EFFECTS OF TEMPERATURE ON MORPHOAGRONOMIC PERFORMANCE, AROMA GENE EXPRESSION AND VOLATILE PROFILE OF AROMATIC RICE

MD. ZAKARIA HOSSAIN PRODHAN

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MD. ZAKARIA HOSSAIN PRODHAN

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Designation: Associate Professor

Name: Dr. Rosna Binti Mat Taha

Designation: Professor

ABSTRACT

Production of aromatic rice with superior agronomic performance and excellent aroma is complicated due to the influences of environmental factors, cultural practice and genotypic condition. Among the environmental factors, the temperature is one of the most important factors that can affect growth, development, and production of aromatic rice as well as the quality of aroma performance. Therefore, selection of an appropriate growth stage and determination of a suitable temperature for proper expression of aroma gene is an important determinant for high-quality aromatic rice cultivation. The effects of the high day-night temperature, heat stress, and low-temperature stress on *badh2* gene expression were investigated by previous researchers but a suitable temperature for proper expression of the *badh2* gene and its relation with volatile compounds and the morphoagronomic performance of aromatic rice are still unrevealed. This study investigated the effects of three different temperatures (ambient or 28°C, 25°C and 20°C) on relative expression of *badh2* gene, volatile profile, 2-Acetyl-1-pyrroline (2AP) concentration, phenotypic aroma expression as well as morphoagronomic performance of five aromatic and one non-aromatic rice genotype. The results indicated that the maximum down-regulation of *badh2* gene (-18.31 fold), highest 2AP concentration (0.14 ± 0.02 ppm) and strong phenotypic aroma (score 4) were observed at 25°C temperature compared to ambient and 20°C temperature. The morphoagronomic traits were also varied on temperature condition where the 25°C facilitated better vegetative growth (Rato Basmati) and yield performance (E 13 genotype). Hence, the expression levels of the aroma gene and 2AP concentration were affected by temperature and regulated the phenotypic expression of an aroma at different growth stages in aromatic rice. This study is useful to develop a sustainable and suitable platform for production of high-quality aromatic rice.

ABSTRAK

Pengeluaran beras aromatik dengan prestasi agronomi yang unggul dan aroma yang sangat baik adalah rumit kerana pengaruh faktor-faktor alam sekitar, amalan budaya dan keadaan genotip. Di antara faktor-faktor alam sekitar, suhu adalah faktor yang paling penting yang boleh memberi kesan kepada pertumbuhan, pembangunan dan pengeluaran beras aromatik serta kualiti prestasi aroma. Selain itu, pemilihan peringkat pertumbuhan yang sesuai dan penentuan suhu yang sesuai untuk ekspresi yang tepat bagi gen aroma, merupakan penentu penting untuk penanaman padi wangi yang berkualiti tinggi. Kesan suhu tinggi siang-malam, tekanan haba, tekanan suhu rendah pada ekspresi gen *badh2* telah dikaji oleh penyelidik sebelumnya tetapi penilaian suhu yang sesuai untuk ekspresi gen *badh2* yang tepat, hubungan antara ekspresi gen, sebatian meruap, prestasi morfo-agronomi beras aromatik masih belum di-dedahkan. Kajian ini melibatkan kesan tiga suhu yang berbeza (ambien atau 28°C, 25°C dan 20°C) pada ekspresi relatif gen *badh2,* pencirian sebatian meruap, penganggaran kepakatan 2-Acetyl-1-pyrroline (2AP), penilaian aroma fenotip bersama-sama dengan prestasi morfoagronomi lima padi aromatik dan satu padi tanpa aromatik. Hasil Kejiran menunjukkan bahawa ekspreci gen *badh2* sehingga -18.31 kali ganda, kepekatan 2AP meningkat sehingga (0.14 ± 0.02 ppm) dan skor aroma adalah 4 (aroma kuat) pada suhu 25°C keadaan ambien diikuti oleh 20°C. Prestasi morfoagronomi juga berubah-ubah mengikut keadaan suhu, yakni penunasan dan kesuburan padi (Rato Basmati) dan hasil pengeluaran (E 13 genotype) adalah lebih tinggi pada suhu 25°C berbanding 20°C dan suhu ambien. Oleh itu, tahap ekspresi gen aroma dan kepekatan 2AP telah dipengaruhi oleh suhu dan dikawal selia ungkapan fenotip aroma yang pada peringkat pertumbuhan yang berbeza dalam beras wangi. Kajian ini adalah berguna untuk membangunkan platform mampan dan sesuai untuk pengeluaran beras wangi yang berkualiti tinggi.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
°C	:	Degree Celsius
2AP	:	2-Acetyl-1-pyrroline
A	:	Absorbance
ANOVA	:	Analysis of variance
BADH	:	Betaine aldehyde dehydrogenase
bet-ald	:	Betaine aldehyde
bp	:	Base pair
cDNA	:	Complimentary DNA
cm	:	Centimeter
cM	:	Centimorgan
CO_2	:	Carbon Dioxide
C_T	:	Threshold cycle
d	:	Day
DNA	:	Deoxyribonucleic acid
F value	:	F distribution value
g	:	Gram
GABA	:	γ-aminobutyric acid
GABald	:	γ-aminobutyraldehyde
GC-FID	:	Gas Chromatography-Flame Ionization Detector
GC-MS	:	Gas Chromatography-Mass Spectrometry
GT	:	Gelatinization temperature
h	:	Hour
ha	:	Hectare

