Figure 4). RNA samples were hybridised with gene-specific colour-coded probes for multiplexed measurement of gene expression. The NanoString Codeset was designed and synthesised by NanoString Technologies.

The genes selected in this study are based on the previous databases and publications and the selected genes were identified from the black pepper transcriptomic data which were differentially expressed among the three different black pepper varieties. The candidate genes selected are the homologues sequences parsed again Genbank database (Table 1) which are detectable and quantifiable by probe-based gene expression analysis. The targeted sequences of each selected genes were listed in Appendix I. The data retrieval was completed with the nCounter Digital Analyzer as illustrated by the manufacturer (Nanostring Technologies, USA). Data analysis and normalization were done using nSolver Analysis Software 3.0 (NanoString Technologies). For the quality control analysis, six positive control probes with step-wise concentrations were used to evaluate the efficiency of the hybridisation reaction and to review the linearity of the assay performance. Meanwhile, the geometric mean of eight negative probes was used to set the background threshold to control the false positive and false negative occurrence. Standard normalisation was performed using the geometric mean of six synthetic ssDNA positive control targets and three black pepper specific housekeeping genes,

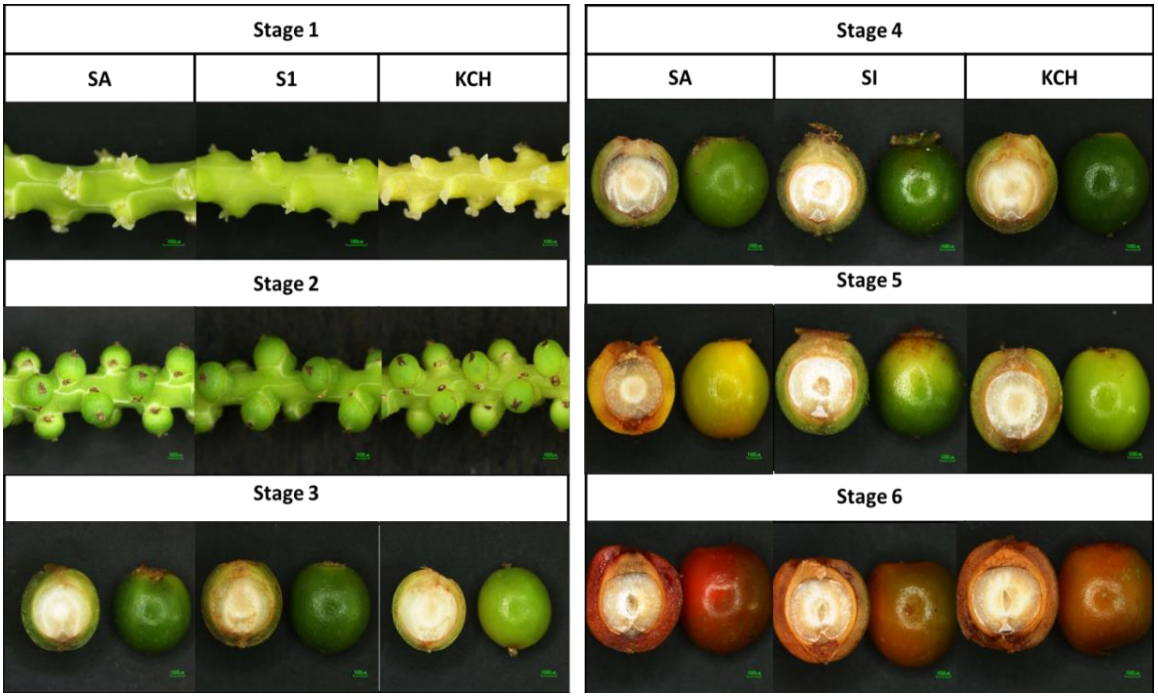
Ubiquinone biosynthesis protein COQ9 (COQ9), Histone 3 (H3) and Elongation factor 1-alpha (EF1a) to obtain the normalisation factor for the measurement of gene expression level (Hu et al., 2015; Khew et al., 2015) . The data analysis was performed with one-way ANOVA at $P<0.05$ followed by Tukey's test to determine the significance of a difference in different stages.

Table 1: Identification of the candidate target genes for probe-based gene expression analysis from the *de novo* transcriptome assembly of black pepper.

Query	Homolog identified	Lowest E-value	Accession (E-value)	Description (E-value)	Greatest identity %
PN_8388.1:1445	AAO	0.6	AP009759	OsAAO	100
PN_10562.1:1183	ARF2	1.70E-20	XM_022874698	DzARF2	84
Pin_8290.1	AG-SHT	1.15E-03	AY464109	HoAG-SHT	96
PN_50799.1:560	TAR4	0.6	XM_025088233	CcTAR4	100
PN_43579.1:408	TAR3	8.27E-12	XM_026591070	PsTAR3	91.3
Pin_7153.1	TIR1	1.15E-03	CP032253	GkTIR1	100
Pin_1852.1	AFB2	0.17	LR699104	ArAFB2	100
Pin_64296.1	PIN8	6.36E-07	CP027622	LuPIN8	92.59
PN_scaf24322.1:231	CKX6	0.17	XM_025808007	AhCKX1	100
PN_C804906.1:490	LOG5	1.40E-21	XM_009393884	MaLOG5	85.57
PN_C797402.1:215	LOG8	8.83E-18	XM_010259318	NcLOG8	82.11
Pin_44880.1	CYP707A1	1.08E-16	MF034882	PsCYP707A1	82.8
Pin_50839.1	PYL4	0.01	XM_030610105	SoPYL4	96.3
PN_scaf50790.2:271	GA20Ox	1.01E-10	XM_027324144	CeGA20Ox	86.67
PN_C803408.2:303	SA-BP2	0.17	XM_026436722	FoSA_BP2	100
PN_scaf7527.2:544	OPR3	2.89E-11	XM_025965708	PhOPR3	96.3
Pin_1076.1	AP2	1.15E-03	KU898265	AfAP2	90.62
Pin_12247.1	GLK1	6.79E-13	XM_030660486	RaGLK1	96.55
Pin_18978.1	AGL8	2.22E-06	AB089153	HcAGL8	100
Pin_4332.1	AG	1.08E-16	MK291509	LcAG	84.88
Pin_12251.1	ERD6	1.60E-14	XM_022131832	HaERD6	89.36
Pin_13705.1	GGT	2.08	LK064661	AaGGT	96.3
Pin_4343.1	ABCC2	7.25E-19	XM_011014264	PeABCC2	83.33
Pin_9031.1	VIN	3.29E-04	XM_008670214	ZmVIN	100
Pin_81110.1	PFP	0.6	CP021038	CmPFP	100
Pin_10717.1	ATHB-13	9.43E-05	XM_008232524	PmATHB-13	88.24
PN_C801706.2:269	ISPD	1.82E-07	XM_030636651	CsISPD	96

PN_C628640.2:7	LSD1	2.70E-24	XM_010063489	EgLSD1	87
PN_S40315.2:831	ODC	2.37E-12	AF323910	NgODC	93.1
PN_CL31.2:194	ATXr2	0.01	LR537132	SaATXR2	100
Pin_18925.1:263	Ubiquinone	4.28E-09	XM_027319026	CeUbiquinone	92.59
Pin_125.1:90	Histone 3	8.83E-18	XM_008223088	PmH3	100
Pip_Elf1a.1:230	EF1a	8.26E-31	AF121261	LiEF1a	91.92

Figure 4: Morphological characteristics of fruits harvested at different stages during the development of black pepper in variety Semengok Aman (SA), Semengok I (SI) and Kuching (KCH).



3.7 Extraction and purification of plant hormones

Fresh flower spike and fruit spike samples used in probe-based gene expression analysis were also used in this experiment. The samples were snap frozen in liquid nitrogen before storage at -80^{o}C until use. Lyophilized fruit samples (50 -100 mg) were crushed to a fine powder with pestle and mortar and suspended in 4 mL of extraction solvent containing 80 % (v/v) acetonitrile and 1 % (v/v) acetic acid. Deuterium-labelled internal standards, D_2-IAA, D_6-iP, D_5-tZ, D_3-DHZ, D_6-ABA, D_2-GA_1, D_2-GA_4, D_2-JA, $^{13}C_6$-JA-Ile, and D_4-Sa, were added to the extraction solvent for the quantification of plant hormones in this study. The suspensions were incubated at 4^{o}C for 1 hour. The extracts were transferred to a polypropylene test tube and centrifuged at 3,000 *g* for 10 min at 4^{o}C. Each pellet was rinsed with 4 mL of 80 % acetonitrile containing 1 % acetic acid, followed by centrifugation at 3,000 *g* for 10 min at 4^{o}C. Supernatants were merged with the extracts before further purification with solid phase extraction. The acetonitrile in the supernatant was

evaporated with a vacuum concentrator until only the acidic aqueous solution remained and centrifuged at 16,000 *g* for 10 min at 4°C to pellet any precipitates. The supernatants were applied to Oasis HLB 1 cc (30 mg) extraction cartridge (Waters Corporation, Milford, MA, USA) equilibrated with 1 mL of acetonitrile, 1 mL of methanol, and 1 mL of 1 % acetic acid, successively. After washing with 1 mL of 1 % acetic acid, hormones were eluted with 2 mL of 80 % acetonitrile containing 1 % acetic acid. Acetonitrile in the eluate was evaporated with vacuum evaporator to remain water and acidified with 1 % acetic acid before applied to Oasis MCX 1 cc (30 mg) extraction cartridge (Waters Corporation, Milford, MA, USA) conditioned with 1 mL of acetonitrile and 1 mL of methanol, successively. After activating the cartridge with 1 mL of 0.1N HCL equilibrated with 1 mL of 1% acetic acid, the acidic fraction containing GA_1, GA_4, IAA, ABA, JA, JA-Ile, and salicylic acid was eluted with 2 mL of 80% acetonitrile containing 1 % acetic acid. Two hundred microliters of the fraction were evaporated to dryness and reconstituted in 50 µL of 1 % acetic acid for analysis of salicylic acid. The MCX cartridge was successively washed with 2 mL of 6 % (v/v) ammonia in water, and the basic fraction containing *trans*-zeatin (tZ) and N^6-(Δ^2-isopentenyl)adenine (iP) was eluted with 1 mL 40 % acetonitrile containing 5 % ammonia and 1 mL 80 % acetonitrile containing 5 % ammonia, successively. The basic fraction was evaporated to dryness and reconstituted in 1 % acetic acid for analysis of tZ, DHZ and iP. Oasis WAX 1 cc (30 mg) extraction cartridge (Waters Corporation, Milford, MA, USA) was conditioned with 1 mL acetonitrile and 1 mL methanol, successively. Acetonitrile in the acidic fraction was evaporated, and the retain water further applied to a WAX cartridge activated with 0.3 mL 0.1 N KOH and equilibrated with 1 mL of 1 % acetic acid. After washing with 1 mL 1 % acetic acid and 3 mL of acetonitrile: formic acid, 97:3 (v/v), successively, eluates were evaporated to dryness and reconstituted in 1 % acetic acid and subjected to analysis of GA_1, GA_4, IAA, ABA, JA, and JA-Ile.

3.8 LC-MS analysis

The liquid chromatography (LC) methods adopted were as in Mikami et al. (2016) in which the plant hormone levels were determined by triple quadrupole liquid chromatography coupled to tandem mass spectrometry (LC-ESI-MS/MS) (Agilent

1260-410). The system was equipped with a ZOBRAX Eclipse XDB-C18 column and XDB-C8 Guard column, and peak areas were determined using MassHunter Workstation software (ver. B.04.00, Agilent Technologies Inc.). Three different LC methods were used for the analysis of the different group of plant hormones. LC "Method 1" was used for the analysis of indole acetic acid, gibberellic acid, jasmonic acid and abscisic acid whereas LC "Method 2" was used for the analysis of trans-zeatin, dihydrozeatin and isopentenyladenine. Meanwhile, LC "Method 3" was used for the analysis of salicylic acid. The detail of the programme in LC methods is described in Table 2. The parameters for the mass spectrometer in the detection of plant hormones are listed in Table 3. The quantification was made by considering recovery rates for each sample by using the deuterium-labelled internal standard. Data were analysed by one-way analysis of variance (ANOVA). Multiple comparisons tests were carried out using the Tukey posthoc test. All statistical tests were performed using SPSS 21.0 statistical package. In all cases, differences were considered significant at a probability level of $P < 0.05$.

Table 2: List of liquid chromatography (LC) methods used for the analysis of plant hormones in black pepper.

Method	Solvent A	Solvent B	Gradient (Proportion of Solvent B)	Flow rate (mL/min)
1	Water with 0.01% (v/v) AcOH	MeCN with 0.05% (v/v) AcOH	3% to 55% in 22 mins	0.4
2	Water with 0.01% (v/v) AcOH	MeOH with 0.02% (v/v) AcOH	3% to 97% in 16 mins	0.25
3	Water with 0.1% (v/v) FA	MeCN with 0.1% (v/v) FA	3% to 98% in 10 mins	0.4

Table 3: Parameters of mass spectrometer analysis in the quantitation of plant hormones in black pepper.

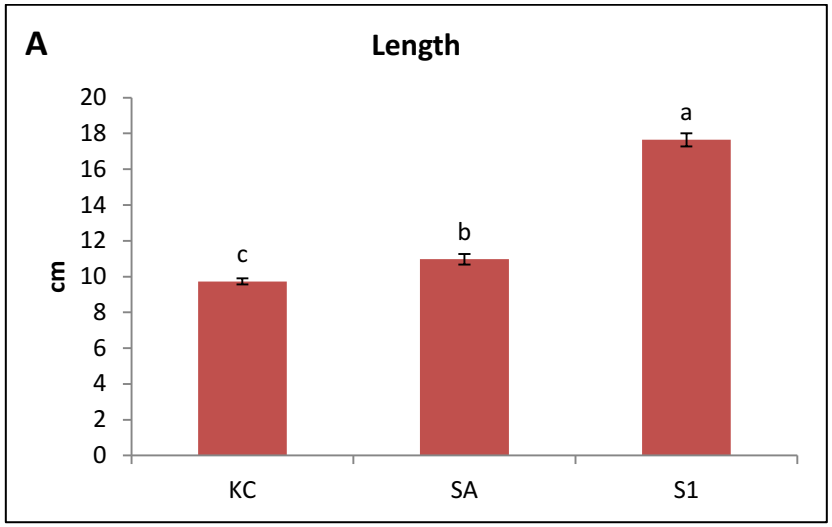
Compound	Retention time (min)	ESI mode	MRM transition (*m/z*)	Fragmentor voltage (V)	Collision energy (V)
IAA D_2-IAA	9.2	positive	176/130 178/132	90	15
ABA D_6-ABA	11.5	negative	263/153 269/159	130	4
GA_1 D_2-GA_1	8.2	negative	347/273 349/275	160	18
GA_4 D_2-GA_4	15.4	negative	331/257 333/259	160	20
JA D_2-JA	13	negative	209/59 211/59	135	11
JA-Ile $^{13}C_6$-JA-Ile	16.2	negative	322/130 338/136	140	17
tZ D_5-tZ	8.1	positive	220/136 225/136, 137	100	12
DHZ D_3-DHZ	8.2	positive	222/136 225/136	140	12
iP D_6-iP	12	positive	204/136 210/137	110	11
SA D_4-SA	5.3	negative	137/93 141/97	90	15

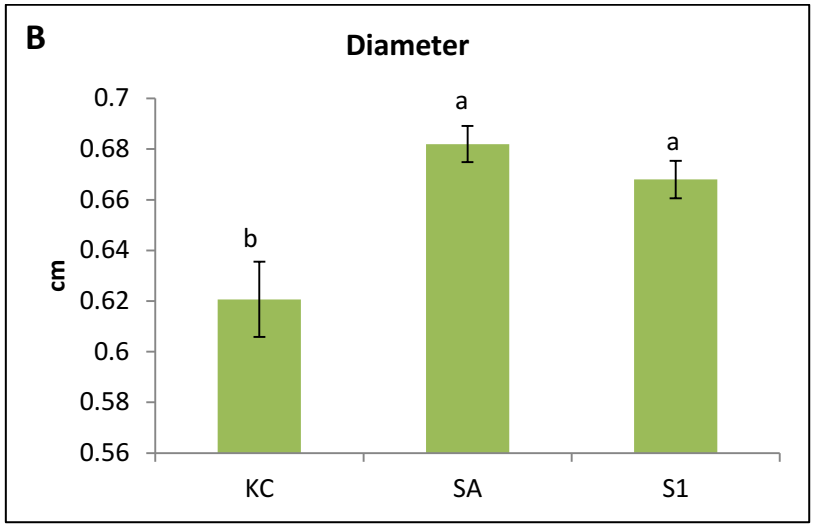
CHAPTER 4: RESULTS

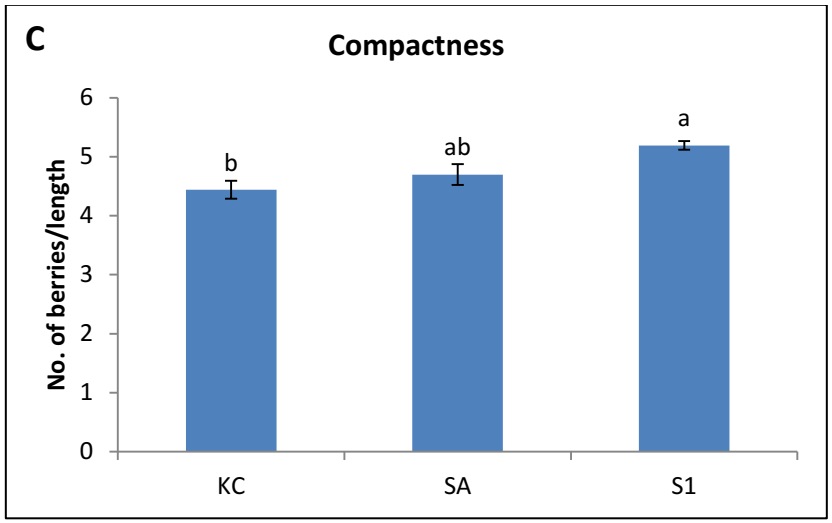
4.1 Morphological comparison on fruit spikes of three different black pepper varieties

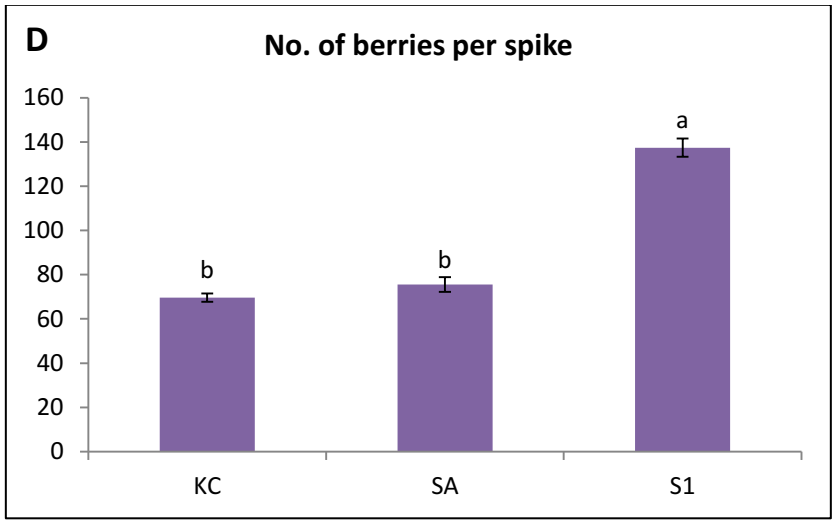
Morphological characteristics of fruit development were compared for three varieties; Kuching (KC), Semongok Aman (SA) and Semongok I (S1). All of the varieties produce drupes with fleshy one-seeded indehiscent fruit. The KC variety has golden-yellow coloured bracts, whereas SA and S1 have green bracts with green inflorescences (Figure 4). Within the first week after anthesis, the ovary enlarges and emerges from the bracts. The young fruit appears as a small green pinhead. After about 1 to 2 weeks, the young fruit emerges further but is still half covered by bracts. The fruit then grow in size, touching or nearly touching each other. The fruit is crispy and can be pressed open easily with finger. After about 3 months, the fruit will grow to its full size and become very hard with a dark green mesocarp. The fruit is firm and difficult to open by pressing with finger at this stage. When reaching the breaker stage, the colour of the mesocarp was change to greenish-yellow while the ripe fruit was yellowish-orange or red. At ripening, the mesocarp was soft and the endocarp can be separated neatly from the mesocarp.

Figure 5 describes the morphological comparison among the fruit setting on three different black pepper varieties. S1 has the longest fruit spike which is about double the length of SA and KC. Moreover, the number of berries per fruit spike and the weight of fruit spike are significant higher in S1 compared to SA and KC. The SA and S1 varieties have the largest fruit diameter and the weight of fruit is almost similar. From all the morphological comparison, KC variety has shown overall poor fruit setting compared to SA and S1, however KC is still widely planted by pepper farmers as the pepper fruit is suitable to produce high value premium white pepper. In summary, there is a great variation on fruit spike length, fruit size and weight of three different black pepper varieties in fruit setting.


A
Length
cm
20
18
16
14
12
10
8
6
4
2
0
c
b
a
KC
SA
S1


B
Diameter
cm
0.7
0.68
0.66
0.64
0.62
0.6
0.58
0.56
b
a
a
KC
SA
S1


C
Compactness
No. of berries/length
6
5
4
3
2
1
0
b
ab
a
KC
SA
S1


D
No. of berries per spike
160
140
120
100
80
60
40
20
0
b
b
a
KC
SA
S1

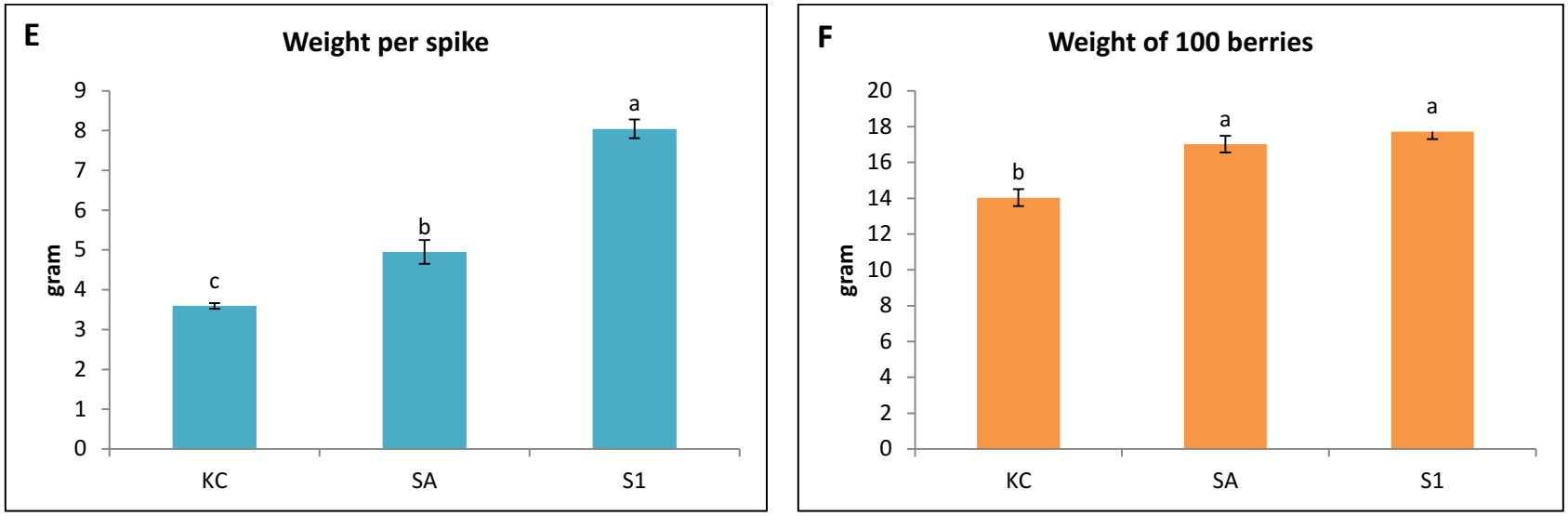


Figure 5: Comparison on the morphological characteristic on fruit setting of three different black pepper varieties. (A) Length of fruit spike; (B) Diameter of a berry; (C) Compactness of fruit setting per spike; (D) Number of berries per fruit spike; (E) Weight per fruit spike and (F) Weight of 100 berries. The error bars represent the standard error from the mean calculated from ten replicates. Statistical significant analysis was based on one-way ANOVA at $p<0.05$. Means followed by the same letter between each variety are not significant different by Tukey's test at $p<0.05$.

4.2 Transcriptome assembly

The Illumina next-generation sequencing generated a total of 810,061,892 pair-end reads from 12 samples of flower and fruit tissues from three black pepper varieties. The sequencing platform delivered 75 GB of data output with an average read length of 93 bp. In this study, two assemblers, SOAPdenovo-Trans and CLC Workbench were run at varying k-mer sizes of between 21 and 50 to determine the most suitable assembler and k-mer size for black pepper *de novo* assembly (assembly statistics shown in Table 4). Although the assembly from the CLC Workbench (k-mer 45) analysis provided higher coverage (about 75%) (Table 4), the total contig number was also significantly higher than that of the other assembler (113,854), suggesting the CLC assembly had high redundancy, thus the SOAPdenovo-Trans assembly at k-mer size 31, with the highest maximum contig size was used for further analysis and annotation.

Table 4: Summary of transcriptome data from two assemblers after redundancy removal

Assembler	**Soap-Trans**				**CLC**
K-mer	**21**	**23**	**27**	**31**	**45**
N50 size	1,641	1,639	1,653	1,654	1,020
N50 no	13,546	14,074	14,587	14,809	26,118
Contig number	61,006	63,669	66,025	66,906	113,854
Genome size	67,404,744	70,065,254	72,708,828	73,742,859	93,269,479
Average length	1,105	1,100	1,101	1,102	819
Min contig	200	200	200	200	295
Max contig	9,932	9,884	9,781	12,965	12,375
Mapping				72%	75%

***Note*:** Table 4 has shown the assembly statistics from two assemblers after the removal of redundant contigs from the initial assembly.