HCl	:	Hydrochloric acid
K	:	Potassium
Kg	:	Kilogram
KOH	:	Potassium hydroxide
KOME	:	Knowledge-based Oryza Molecular Biological Encyclopaedia
m	:	Meter
Mb	:	Megabyte
Mha	:	Million Hectare
min	:	Minute
ml	:	Milliliter
mM	:	Micromole
mRNA	:	Messenger RNA
MS	:	Mass spectrometry
Mt	:	Metric Tonne
MT	:	Million Tonne
N	:	Nitrogen
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
NCBI	:	National Centre for Biotechnology Information
ng	:	Nanogram
P	:	Phosphorus
p value	:	Probability value for sample
PCR	:	Polymerase Chain Reaction
ppb		Parts per billion
ppm	:	Parts per million
RAPD	:	Random Amplification of Polymorphic DNA

RFLP	:	Restriction fragment length polymorphism
RNA	:	Ribonucleic acid
rpm	:	Rotation per minute
RTqPCR	:	Real-Time Quantitative Polymerase Chain Reaction
S	:	Sulfur
SNP	:	Single nucleotide polymorphism
SSR	:	Simple sequence repeat
t	:	Tonne
T value	:	Student's t-distribution value
t/ha	:	Tonnes per hectare
V	:	Voltage
µl	:	Microliter

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Rice (*Oryza sativa* L.) is the most important cereal crop and the most widely consumed staple food for more than half of the world's population. Rice can be grown at altitude ranges from 3.048 m (10 feet) below the sea level to 3048 m (10,000 feet) above the sea level and at different parts of the world with latitude from 39°S South (Australia) to 50°N North (China). It grows at upland on mountain slopes, plain lands, rivers banks and wetland in valley bottoms. It is also produced in all the continents except Antarctica, with a total land area of 150 million hectares (Mha), approximate production of 573 million tonnes (MT) along with an average productivity of 3.88 tonnes per hectare (t/ha). Rice is the 3rd highest produced agricultural commodity worldwide after sugarcane and maize (FAOSTAT, 2012; Shamim, 2013).

Rice belongs to the genus *Oryza* under the tribe *Oryzeae* of the family Gramineae or Poaceae. The genus *Oryza* contains 22 wild species, and two cultivated species i.e. *Oryza sativa* (commonly known as Asian rice) cultivated worldwide, and *Oryza glaberrima* (known as African rice) grown in a limited area in West Africa. The Asian rice (*Oryza sativa*) also possesses two subspecies namely *Oryza sativa* L. subsp. *indica* originated in India and *Oryza sativa* L. subsp. *japonica* originated from the Eastern part of Asia. However, based on the isozyme loci, rice was classified into VI different sub-groups, and largely two types of rice (aromatic and non-aromatic) were distributed in those sub-groups (Glaszmann, 1987). Most of the rice varieties belong to different sub-group was non-aromatic while only a few varieties of Group I (*indica*) and VI (*japonica*) and almost all of the cultivars belong to Group V were aromatic including Jasmine and Basmati type rice (Singh et al., 2000a). Moreover, depend on the grain quality traits, aromatic rice was classified into three broad categories, namely, the

Basmati type, Jasmine type and non-Basmati/Jasmine type aromatic rice (Singh et al., 2000b). Basmati type aromatic rice is available in India and Pakistan, Jasmine type is produced in Thailand while non-Basmati/Jasmine type aromatic rice grown in most of the rice growing region such as Khao Dawk Mali and Siamati in Thailand, Bahara in Afganistan, Sadri in Iran, Della, Texamati and Kasmati in the USA (Singh et al., 2000a). Non-Basmati/Jasmine type aromatic rice can be very short, fine-grained and highly scented which may contain weak stem, prolong growth duration, low grain weight, and poor grain yield. Jasmine type aromatic rice is long grain rice which becomes sticky, express sweet flavor, moist and soft when cooked. Basmati type aromatic rice is also long grained but possesses a unique combination of grain quality, elongation, cooking and eating quality.

Aromatic rice is a small but important subgroup of rice which is highly regarded for their excellent grain quality and considered auspicious. Sekhar and Reddy (1982) mentioned that aromatic rice is considered as high-quality rice for its aromatic flavor, superior nutrient value, and better amino acid profile. They added that Basmati-370 (aromatic rice) contained higher lysine, leucine, phenylalanine, and methionine content compared to non-aromatic rice varieties. Recently, Haris (2013) found that aromatic rice contained lower levels of arsenic than non-aromatic rice. He suggested that switching to aromatic rice will not only reduce arsenic intake but will also help to increase (as much as 69%) intake of beneficial zinc and selenium elements.

The grain quality of aromatic rice influenced by genetic and environmental conditions, for example, the grain quality of Basmati type aromatic rice varieties best expressed only when it grew in the North-western region of the Indian sub-continent and Pakistan, but when it cultivated outside these traditional Basmati areas, the grains

did not possess the same quality (Shamim, 2013). It also said that Basmati rice was best grown and produced the best quality grains under warm, humid and valley-like environmental conditions. Thus, the grain quality of aromatic rice highly depends on environmental condition which may also be able to influence the end product at different levels by being present differently throughout the life cycle of the rice plant. Moldenhauer and Slaton (2001) classified the life cycle of rice plant into three different growth stages i.e. vegetative (germination to panicle initiation), reproductive (panicle initiation to flowering) and ripening (flowering to mature grain) stage. Shamim (2013) and Singh et al. (2000a) mentioned that different temperature condition at different growth stages was suitable for high-quality Basmati rice production. They stated that during vegetative growth stage, high humidity (70 to 80%) and temperature ranges of 25 to 35°C were favorable. At the initial flowering stage, bright and clear sunny day with a temperature range of 25 to 32°C were suitable. Comparatively, cooler night temperature (20 to 25°C) with moderate humidity and gentle wind velocity were observed to be necessary at flowering stage and ripening stage for proper grain and aroma development. However, an unusual rise of temperature during any one of these growth stages might affect growth and productivity of aromatic rice plants. The impact of increasing temperature could observe at a later phase of plant development, but it was a cumulative effect of different temperature present at various growth stages. Moreover, physiological changes of rice plant due to increasing temperature at vegetative and the flowering stage would be able to alter the grain filling stage and subsequently would affect ripening stage which might change the grain quality of aromatic rice (Shrivastava et al., 2012). Rohilla et al. (2000b) also stated that grain quality of aromatic rice is highly influenced by the temperature while critically low temperature (below 20°C) and high temperature (above 30°C) both are destructive and adversely affects grain quality. However, within the range of optimum temperature (20°C to 30°C) for rice growth and

development (Yoshida, 1981) the growth rate increase linearly until 25°C and after 26°C rice grain yield decrease progressively (Baker & Allen, 1993; Baker, 2004). In a study, Wang et al. (2014) stated that gene expression is also affected by temperature and they observed that 50.4% genes of rice expressed at 25°C while slightly fewer genes expressed at 30°C. Additionally, they found a significant number of genes present in ribosome pathway up-regulated at 25°C and suggested that translation were more robust at 25°C temperature. However, in the 38 rice producing countries of the world (FAOSTAT, 2012), the daily mean temperature ranges from 18°C to 35°C and in tropical countries like Malaysia where the day-night averages temperature ranges from 27°C to 29°C as ambient temperature (surrounding environmental temperature of rice growing area) can produce rice but fewer aromatic rice (Golam et al., 2010). Moreover, the present ambient temperature at most of the aromatic rice-growing region is already close to the maximum limit of optimum temperature for aromatic rice production. Furthermore, during the past 100 years, the global average air temperature has been increased by 0.74 ± 0.18°C which will likely be increased by 1.1 to 6.4°C throughout 21st century as stated in regional climate projections reports (4th Assessment Report) of the Intergovernmental Panel on Climate Change (Solomon et al., 2007). Therefore, searching for the places or the development of suitable environmental condition with appropriate temperature during growing season can widen the possibility of high-quality aromatic rice production.