4.3 Annotation and gene ontology analysis

The set of 66,906 assembled contigs was BLASTX parsed against the non-redundant (nr) NCBI protein database with a cut-off E value of 1.0^{-3}, returning matches for 43,115 contigs while 23,791 were without matches (Figure 6). From the BLASTX results, *Vitis vinifera*, *Nelumbo nucifera*, *Theobroma cacao*, *Elaeis guineensis* and *Phoenix dactylifera* were the plant species with the highest number of matches to the pepper transcriptome assembly (Figure 7). The InterProScan, Annex and GO annotation query resulted in a 15% increase in the number of annotated sequences.

A total of 37,377 unigenes (55.8%) were assigned with at least one gene ontology category (Figure 8). Then, the plant-specific GO slim was used to categorise the unigenes into varying functional groups. Under the cellular component category, 46.5% of unigenes were categorised as cell, membrane and organelle. Meanwhile, for the molecular function category, binding and catalytic activity were the two most abundant groups. Next, concerning the biological process category, the most significant proportion was under metabolic process and cellular process (Figure 8). GO annotations showed a high number of expressed genes associated with biosynthetic processes (5,122), reproduction (556), anatomical structure morphogenesis (512), flower development (298), response to stimulus (4,112) and signalling activity (1,161). Genes involved in other important biological processes such as flower development, stress response, signal transduction, cell differentiation, pollen-pistil interaction and fruit ripening were also identified. A total of 5,874 unigenes were mapped in 150 KEGG pathways. The pathways with the highest unigene representation were purine metabolism (map00230; 447 unigenes; 7.6%), followed by starch and sucrose metabolism (map00500; 362 unigenes, 6.2%) and phenylpropanoid biosynthesis (map00940; 241 unigenes, 4.1%) (Appendix II).

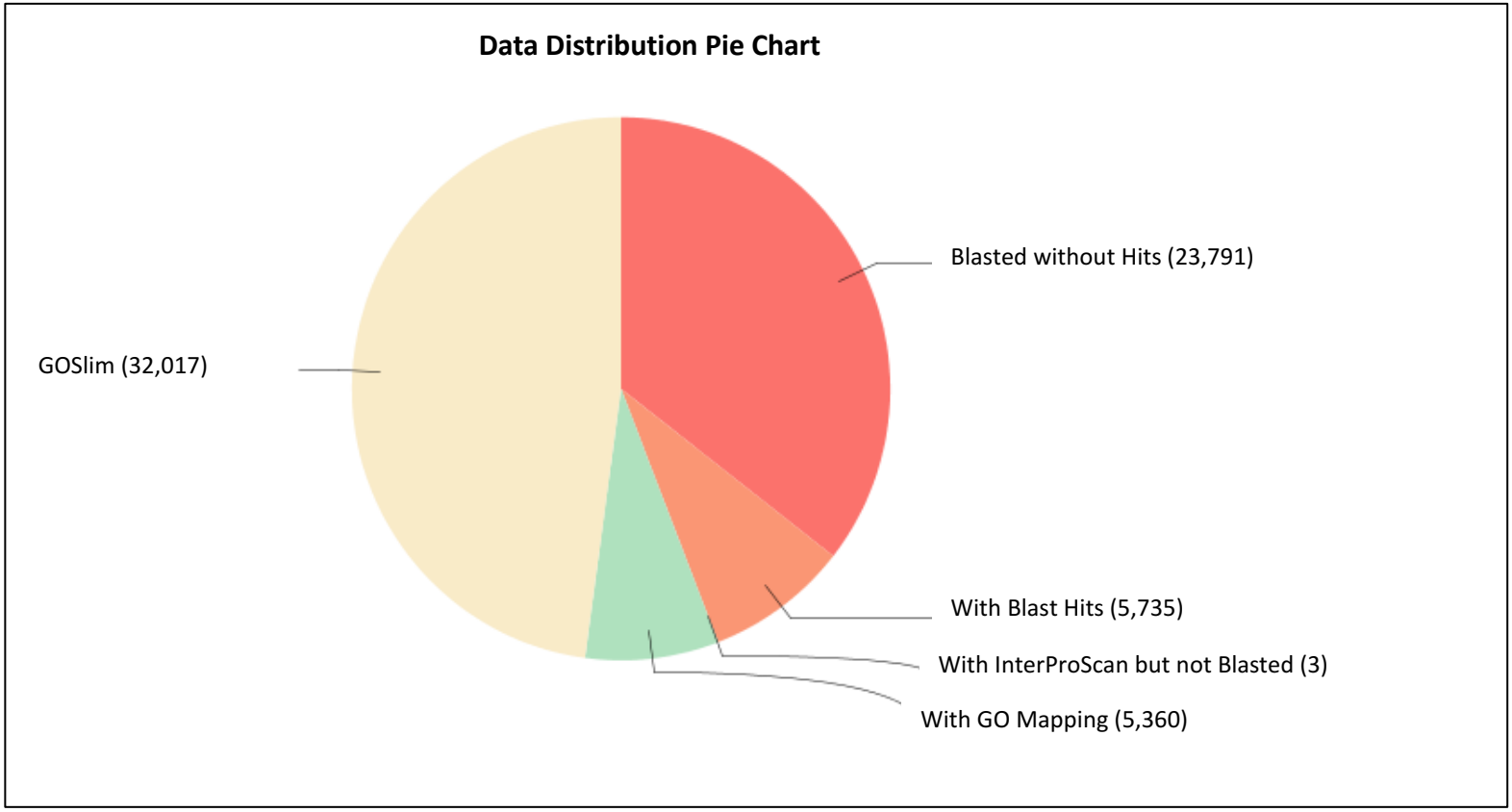


Figure 6: Data distribution pie chart for the visualisation of annotated and non-annotated sequences in black pepper flower and fruit transcriptome.

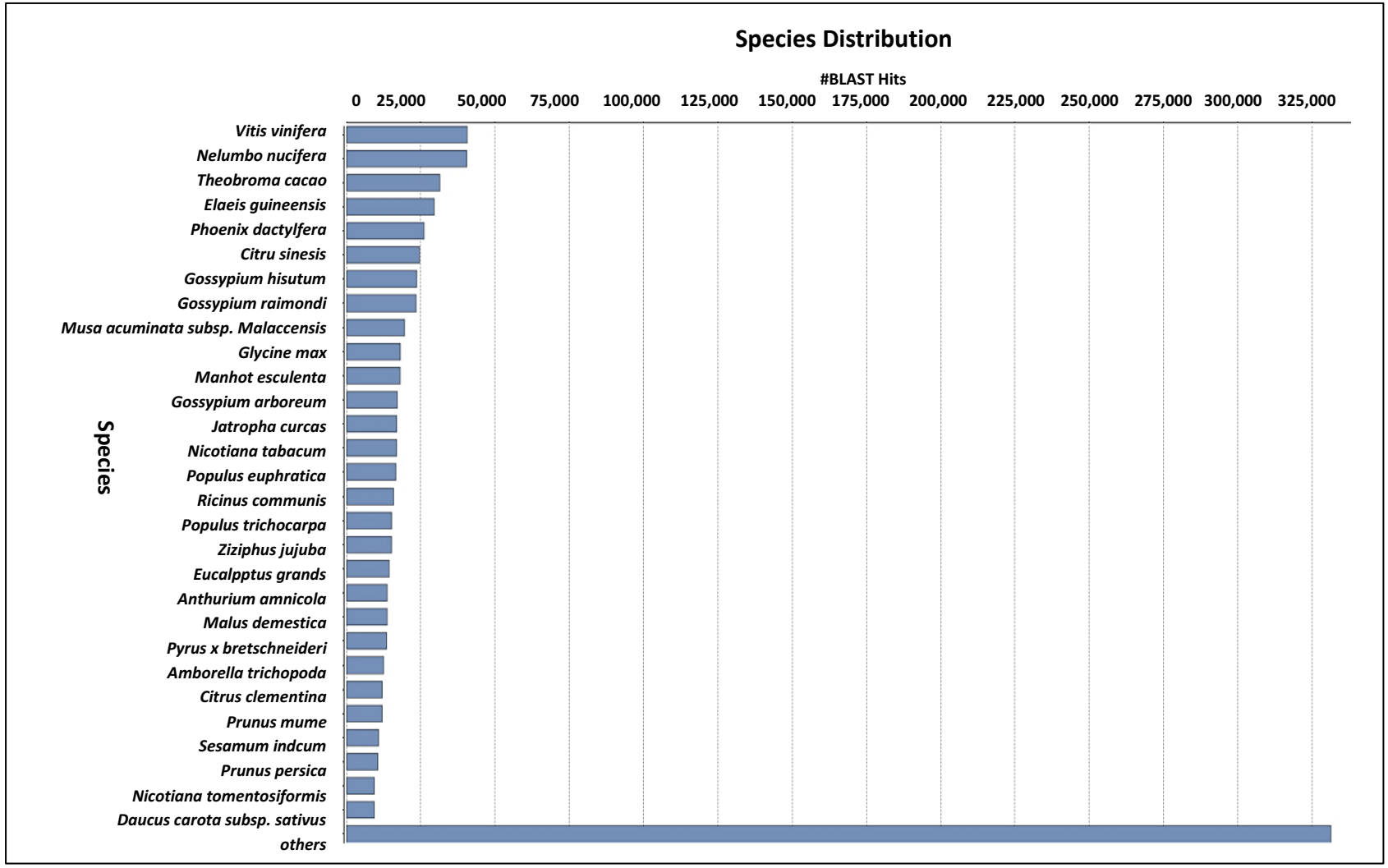


Figure 7: Species distribution based on BLASTX hits in the transcriptome assembl

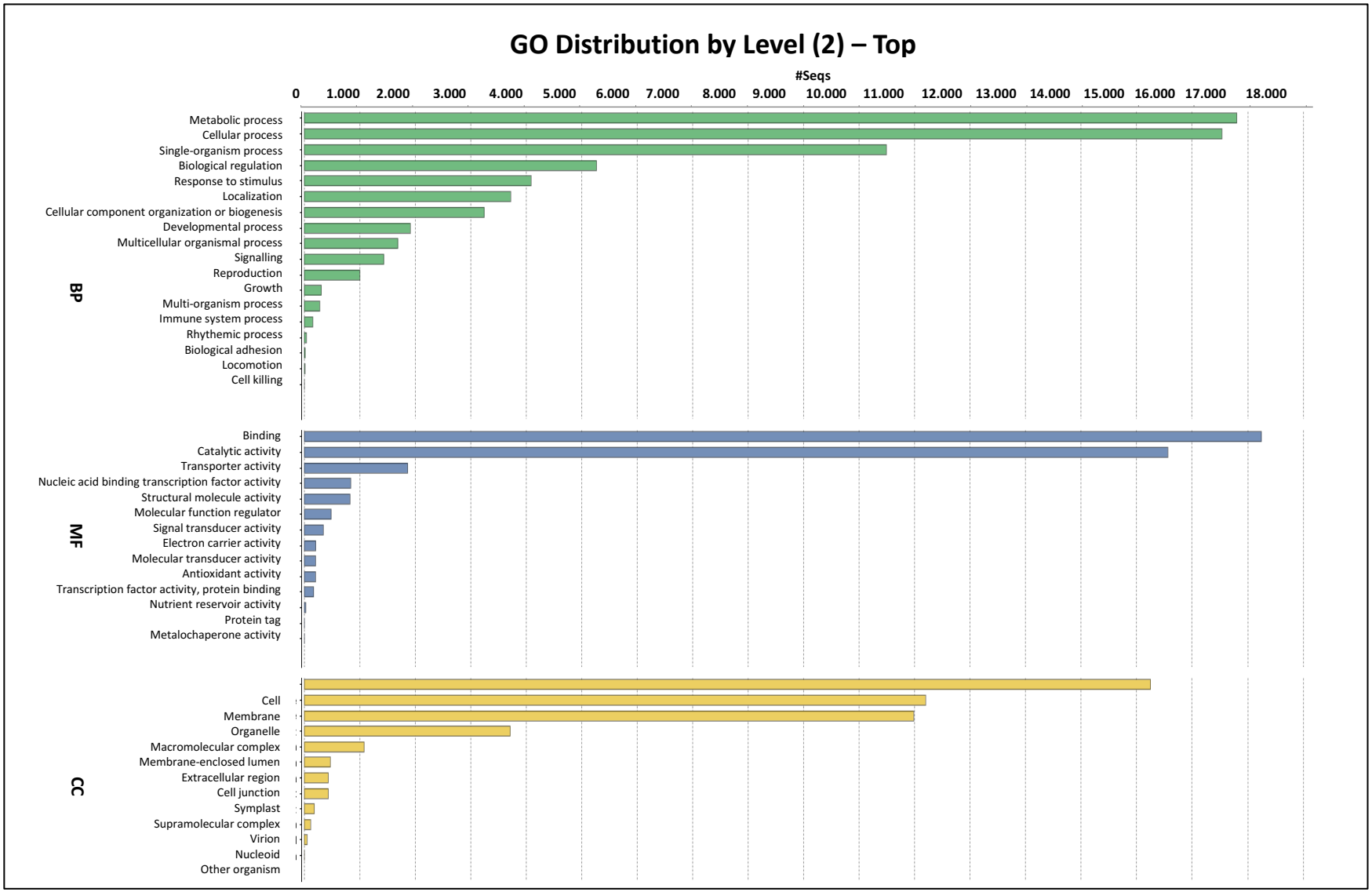


Figure 8: Blast2GO characterization of top 20 GO terms in the three categories of Biological Process, Molecular Function, and Cellular Component.

4.4 Differential gene expression in transcriptome

In this study, FPKM method was used to calculate the expression of unigenes in black pepper flower and fruit. The transcriptome expression analysis identified a total of 66,906 unigenes, with 7,721 and 919 showing differential expression between flower and fruit tissues, respectively. The unigenes were considered sufficiently differently expressed when the absolute value of log2FC more than 2 and statistically significant when FDR less than 0.05. From the analysis, SA flower yields a higher number of up-regulated genes when compared between SA and KC as well as between SA and S1 (Figure 9). Regarding the number of differentially expressed genes which are up-regulated in flower between each variety or exclusively expressed in the flower of each variety, SA had a higher number compared to KC and S1. Overall, the differential gene expression analysis on the flower in black pepper showed the variety KC and S1 have a more similar set of differentially expressed genes than in the SA variety based on the number of commonly up-regulated genes and exclusively expressed genes in each variety.

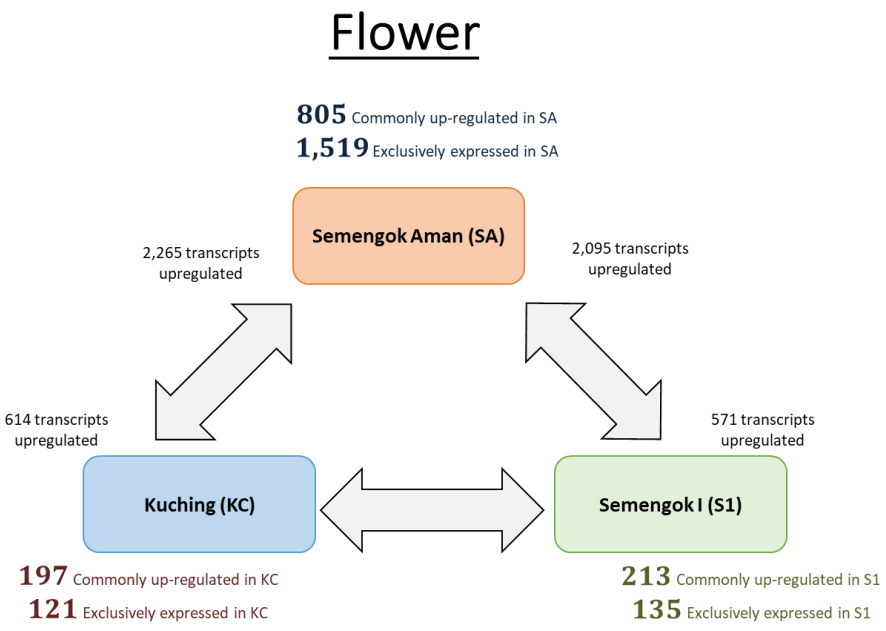


Figure 9: Comparison of numbers of genes commonly up-regulated in between each variety and number of genes exclusively expressed in flowers of each variety of black pepper (SA, S1 & KC).

Similar to the differential genes expression analysis in flower, SA fruit has higher number of up-regulated genes than in KC and S1 (Figure 10). The number of exclusively expressed genes in SA is the highest followed by KC and S1 variety. The comparison of the differentially expressed genes in fruit sample of three different black pepper varieties revealed that more differentially expressed genes were found in the comparison between SA and KC rather than between SA and S1 and between KC and S1. Therefore, the results suggested that fewer changes in the expression of genes in fruit samples than in flower samples in three different black pepper varieties. The study also reveals that the expression of genes is variety specific as there are a large amount of genes were exclusively expressed in each individual variety.

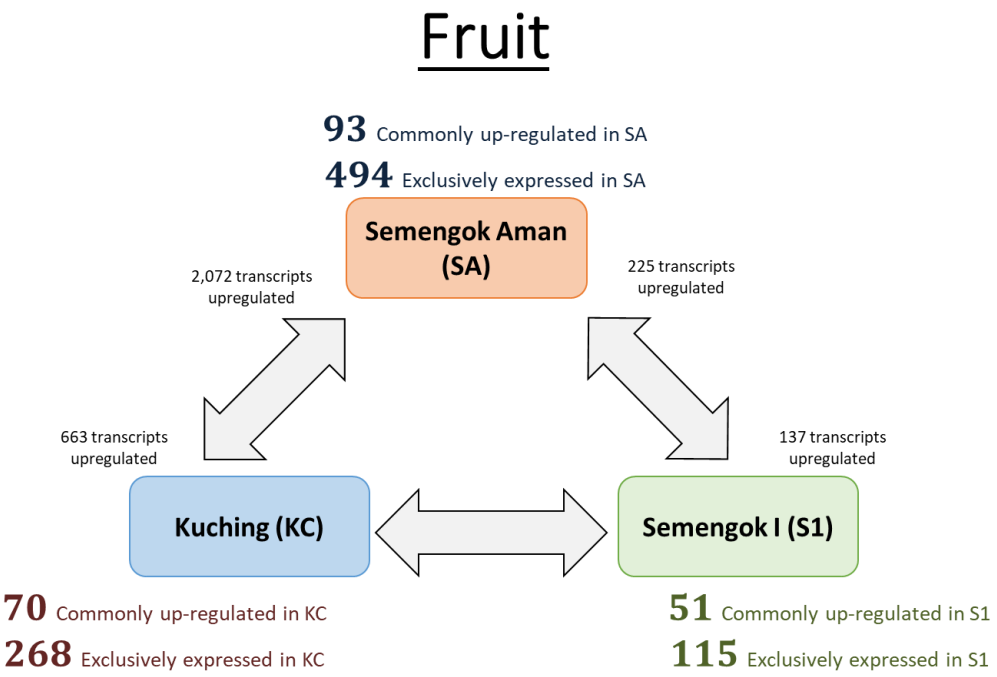


Figure 10: Comparison of numbers of genes commonly up-regulated in between each variety and numbers of genes exclusively expressed in flowers of each variety of black pepper (SA, S1 and KC).

4.5 Gene ontology and pathway enrichment analysis

In order to understand the function of genes that were differentially expressed between flower and fruit and between different varieties of black pepper, GO enrichment and KEGG pathway enrichment were performed on the set of up-regulated genes in different group of comparison with the transcriptome annotation as background (Figure 11). Ten GO terms were enriched in the flower transcriptome of the SA variety while thirteen GO terms and fourteen GO terms were enriched in the transcriptome from the KC and S1 variety, respectively (Figure 11). In the SA variety, the biological process GO term of carbohydrate metabolic process was the most enriched term and coincided with the molecular function terms of catalytic activity and cell cellular component of thylakoid. The SA variety was also enriched for the biological process terms of embryo development and DNA metabolic process, which is consistent with the enriched molecular function terms of nuclease activity and structural molecule activity. For the KC variety, functional enrichment analysis showed that the biological process terms of secondary metabolic process was specifically enriched, coinciding with the molecular function terms of response to extracellular stimulus and cell cellular component of cytosol. In the S1 variety, eight GO terms (nuclear envelope, signal transduction, protein binding, transport, motor activity, cytoskeleton, nucleotide binding and regulation of gene expression, epigenetic) were uniquely enriched. Distinction in the gene ontology enrichment of the differential genes expressed in each variety might be the determinant that contributes to the morphological difference in each variety.

SA		KC		S1	
SA vs KC	**SA vs S1**	**KC vs SA**	**KC vs S1**	**S1 vs SA**	**S1 vs KC**
GO:0003824 catalytic activity	GO:0004518 nuclease activity	GO:0009058 biosynthetic process	GO:0005840 ribosome	GO:0005635 nuclear envelope	GO:0003774 motor activity
GO:0009790 embryo development	GO:0006259 DNA metabolic process	GO:0003824 catalytic activity	GO:0005829 cytosol	GO:0007165 signal transduction	GO:0005856 cytoskeleton
GO:0005975 carbohydrate metabolic process	GO:0005840 ribosome	GO:0019748 secondary metabolic process	GO:0005773 vacuole	GO:0009058 biosynthetic process	GO:0000166 nucleotide binding
	GO:0006412 translation	GO:0006629 lipid metabolic process	GO:0006412 translation	GO:0005515 protein binding	GO:0005635 nuclear envelope
	GO:0009579 thylakoid	GO:0005773 vacuole	GO:0005198 structural molecule activity	GO:0006810 transport	GO:0005515 protein binding
	GO:0005198 structural molecule activity	GO:0005198 structural molecule activity		GO:0019825 oxygen binding	GO:0007165 signal transduction
	GO:0005773 vacuole	GO:0019825 oxygen binding		GO:0040029 regulation of gene expression, epigenetic	
		GO:0009991 response to extracellular stimulus		GO:0003774 motor activity	

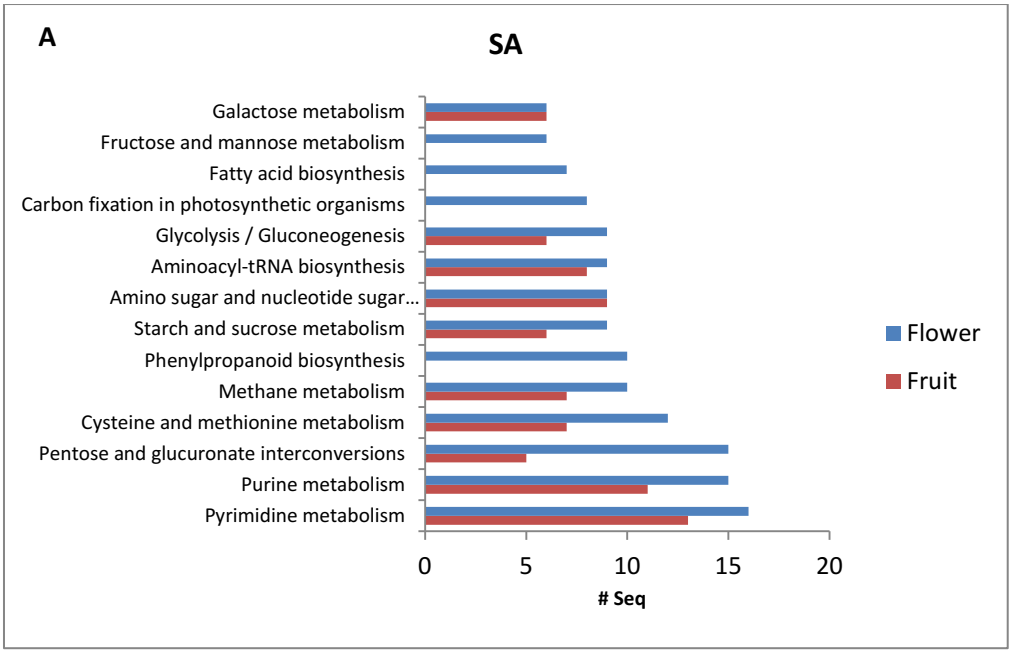
Figure 11: Gene ontology term enrichment in flower differentially expressed genes of three black pepper varieties. Orange: GO terms enriched in three different varieties. Orange: SA. Blue: KC; Green: S1. The GO terms shared between the varieties were in the same color fonts.

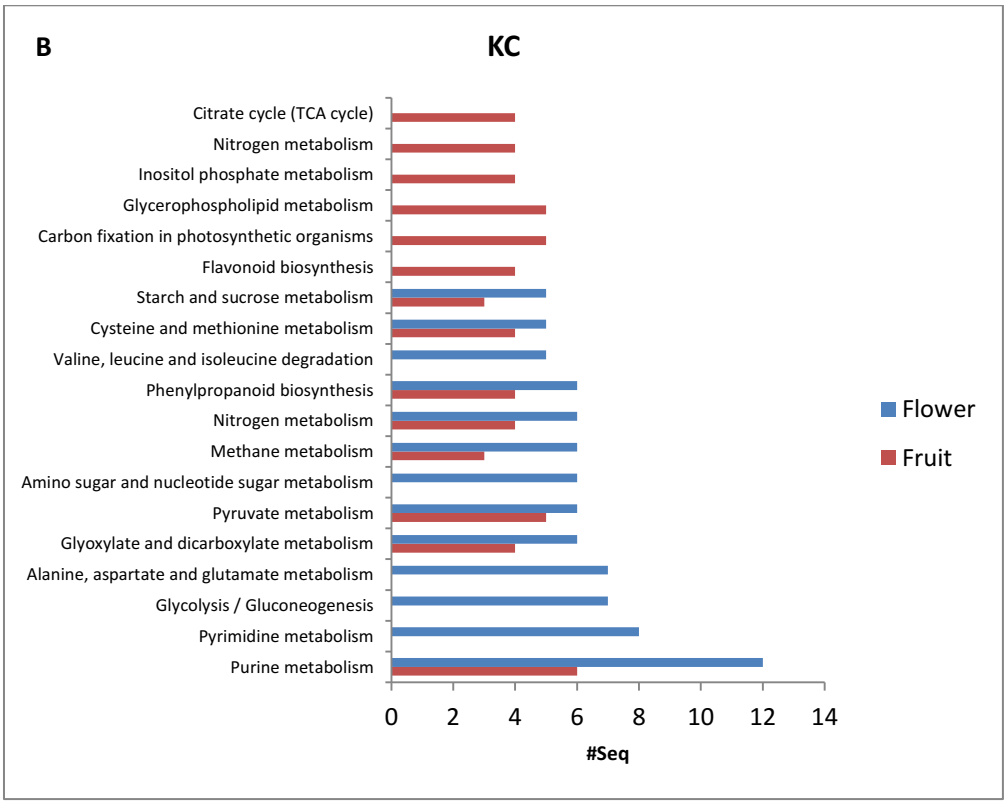
For the GO enrichment analysis of fruit transcriptome data, fewer terms were enriched compared to the numbers seen in flowers. Only six GO terms (DNA metabolic process, translation, ribosome, structural molecule activity, nucleolus and lipid metabolic process) were enriched in SA fruit (Figure 12). The biological process terms of catabolic process and secondary metabolic process were specifically enriched in the KC variety which is coincided with molecular function terms of oxygen binding, catalytic activity and transporter activity. The molecular function term of structural molecule activity and cell cellular component of ribosome were enriched in KC and S1 but not in SA fruit.