Aroma of rice is controlled by a major gene known as *Badh2* gene which only express aroma at homozygous recessive condition (*badh2*/*badh2*). The aroma gene also known as *FGR*/*fgr* or *Badh2*/*badh2* or *OsBadh2*/*osbadh2* gene possessed 15 exons and 14 introns. The 8-bp (5´-GATTATGG-3´) deletion in exon 7 (Bradbury et al., 2005b) or the 7-bp (5´-CGGGCGC-3´) deletion in exon 2 (Shi et al., 2008) of aroma gene present

on chromosome 8 expressed popcorn-like aroma. Therefore, gene expression analysis of *badh2* gene using reverse transcription quantitative PCR (Real Time qPCR) could explore the influences of temperature on aroma gene (Kim et al., 2003). Moreover, the relative quantification method used in RTqPCR data analysis i.e. relative expression of a target gene compared to the control sample normalized by a reference gene could explain the fold changes of the gene of interest (Livak & Schmittgen, 2001). For this reason, gene expression analysis could be useful to study the effect of temperature on an aroma gene.

Biochemical analysis of aromatic rice showed that aroma quality of rice depends on the composition of volatile compounds in rice grain. Previously, Yajima et al. (1979) detected 114 volatile compounds, Jezussek et al. (2002) reported more than 200 volatile compounds and Yang et al. (2007) found more than 300 volatile compounds in aromatic and non-aromatic rice cultivar. Among the volatile compounds, 2-Acetyl-1-pyrroline (2AP), a potent volatile flavor component was considered as the main aroma compound of aromatic rice (Buttery et al., 1983; Widjaja et al., 1996; Yoshihashi et al., 2002). Chen et al. (2008) mentioned that homozygous recessive allele of *Badh2* gene (*badh2/ badh2)* induced the formation of 2AP and rice becomes aromatic. Hence, the quantitative analysis of 2AP can correlate aroma gene expression and phenotypic aroma of rice. To quantify 2AP content in aromatic and non-aromatic rice, most of the researchers (Buttery et al., 1988; Laksanalamai & Ilangantileke, 1993; Maraval et al., 2008; Tanchotikul & Hsieh, 1991; Yang et al., 2007) identified and characterized 2AP only on cooked rice while few researchers (Itani et al., 2004; Mahatheeranont et al., 2001; Vercellotti et al., 1988) quantified it in raw rice. However, qualitative analysis of volatile compound and quantitative analysis of 2AP in raw rice was more accurate but complicated than in cooked rice. Later on, Mahatheeranont et al. (2001) stated a simple

solvent extraction method for isolation of 2AP along with other volatile compounds from uncooked brown rice. This solvent extraction method could be used to identify volatile compounds and quantify 2AP in freshly harvested raw rice without changes of chemical composition and aroma quality of rice grains.

Most of the aromatic rice varieties demonstrated lower agronomic performance and associated with undesirable agronomic characters, such as low yield, poor grain quality, lodging, and seed shattering. Therefore, breeders wish to develop high yielding aromatic rice varieties with superior grain quality (Berner & Hoff, 1986). However, production of improved aromatic rice variety with the better agronomic performance was observed to be a difficult task for rice breeder because it related to genotypic constitution of rice varieties and influences of growing environment. Environmental factors might affect the agronomic performance of aromatic rice by affecting agronomic traits, aroma quality and finally the grain yield. Rice grain yield is also a complex character which depends on several agronomic characters such as days to flowering, days to maturity, grain filling period, the number of fertile tillers, plant height, the number of fertile grain per panicle, panicle length, 1000 grain weight, and grain yield per plant (Halil & Necmi, 2005). Several researchers pointed out that crop yield should increase to meet the global food demand and nourishment of the increasing population. Moreover, several studies (Jagadish et al., 2010; Nagarajan et al., 2010; Nguyen, 2005; Peng et al., 2004) stated that the production and distribution of aromatic rice into different parts of the world would be affected by the global climate changes. Considering the potential impact of climate change on aromatic rice production, development of appropriate strategies for adaptation and mitigation of sustainable aromatic rice production for long-term food security is an important task. The adaptation of aromatic rice production in changing climate involves adjustments of

some important factors to decrease the vulnerability of rice production while mitigation will focus on the reduction of greenhouse gasses from rice production purpose. The scientists are trying to develop different types of technological systems which can be used shortly for enhancing aromatic rice production with the ability to adapt and mitigate the effects of global climate changes.

Recently, a technology for protected agriculture has been developed where the facilities of greenhouses and natural sunlight could utilize by controlling several environmental factors (temperature, humidity, CO_2 concentration, etc.). This technology is known as plant factory which is being used in Asia especially in Japan for commercial production of leaf vegetables. The yield of tomato plants has also increased by using this technology in Netherlands as well as in another part of the Europe. The concept of plant factory is a new facility to grow plants under the controlled environment which are computer based, can ensure automated control of optimum plant growth conditions including temperature, light, CO_2 and nutrient source. A plant factory is a food production technology where the labor-saving devices used and the crops remain unaffected by adverse weather condition. Moreover, it can be used to produce high-quality plants with high nutritive values by controlling nutrient solution with hydroponic cultivation (Kozai, 2013; Yamori et al., 2014). Though the possibility of rice production in plant factories is under consideration (cost-benefit analysis) but theoretically, by using natural sunlight and supplemental light, aromatic rice can be grown more than 2-3 times per year which can minimize production cost and increase production of aromatic rice. Moreover, in plant factories optimum temperature can be controlled. So, investigation of suitable temperature and other necessary components for production of aromatic rice is important.

Previous researchers have studied the effects of cold stress (Ghadirnezhad & Fallah, 2014), high-temperature stress (Aghamolki et al., 2014; Islam, 2011; Rani & Maragatham, 2013; Shrivastava et al., 2012), salinity stress (Fitzgerald et al., 2008; Gay et al., 2010; Wijerathna et al., 2014), accelerated ageing condition (Pisithkul et al., 2010) and shading (Mo et al., 2015) on some agronomic traits, volatile profiles, and the 2AP concentration of aromatic rice varieties. Some researchers investigated the genetic and molecular basis of aroma (Sakthivel et al., 2009) as well as the genetic and environment interaction on yield performance (Wirnas et al., 2015). They suggested that selection of superior aromatic rice genotypes would facilitate better aromatic rice production in changing environmental condition. Some researchers determined the critical temperature for different growth stages including low, high and optimum temperatures for proper development of rice plant (Yoshida, 1978). Till now, no comprehensive study was conducted to evaluate the effect of temperature on aroma of aromatic rice by phenotypic analysis (organoleptic test), molecular analysis (aroma gene expression), biochemical analysis (GC-MS analysis) as well as agronomic performance analysis. Such types of investigation are necessary to produce aromatic rice in the places where the optimum temperature can be obtained during rice growing season and in the plant factories where environmental condition can be controlled. These facilities could be used to grow aromatic rice all over the world to satisfy nutritive food demands for the increasing population.

Based on the above information, the aims of this study were to evaluate suitable temperature for better agronomic performance, aroma gene expression, and high-quality aroma rice which eventually ensure good quality aromatic rice production in the changing climatic condition.

To achieve these aims the following objectives were considered:

- To observe the effects of temperature on the morpho-agronomic performance of aromatic and non-aromatic rice.
- To analyze the sequence of *Badh2/badh2* gene for getting information about the genetic constitution of aromatic rice.
- To evaluate the effects of temperature on aroma gene expression at different growth stages of aromatic rice.
- To evaluate the effects of temperature on volatile aroma compounds for analyzing aroma characteristics of aromatic rice.
- To analyze the concentration of 2AP that represents aroma status in aromatic rice.

The gathered information will help to develop a sustainable and suitable platform for the production of high-quality aromatic rice under different temperature conditions.

In this thesis, an introduction of the research work has been described in Chapter 1 and further extention of current information with the literature review presented in Chapter 2 which described the importance and comprehensive description of the study. The morpho-agronomic performance of five aromatic and a non-aromatic rice genotypes were evaluated under three temperature conditions (ambient or 28°C, 25°C and 20°C) to observe the effects of temperature on yield and yield components of aromatic rice varieties at different growth stages (Chapter 3). The sequence of dominant *Badh2* gene (present in non-aromatic genotype) and recessive *badh2* gene (present in aromatic genotypes) was analyzed to assess the possible genetic reason for the aromatic

condition of the studied genotypes (Chapter 4). Moreover, the relative expressions of aroma gene (*Badh2*/*badh2*) were examined at different growth stages using RTqPCR to select appropriate growth stage for the expression of aroma gene (Chapter 5). In addition, volatile compounds were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) and the concentration of 2AP was quantified by Gas Chromatography-Flame Ionization Detector (GC-FID) to evaluate the effects of temperature on volatile profile (Chapter 6). Hence, after careful discussion of the findings (Chapter 7) some conclusions were drawn with recommendations (Chapter 8) for future work.