The KEGG pathways enrichment analysis of the gene differentially expressed between each variety in flower and fruit were those related to energy production and to nucleotide formation, to be significantly enriched (Figure 13). Purine metabolism, cysteine and methionine metabolism and pyrimidine metabolism were amongst the enriched pathways correlated with the ongoing growth and development of fruit. The pathways related to energy metabolism, including pentose and glucoronate interconversions, amino sugar and nucleotide sugar metabolism, glycolysis/ gluconeogenesis, starch and sucrose metabolism, carbon fixation in photosynthetic organisms, fructose and mannose metabolism and galactose metabolism were highly enriched in the flower and fruit of each black pepper variety. SA and KC have share several same KEGG pathways in between flower and fruit, but this is not the case for S1 variety as the KEGG pathways enriched in flower is distinct from fruit.

SA		KC		S1	
SA vs KC	SA vs S1	KC vs SA	KC vs S1	S1 vs SA	S1 vs KC
GO:0006259 DNA metabolic process	GO:0006629 lipid metabolic process	GO:0019825 oxygen binding	GO:0005215 transporter activity	GO:0005198 structural molecule activity	
GO:0006412 translation		GO:0019748 secondary metabolic process	GO:0009056 catabolic process	GO:0009058 biosynthetic process	
GO:0005840 ribosome		GO:0003824 catalytic activity		GO:0005840 ribosome	
GO:0005198 structural molecule activity				GO:0006950 response to stress	
GO:0005730 nucleolus				GO:0009987 cellular process	

Figure 12: Gene ontology term enrichment in fruit differentially expressed genes of different black pepper varieties. Orange: GO terms enriched in three different varieties. Orange: SA. Blue: KC; Green: S1. The GO terms shared between the varieties were in the same color fonts


A
SA
Galactose metabolism
Fructose and mannose metabolism
Fatty acid biosynthesis
Carbon fixation in photosynthetic organisms
Glycolysis / Gluconeogenesis
Aminoacyl-tRNA biosynthesis
Amino sugar and nucleotide sugar...
Starch and sucrose metabolism
Phenylpropanoid biosynthesis
Methane metabolism
Cysteine and methionine metabolism
Pentose and glucuronate interconversions
Purine metabolism
Pyrimidine metabolism
0
5
10
15
20
Seq
Flower
Fruit


B
KC
Citrate cycle (TCA cycle)
Nitrogen metabolism
Inositol phosphate metabolism
Glycerophospholipid metabolism
Carbon fixation in photosynthetic organisms
Flavonoid biosynthesis
Starch and sucrose metabolism
Cysteine and methionine metabolism
Valine, leucine and isoleucine degradation
Phenylpropanoid biosynthesis
Nitrogen metabolism
Methane metabolism
Amino sugar and nucleotide sugar metabolism
Pyruvate metabolism
Glyoxylate and dicarboxylate metabolism
Alanine, aspartate and glutamate metabolism
Glycolysis / Gluconeogenesis
Pyrimidine metabolism
Purine metabolism
0
2
4
6
8
10
12
14
#Seq
Flower
Fruit

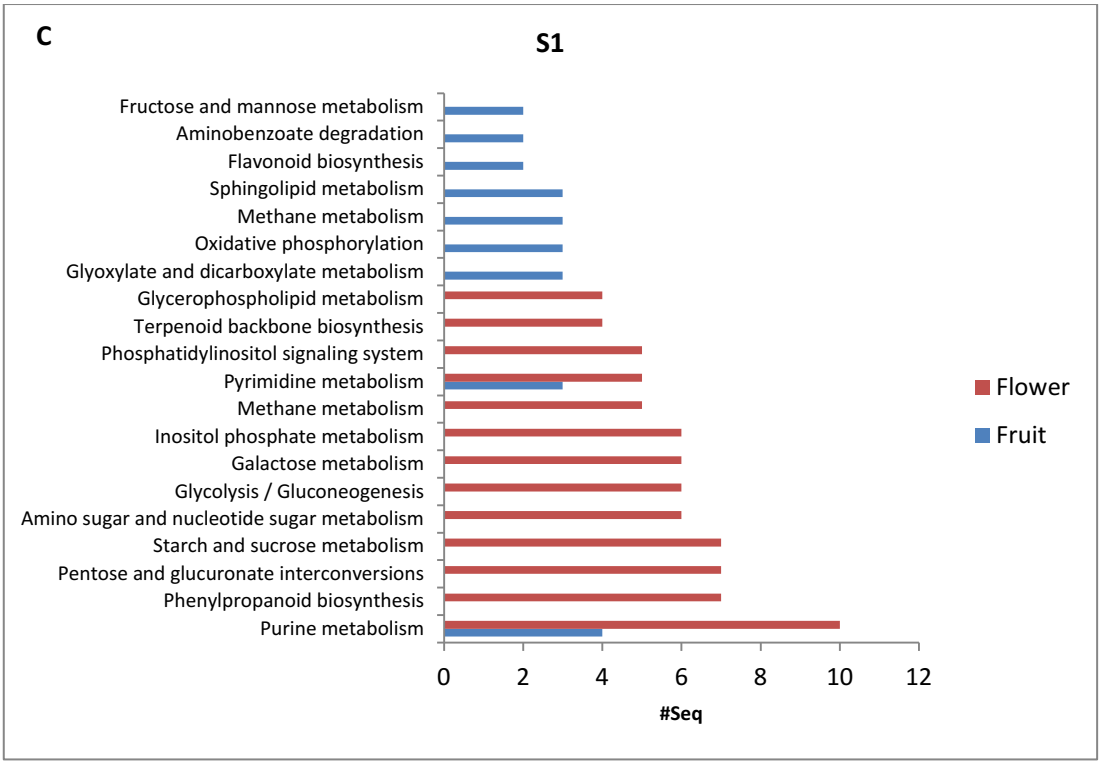
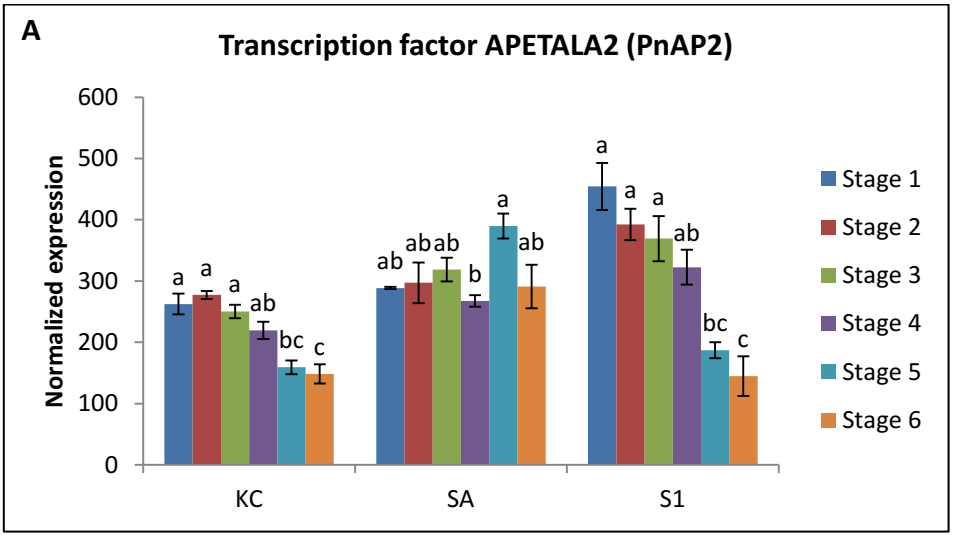


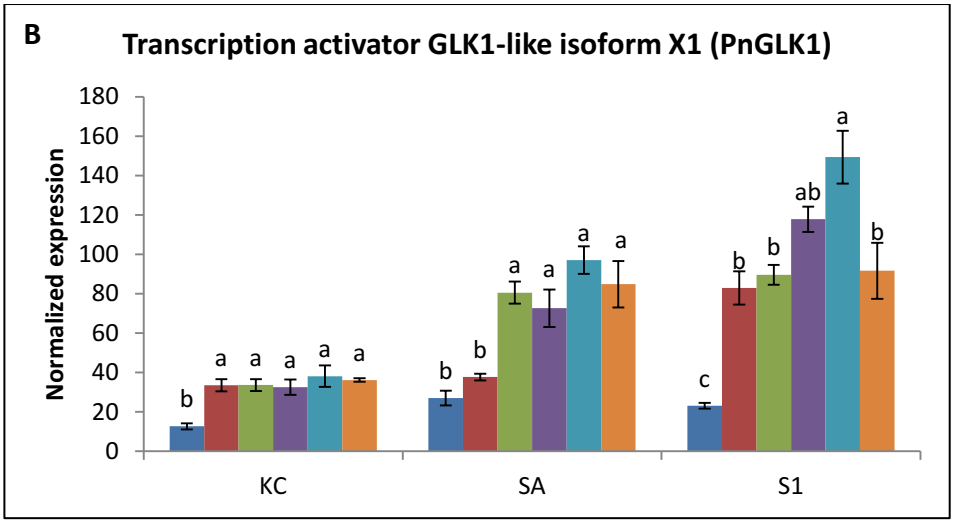
Figure 13: KEGG pathways enrichment analysis of differentially expressed genes in the three black pepper varieties. (A) KEGG pathways enriched in SA; (B) KC and (C) S1variety.

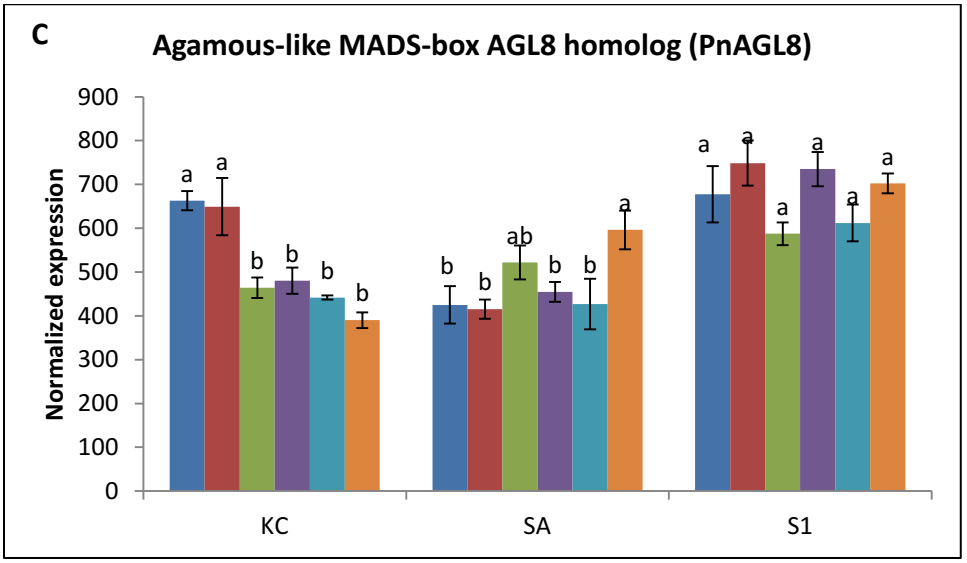
4.6 Expression analysis on flower formation genes

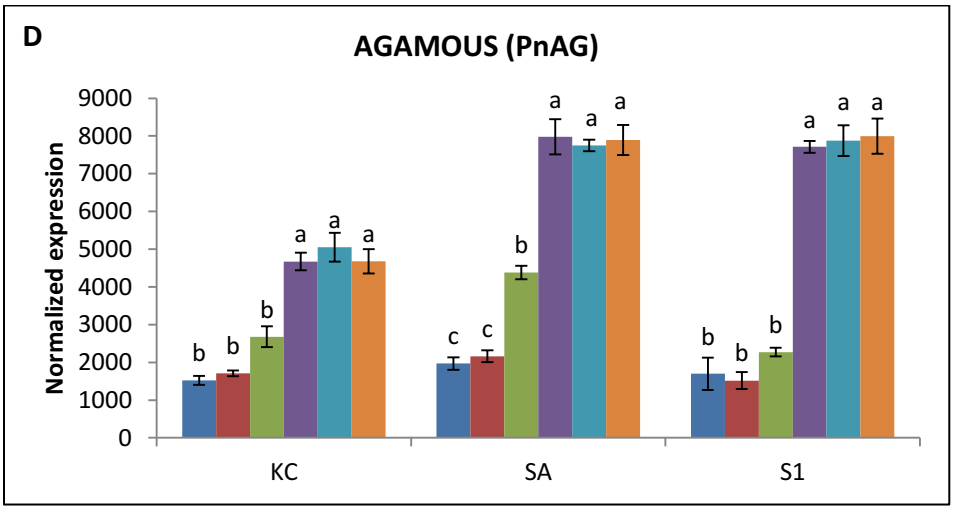
To identify the gene expression patterns of flower formation genes in three different black pepper varieties, four genes that identified from the transcriptome with absolute value of log2FC more than 2 and statistically significant when FDR less than 0.05 were further analysed in six different fruit development stages (Figure 14). Overall, three different black pepper varieties have distinct gene expression profiles throughout the fruit development stages in most of the studied genes. All varieties exhibit high expression of transcription factor APETALA2 (PnAP2) at early stage of fruit development but gradually decrease as fruit matured in KC and S1 varieties but remained high in SA variety (Figure 14A). The expression of agamous-like MADS-box AGL8 homolog (PnAGL8) remained high across the fruit development stages in SA and S1 varieties but was slightly declined in KC varieties. The AP2 regulating the network of flowering time control by directly binding and activated the AGL8.

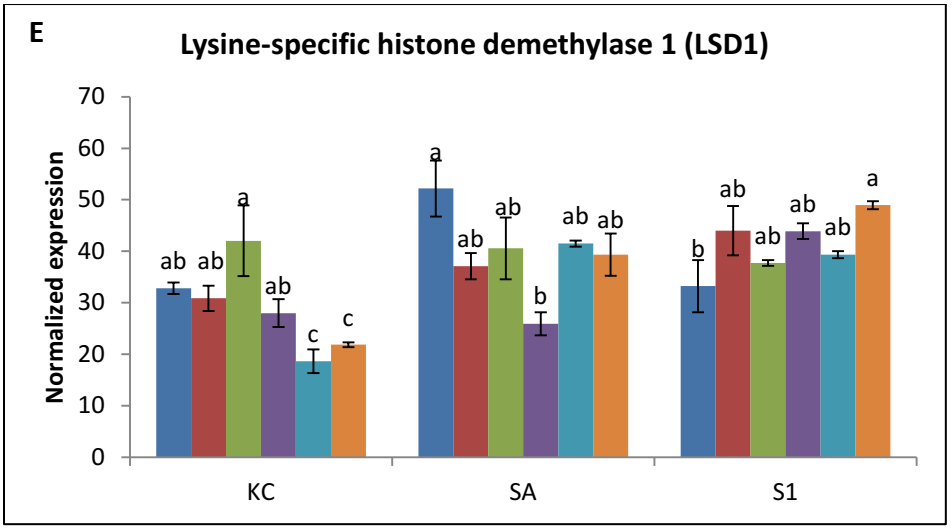
Transcription activator GLK1-like isoform X1 (PnGLK1) implicated in coordinating the development and maintenance of chloroplasts were significantly highly expressed at the late stage of fruit development in SA and S1 varieties except that the expression of PnGLK1 was levelled off across fruit development stages in KC variety. The AGAMOUS (PnAG) that acts as key regulator in flower and fruit development also has a same expression pattern as PnGLK1 in all three black pepper varieties. High expression of lysine-specific histone demethylase 1 (PnLSD 1) and histone-lysine N-methyltransferase (PnATXR2) that involved in the methylation of lysine residue of histone H3 tail for flora transition were observed across the stages in all three varieties with some stages showed a significant different in the expression levels. All the gene expression analysis throughout this study was normalised against the housekeeping genes of ubiquinone biosynthesis protein (COQ9), histone 3 (H3) and elongation factor 1-alpha (EF1a) with no significant difference across fruit development stages in all three varieties (Figure 14 G to I).

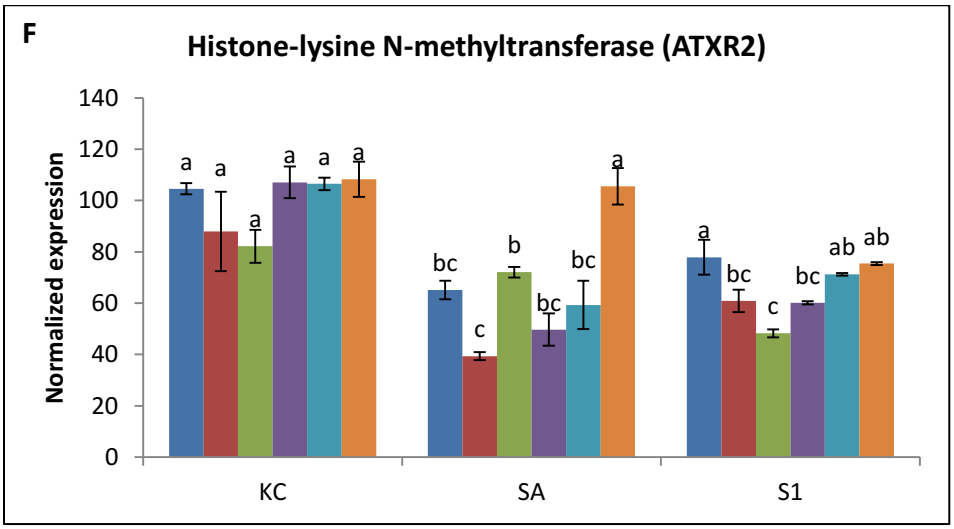


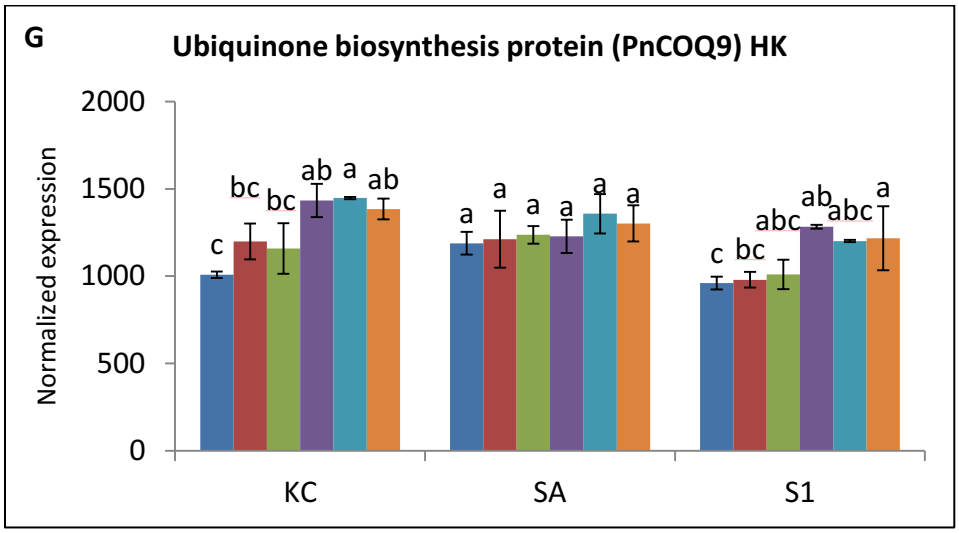

B
Transcription activator GLK1-like isoform X1 (PnGLK1)
Normalized expression
180
160
140
120
100
80
60
40
20
0
KC
SA
S1

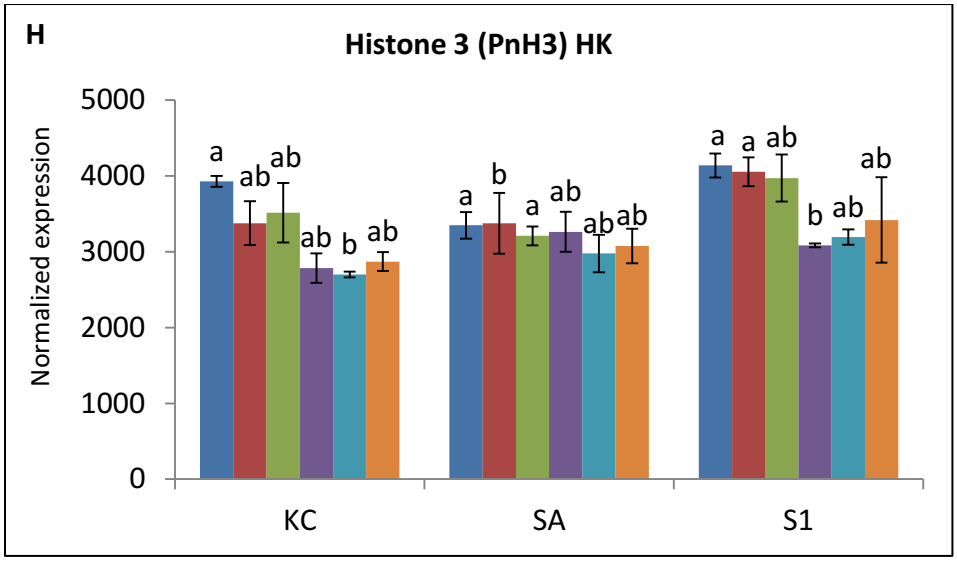

C
Agamous-like MADS-box AGL8 homolog (PnAGL8)
Normalized expression
900
800
700
600
500
400
300
200
100
0
KC
SA
S1


D
AGAMOUS (PnAG)
Normalized expression
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
KC
SA
S1


E
Lysine-specific histone demethylase 1 (LSD1)
Normalized expression
70
60
50
40
30
20
10
0
ab ab a ab c c
a ab ab b ab ab
b ab ab ab ab a
KC
SA
S1


F
Histone-lysine N-methyltransferase (ATXR2)
Normalized expression
140
120
100
80
60
40
20
0
a a a a a a
bc c b bc bc a
a bc c bc ab ab
KC
SA
S1


G
Ubiquinone biosynthesis protein (PnCOQ9) HK
Normalized expression
2000
1500
1000
500
0
c bc bc ab a ab
a a a a a a
c bc abc ab abc a
KC
SA
S1



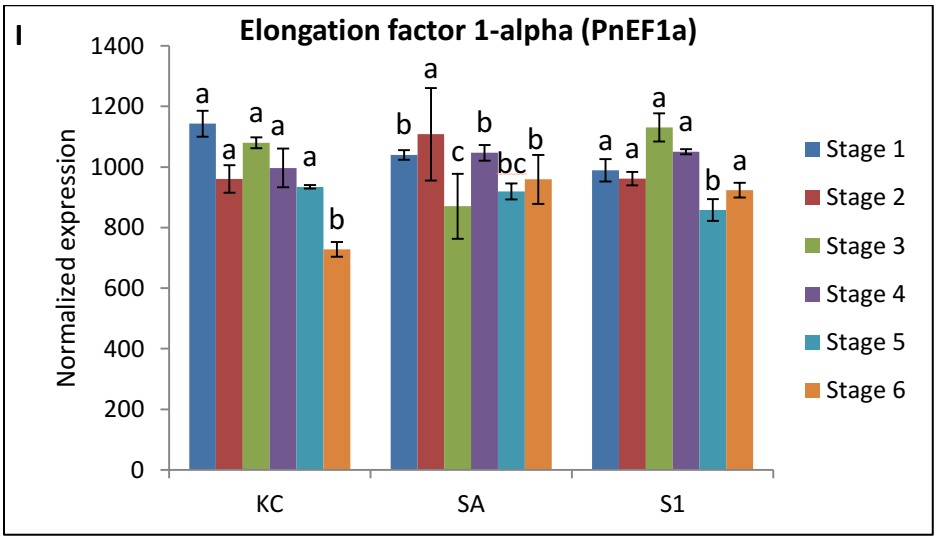
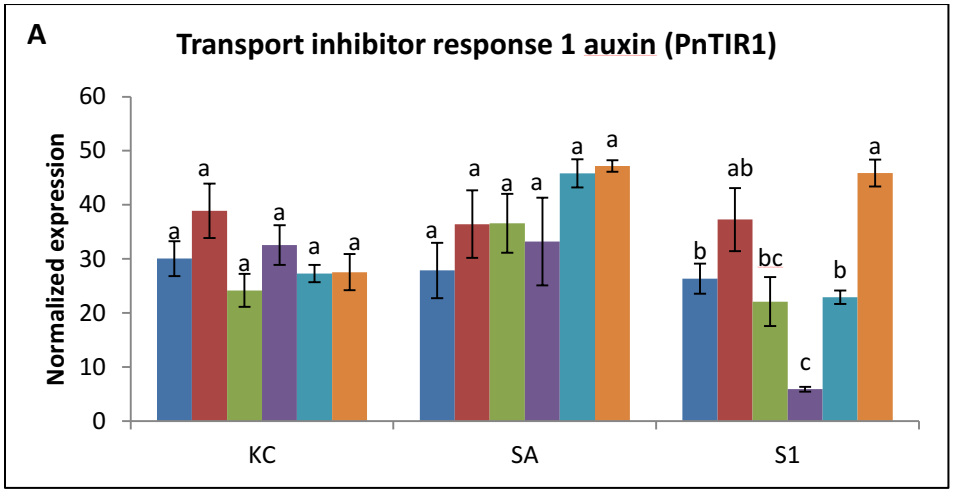


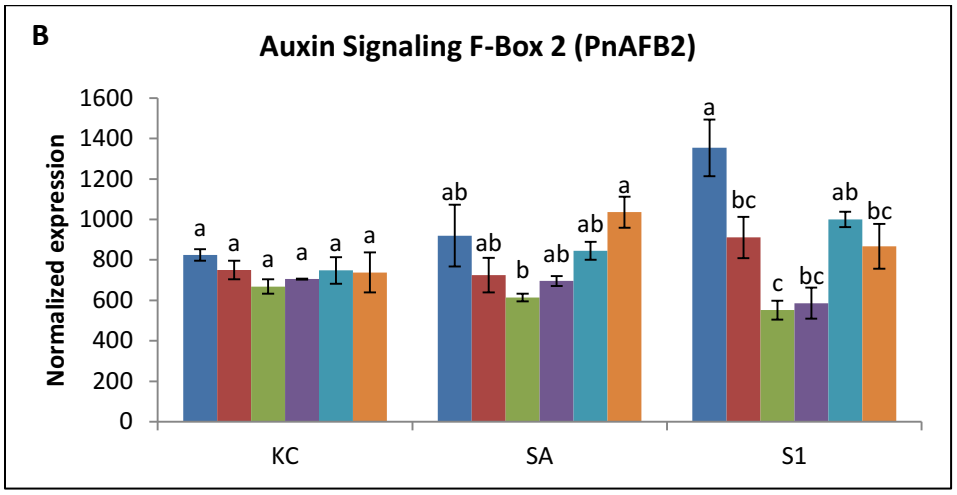
Figure 14: Expression profiles of six stages of fruit specific transcripts in three varieties of black pepper using probe-based gene expression analyses. Panels A to D are gene expression profiles related to flower formation genes and panels E to G are for three housekeeping genes. The transcript counts (panels A to F) were normalised using six synthetic ssDNA positive control targets and three housekeeping genes (panels G to I). The error bars represent the standard error from the mean expression level calculated from three biological replicates. Statistical significant analysis was based on one-way ANOVA at $p<0.05$. Means followed by the same letter between stages of each variety are not significant different by Tukey's test at $p<0.05$.