CHAPTER 2: LITERATURE REVIEW

Rice is such an important food crop in the world that the Food and Agriculture Organization (FAO) of the United Nations had declared 1966 as the Year of Rice. Moreover, the year 2004 was also declared as the International Year of Rice by the United Nations General Assembly (UNGA). Rice is the only food crop that honored twice by the United Nations as a special tribute to rice as an important element of food security. Besides being an essential food, rice is also an important factor for enriching culture, lifestyles, social status, economic development, political stability, global unity and functional ecosystem which lead to detail investigation about rice (Shamim, 2013).

2.1 Taxonomy and Origin of Rice

The morphology, physiology, agronomy, genetics, biochemistry and taxonomy of rice has intensely studied for a long time, and more than 40,000 varieties of rice had reported worldwide (Tripathi et al., 2011). The taxonomic positions of cultivated rice (*Oryza sativa* L.) as below:

Kingdom	Plantae
Division	Magnoliophyta
Class	Liliopsida
Order	Poales
Family	Gramineae or Poaceae
Tribe	Oryzeae
Genus	*Oryza*
Species	*sativa*

Rice belongs to the genus *Oryza* and the tribe Oryzeae which contain 12 genera including *Oryza*. Morishima and Oka (1960) suggested that the genus *Oryza* could be

divided into three broad groups such as *O. sativa* and its relatives, *O. officinalis* and its relatives and the other more distantly related species. The genus *Oryza* has been observed to contain 24 recognized species, among them were 22 wild species and 2 cultivated species namely *O. sativa* and *O. glaberrima*, which derived from perennial wild progenitors *Oryza rufipogan* and *Oryza longistaminata*, respectively (Sanchez et al., 2013; Vaughan et al., 2003). Between the two cultivated species, *O. sativa* is the most widely grown species worldwide including in Asian, North and South American, European Union, Middle Eastern and African countries while *O. glaberrima* is grown solely in West African countries. Based on the multivariate analysis of allelic variation at 15 isozyme loci present in 1,688 rice varieties collected from different countries, Glaszmann (1987) stated that 95 percent of the varieties fell into six distinct groups while the remaining 5 percent scattered over intermediate positions. This classification did not consider morphological data of the varietal group, but when the morphological traits of these six groups compared with the varietal groups classified by morphological characters, Group I corresponded to the *indica*, Groups II, III, IV, and V were atypical but were also classified as *indicas* in the conventional classification. The Group V included the aromatic rice (Basmati) from the Indian subcontinent, and Group VI contained the *japonica* rice. The Group VI also included the *bulu* and *gundil* varieties formerly classified into *javanicas*. However, Group I, V, and VI were observed to contain aromatic rice while most of the rice variety belong to Group V were aromatic included Basmati, Ambemohar, Kataribhog, Hansraj, Barah, Lawangin, Sadri, Badshahbhog, Prasadbhog, Tulsimanjri, Bindli, Nama Tha Lay etc. These aromatic rice genotypes demonstrated excellent lengthwise elongation after cooking. Recent molecular characterization of Basmati type rice revealed that it was relatively closer to the *japonica* group than the *indica* group (Garris et al., 2005; Kovach et al., 2009). Besides, Nagaraju et al. (2002) studied genetic relationship using simple sequence

repeats (SSR) and fluorescent labeled inter-SSR-PCR (FISSR) primers and mentioned that aromatic rice could be three types as traditional Basmati (TB), evolved Basmati (EB) and non-Basmati (NB) varieties where traditional Basmati was distinctly different from non-Basmati rice (Fig. 2.1).

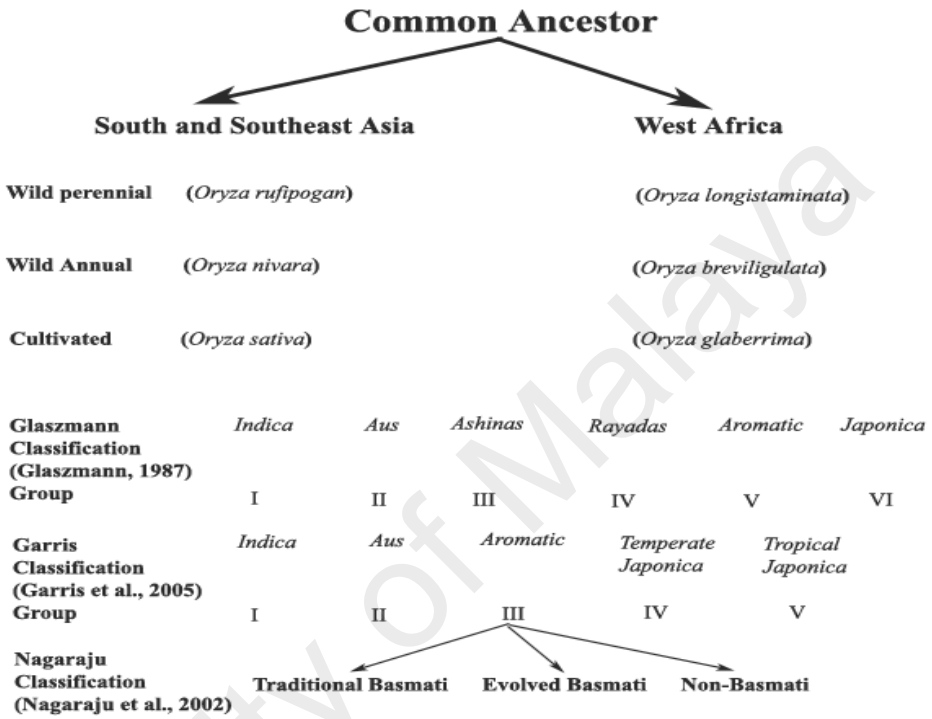

Figure 2.1: Classification and evolutionary pathway of aromatic rice.

Source: Siddiq et al. (2012)

The center of origin of aromatic rice was in the foothills of the Himalayas in Uttar Pradesh and Bihar in India and Tarai region of Nepal where many aromatic cultivars are still grown. The aromatic rice varieties were then spread northwestward to Punjab in India and Pakistan, Afghanistan, Iran, and Iraq, northeastward to Bangladesh and Myanmar and the Indian states of Orissa, Bengal, Assam, and Manipur. The westward distribution occurred to other states of India such as Rajasthan, Madhya Pradesh, Maharastra and Gujarat while the easternmost limits of aromatic rice were Myanmar (Singh et al., 2000a). Now, the aromatic rice has been introduced into the

global market, and aromatic rice from India and Pakistan consists of Basmati types, from Thailand Jasmine types and other countries non-Basmati/Jasmine type aromatic rice (Singh et al., 2000a).

2.2 Morphology, Floral Biology and Growth Stages of Rice

The information on morphology, floral biology, and growth stages of rice including structural and functional aspects is considered as an essential part for plant breeders to plan and execute breeding strategies as well as to evaluate the changes due to experimental treatments. The inflorescence of rice formed on panicle which is the top part of a rice plant. The inflorescence consists of single floret known as spikelet which born on pedicels (Fig. 2.2) of secondary branches. The secondary branches born on primary branches originated from the central axis of the panicle. The spikelet consists of lemma and palea which enclose the sexual organs i.e. six stamens arranged in whorls and a pistil at the center. The stamen consists of bilobed anthers borne on slender filaments while the pistil consists of an ovary, style and feathery bifid stigma (Chang & Bardenas, 1965).