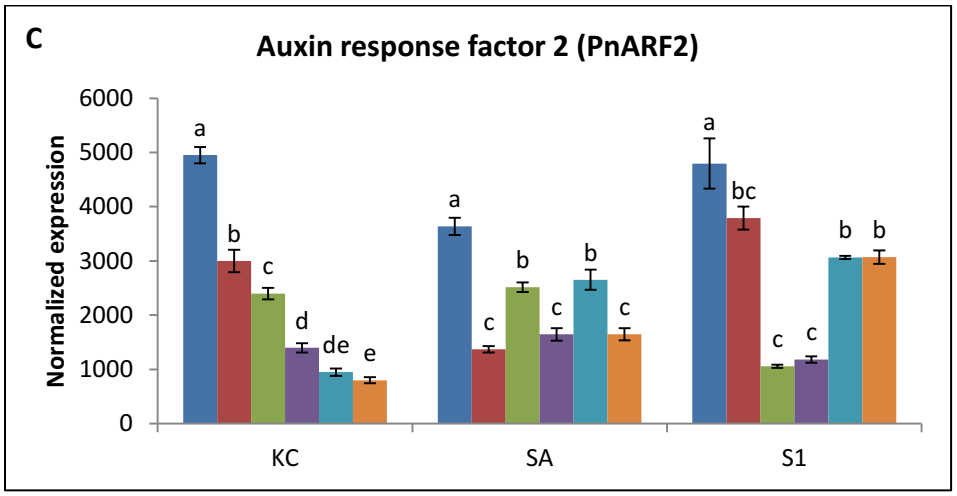
4.7 Expression analysis on plant hormones related genes

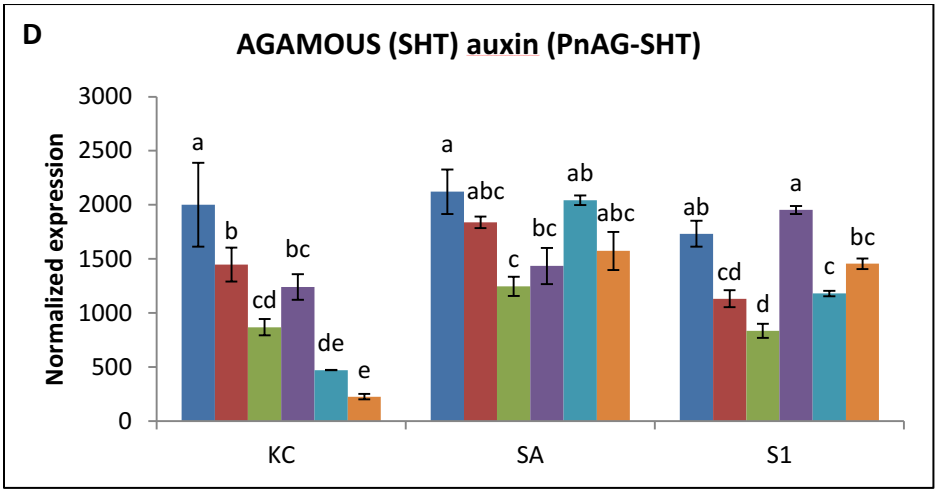
4.7.1 Genes related to auxin metabolism and signalling during the fruit development in black pepper

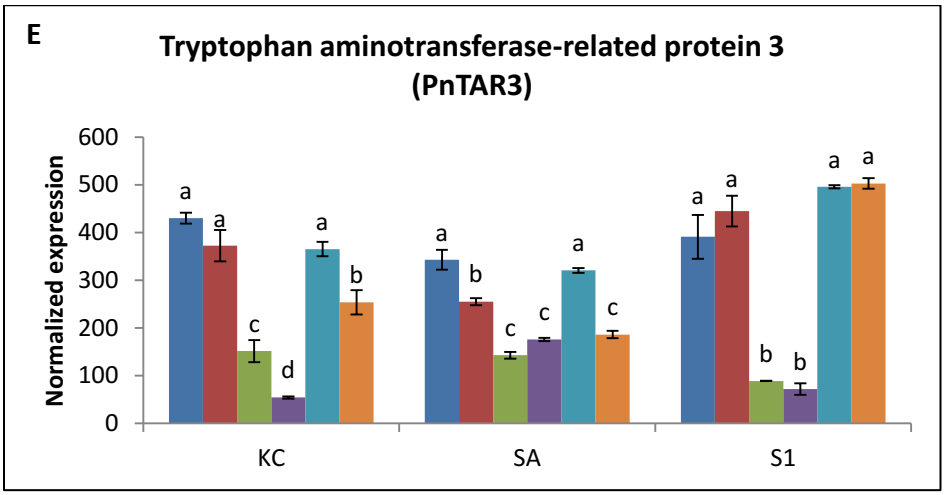
During the flower and fruit development in black pepper, two genes that codes for the auxin receptor, transport inhibitor response 1 auxin (PnTIR1) and auxin signalling F-box 2 (PnAFB2) remained high across stages in different varieties of black pepper except that a bottomed out expression of PnTIR1 in S1 variety at fruit maturation stage (Figure 15A&B). Furthermore, the recognized auxin signalling component, auxin response factor 2 (PnARF2) that regulate the expression of auxin-responsive gene, AGAMOUS (SHT) auxin (PnAG-SHT) were both showed a gradual decrease expression pattern in KC variety but a fluttered expression across the fruit development stage in SA and S1 varieties (Figure 15C&D). Under the tryptophan-dependent pathway of auxin biosynthesis, two genes that mediate the conversion of tryptophan to indole-3-acetic acid (IAA), tryptophan amino transferase-related protein (PnTAR3) and tryptophan amino transferase-related protein 4 (PnTAR4) were further analysed in this study in which the PnTAR3 exhibited high expression at early and late stage of fruit development but low expression at fruit expansion and fruit maturation stage (Stage 3 and 4) in all three varieties (Figure 15E). Meanwhile, the PnTAR4 was highly expressed at fruit ripening stage in KC variety and SA varieties but low expression across stages in S1 variety (Figure 15F). Study on the expression of the auxin-transporter gene, auxin efflux carrier component 8 (PnPIN8) reveals high expression at fruit maturation stage in KC and S1 varieties but gradually decreased as the fruit ripened in both varieties. Meanwhile, the expression of PnPIN8 was not identified in SA variety although it was detected in both KC and S1 varieties which suggested that the level of transcription for PnPIN8 in SA variety is below the detection limits of the assay (Figure 15G).

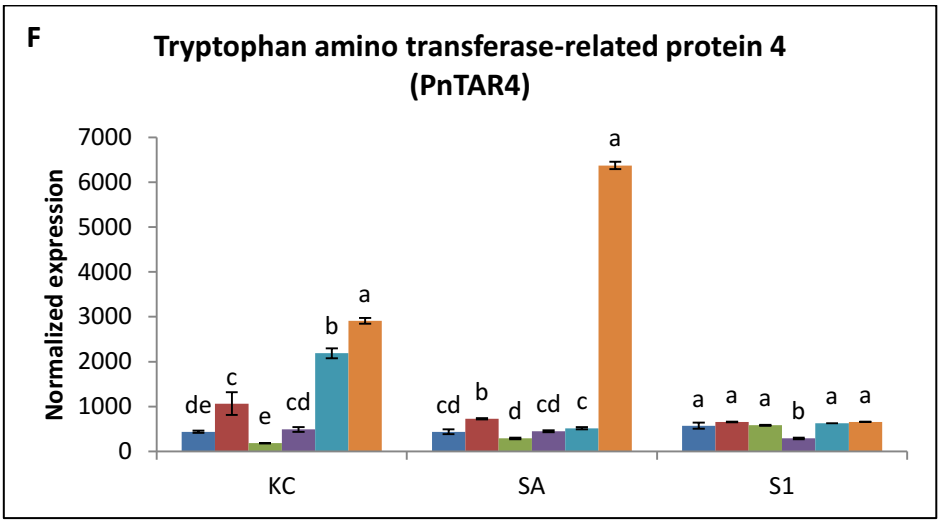

A
Transport inhibitor response 1 auxin (PnTIR1)
Normalized expression
60
50
40
30
20
10
0
KC
SA
S1


B
Auxin Signaling F-Box 2 (PnAFB2)
Normalized expression
1600
1400
1200
1000
800
600
400
200
0
KC
SA
S1


C
Auxin response factor 2 (PnARF2)
Normalized expression
6000
5000
4000
3000
2000
1000
0
KC
SA
S1


D
AGAMOUS (SHT) auxin (PnAG-SHT)
Normalized expression
3000
2500
2000
1500
1000
500
0
a
b
cd
bc
de
e
a
abc
c
bc
ab
abc
ab
cd
d
a
c
bc
KC
SA
S1


E
Tryptophan aminotransferase-related protein 3
(PnTAR3)
Normalized expression
600
500
400
300
200
100
0
a
a
c
d
a
b
a
b
c
c
a
c
a
a
b
b
a
a
KC
SA
S1


F
Tryptophan amino transferase-related protein 4
(PnTAR4)
Normalized expression
7000
6000
5000
4000
3000
2000
1000
0
de
c
e
cd
b
a
cd
b
d
cd
c
a
a
a
a
b
a
a
KC
SA
S1

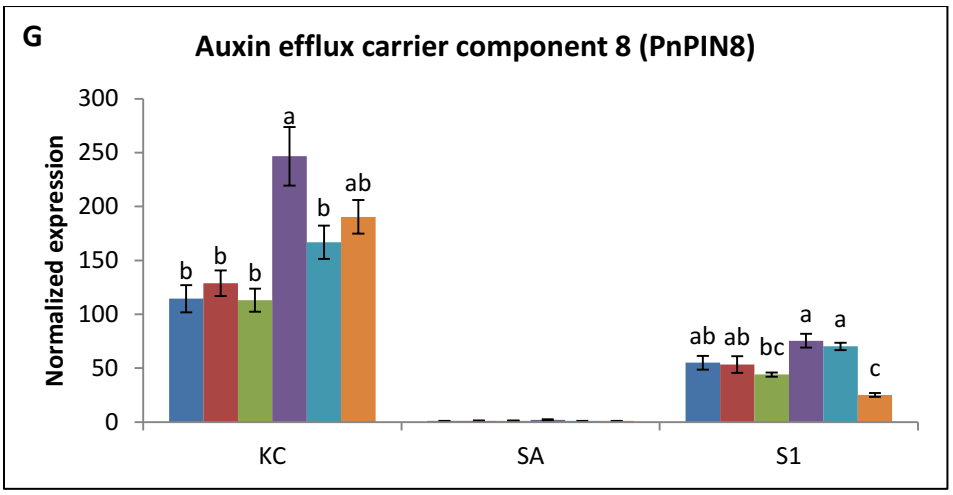
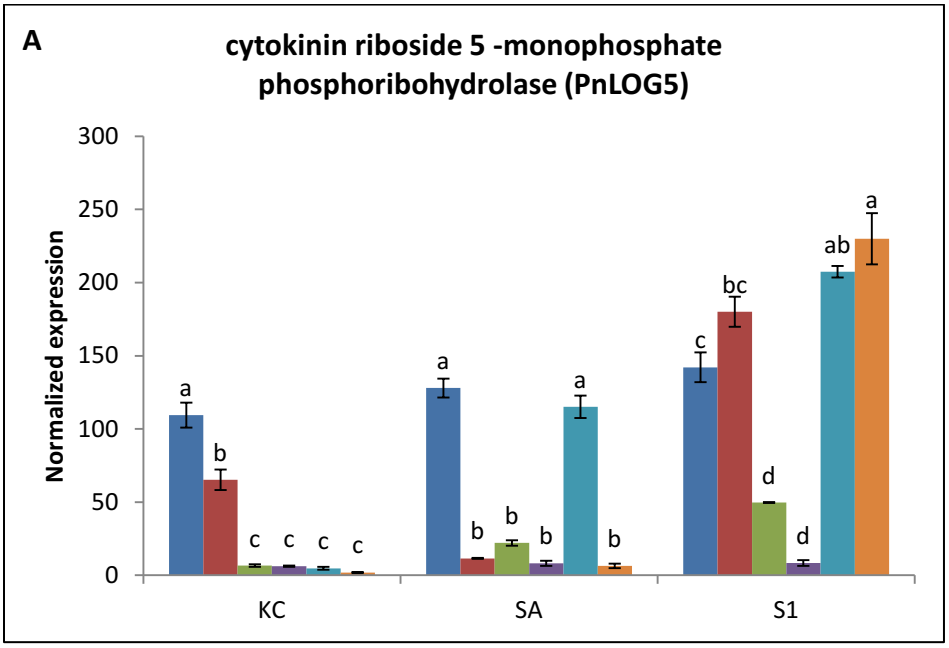


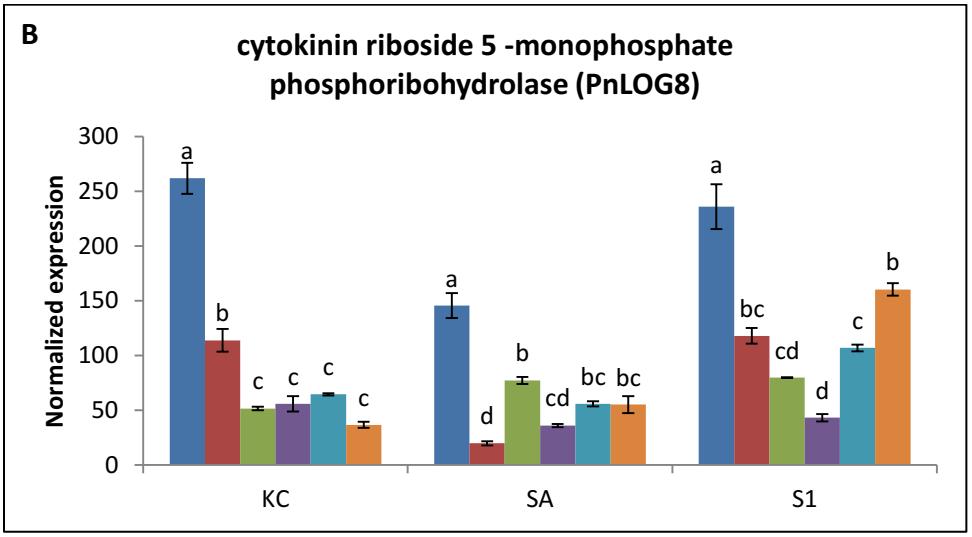
Figure 15: Expression profiles of auxin-related genes in six stages of fruit development in three varieties of black pepper using probe-based gene expression analyses (Panels A to G). The transcript counts were normalised using six synthetic ssDNA positive control targets and three housekeeping genes (Figure 14: panels G to I). The error bars represent the standard error from the mean expression level calculated from three biological replicates. Statistical significant analysis was based on one-way ANOVA at $p<0.05$. Means followed by the same letter between stages of each variety are not significant different by Tukey's test at $p<0.05$.

4.7.2 Genes related to the regulation of cytokinins during fruit development in black pepper

Two genes that involved in the conversion of inactive cytokinin ribotides to active cytokinins, cytokinin riboside 5-monophosphate phosphoribohydrolase (PnLOG5) and cytokinin riboside 5-monophosphate phosphoribohydrolase (PnLOG8) were further investigated in this study. The expression of PnLOG5 and PnLOG8 are generally high at the early stage of fruit development than the late stage in all three varieties but declined as the fruit ripened in KC variety (Figure 16A&B). In addition, S1 variety reached a low expression of PnLOG5 and PnLOG8 at fruit maturation stage but the expression was later increased sharply at fruit ripening stage. Study on the transcription expression of cytokinin oxidase dehydrogenase (PnCKX) that catalysis the irreversible degradation of cytokinin to regulate the cytokinin levels has exhibited distinct expression profile patterns in all three varieties (Figure 16C). KC and S1 varieties have generally high expression of PnCKX at early stage of fruit

development. However, the expression of PnCKX declined as fruit grow in S1 variety but not in KC and SA variety. All three varieties have high PnCKX expression at breaker stage of fruit development but the expression of PnCKX decreased as fruit ripened in KC and SA varieties.





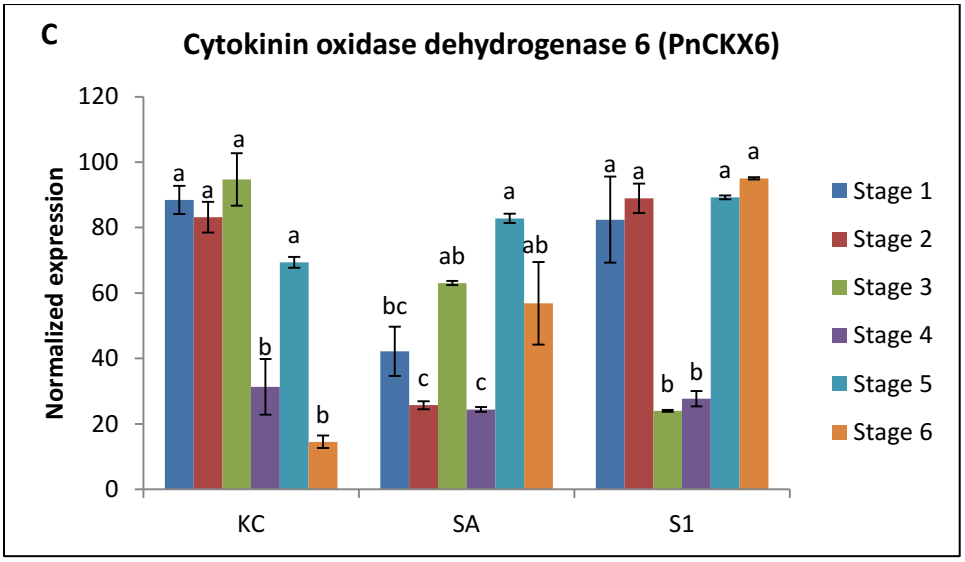
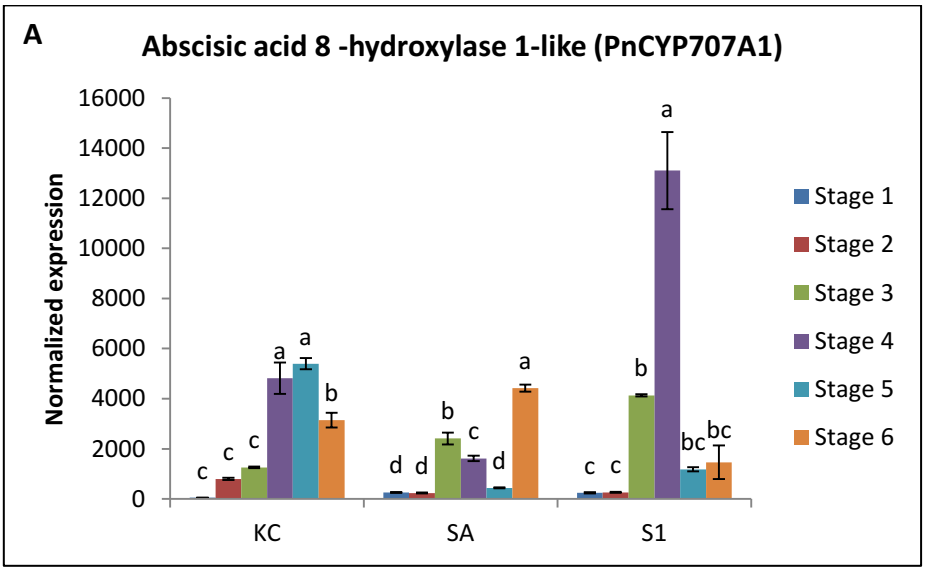


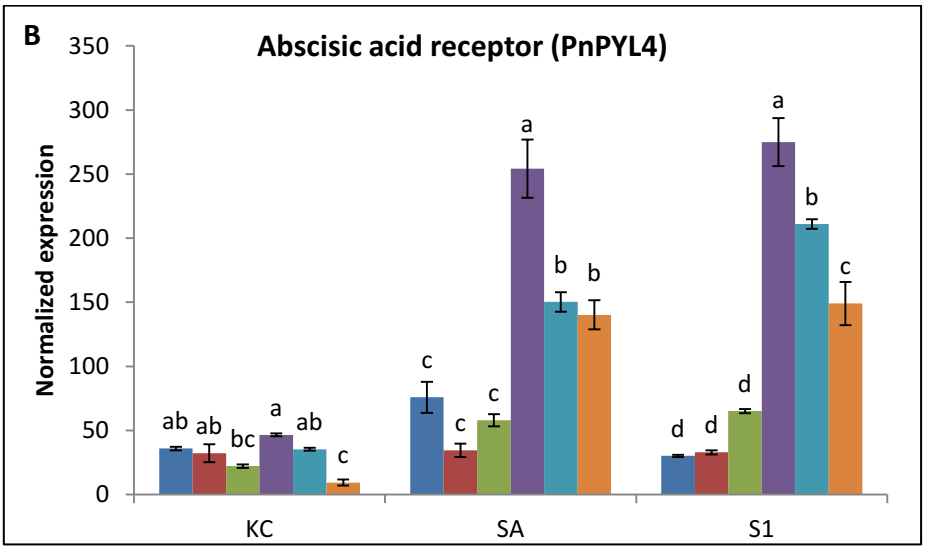
Figure 16: Expression profiles of cytokinin-related genes in six stages of fruit development in three varieties of black pepper using probe-based gene expression analyses (Panels A to C). The transcript counts were normalised using six synthetic ssDNA positive control targets and three housekeeping genes (Figure 14: panels G to I). The error bars represent the standard error from the mean expression level calculated from three biological replicates. Statistical significant analysis was based on one-way ANOVA at $p<0.05$. Means followed by the same letter between stages of each variety are not significant different by Tukey's test at $p<0.05$.

4.7.3 Genes regulated abscisic acid signalling during the fruit development in black pepper

Study on the transcription expression of abscisic acid 8-hydroxylase-1-like (PnCYP707A1) which encode enzyme in the oxidative catabolism of abscisic acid (ABA) to negatively regulate ABA levels. PnCYP707A1 has exhibited low expression at early stage of fruit development in all three varieties but the expression gradually increased as the fruit matured in KC and SA varieties (Figure 17A). Conversely, S1 variety showed sharp decreased of PnCYP707A1 expression from fruit maturation stage to fruit ripening stage and this might linked to the abscisic acid levels in the fruit that determined by the enzyme encoded by PnCYP707A1. ABA signalling is mediated by the ABA receptor for subsequent genes responses and the study of abscisic receptor PYL4 –like (PnPYL4) has revealed a significantly high expression of PnPYL4 at the fruit maturation stage in SA and S1 variety (Figure

17B). Meanwhile, the expression profile of PnPYL4 was similar in SA and S1 varieties but distinct in KC variety. Generally, the expression of PnPYL4 was low across fruit development stages in KC variety. In addition, the indole-3-acetaldehyde oxidase (PnAAO) gene that facilitated the oxidization of indole-3-acetaldehyde for the biosynthesis of ABA was strongly expressed at fruit setting (Stage 2) and fruit ripening stage (Stage 6) than other developmental stages in SA and S1 varieties while KC variety shows a steadily grew pattern which peaks at fruit maturation stage (Figure 17C).





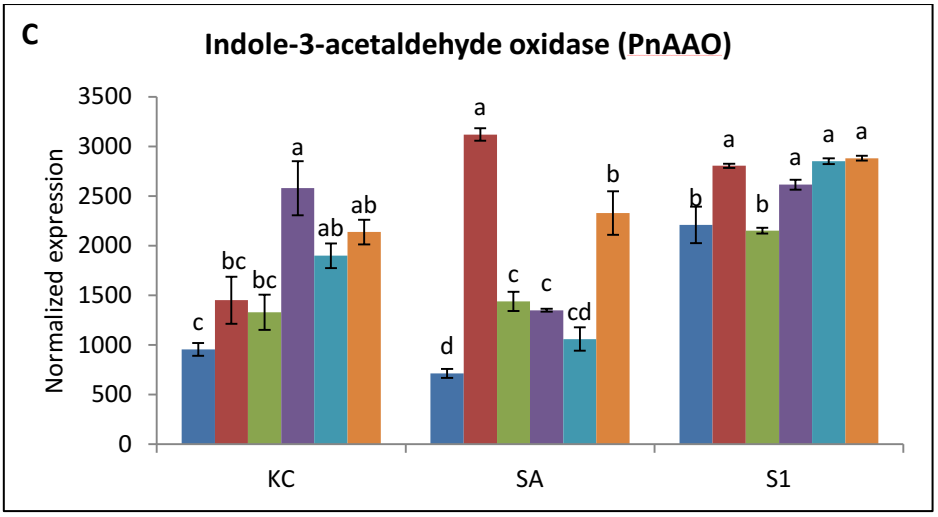
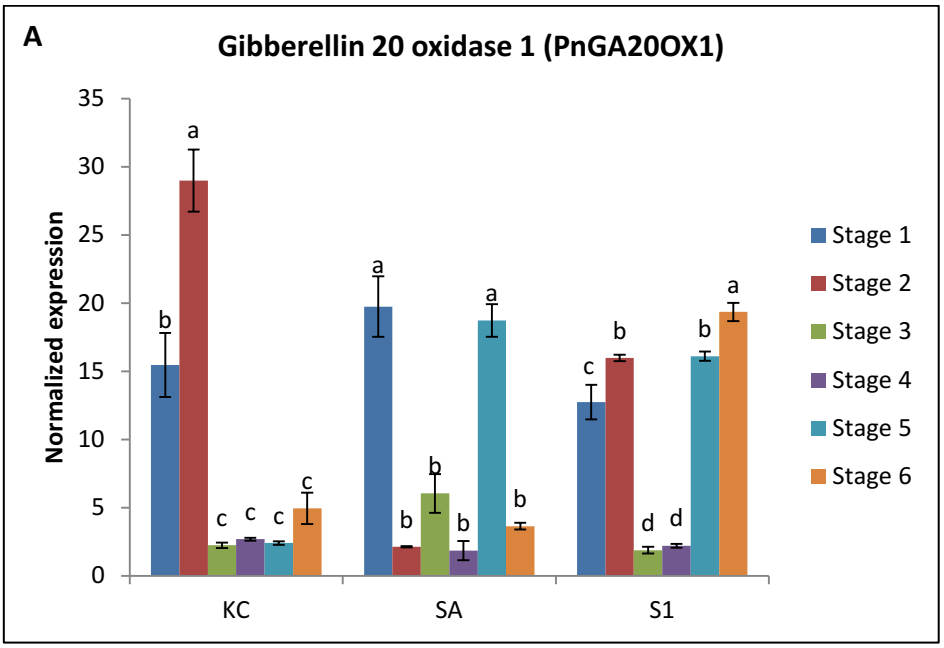


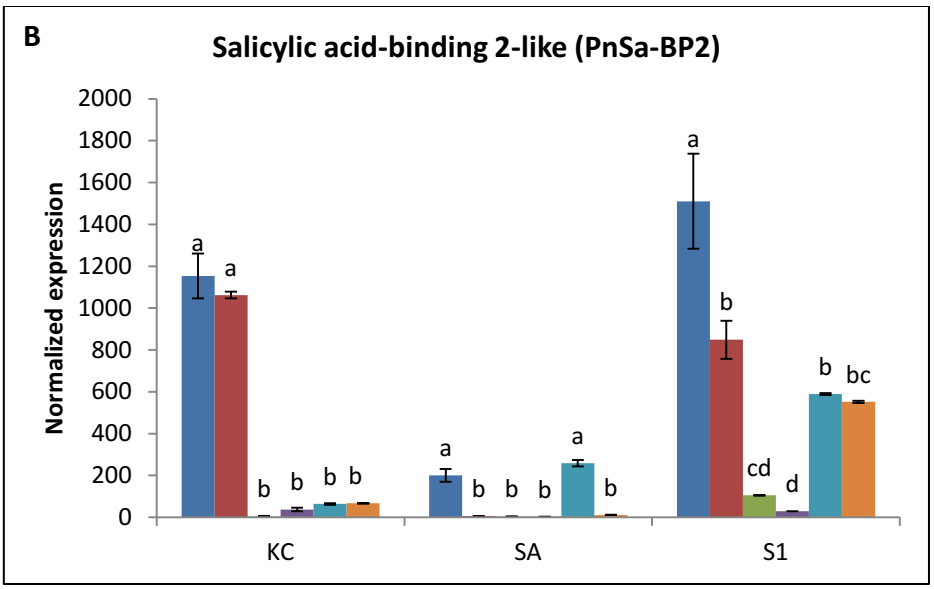
Figure 17: Expression profiles of abscisic acid-related genes in six stages of fruit development in three varieties of black pepper using probe-based gene expression analyses (Panels A to C). The transcript counts were normalised using six synthetic ssDNA positive control targets and three housekeeping genes (Figure 14: panels G to I). The error bars represent the standard error from the mean expression level calculated from three biological replicates. Statistical significant analysis was based on one-way ANOVA at $p<0.05$. Means followed by the same letter between stages of each variety are not significant different by Tukey's test at $p<0.05$.

4.7.4 Genes related to gibberellin and salicylic acid metabolism and signalling during the fruit development in black pepper

Gibberellin 20 oxidase 1 gene (PnGA20OX1) that regulates the biosynthesis of gibberellin (GA) through feedback from bioactive GAs was investigated in this study. PnGA20OX1 has exhibited a high expression at the fruit set stage in KC variety but the expression was decreased at the subsequent stage. Meanwhile, SA has a low expression of PnGA20OX1 from fruit set stage to fruit maturation before it elevated at breaker stage. S1 variety has a low expression of PnGA20OX1 at fruit expansion and maturation stage compared to early and late stage of fruit development (Figure 18A). For the expression of salicylic acid-binding 2-like (PnSa-BP2) which is important to convert the inactive form of methyl salicylate to salicylic acid was found highly expressed at the early stage of fruit development in KC and S1 varieties but has comparatively low expression in all stages in SA variety (Figure 18B). In addition, the PnSa-BP2 was also found expressed highly at fruit ripening

stage in S1 variety. OPDA-reductase 3 (PnOPR3) which is used as an indicator of jasmonic acid (JA) production in grapevine (Böttcher et al., 2015) has shown similar expression pattern in KC and SA varieties with high expression at fruit setting stage and fruit ripening stage. The PnOPR3 expression profiles are distinct in S1 variety as the expression was gradually increased as the fruit ripened (Figure 18C).





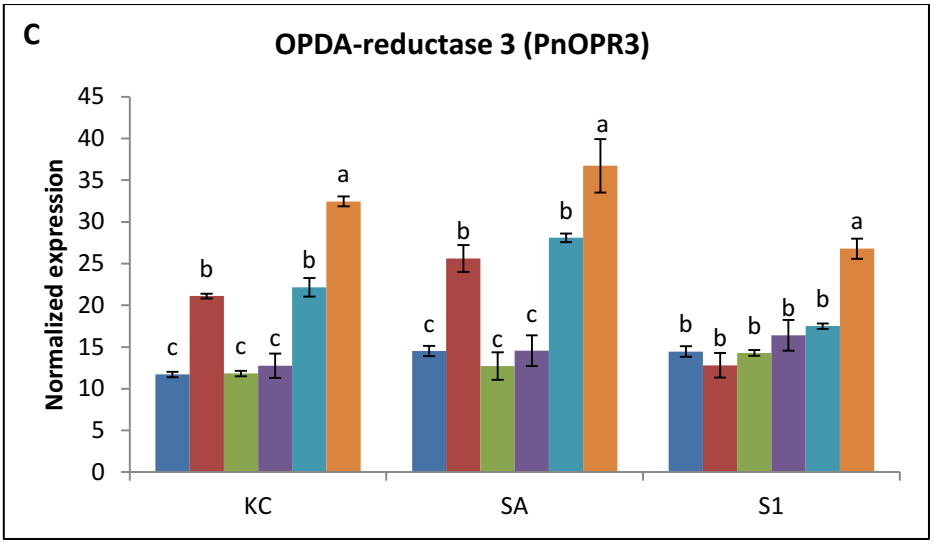
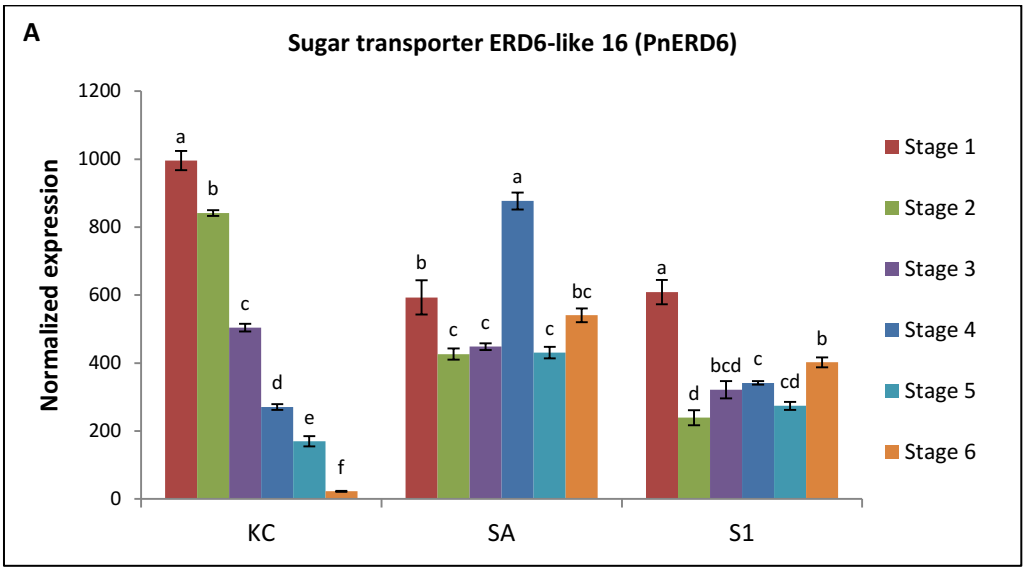


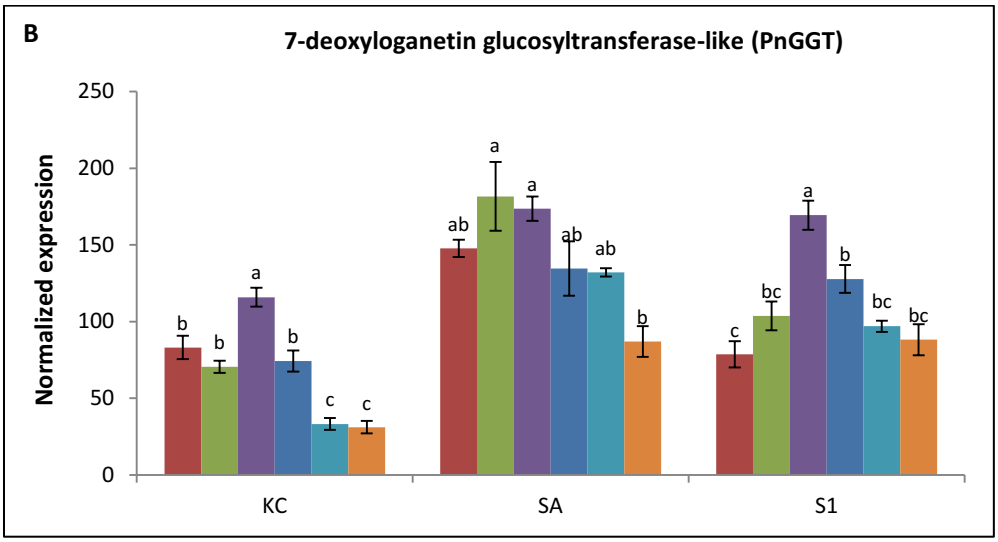
Figure 18: Expression profiles of other plant hormones related genes in six stages of fruit development in three varieties of black pepper using probe-based gene expression analyses (Panels A to C). The transcript counts were normalised using six synthetic ssDNA positive control targets and three housekeeping genes (Figure 14: panels G to I). The error bars represent the standard error from the mean expression level calculated from three biological replicates. Statistical significant analysis was based on one-way ANOVA at $p<0.05$. Means followed by the same letter between stages of each variety are not significant different by Tukey's test at $p<0.05$.

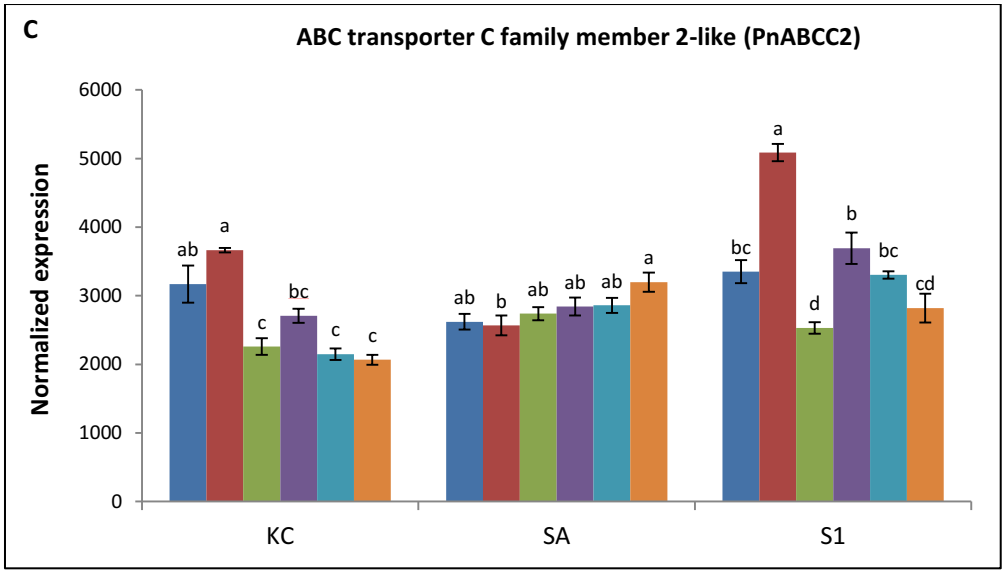
4.8 Expression analysis of genes related to sugar-transporter and carbohydrate metabolism

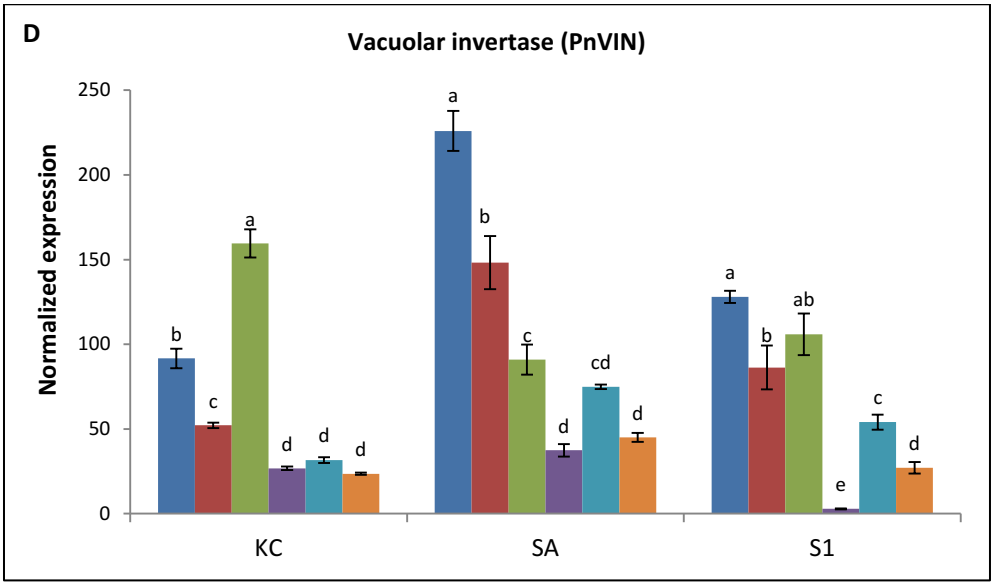
Sugar-transporter genes and carbohydrate metabolism genes are essential to fruit development. Six differentially expressed genes related to sugar-transport and carbohydrate metabolism were identified in the transcriptome and analysed in this study (Figure 19). Each pepper variety produced a distinct gene expression profile. The profiles for sugar transporter ERD6-like 16 (PnERD6) showed a general decrease in expression as the fruit ripened in KC variety; however, this was not the case for the SA and S1 varieties (Figure 19A). The gene expression of 7-deoxyloganetin glucosyltransferase-like (PnGGT) which is important for secondary metabolites metabolism was highly expressed at the fruit expansion stage in in all three varieties as well as fruit setting stage in SA variety (Figure 19B). On the other hand, the homeobox-leucine zipper (PnATHB-13) gene that implicated in pollen

germination was only highly expressed at flowering stage in all three varieties as well as fruit setting stage in SA variety (Figure 19F). There were significant differences in gene expression at some stages for each of the varieties for the ABC transporter C family member 2 -like (PnABCC2) (Figure 19C), vacuolar invertase (PnVIN) for flower development (Figure 19D) and pyrophosphate-fructose-6-phosphate 1- phosphotransferase subunit alpha (PnPFP) (Figure19E) that modulating carbon metabolism, while there were no significant differences across the fruit development stages for the housekeeping genes.






C
ABC transporter C family member 2-like (PnABCC2)
Normalized expression
6000
5000
4000
3000
2000
1000
0
ab a c bc c c
ab b ab ab ab a
bc a d b bc cd
KC
SA
S1


D
Vacuolar invertase (PnVIN)
Normalized expression
250
200
150
100
50
0
b c a d d d
a b c d cd d
a b ab e c d
KC
SA
S1

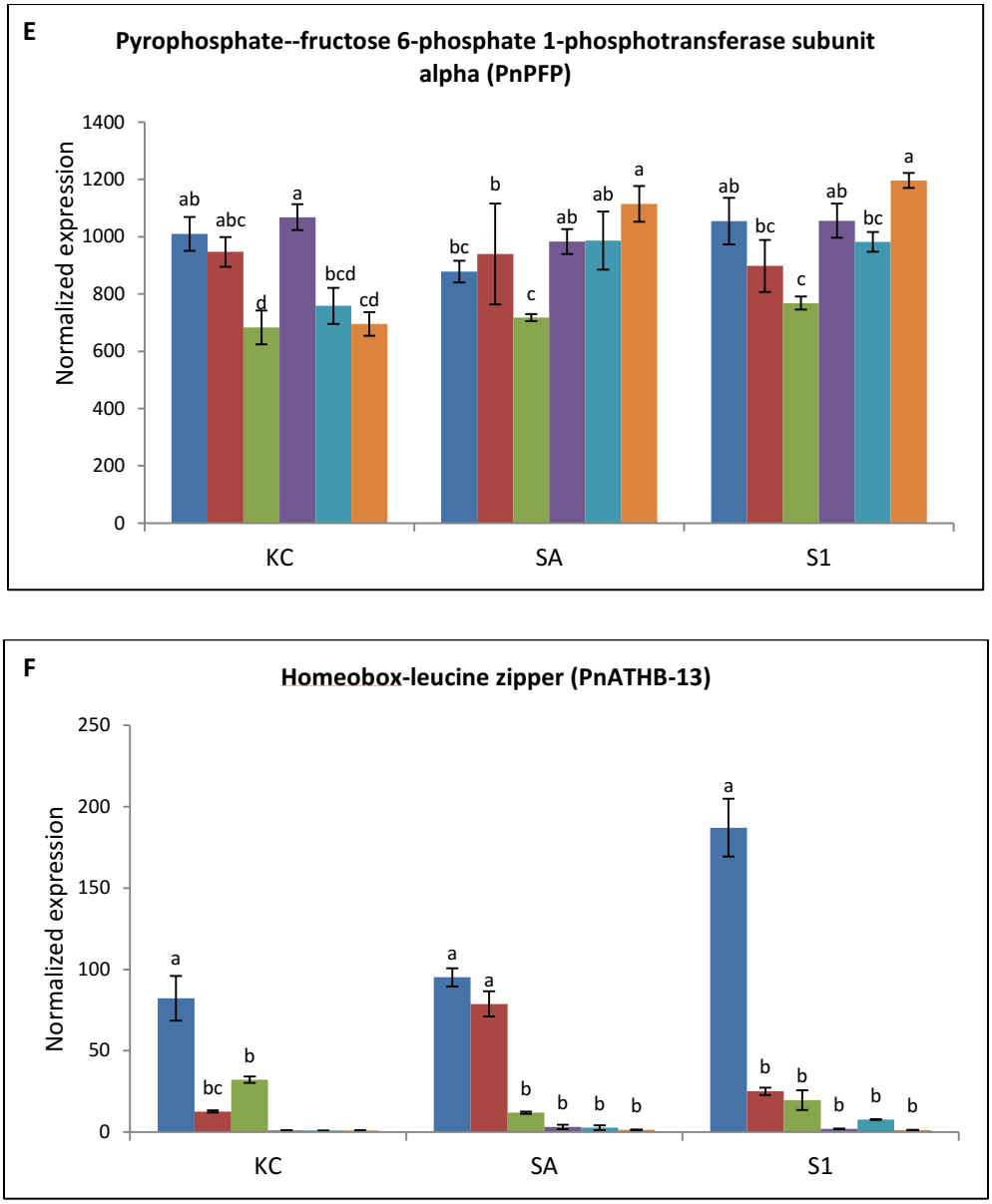
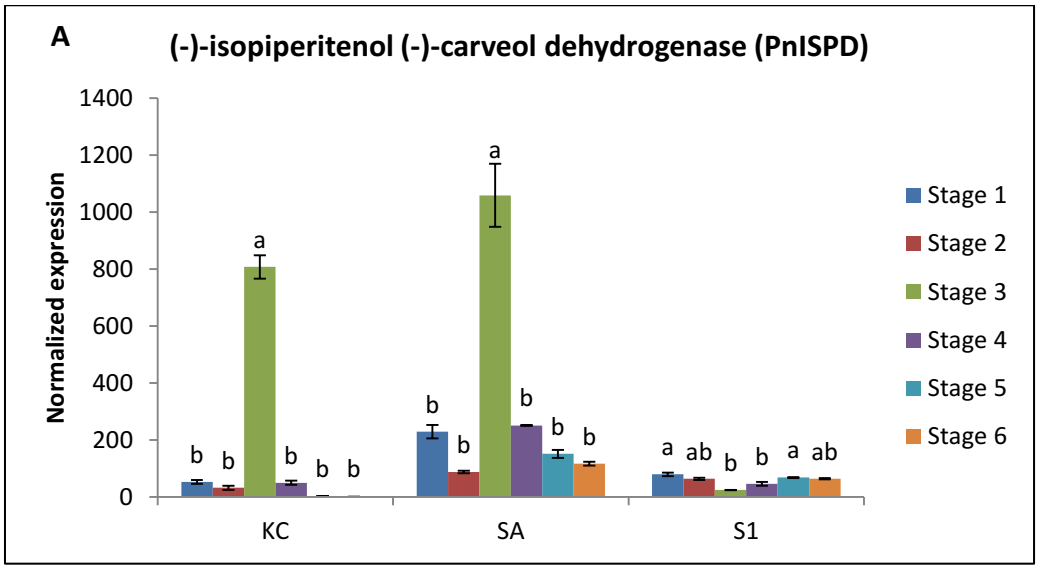


Figure 19: Expression profiles of genes related to sugar-transporter and carbohydrate metabolism in six stages of fruit development in three varieties of black pepper using probe-based gene expression analyses (Panels A to F). The transcript counts were normalised using six synthetic ssDNA positive control targets and three housekeeping genes (Figure 14: panels G to I). The error bars represent the standard error from the mean expression level calculated from three biological replicates. Statistical significant analysis was based on one-way ANOVA at $p<0.05$.Means followed by the same letter between stages of each variety are not significant different by Tukey's test at $p<0.05$.

4.9 Expression analysis on piperine related genes

The special pungent flavor of black pepper is contributed from the piperine. In this study, five genes that involved in the piperine biosynthesis pathway revealed from the transcriptome were further analyzed in different fruit development stages of different black pepper varieties (Figure 20). Significant high expression of (-)-isopiperitenol (-)-carveol dehydrogenase (PnISPD) and Ornithine decarboxylase (PnODC) were observed during the fruit expansion stage in KC and SA varieties compared to low expression of PnISPD and PnODC across stages in S1 varieties (Figure 20A). ISPD was important to catalyze the oxidation of isopiperitenol while ODC is the enzyme that catalyzes the conversion of ornithine to polyamine. The lysine histidine transporter-like 8 (PnAATL1) gene that code for enzyme facilitates lysine and histidine transportation across cellular membrane was significantly highly expressed at flower stage in all three varieties but had low transcript level when fruit ripened in KC and SA varieties. In S1 varieties, the expression of PnAATL1 was elevated at breaker stage after low expression at fruit maturation stage (Figure 20B)



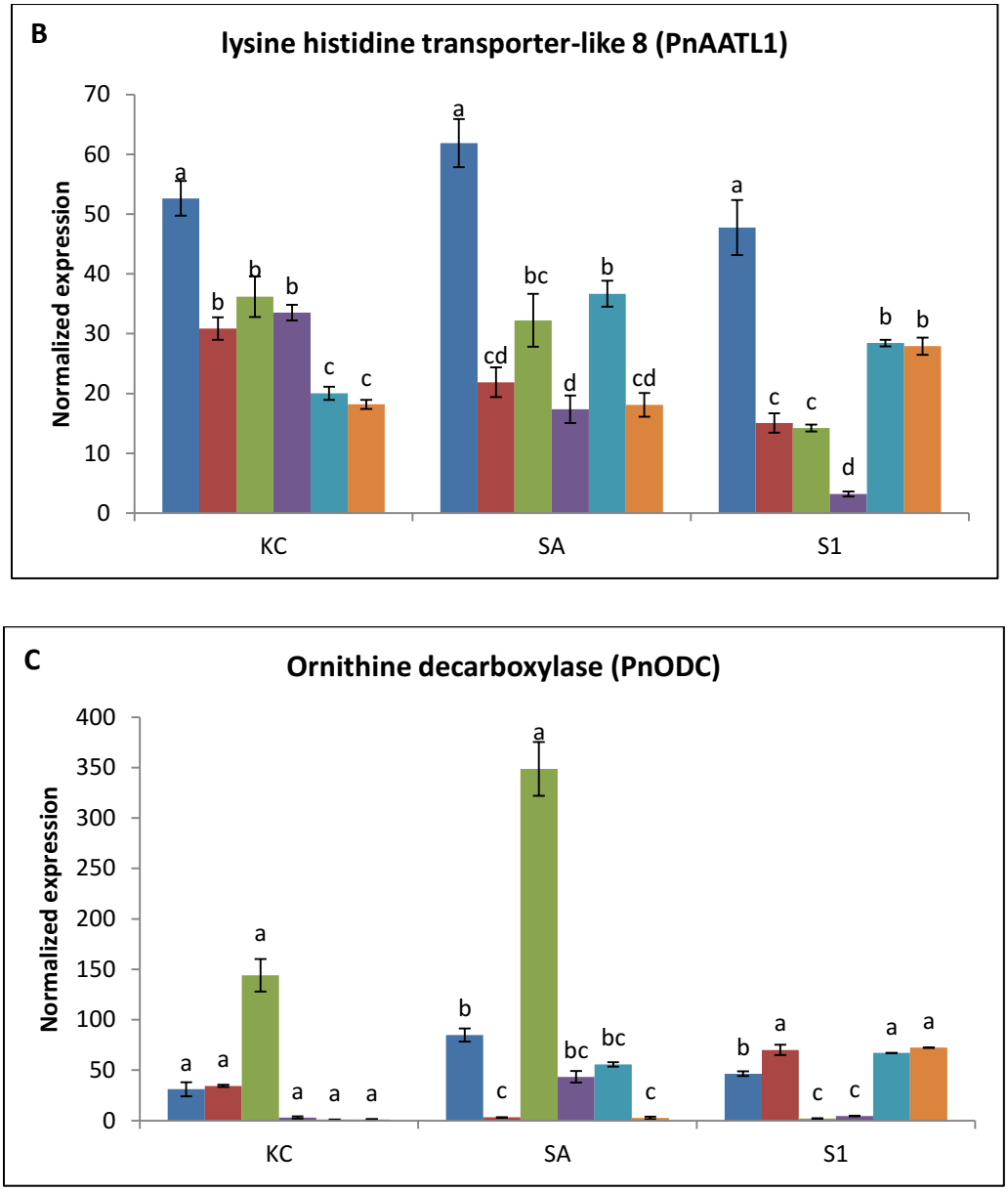


Figure 20: Expression profiles of piperine biosynthesis genes in six stages of fruit development in three varieties of black pepper using probe-based gene expression analyses (Panels A to E). The transcript counts were normalised using six synthetic ssDNA positive control targets and three housekeeping genes (Figure 14: panels G to I). The error bars represent the standard error from the mean expression level calculated from three biological replicates. Statistical significant analysis was based on one-way ANOVA at $p<0.05$.Means followed by the same letter between stages of each variety are not significant different by Tukey's test at $p<0.05$.

4.10 Plant hormones quantification in flower and fruit development

4.10.1 The auxin profiling

The endogenous levels of plant hormones was analyzed at six fruit developmental stages of three different black pepper varieties. On a dry weight basis, the concentration of the plant hormone auxin, IAA has increased from a low level at flowering to maximum at fruit expansion stage in three different black pepper varieties (Figure 21). The levels of IAA were later decreased as fruit matured before slight increased at fruit ripening stage in all three varieties. Overall, three different black pepper varieties exhibited a similar IAA accumulation pattern over the fruit development.

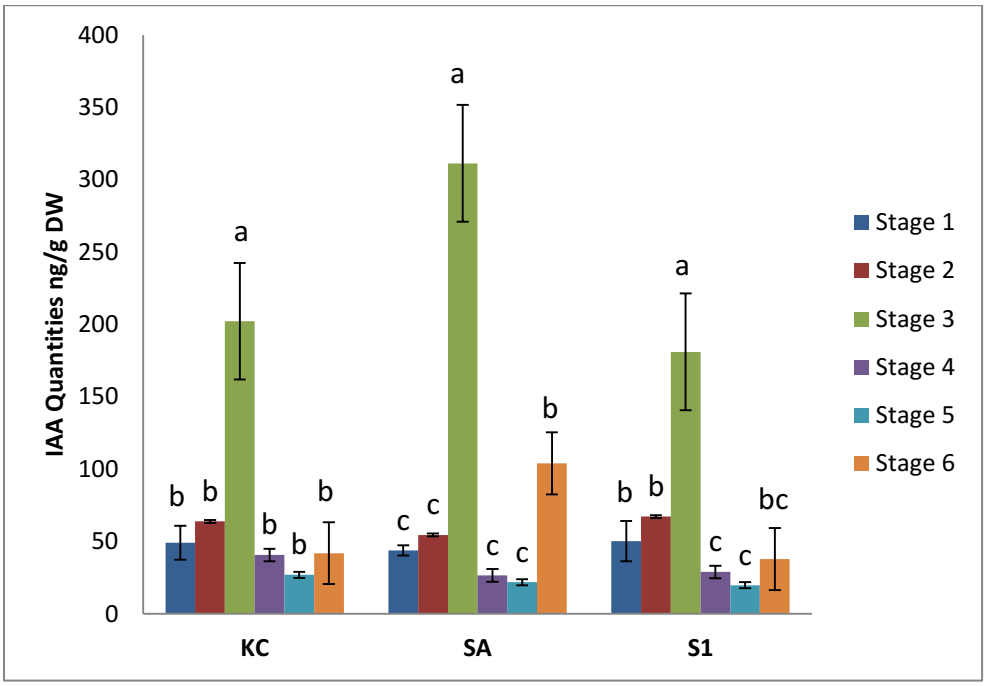


Figure 21: Endogenous concentration of indole-3-acetic acid (IAA) during fruit development in black pepper. Data are means ± SE of n = 3. Statistical comparisons were performed by one-way ANOVA followed by a posthoc Tukey HSD test. Different letters indicate significant difference between the developmental stages at $P < 0.05$.

4.10.2 The cytokinin profiling

In plant, the initial step in cytokinin biosynthesis is to produce isopentenyladenine (iP) nucleotides as cytokinin precursors (Sakamoto et al., 2006). The concentration of iP was remained low at early stage of fruit development but increase sharply as fruit ripened in SA variety (Figure 22). After fruit maturation, the iP concentration pattern was differing among the three varieties as SA variety exhibited a significantly high concentration of iP at fruit ripening stage than two other varieties. Quantification of another active form of cytokinin, trans-Zeatin (tZ) was found has high concentration at fruit expansion stage but dropped in subsequent stages in KC and SA varieties (Figure 23). The trends of tZ profiles were similar in all three varieties, except that KC and SA varieties have significantly high levels of tZ concentration at fruit expansion and maturation stage. In addition, another naturally occurring cytokinin, dihydrozeatin (DHZ) was not detected in all samples in this study and it is suggested that DHZ is absent in black pepper.

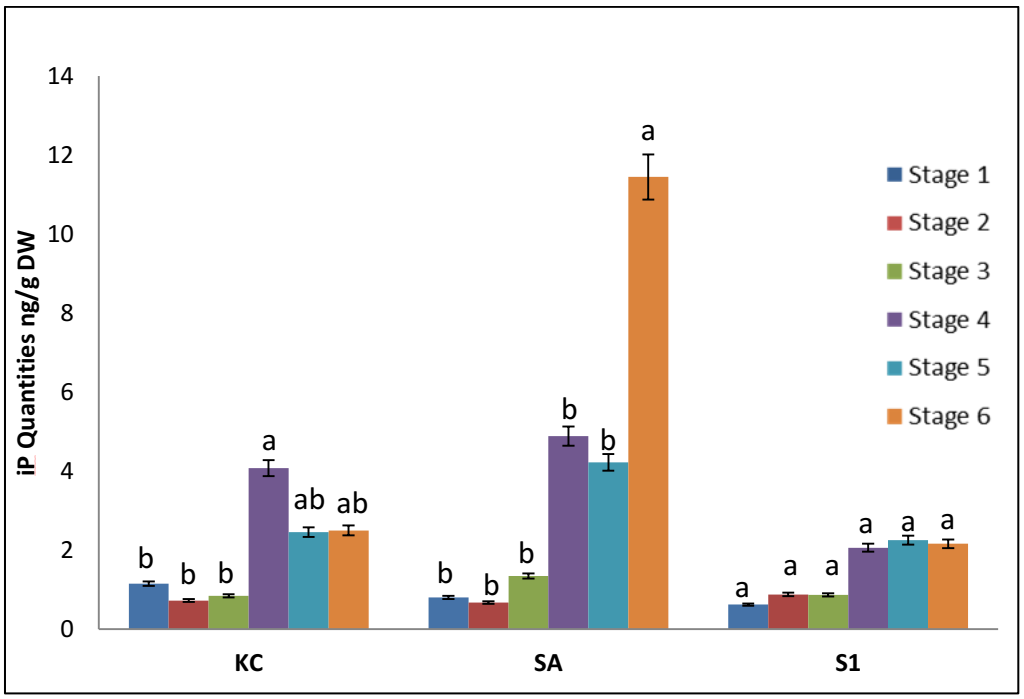


Figure 22: Endogenous concentration of isopentenyladenine (iP) during fruit development in black pepper. Data are means ± SE of n = 3. Statistical comparisons were performed by one-way ANOVA followed by a posthoc Tukey HSD test.

Different letters indicate significant difference between the developmental stages at $P < 0.05$.

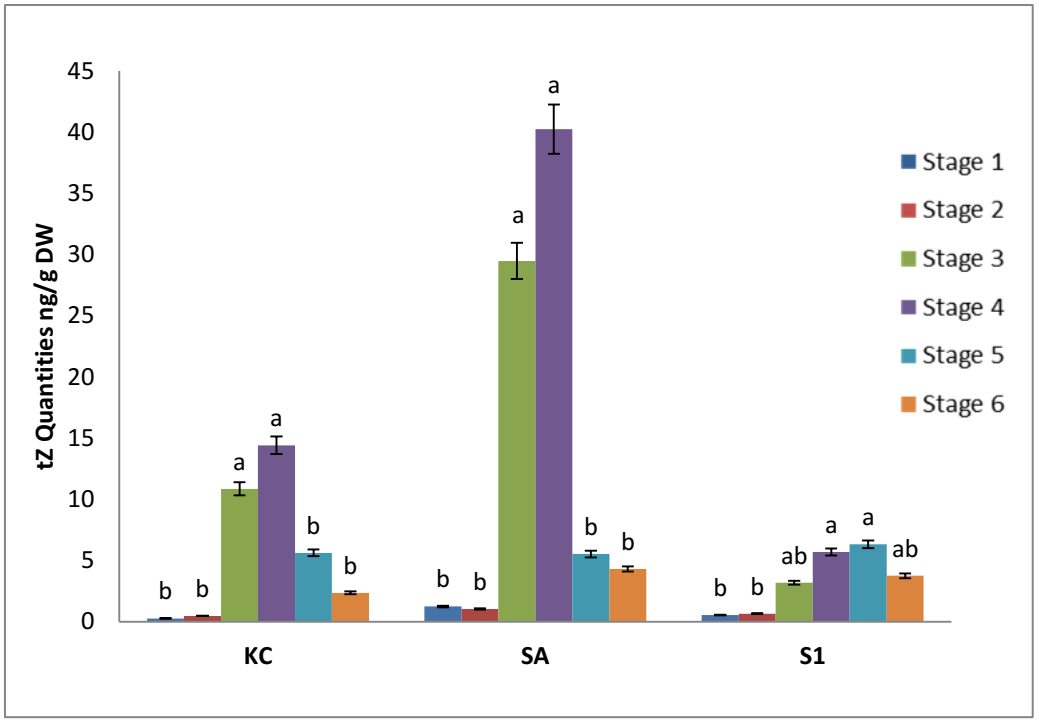


Figure 23: Endogenous concentration of trans-Zeatin (tZ) during fruit development in black pepper. Data are means ± SE of n = 3. Statistical comparisons were performed by one-way ANOVA followed by a posthoc Tukey HSD test. Different letters indicate significant difference between the developmental stages at $P < 0.05$.

4.10.3 The abscisic acid profiling

Abscisic acid (ABA) is known to play an important role in fruit ripening in both climacteric and non-climacteric fruits. In this study, the ABA concentration increased markedly at ripening stage in KC and SA varieties but remained low at earlier stages of fruit development (Figure 24). S1 variety showed a different ABA profile compared to KC and SA varieties as the concentration of ABA was peaked at fruit expansion stage then declined at fruit maturation stage before the ABA content elevated again at ripening stage.

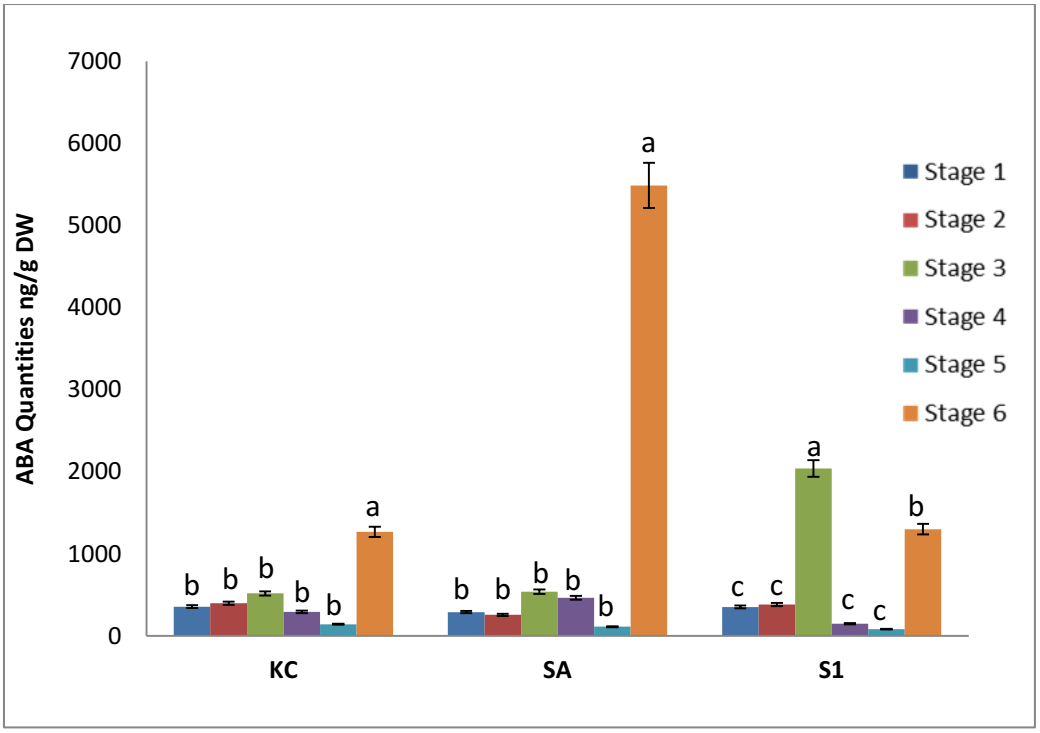


Figure 24: Endogenous concentration of abscisic acid (ABA) during fruit development in black pepper. Data are means ± SE of n = 3. Statistical comparisons were performed by one-way ANOVA followed by a posthoc Tukey HSD test. Different letters indicate significant difference between the developmental stages at $P < 0.05$.

4.10.4 The jasmonic acid profiling

Changes of the jasmonic acid (JA) content over the fruit development in black pepper were measured in this study. In KC variety, high concentration of JA and bioactive form of JA, jasmonyl-isoleucine (JAIle) were accumulated at flowering stage than the rest of the fruit development stages in KC variety (Figure 25A and 25B). The JA and JAIle accumulation profiles were different in SA variety as high concentration of JA was recorded in flower and fruit expansion stage, whereas JAIle was highly accumulated at flower stage only. For S1 variety, high levels of JA was accumulated at flower and fruit set stage then decreased the levels toward the fruit ripened stage. The JAIle accumulation profile in S1 variety exhibited high level at flower and fruit expansion stage compared to the other fruit development stages in S1 variety (Figure 25A and 25B). From the analysis of JA and

JAIle profiles revealed that, the JA content accumulated in all samples are between 15 to 40 fold higher than the JAIle accumulation levels.

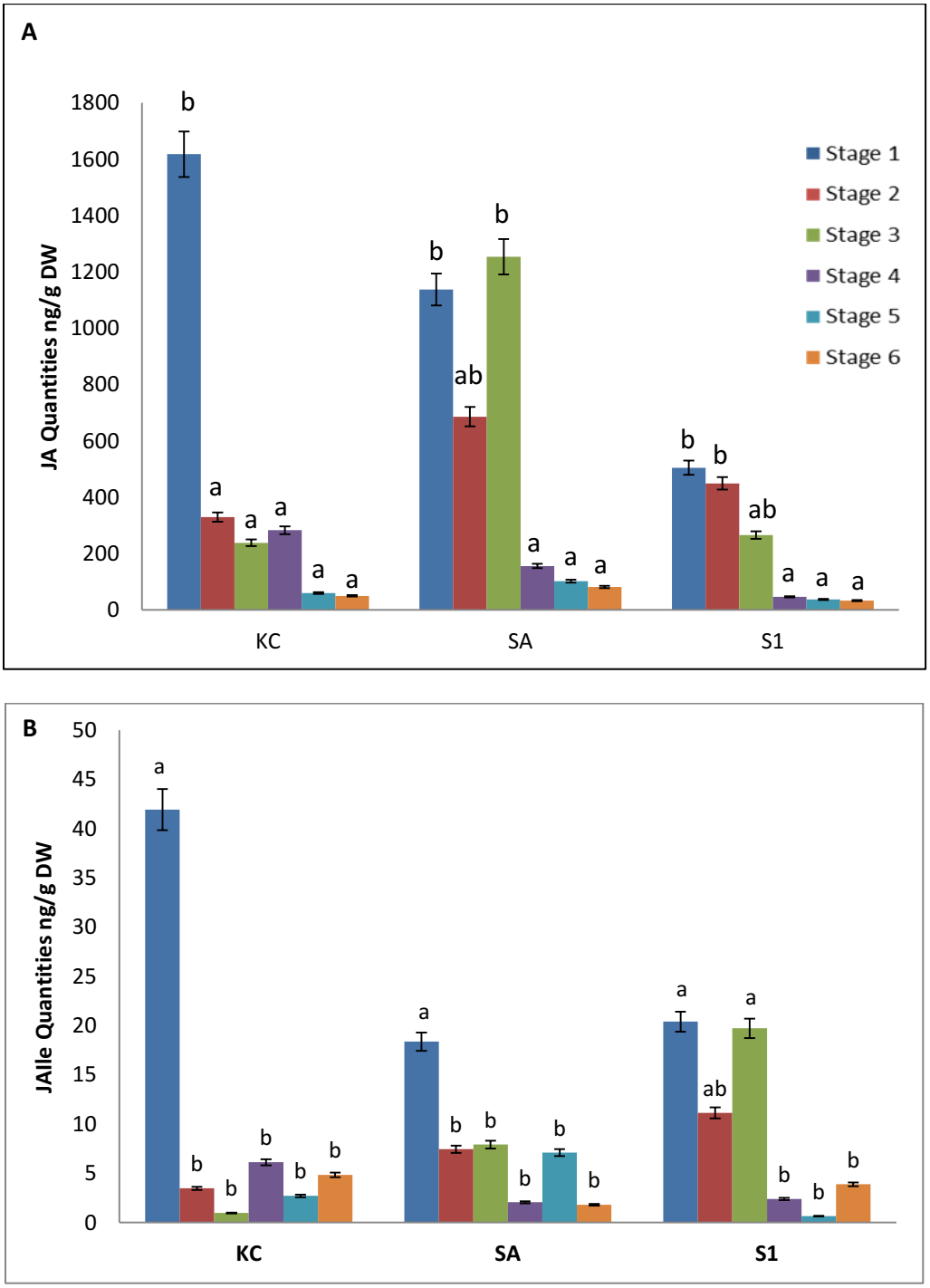


Figure 25: Endogenous concentration of (A) jasmonic acid (JA) and (B) jasmonoyl-isoleucine (JAIle) during fruit development in black pepper. Data are means ± SE of n = 3. Statistical comparisons were performed by one-way ANOVA followed by a

posthoc Tukey HSD test. Different letters indicate significant difference between the developmental stages at $P < 0.05$.

4.10.5 The salicylic acid and gibberellins profiling

The plant hormones quantification method used in this study has detected ten types of plant hormones included with salicylic acid. Overall, the salicylic acid concentration was decrease over the fruit development stages with a dramatic dropped was observed in KC variety from flowering stage to fruit formation stage (Figure 26). Meanwhile, gibberellins (GAs), GA_1 and GA_4 were detected at the early stage of fruit development but not in the late stage of fruit development. Both GA_1 and GA_4 was peak at fruit expansion stage in all varieties except that GA_4 has slightly higher concentration at fruit set stage in KC variety (Table 5). In KC variety, the level of GA_1 is relatively higher compared with SA and S1 varieties whereas GA_4 is vice versa.

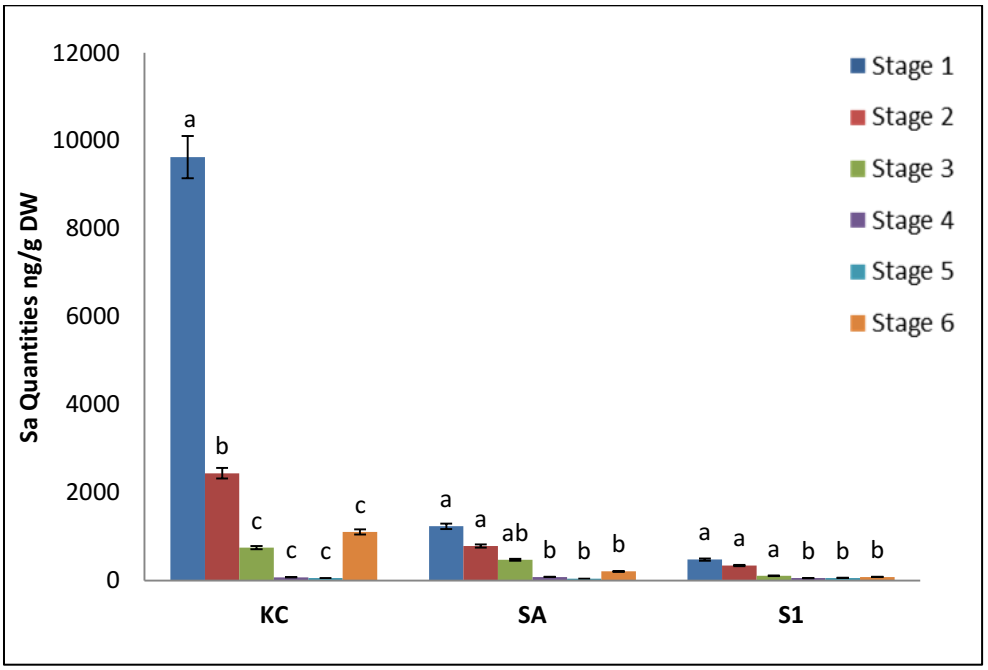


Figure 26: Endogenous concentration of salicylic acid (Sa) during fruit development in black pepper. Data are means ± SE of n = 3. Statistical comparisons were performed by one-way ANOVA followed by a posthoc Tukey HSD test. Different letters indicate significant difference between the developmental stages at $P < 0.05$.

Table 5: Changes in the endogenous level of gibberellins (GA_1 and GA_4) in the fruit development of 'Kuching (KC)', 'Semengok Aman (SA)'and 'Semengok 1(S1)' variety of black pepper.

Stages	Gibberellins levels (ng/g DW)					
	GA_1			GA_4		
	KC	**SA**	**S1**	**KC**	**SA**	**S1**
1	ND	ND	ND	28.17 ± 5.94[a]	105.06 ± 9.83[b]	78.24 ± 12.10[b]
2	2.08 ± 0.55[c]	ND	ND	34.56 ± 3.36[a]	157.15 ± 10.18[b]	79.84 ± 12.85[b]
3	100.98 ± 7.69[a]	54.26 ± 8.34[a]	34.07 ± 4.07[a]	31.94 ± 3.22[a]	524.22 ± 13.73[a]	313.99 ± 18.45[a]
4	13.71 ± 2.04[b]	ND	ND	ND	ND	ND
5	ND	ND	ND	ND	ND	ND
6	ND	ND	ND	ND	ND	ND

ND not detected

Note: Data are means ± SE of n = 3. Statistical comparisons were performed by one-way ANOVA followed by a posthoc Tukey HSD test. Different letters indicate significant difference between the developmental stages at $P < 0.05$.

CHAPTER 5: DISCUSSIONS

5.1 The fruit and flowers of different varieties of black pepper have several overlapping but also uniquely expressed transcripts

The present study provides the first flower and fruit transcriptomic view of different varieties of *P. nigrum* L. and provides a report of the identification of genes and gene categories associated with flower and fruit development in black pepper. With the absence of a complete genome sequence for black pepper or other closely related species, the *de novo* assembly was crucial to provide useful and more comprehensive genetic information. The transcriptome not only influenced the data coverage but also improved the number of genes sequences available for downstream applications such as the study of tissue-specific gene expression (Feldmeyer et al., 2011). Based on paired-end sequencing of RNA-Seq libraries prepared from mRNA isolated from flower and fruit tissues, a SOAPdenovo-Trans assembly and a CLC Workbench assembly were compared. Here, a better *de novo* assembly of the black pepper flower and fruit transcriptome was afforded through SOAPdenovo-Trans with highest maximum contig size as shown in Table 4. The relative superiority of SOAPdenovo-Trans to CLC Workbench was reported earlier (Jazi et al., 2017, Chakraborty et al., 2015). The SOAPdenovo-Trans includes the feature of an error-removal model from Trinity and the robust heuristic graph traversal approach from Oases. Moreover, it simplifies the scaffolding graphs through a strict transitive reduction method (Xie et al., 2014). Previous studies have shown that SOAPdenovo-Trans provided higher contiguity, lower redundancy and faster execution (Chen et al., 2016, Vijay et al., 2013). The assembly of *P. nigrum* transcriptome reported here compared favourably with transcriptomes produced with NGS in previous studies. The average length of the *P. nigrum* assembly was 1,102 bp (Table 2) which was higher than the 449 bp reported for *P. nigrum* cv. Panniyur 1 (Joy et al., 2007). The N50 length was similar to that reported from the assembly by Hu et al. (2015) of the fruit transcriptome from *P. nigrum* cv. Reyin 1.

Gene annotation is another important aspect to understand the biological function from NGS data and for comparison with data from other studies and

species. In this thesis study, BLAST2GO with comprehensive functional annotation for this non-model plant was used for gene annotation. Among all the databases, including KEGG, GO, Inter-Pro and NR, annotated unigenes accounted for 64.44% of the total number of unigenes in the current black pepper transcriptome (Figure 6). Considering the shortage of sequence data for *P. nigrum*, the level of annotation seems reasonable, similar to values reported previously, i.e. 65.43% (Hu et al., 2015). As in reports for other fruit plants, an enormous number of genes (24,011) was annotated with categories related to binding and catalytic activity (Guo et al., 2015, Hu et al., 2015). About 2,400 transcripts were annotated with "DNA binding" and from the number; around 800 were further annotated precisely with GO term "sequence-specific DNA binding transcription factor activity" (Figure 8). This result shows that the sequences might represent the expressed transcription factors involved in the regulation of gene expression during flower and fruit development. Furthermore, the *P. nigrum* flower and fruit transcriptome were annotated to encode proteins associated with terms related to biosynthetic process, reproduction, anatomical structure morphogenesis, flower development and secondary metabolic process, showcasing the active processes happening in the fruit development. Therefore, GO annotation identified a broad set of candidate genes which are believed to be involved in the control of fruit development and the bioactive secondary metabolites synthesis.

A differential gene expression dataset was established to elucidate the molecular mechanisms and regulation pathways of flower and fruit in black pepper through the comparison among three different varieties, namely Semengok Aman (SA), Kuching (KC) and Semengok I (S1). The 'Semongok Aman' *P. nigrum* variety shows more uniform ripening than other commonly planted varieties; however, it has shorter fruit spikes and a thick fruit pericarp. The pepper variety 'Kuching' has a thinner pericarp, which is important for premium white pepper production, but has poor fruit setting with comparatively lower yield (Paulus, 2011). The pepper variety 'Semongok I' has potentially the highest yield as it has the longest fruit spikes and large berry size; however, the fruit ripening is not as even as that of 'Semongok Aman'.

From the gene expression analysis of the transcriptome has indicated that a large proportion of genes were exclusively expressed in SA variety, which were not expressed in the KC and S1 varieties. This made a substantial difference between the expression of genes in the SA variety than the KC and S1 varieties. Furthermore, analysis of SA flower was found to have more changes in genes expression compared to those of KC and S1 (Figure 9). The majority of the differentially expressed genes are represented under the biological process terms of carbohydrate metabolic process, embryo development and DNA metabolic process. These biological processes are the primary metabolisms in flower and integrate with each other for the growth and development of flower (Rodrigues et al., 2018).

Previous studies have presented the involvement of primary metabolism in flowers constitutes the physiological and ecological functions associated with flower development and fruit set (Borghi & Fernie, 2017). In *Arabidopsis*, transient starch accumulation in stamen development is coordinated with the expression of carbohydrate metabolism and transport genes (Hedhly et al., 2016). On the other hand, in *Lilium pumilum*, high energy potential was showed during floral opening and senescence which demonstrated the employment of carbohydrate metabolism in floral structures (Santos et al., 2016). For the genes regulating the secondary metabolic process and response to extracellular stimuli were observed upregulated in KC flower compared to SA and S1 flower (Figure 11). Plant secondary metabolites are not only found in leaves but are also common in floral rewards such as nectar (Adler, 2000). For example, a broad survey revealed that virtually all plant species' floral nectar contained non-protein amino acids, 36% contained phenolics and 8% contained alkaloids (Baker, 1977). The existence of the secondary metabolites in the flower is plant defence against diseases and herbivores (Lucas et al., 2016). Furthermore, a high number of genes was noticed in the category of signal transduction and cell-cell signalling in S1, indicating the small molecule like plant hormones (Song et al., 2017) in regulating the growth of black pepper and is being necessary to signal the flower formation, fruit set, fruit maturation and ripening (Obroucheva, 2014).

KEGG pathway analysis is widely used to clarify the physiological functions of genes through association with a biochemical network (Kanehisa and

Goto, 2000, Makkar et al., 2007). Further enrichment analysis of the gene data from the flower and fruit of three different varieties of black pepper indicates that 'nucleotide metabolism' and 'carbohydrate metabolism' are enriched in these tissues in black pepper (Figure 13). Purine nucleotides are known to play an important role as a base for nucleic acid synthesis and to become the initial part for the synthesis of many primary and secondary products like sucrose, polysaccharides and phospholipids (Stasolla et al., 2003). The presence of the differentially expressed genes from the transcriptome is consistent with the assayed black pepper tissues that are undergoing active fruit growth and development. Similarly, a large group of unigenes annotated to be involved in starch and sucrose metabolism is in line with the carbohydrate metabolism category that playing an essential role in fruit maturation. Furthermore, the growth of fruit is known to be highly dependent on the carbohydrate supply, and carbohydrate metabolism of mainly starch and sucrose, which ultimately contribute to plant fruit development and maturation (Wang et al., 2016). These black pepper fruit development results align with the findings reported from the grape berry that starch metabolism has great influence on the quality and flavour in fruit (Zhu et al., 2017). Furthermore, the enzyme activities involved in starch-sucrose metabolism may delay the senescence in lotus pods and seeds (Luo et al., 2017). On the other hand, a large proportion of genes were homologs of those shown to be involved in phenylpropanoid biosynthesis in other plant studies, which may be correlated with the rich range of phytochemical compounds found in black pepper that give unique flavour and aroma in the fruit (Hu et al., 2015).

5.2 Flower meristem identity genes control flower formation

Black pepper production is hampered by the non-synchronous nature of flowering and uneven fruit ripening which reduces the fruit quality in black pepper. Therefore, it is critical to explore the molecular mechanism underlying flower formation for better understanding and application to the management of flowering time to increase black pepper productivity and quality. MADS-box gene families are important regulatory factors to control flower transition in plant (Theißen et al., 2016). The Agamous-like MADS-box AGL8 (AGL8) homolog had been reported

from earlier studies in the model plant *Arabidopsis* to regulate the establishment of carpels during fruit development (Mandel and Yanofsky, 1995) and is categorized as flower meristem identity genes. AGL8 is implicated in the network of flowering control by directly binding and being activated by transcription factor APETALA2 (AP2), which is both the flower repressor and A-class flower identity gene (Mandel et al., 1995). In this study, the expression profiles of the pepper homologs of PnAP2 and PnAGL8 also implicated their role in flowering with their high expression at flowering stage with complete emergence of stigma in KC and S1 varieties (Figure 14A & C).

In SA variety, high expression of PnAP2 was shown at breaker stage, whereas PnAGL8 was highly expressed at fruit ripening stage (Figure 14 A&C). The involvement of PnAP2 and PnAGL8 at late stage of fruit development remains unknown and therefore more study need to be carried out to identify the reason that contributed to the distinct expression profile of PnAP2 and PnAGL8 in SA varieties than KC and S1 varieties. The expression The pepper homolog of another C-type MADS-box gene, Agamous (PnAG) demonstrated high expression at the late stage of fruit development in all three varieties which supports the results from PnAGL8 as well as the previous study in grapevine (Giménez et al., 2015) that AG has a regulatory role in flower development and also acts as a key regulator in fruit development. The current result is more in agreement of the later in regulating fruit development in black pepper.

Golden two-like (GLK) families have been implicated in coordinating the development and maintenance of chloroplasts (Zubo et al., 2018). Study of the transcription activator of GLK1 pepper homolog (PnGLK1) in this study showed low transcript levels at the flower stage. Previous study has suggests that GLK may act as a negative regulator of flowering by delaying the flowering time in Arabidopsis (Zubo et al., 2018). Low expression of PnGLK1 at flower stage in this study might be due to reduce negative regulation of flower formation in black pepper. Function characterization of PnGLK1 need to be carried out to further identify the exact role of PnGLK1 in flower stage. Meanwhile, another study has reported that high expression of GLK in tomato will enhance fruit photosynthesis rate and chloroplast development, which contribute to increased carbohydrates and

carotenoids in the fruit, leading to uniform fruit ripening (Powell et al., 2012). Therefore, increase in expression of PnGLK1 toward the fruit ripening stage in SA and S1 varieties in black pepper (Figure 14 B) might proposed its putative role in the fruit development and ripening activity in black pepper.

The lysine-specific histone demethylase (PnLSD1) and histone-lysine N-methyltransferase (PnATXR2) were found to express throughout the fruit development stages in all three varieties (Figure 14E&F). From the earlier studies have reported that the levels of methylated lysines are dynamically controlled by histone methyltransferases and demethylases (Klose et al., 2006; Shilatifard, 2008). Histone lysine methylation may be coupled to activation or repression of gene transcription, depending on the methylated residue and the degree of methylation (Lan et al., 2008). In Angiosperms, histone lysine methylation plays an important role in control of developmental transition from vegetative to a reproductive phase (i.e. flowering) (Yang et al., 2010).

5.3 Regulatory interaction between plant hormones related genes and plant hormones accumulation in black pepper flower and fruit development

5.3.1 Auxin accumulated at fruit expansion stage and the signalling involved complex regulatory mechanisms

The importance of plant hormones during flower and fruit development has been well documented in model plants *Arabidopsis* and tomato (Ozga & Reinecke, 2003; Srivastava & Handa, 2005). However, the knowledge of the roles of different plant hormones during flower formation, fruit growth, maturation and ripening in black pepper are scarce. Therefore, to better understand the plant hormonal regulation of flower and fruit development in black pepper, the changes in transcripts level of the genes related to plant hormones accumulation were measured from flowering to fruit ripening stages in three different black pepper varieties.

Auxin is a key plant hormone in fruit growth and development by promoting cell division and elongation (Schimmel et al., 2015). High concentration of endogenous auxin (IAA) at the fruit expansion stage in black pepper (Figure 21) was believed due to the continued fruit expansion through the regulation of cell division and enlargement by auxin. Previous studies suggest that the formation of seeds is closely linked with the fruit cell expansion in most of the angiosperm species (Gillaspy et al., 1993). The auxin produced in seeds has been suggested to be transported to the surrounding tissues to encourage the cell division and expansion in fruit growth (Ozga et al., 2002; Tiwari et al., 2013). Therefore, high concentration of IAA at fruit expansion stage in black pepper is in line with the development of the seeds in the fruit.

The expression profiles of seven selected genes related to auxin were found distinct from the hormonal profile of auxin accumulation at various stages of fruit development in black pepper (Figure 15). Yang et al. (2012) has reported that the expression of genes specific to auxin biosynthesis is not always correlated with the detected auxin concentrations. The difference in these profiles is believed to be mainly contributed by the high degree of complexity within the auxin-signaling network in regulating the fruit growth and development (Paponov et al., 2008; Sauer et al., 2013). Transport inhibitor response 1 auxin (PnTIR1) and auxin signalling F-box 2 (PnAFB2) were found expressed highly at all stages of flower and fruit development in majority of the black pepper varieties (Figure 15A&B), except a low expression of PnTIR1 were detected at fruit maturation stage in S1 variety (Figure 15A). Identifying F-box proteins, TIR1/AFB as auxin receptors was a great help in understanding the molecular mechanisms in the pathways of auxin signaling. The interaction of auxin with auxin receptors TIR1/AFB promotes ubiquitin-ligase to target AUX/IAA proteins for degradation, and allow auxin response factor 2 (ARF2) transcription factors to positively regulate the transcription of downstream genes (Dharmasiri et al., 2005). The current result of the expression of PnTIR1 and PnAFB2 suggested that the auxin receptors are present in all stages of flower and fruit development in black pepper to interact with the auxin for the downstream genes regulation.

Auxin responsive transcription factors (ARFs) have an important function in plant reproductive development. A genetic change in the ETTIN gene that also encodes an ARF affects the development of floral meristem and floral organs in *Arabidopsis* (Sessions et al., 1997; Sessions & Zambryski, 1995). Other members of the ARF gene family have also been involved in numerous aspects of reproductive development. In *Arabidopsis thaliana*, ARF1 and ARF2 control the abscission and senescence in floral organ (Ellis et al., 2005), while ARF8 acts as a fruit initiation repressor (Goetz et al., 2006). Moreover, ARF6 and ARF8 were also reported to play a role in the maturation of stamen and gynoecium in *Arabidopsis* (Nagpal et al., 2005). Therefore, the high expression of PnARF2 at the flower stage in black pepper (Figure 15C) might correspond with the development of the reproductive system for the flower formation. Nevertheless, ARF2 was also suggested as a repressor of cell division in ARF mutant fruits as the period of cell division have been prolonged in the *Arabidopsis* (Schruff et al., 2006). This finding supports the result of a decrease in PnARF2 expression as fruit grows and matures in KC variety (Figure 15C) is to prevent the suppression of the cell division during fruit development. SA and S1 varieties have distinct PnARF2 expression profiles than KC varieties and this may suggest that PnARF2 might involve in different role in regulating fruit development in different varieties of black pepper.

For the tryptophan-dependent pathway for auxin biosynthesis, the tryptophan amino tansferase-related protein (TAR) gene encodes aminotransferase converting tryptophan to indole-3-pyruvic acid (IPA) for the indole-3-acetic acid (IAA) biosynthesis. Knockdown of TAR gene in *Arabidopsis* results in a great decrease in free IAA levels, suggesting that IPA-dependent IAA biosynthesis is an important pathway to free IAA biosynthesis (Mano & Nemoto, 2012). In wheat, overexpression of TAR improved the grain yield and is important to the overall wheat growth (Shao et al., 2017). Expression analysis of PnTAR3 in all three black pepper varieties has revealed high expression at early and late stage of fruit development (Figure 15E). Meanwhile, PnTAR4 exhibited a distinct expression profile from PnTAR3 with high expression of PnTAR4 was detected at ripening stage in KC and SA varieties (Figure 15F). This result indicated that different

genes in TAR family may have different role in regulating the fruit development in black pepper.

In concern to the transportation of auxin within plant, the auxin efflux carrier component (PIN) genes encode integral membrane proteins are the most studied auxin transporter genes family. In *Arabidopsis*, there are eight members of the PIN protein family and most of them have been demonstrated to transport auxin either in *Arabidopsis* or in heterologous systems (Löfke et al., 2013). In this study, the expression of PnPIN8 was detected at all stages in KC and S1 varieties but not in the SA varieties (Figure 15G). This result indicated that the different varieties of black pepper might have different PnPIN regulatory genes in facilitating the transportation of auxin in plant. Synergistic interaction between PIN proteins has been shown earlier in functional overlapping in regulating different developmental processes, like tropism, embryo formation and organ development in *Arabidopsis* (Vieten et al., 2005). High expression of PnPIN8 was detected at fruit maturation stage in KC and S1 varieties which are believed that high transportation of auxin was happening at fruit maturation stage (Figure 15H).

5.3.2 Integration of gibberellin and auxin in regulating fruit expansion in black pepper

Crosstalk between auxin with other plant hormones in regulating the flower and fruit development has been demonstrated in *Lycopersicon esculentum* in which the auxin seems to be able to enhance biosynthesis of gibberellin (Koshioka et al., 1994), in turn, experiments with tomato have shown that the gibberellin application may lead to an increase in auxin levels (Sastry & Muir, 1963). In the present study, GA_4 was shown as the major gibberellin (GA) in black pepper (Table 5). In rice, GA_4 has been reported to be more active than GA_1 to control many aspects of growth and development in plants (Magome et al., 2013). This is similarly reported in strawberry fruits that GA_4 was detected to be high during fruit development (Achard et al., 2007).

The current results support the finding that GA, with high levels at the fruit expansion stage, may play a role in promoting cell division and expansion together

with auxins (Honda et al., 2017; Martins et al., 2018). Higher concentration of GA_4 in SA and S1 variety (Table 5) could be correlated to the fruit size of these two varieties in comparison to the KC variety (Figure 5) as demonstrated in figure 5F with SA and S1 have significant heavier weight of 100 berries. Higher level of GA_1 than GA_4 in KC variety might be due to the decrease GA bioactivity by the enzyme that participate in GA homeostasis as demonstrated in rice (Magome et al., 2013).

Study of the transcriptional profiles of PnGA20OX1 which is essential in regulating the levels of active GA in Arabidopsis (Rieu et al., 2008) was found inconsistent with the GA accumulation profiles in all three different varieties. This result indicated that PnGA20OX1 might not be the key regulator in regulating the GA levels in black pepper and more genes involved in the GA biosynthesis need to be elucidated to determine the molecular mechanism. High expression of PnGA20OX1 was detected at fruit maturation stage in SA variety and fruit ripening stages in S1 varieties (Figure 18A). This result was reported earlier in morning glory that there is a possibility of some stimulants being translocated into the seed coat through the phloem, which triggers the expression of the GA20OX1 gene toward the late stage of fruit development (Nakayama et al., 2005).

5.3.3 Cytokinins regulate fruit expansion and ripening in black pepper through different molecules

After flower pollination, cytokinin levels were increased in fruit to promote fruit set and early fruit growth with high rate of cell division (Honda et al., 2012). Therefore, cytokinins are generally considered to have an important role to stimulate the cell division during fruit development. In *Arabidopsis*, cytokinins induce the medial region of the developing gynoecia at the early proliferation stage and facilitate the formation of fruit valve margins at the later stage (Marsch-Martínez et al., 2012). In the present study, the concentration of trans-Zeatin (tZ) and isopentenyladenine (iP), the two most common active cytokinins in plants, were detected at various stages of fruit development in three different black pepper varieties. The content of tZ was peak at fruit expansion and maturation stage in KC and SA varieties (Figure 23) suggesting that tZ is involved in the cell division during fruit expansion. The high accumulation of tZ at fruit expansion stage was also

observed in other plant species, including kiwifruit (Pilkington et al., 2013), maize (Yonekura-Sakakibara et al., 2004) and grapes (Böttcher et al., 2015b). For S1 variety, high accumulation of tZ level was detected at later stage than SA and KC varieties (maturation stage and breaker stage) which indicated that the role of tZ might be difference in S1 variety than KC and SA. Moreover, the difference in the maximal tZ contents among the three black pepper varieties might be linked to the different degrees of relevance or functional adaptations of this hormone in the fruit growth and maturation in each variety, as has been shown in grapes (Böttcher et al., 2015a). In order to support the specification of function, application of tZ cytokinin to black pepper plants in future study might reveal the functional role of different levels of tZ in the fruit development process.

High iP content was accumulated at fruit ripening stage in SA and S1 varieties (Figure 22). Involvement of cytokinins in a late stage of fruit development is less well studied. Some study of the elevated cytokinin contents in the ripening fruits of kiwi (*Actinidia deliciosa*) (Pilkington et al., 2013) and grapes (*Vitis vinifera* L.) (Böttcher et al., 2013) have suggested that cytokinins are also responsible for regulating the ripening process. In KC variety, the iP level is high at fruit maturation stage but decreased in level towards fruit ripening. This result suggested that the iP accumulated earlier in KC variety than other two varieties and this may contributed to the difference in fruit ripening between the varieties. In this study, the accumulation of tZ was predominantly at the black pepper fruit growth and maturation stage (Figure 23) while iP was detected at ripening stage (Figure 22). Although both tZ and iP are categorized as cytokinins and they only vary in the hydroxylation of the side chain, their difference in signaling outputs and their biological effects may mean that these act as independent molecules to have different roles in fruit development in black pepper. In order to verify this, exogenous treatment with different cytokinins may further reveal the particular role of tZ and iP in regulating the black pepper fruit development.

The LOG-dependent pathway is the dominant cytokinin-activating mechanism in rice (Kurakawa et al., 2007) and *Arabidopsis* (Kuroha et al., 2009) to convert inactive cytokinin ribotides to active cytokinin nucleobases. This LOG-dependent pathway also appeared to be active in black pepper fruit development as

high expression of cytokinin riboside 5-monophosphate phosphoribohydrolase (PnLOG5) and cytokinin riboside 5-monophosphate phosphoribohydrolase (PnLOG8) homologs were observed at the flower stage in different varieties of black pepper except in S1 variety with high expression of PnLOG5 at ripening stage (Figure 16A&B). The distinct expression pattern of both PnLOG genes indicated a highly complex system involved in the cytokinin regulation processes for the normal growth of fruit in black pepper.

Another crucial part of the regulation of local cytokinin concentrations is through the irreversible degradation of cytokinins by cytokinin oxidase dehydrogenase (CKX) enzymes (Werner et al., 2006). In *Arabidopsis*, strong expression of CKX5 was found in stamen, primordial and developing pollen, while CKX6 was expressed in the gynoecium (Frébort et al., 2011). This indicated that CKXs do play an important role in reproductive organ development in plants. In this study, high PnCKX6 transcript level was observed at flower and fruit set stage in KC and S1 varieties but not in SA variety. This indicated the differential expression of PnCKX6 in different varieties of black pepper. High expression level of PnCKX6 at flower and fruit set stage is corresponded with low level of cytokinin with high degradation of cytokinin by enzyme coded by PnCKX6 gene. Therefore, it is suggested that PnCKX expression may contribute to cytokinin homeostasis to control the cytokinin levels during fruit development. In future, study on the mutants impaired with PnCKX genes in black pepper plant may further reveal the regulation role of PnCKX genes in controlling the cytokinin levels.

5.3.4 Abscisic acid play role in fruit ripening and regulated by ABA-binding protein

Upon fruit maturation, abscisic acid (ABA) plays an important role to regulate the fruit development process in both climacteric and non-climacteric fruit. Study from the tomato fruits has found that ABA induces ethylene biosynthesis as well as the ripening process (Zhang et al., 2009). Study of the ABA accumulation profile indicated that the ABA levels were peaked at fruit ripening stage in KC and SA varieties (Figure 24). Meanwhile, a relatively high level of ABA was observed at the fruit expansion stage in the S1 variety. Previous study in strawberry has

demonstrated that high levels of ABA at early fruit development stage will inhibit the growth of the fruits (Liao et al., 2018). CYP707A1 which encode abscisic acid 8-hydroxylase has been reported to act as a negative regulator in ABA biosynthesis to catalyze ABA catabolism and regulate the ABA levels across fruit development stages (Wang et al., 2017). Therefore, the high expression of PnCYP707A1 observed at the fruit expansion and maturation stages in S1 variety (Figure 17A) may serve to prevent the inhibitory effect of high levels of ABA at fruit expansion stage in S1variety (Figure 24). The transcript expression analysis of abscisic acid receptor PYL4-like (PnPYL4), an ABA receptor, has high expression at fruit maturation stage and gradually decreased as fruit ripened (Figure 17B). An increase of PnPYL4 gene expression at the beginning of fruit maturation and ripening may be necessary to accumulate sufficient ABA receptor for the binding of ABA to convert the signals into the right cellular responses (Guo et al., 2017). Therefore, PnPYL4 is also a key limiting step on the response to ABA for the fruit ripening in black pepper. Study from sweet cherry fruit support the current result that the ABA activity not only reliant on the ABA content levels, but is also linked to the activity of ABA-binding protein (Ren et al., 2011).

Meanwhile, substantially high expression of indole-3-acetaldehyde oxidase (PnAAO) was detected at fruit set and fruit ripening stage in SA and S1 varieties (Figure 17C). AAO1 was anticipated to work in IAA biosynthesis in early research and other AAO genes have been shown to be involved in biosynthesis of abscisic acid (ABA) (Di et al., 2016). However, it has been suggested recently that AAO genes are irrelevant to IAA biosynthesis (Mashiguchi et al., 2011). Lack of correlation between PnAAO (Figure 17C) and changes in IAA level (Figure 24) in black pepper may give a negative indication for the involvement of PnAAO in IAA biosynthesis, likewise reported in rice (Abu-Zaitoon et al., 2019). Previous study in *Arabidopsis* has reported that AAO genes encode the enzyme that catalyzes the last step of ABA biosynthesis (Seo et al., 2004). High transcription of PnAAO genes at fruit ripening stage in SA and S1varieties might correlated with the involvement of PnAAO genes in ABA biosynthesis which highly accumulated at fruit ripening stage. Meanwhile, high expression of PnAAO gene was also observed at fruit setting stage is SA and S1 varieties. Study from avocado fruit has reported that

AAO activity may be part of the syndrome associated with the appearance of a fruit size phenotype. Therefore, the current high expression of PnAAO in SA and S1 varieties might be correlated with the bigger fruit size observed in these two varieties in compared to KC variety.

5.3.5 Involvement of jasmonic acid and salicylic acid at black pepper flowering stage

As for other plant hormones, the homeostasis of jasmonic acids (JAs) is well regulated to control the fruit development process. In the present study, high levels of JA and jasmonyl-isoleucine (JA-Ile) were detected in black pepper flowers and at the early stage of fruit development but decreased in levels as fruit grow and ripened (Figure 25). JAs have been known to play an important role in flower development in many plant species like rice, tomato and *Arabidopsis* (Yuan & Zhang, 2015). The JAs content was also reported high in the young fruit of grape berries and the levels go down as fruit ripens (Böttcher et al., 2015b). The JA biosynthesis OPDA-reductase 3 (PnOPR3) gene that converts 12-oxophytodienoate (OPDA) into JA showed a high expression at fruit set stage (Figure 18C). Previous study has indicated that the expression of OPR3 was induced by JA through feedback regulation of gene expression in *Arabidopsis* (Müssig et al., 2000). In this study, high levels of JA was detected at the flower stage then followed by a high expression of PnOPR3 at fruit set stage which support the fact of feedback regulation. In addition, high expression of PnOPR3 was also observed at fruit ripening stage in all three black pepper varieties (Figure 18C). It is suggested that the increase of PnOPR3 at the fruit ripening stage might be due to the elevated levels of JA in the maturing pericarp as has been reported in soybean seed (Simpson & Gardner, 1995). Furthermore, other post-transcriptional molecular events might also take place to control the JAs levels in certain stages which lead to the JAs level not correlating with the PnOPR3 expression at the late stage of fruit development (Garrido-Bigotes et al., 2018). Therefore, more study in functional characterization is needed to identify the main factor that contributed to the distinct expression profile of PnOPR3 in black pepper JA biosynthesis at late stage of fruit development.

A delicate control of the flowering in an interactive network ensures the initiation of flowering at the right time. Salicylic acid is known to promote the flowering in plants (Cleland, 1974; Cleland & Ajami, 1974; Goto, 1981). Study in *Arabidopsis* salicylic acid impaired plants showed delayed flowering compared to the corresponding wild-type plants. In the present work, high levels of salicylic acid were detected at flowering and early stage of fruit development. The KC variety exhibited comparatively higher levels of salicylic acid at the flower stage than the SA and S1 varieties (Figure 26). The expression profiles of PnSa-BP2 were mostly similar with the salicylic acid accumulation levels at flower stage in all three varieties. However, high expression of PnSa-BP2 was detected at fruit breaker and ripening stage in the S1 variety (Figure 18B). Sa-BP2 is known to facilitate the hydrolysis of methyl salicylate to salicylic acid, and its enzymatic activity is required for acquired resistance in plants besides for flowering (Kumar & Klessig, 2003). However, in this study, high expression of PnSa-BP2 in S1 variety was not associated with high level of salicylic acid accumulation. Therefore the role of PnSa-BP2 at black pepper plant resistance still need to be further verified.

5.4 Sugar metabolism and transporter genes regulating fruit size, secondary metabolites formation, flowering time and pollen germination in black pepper

Sucrose metabolisms is essential to fuel plant processes including signalling, yield formation and nucleic acid synthesis and transported to the sinks through the predominant sucrose-controlled phloem transport pathway (Ruan, 2014). As the first product of photosynthesis, sugar is translocated from the photosynthetic part to non-photosynthetic sinks, including reproductive organs (i.e. flower) and fruit in plants (Wang and Ruan, 2016). Analysis of sugar transporter ERD6-like 16 (PnERD6), a sugar transporter gene showed contrasting patterns of expression among the three different black pepper varieties across the fruit developmental stages (Figure 19A). The pattern of decreasing expression through the fruit developmental stages in KC variety (and the high expression of ERD6 over the fruit developmental stages in SA and S1 varieties) is believed to connect with different sugar level in different stages of fruit development (Shanmugam et al.,

2017). Study on the sugar content level in different varieties will further shed light on the sugar transportation in black pepper.

Next, the analysis of 7-deoxyloganetin glucosyltransferase-like (PnGGT) gene in this study revealed high expression of PnGGT at fruit expansion stage in all three varieties (Figure 19B). Glucosyltransferases have been reported earlier to be linked to secondary metabolite metabolism by catalyzing carbohydrate moieties into natural compounds (Tiwari et al., 2016, Asada et al., 2013). Active response of PnGGT at fruit expansion stage might be link to the formation of secondary metabolites at fruit expansion stage in black pepper. Further study of the PnGGT impaired mutant plants may provide more understanding of the role of PnGGT in secondary metabolites formation.

Sucrose metabolism is also a critical step in reproductive organ formation, and previous study has reported that silencing vacuolar invertase (VIN) gene in cotton has led to a failure in pollination (Wang and Ruan, 2016). In this study, PnVIN pepper homolog gene was highly expressed at the flowering stage in SA and S1 varieties, suggesting a role of PnVIN in the black pepper flower development process (Figure 19 D). Furthermore, a study reported by Heyer et al. (2004) showed that flowering time control is strongly affected by the different levels of sugar in the apex and VIN play a crucial role in this network. VIN coordinating carbon dioxide uptake through sugar-mediated signaling pathways and affects the sugar levels in apex (Bolouri Moghaddam and Van den Ende, 2013). High expression of PnVIN at flowering stage in SA variety that exhibited a more uniform ripening characteristic suggests a role for PnVIN in controlling the flowering time i.e. inducing the transition to flowering and it may be a potential gene for manipulating the synchronization of flowers in black pepper. Improving the flowering trait in black pepper could be achieved through targeted PnVIN gene overexpression in the plants.

The black pepper pyrophosphate fructose-6-phosphate 1-phosphotransferase (PnPFP) was ubiquitously expressed in various stages of fruit development in all three different black pepper varieties (Figure 19E). This result is associated with previous research in rice as OsPFP is expressed in different stages of grain filling

through modulating carbon metabolism (Duan et al., 2016). Another gene which has an active role in the sucrose signalling pathway, homeobox leucine zipper (PnATHB-13) is a transcription factor (Hanson et al., 2001) that was highly expressed only at early stage of fruit development in all three black pepper varieties (Figure 19F). Previous reports have indicated that AtHB-13 is expressed in stigma and anther (Ribone et al., 2015). Hence, it is tempting to speculate that this transcription factor could be involved in communication between pollen and stigma, in particular by playing a role in the generation of the pollen coat. Moreover, it has been suggested that molecules located on the extracellular surfaces of both pollen and stigma are responsible for such recognition (Edlund et al., 2004). Therefore, high expression of pepper homolog gene PnATHB-13 at the flower stage of black pepper was believed to have possible association with pollen germination as reported earlier in *Arabidopsis*. ATHB-13 regulate pollen germination through modifying the expression of pollen coat genes and genes involved in cell development and organization as well as proteins and lipids transportation (Komarova et al., 2008, Bock et al., 2006).

5.5 Expression of the piperine related genes were associated with the biosynthesis of secondary metabolites in different varieties

Black pepper is valued for the presence of piperine that gives the flavor of pungency (Gorgani et al., 2017). Piperine (1-piperoylpiperidine) belongs to the most important group of nitrogenous secondary metabolites referred to as alkaloids (Hu et al., 2015). Study on the biosynthesis of piperine has shown that piperine is derived from the primary metabolism of lysine/ornithine in the *Punica granatum, a Piper species* (Szőke et al., 2013). However, information on the molecular mechanism on piperine biosynthesis in black pepper is still confined. In this study, the unigenes of homologs involved in the lysine/ornithine metabolism related pathway were manually identified from the transcriptome. Among them, three genes were further profiled in six different fruit development stages in three different varieties (Figure 20).

Lysine histidine transporter-like 8 (PnAATL1) pepper homolog gene facilitate lysine and histidine transportation across cellular membrane in higher

plants (Zhao et al., 2012). PnAATL1 expression was highly expressed at flower stage in all three varieties and the expression was also detected at various stages of fruit development (Figure 20B). Generally, it is assumed that more active transportation of lysine products is happening at the early stage of fruit development and remained active throughout the development stages. This assumption is based on the study in pear fruits that some transporter genes were involved in the early stages of fruit cell expansion for the generation of high osmotic stress in vacuoles of young fruits, while some transporter genes are increases at late stage of fruit development for the accumulation of sugar and organic acid for fruit maturation and dispersal (Shiratake and Martinoia, 2007).

The expression of isopiperitenol (-)-carveol dehydrogenase (PnISPD) and Ornithine decarboxylase (PnODC) increased markedly at the fruit expansion stage in KC and SA varieties but not in the S1 variety (Figure 20A&D). Previous studies have confirmed that ISPD was important to catalyzes the oxidation of isopiperitenol (Ringer et al., 2005), whereas ODC is the enzyme that catalyzes the conversion of ornithine to polyamine (Kaur-Sawhney et al., 2003, Agudelo-Romero et al., 2014). Both genes are involved in the biosynthesis of aromatic compounds (Agudelo-Romero et al., 2013). High expression of PnISPD and PnODC at fruit expansion stage might be an indicator showing that active aromatic compounds biosynthesis was happening at fruit expansion stage in KC and SA varieties than S1 variety (Figure 20A&D). Therefore, aromatic compounds characterization at fruit expansion stages in different varieties of black pepper need to carried out to confirm the current result.

The three different varieties investigated in this study have different fruit morphological characteristics as well as different maturation time based on the field morphological observation. Therefore, study on the genes related to flower, sugar, hormones and piperine may provide clues on the difference in fruit development among the three varieties. The differential expression of the genes on the three different varieties, making them as important candidates for the growth of fruits with distinct genotypes. The genes characterized in these three varieties through different fruit development stages may serve as the target for any black

pepper improvement programs for development of high yield and better quality of black pepper.

CHAPTER 6: CONCLUSIONS

The findings in this study give the first report on the molecular mechanism of flower and fruit development in various black pepper varieties. The assembled transcriptome of black pepper flower and fruit has presented a global description of expressed genes in black pepper flower and fruit development. The potential role of different growth regulators and genes could be inferred through confluence with the comprehensive quantification of various plant hormones including:

(1) Purine metabolism, starch and sucrose metabolism, signal transduction and secondary metabolite biosynthesis are the main GO terms enriched in the transcriptome.

(2) The transcriptomic analysis has helped to mine the dataset on flowering in black pepper. Some candidate genes associated with important regulatory pathways were selected and analysed at different flower and fruit developmental stages. The current findings indicated that PnAP2 &PnAGL8 might be significant regulators of flower meristem identity in black pepper, while PnAG might be necessary for the fruit development and PnGLK1 for fruit ripening. These results provide essential information for further functional research and key genes selection to understand the flower and fruit development mechanisms in black pepper.

(3) The expression patterns of six genes related to sugar-transport and carbohydrate metabolism were revealed by gene expression analysis using the probe-based method. The results indicated that carbohydrate metabolism in black pepper fruit is developmentally regulated. The study of the gene PnERD6 might have a role in regulating the sugar content level in black pepper, while PnGGT might link to the formation of secondary metabolites in the fruit and PnVIN potentially has a role in regulating flowering time in black pepper. This study serves as a platform for further studies to understand sugar transport and carbohydrate metabolism in black pepper fruit for quality improvement.

(4) The presence of piperine in black pepper is a unique character in the plant that needs further study on the specific molecular-genetic mechanisms. A

total of three genes related to piperine were analyzed in three different varieties in this study. The transportation of lysine products is believed more active at flower stage but remained active throughout the different developmental stages. Also, high expression of PnISPD and PnODC genes at fruit expansion stage in KC and SA varieties may hints the active aromatic compounds synthesis at the particular stage in these two varieties compared to S1 variety.

(5) JA and salicylic acid play decisive roles in flowering and fruit set, whereas auxin, GA and cytokinins play roles predominantly in early fruit development stages through cell division and expansion. ABA appears to play a role in fruit maturation and ripening in the fruit development process. Involvement of more than one hormone in the regulation pathway indicating possible crosstalk between the plant hormones in the fruit development stages in black pepper. Additional analysis involving the application of exogenous plant hormones or antagonists will be needed to determine the significance of this study.

(6) The patterns of hormonal gene expression and hormone levels vary considerably between the varieties and in different stages of fruit development. The difference found in the auxin-signalling network suggested that a high degree of complexity of regulation was involved in the auxin regulation mechanisms. Several differentially expressed genes like PnLOG and PnOPR3 might be involved in more than one hormone pathway, further supporting the possibility of crosstalk between plant hormones. Study of the ABA biosynthesis genes further suggested that the activity of ABA does not only rely on the ABA accumulation levels but is also dependent on the activity of the ABA-binding protein. Functional genetic studies could be used to manipulate the expression of the primary genes in the target pathways to evaluate the function of the specific genes further.

Overall, distinctions in patterns and levels of gene expression, as well as plant hormones accumulation between the three different varieties were observed at different stages of fruit development. These findings are likely the critical determinants in flower and fruit development in black pepper. Therefore, this thesis

study has achieved the first aims of the study in understanding the genetic mechanisms in black pepper flower and fruit development. This was achieved through a comprehensive analysis of the black pepper transcriptome and established the gene expression profiles on selected transcripts in flower formation, sugar metabolism and transportation, as well as piperine biosynthesis. The information provides valuable gene resources for future crop and fruit quality improvement. Meanwhile, the second aim of the thesis study was achieved through the analysis of the regulatory interaction of plant hormones related genes on plant hormones accumulation in black pepper flower and fruit development. The outcome from the plant hormones study has provided an understanding of another level of regulation in the development mechanism, which can be applied to improve the flowering and fruiting in black pepper.

APPENDIX I

Targeted Sequences for probe-based gene expression analysis

Target Identifier	Abbreviation	Targeted sequence
PN_8388.1:1445	PnAAO	ACAGGCTCAGAAAGATTAACAATTACTCTAGAAGAGTTCCTCAAGAGTCTTCCTAATAATACCCAGG CATTGCTCCTTAGCATCTACATTCCAAACTGGG
PN_10562.1:1183	PnARF2	ACAAACCAAGAACTAGCCCATCTGAGTTTATCATTCCATATGATCAGTACATGGAATCTGTGGAGAG CAATCATTTCATAGGAATGCGATTCAAAATGAG
Pin_8290.1	PnAG-SHT	TTCTTCAACCAAATCTGCTGGAACCCAATGTCAACTACTCCCAGGAACAGACAACCCTTCAACTTGG GTGATGAGATTCTCCACCGTGATTCTCCAACCG
PN_50799.1:560	PnTAR4	GGAGTAGGATCCATGCAACTGATTAACGCTGCTGTCCGAACTCTCTCTGCGTCAAATGGTTCACAAC CTTCAACACCATCAAAAATTGTGGCAACTCCTC
PN_43579.1:408	PnTAR3	TGCTCTCAATTCTCTGATTGCCCAGCTGATGCCGATAGTGGAAATCCGTTGTTTTTGGAACCATTTTG GATGCGGTACAAGGCTAGCAGTGCAGTCATGG
Pin_7153.1	PnTIR1	TCTGCAACAAAAGGGTAAGATGGGAACACCCGAAGTTCCTCTAAGCTCTTGCAATTGTCAGCGACA GCTTGGAGTCCAGTGTCCTCTATAAGGTCCAGGA
Pin_1852.1	PnAFB2	GCTAAGAAAATAGTAATACAATGAACAATGTCTAACCTCGCCTACCAGAAAAAAGCCTGGTCATGGG ACGAGAACCTCGACATTTTACAAACCTCCAACT
Pin_64296.1	PnPIN8	ATGTCTCCATAGCCACATTGTCCAATACTTTGGTGGTGGGCGTGCCATTGTTGAGGGCAATGTATGG GTCTCTGGCACAGGATCTGGTGATTGAAGTGTT
PN_scaf24322.1:231	PnCKX6	GCAGAACCCTCTTTTGTCTAGAAGTGGCAAAATATGCATACACAGATGAAGGAGACAAACTGGACCA GGAAGTTAAAAGCCTTTTGGCCAAGCTGAGTTA
PN_C804906.1:490	PnLOG5	GCTGTGGCGGACATGCACCAGAGGAAGGCCGAAATGGCATGCAACTCCGATGCCTTCATTGCCTTG CCCGGTGGATATGGAACGCTAGAAGAGCTGCTTG
PN_C797402.1:215	PnLOG8	AGATAGATTTGATATATGGAGGAGGGAGCGTTGGCCTCATGGGATTGATATCCAAAACTTTACACAC TGGTGGATGCCATGTCCTTGGAGTTATTCCTGC
Pin_44880.1	PnCYP707A1	ATACCTCATCCCAAAAGGCTGGAAAGTTCTCCCTCTCTTTAGGAACATTCATCATAGCCCAGCCAAC TTTAAAGATCCAGAAAAGTTCGATCCAACCAGA
Pin_50839.1	PnPYL4	CGCACTCGCAGATTCGCAAGTGCACACCCCTCGCCCTCGCCGGCAGAGCGCCGGAGTCGGCCGC CCGACACCACGCCCACACGATCGGCCCCAACCAGTG
PN_scaf50790.2:271	PnGA20Ox	GTGTTTCTGGGTGAACTCGAGCAGTTGTGACCATGTGAAATCCGGGAACTTCTGTGGTCCATCCCT GACGAGGAGATCCTGTGGGGGCTTCACTATTTTG
PN_C803408.2:303	PnSa-BP2	CCGGCCGGAAAGCCTAACTGACCTCAATGGGTTTCCAGACTTTGTGAAACCATTGGTGGATGTGAT GGAAGGTCTGCCTGCTTCTGAGAAGGTGATCCTT
PN_scaf7527.2:544	PnOPR3	TCTGGCCGTAGGCCGTGTACCGCGGCTGGGTCACGTGGAGGTAGGCCAGACCGAACCCGTTCAAG GCGCTCACCACGGCCAGGCCCAGCCCGAGCGGGTC

Pin_1076.1	PnAP2	CCCCACTTCTCATCAGTCTCCCTTAAGCTTGGTGGTGGACGGTACATGAAAGCCGGAGAAAGCTCC ATGGTTCACGTGATGCAACACCATTTTAATCCCC
Pin_12247.1	PnGLK1	AACTTCGACGACCTCTTCGTCGGGATCGAGGATGGCGACCTTCCGGAACTGGAGATGGACTCGGAT ATCCTTGCCGAGTTCTCGTCGGTCGAGGAGTCGA
Pin_18978.1	PnAGL8	GGCTAGAATCTTGGAGCGCTACGAGAGACACACTTTTTCTCAGAGAGAACCTTCCATGACAAGTATG GAATCAGAGGGGCCTTGGTCTCTACAGTATGGT
Pin_4332.1	PnAG	AGCAAGAGTCAAACAAACTTCGGCAGCAAATTGGGCTTCTTCAAAATGCAAATAGGCATTTAATGGG CGAGTCTCTTAGCTCCATGACTATCAAGGAACT
Pin_12251.1	PnERD6	TAGGAGGTTTAGAGGTAAGAATGCTGATATTTCCATTGAGGCAGCTGAAATCCAGGATTACATAGAA ACGCTAGAAAGACTACCCAAGGCCCAAGTTTTA
Pin_13705.1	PnGGT	CCATAAGTTGCAAGCCATGGACGCAAGCCGAGGGTCGTCAATCTGGTTGCACATCAAGGACAGAGG ACCAACTGTGTATATCTTGGGAAAGCTCCCTTTT
Pin_4343.1	PnABCC2	GCTTATAAGGCCTATGATAGGATGGCCCAGATAAATGGAAACTCAATGGATAATAATGCCAGATTTA CACTGGTGAACATGAGTGCAAACCGATGGTTGG
Pin_9031.1	PnVIN	TCGATTTCTATCCGGTGGCGGCTAATGGGAGCGGCAAGGGGCTTGATACTTCGGCGGTTGGGGCC GGGTTGAAGCATGTCCTCAAGGCCAGCCTTGACGA
Pin_81110.1	PnPFP	TGGACTCGACGGCGAGCGTGATGCAGACGTCGATCTTTTGAGGAATGATCTATGGTTATCATCATCT GCATAACAGAGGAGAGACTCCTCGCAGATCTCT
Pin_10717.1	PnATHB-13	CGTTGCTGAAACCCACTGCCGTAGCCCAACTTTTCCAACCGTCTACCAGGCCGGATCTCCAGTGCC CAAAGTTGGAGCAACCTATTCAAGATGAGAGCTT
PN_C801706.2:269	PnISPD	GGGCCAAAATGTGGCGGCTTCAATCGGCCATGTCCGGTGCAAGTACGTACACTGCGACGTCTCCG ACGAGTCCCAAATCAAGCAGCTGGTGGAACAAACC
PN_C628640.2:7	PnLSD1	CCGATCGGGACGCCGATGAAGCGGTCGCTGATGAACATGAAGGGGTACCTCGAGGAGGCGGGTC ACCTCACCAAGCTCAAACCGTAGGACGCCTGGCTCC
PN_S40315.2:831	PnODC	CGTTTACATTGGTGACAACGGTGATAGGGAAGCGAATGAGGGGTGGAGTGAGGGAATATTGGATCA ACGACGGAACGTATGGTGCTCTCAACTGCATCAT
PN_CL31.2:194	PnATXr2	CAAGGACTACTATAAGCAGCTTCTGAGAGAGAGGGAGATATCATTTCTGAGAGTGGAGCAGAATGG CAAACACGGAAAAGGTTTGTATGCGGATGTCGAA
Pin_18925.1:263	Ubiquinone	GAAATTTGGCATTTAGCTGGTGACTCCTCCTCTGACATGGACTGGTATGTCAAGCGCACAATTTTAG GAGGCATCTACTCAACGGCTGAAGTATATATGA
Pin_125.1:90	Histone 3	CCACCAAGGCTGCAAGGAAGTCAGCCCCGACGACAGGTGGTGTCAAGAAGCCTCACCGCTACCGC CCTGGAACTCCTGATCCGCAAGCTACCTTTCCAGC
Pip_Elf1a.1:230	EF1a	CGGTGGTTTTGAGGCTGGTATCTCTAAGGATGGTCAGACCCGAGAGCATGCCCTGCTTGCCTTCAC TCTGGGGGTGAAGCAGATGATTTGTTGTTGCAAC

APPENDIX II

KEGG Pathways

Pathway	Pathway ID	#Enzs in Pathways
Glyoxylate and dicarboxylate metabolism	map00630	23
Tryptophan metabolism	map00380	13
Pyruvate metabolism	map00620	28
Caffeine metabolism	map00232	3
Monobactam biosynthesis	map00261	5
Lipopolysaccharide biosynthesis	map00540	6
Fluorobenzoate degradation	map00364	1
Valine, leucine and isoleucine biosynthesis	map00290	7
Glycosphingolipid biosynthesis - ganglio series	map00604	3
Styrene degradation	map00643	4
Lipoic acid metabolism	map00785	1
Valine, leucine and isoleucine degradation	map00280	17
Amino sugar and nucleotide sugar metabolism	map00520	40
Toluene degradation	map00623	2
N-Glycan biosynthesis	map00510	15
Monoterpenoid biosynthesis	map00902	1
Metabolism of xenobiotics by cytochrome P450	map00980	5
Flavonoid biosynthesis	map00941	12
mTOR signaling pathway	map04150	1
Aminoacyl-tRNA biosynthesis	map00970	23
Aminobenzoate degradation	map00627	9
Other types of O-glycan biosynthesis	map00514	2
Phosphonate and phosphinate metabolism	map00440	3
Novobiocin biosynthesis	map00401	5
Tropane, piperidine and pyridine alkaloid biosynthesis	map00960	7
Oxidative phosphorylation	map00190	7
Taurine and hypotaurine metabolism	map00430	4
Phosphatidylinositol signaling system	map04070	15
Glycosaminoglycan biosynthesis - keratan sulfate	map00533	1
Inositol phosphate metabolism	map00562	19
Polyketide sugar unit biosynthesis	map00523	3
beta-Alanine metabolism	map00410	17
Fatty acid degradation	map00071	13
Linoleic acid metabolism	map00591	3
Drug metabolism - other enzymes	map00983	14
Flavone and flavonol biosynthesis	map00944	5
Brassinosteroid biosynthesis	map00905	1
Th1 and Th2 cell differentiation	map04658	1

Fatty acid biosynthesis	map00061	9
Porphyrin and chlorophyll metabolism	map00860	24
Fructose and mannose metabolism	map00051	20
Sesquiterpenoid and triterpenoid biosynthesis	map00909	2
Arginine and proline metabolism	map00330	19
D-Arginine and D-ornithine metabolism	map00472	1
Lysine degradation	map00310	10
Biotin metabolism	map00780	8
Lysine biosynthesis	map00300	10
Pantothenate and CoA biosynthesis	map00770	15
Biosynthesis of terpenoids and steroids	map01062	1
Nicotinate and nicotinamide metabolism	map00760	13
Purine metabolism	map00230	51
Vitamin B6 metabolism	map00750	7
Arginine biosynthesis	map00220	19
Benzoate degradation	map00362	6
Methane metabolism	map00680	18
One carbon pool by folate	map00670	14
Nitrogen metabolism	map00910	16
C5-Branched dibasic acid metabolism	map00660	5
Terpenoid backbone biosynthesis	map00900	25
Ubiquinone and other terpenoid-quinone biosynthesis	map00130	10
Butanoate metabolism	map00650	14
Chloroalkane and chloroalkene degradation	map00625	3
Primary bile acid biosynthesis	map00120	3
Biosynthesis of vancomycin group antibiotics	map01055	1
Mucin type O-glycan biosynthesis	map00512	1
Steroid biosynthesis	map00100	9
Glycosaminoglycan degradation	map00531	5
Geraniol degradation	map00281	4
Streptomycin biosynthesis	map00521	8
Pentose phosphate pathway	map00030	18
Peptidoglycan biosynthesis	map00550	2
Other glycan degradation	map00511	7
Anthocyanin biosynthesis	map00942	1
Limonene and pinene degradation	map00903	2
Insect hormone biosynthesis	map00981	1
Citrate cycle (TCA cycle)	map00020	16
Acarbose and validamycin biosynthesis	map00525	1
Glycolysis / Gluconeogenesis	map00010	26
Mannose type O-glycan biosynthesis	map00515	3
Glutathione metabolism	map00480	21
Benzoxazinoid biosynthesis	map00402	1
Glycosaminoglycan biosynthesis - heparan sulfate / hepar	map00534	5
Cyanoamino acid metabolism	map00460	8

Betalain biosynthesis	map00965	3
Photosynthesis	map00195	2
Biosynthesis of antibiotics	map01130	175
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	map00563	1
Neomycin, kanamycin and gentamicin biosynthesis	map00524	1
Selenocompound metabolism	map00450	10
Synthesis and degradation of ketone bodies	map00072	3
alpha-Linolenic acid metabolism	map00592	10
Steroid degradation	map00984	2
Stilbenoid, diarylheptanoid and gingerol biosynthesis	map00945	4
Carotenoid biosynthesis	map00906	6
Fatty acid elongation	map00062	8
Galactose metabolism	map00052	16
Phenazine biosynthesis	map00405	1
Biosynthesis of unsaturated fatty acids	map01040	9
D-Alanine metabolism	map00473	2
Phenylalanine metabolism	map00360	19
Atrazine degradation	map00791	2
Sphingolipid metabolism	map00600	11
Penicillin and cephalosporin biosynthesis	map00311	1
Tyrosine metabolism	map00350	18
Histidine metabolism	map00340	13
Cysteine and methionine metabolism	map00270	38
Folate biosynthesis	map00790	11
Biosynthesis of siderophore group nonribosomal peptide	map01053	2
Glycine, serine and threonine metabolism	map00260	26
Starch and sucrose metabolism	map00500	29
Alanine, aspartate and glutamate metabolism	map00250	25
T cell receptor signaling pathway	map04660	2
Glycosphingolipid biosynthesis - globo and isoglobo serie	map00603	6
Ethylbenzene degradation	map00642	1
Pyrimidine metabolism	map00240	30
Aflatoxin biosynthesis	map00254	1
Isoquinoline alkaloid biosynthesis	map00950	19
Indole alkaloid biosynthesis	map00901	2
Phenylpropanoid biosynthesis	map00940	14
Caprolactam degradation	map00930	4
Naphthalene degradation	map00626	1
Various types of N-glycan biosynthesis	map00513	10
Phenylalanine, tyrosine and tryptophan biosynthesis	map00400	25
Sulfur metabolism	map00920	15
Steroid hormone biosynthesis	map00140	6

Glycosaminoglycan biosynthesis - chondroitin sulfate / de	map00532	3
Glycerolipid metabolism	map00561	19
Arachidonic acid metabolism	map00590	6
Drug metabolism - cytochrome P450	map00982	7
Isoflavonoid biosynthesis	map00943	1
Diterpenoid biosynthesis	map00904	4
Retinol metabolism	map00830	6
Ether lipid metabolism	map00565	5
Zeatin biosynthesis	map00908	3
Pentose and glucuronate interconversions	map00040	13
D-Glutamine and D-glutamate metabolism	map00471	2
Glucosinolate biosynthesis	map00966	1
Riboflavin metabolism	map00740	8
Glycerophospholipid metabolism	map00564	24
Thiamine metabolism	map00730	9
Cutin, suberine and wax biosynthesis	map00073	2
Carbon fixation pathways in prokaryotes	map00720	19
Carbon fixation in photosynthetic organisms	map00710	21
Ascorbate and aldarate metabolism	map00053	12
Biosynthesis of ansamycins	map01051	1
Carbapenem biosynthesis	map00332	2
Chlorocyclohexane and chlorobenzene degradation	map00361	1
Glycosphingolipid biosynthesis - lacto and neolacto series	map00601	4
Propanoate metabolism	map00640	15

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