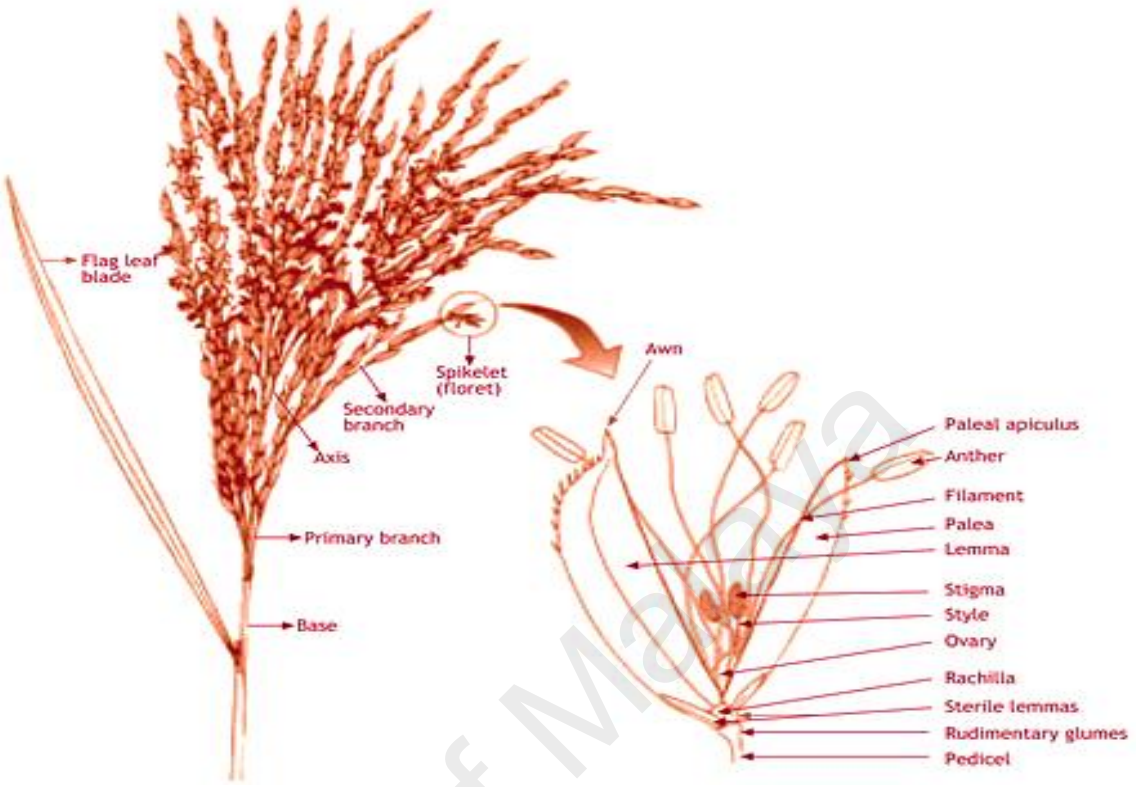

Figure 2.2: Parts of spikelet of the rice plant.

Source: Ricepedia (Partnership, 2013), Chang and Bardenas (1965)

The reproductive phase of rice starts with panicle initiation followed by panicle emergence and anthesis (spikelet opening and dehiscence of anther). During anthesis, lemma and palea get separate, filaments of stamens elongate and protrude out so that anthers dehisce for releasing the pollen. This process greatly influenced by weather conditions. Under the favorable condition, all the spikelets on a panicle complete their flowering stage after fertilization and ovary start to develop into a caryopsis.

However, the growth phase of the rice plant could be divided into three stages namely; (a) vegetative stage, (b) reproductive stage and (c) ripening stage (IRRI, 2002; Moldenhauer & Slaton, 2001) described as follows:

(a) ***Vegetative stage***

The vegetative phase of the rice plant begins with seed germination and emergence of the radicle or coleoptile from the germinating embryo. This stage continues to the pre-tillering stage during which seminal and lateral roots and the first few leaves develop. Then the tillering stage is started with the appearance of the first tiller from the axillary bud in one of the lowermost nodes. The tiller number increases in a continuous process as a sigmoid curve until the maximum tiller number reached. The visible elongation of lower internodes may begin considerably earlier than the reproductive phase or at about the same time.

(b) ***Reproductive stage***

The reproductive stage of rice starts with the initiation of panicle primordium at the tip of the growing shoot. This phase might begin before the maximum tiller number achieved. During this stage, development of panicle remains to continue, and the young panicle primordium becomes visible as a hyaline structure with a fuzzed tip and the developing spikelets become distinguishable. The increasing young panicle becomes detectable inside upper leaf sheaths with upward extension as a bulge in the rapidly elongating culm known as the booting phase. As the auricles of the flag leaf become directly opposite to the auricles of the next lower leaf, then the meiosis occur in the microsporocytes (pollen mother cells) of the panicle. After this step, the panicle emerges from the flag leaf sheath and is called heading or flowering phase. The anthesis of rice flower begins with the protrusion of the first dehiscing anthers in the terminal spikelets on the panicle branches. The pollination, fertilization, development of fertilized egg and formation of endosperm becomes visible at the reproductive stage.

(c) ***Ripening stage***

The ripening stage is the last stage of grain development, and this is a continuous process which can explain by some agronomic terms such as milk stage, soft dough stage, hard dough stage and fully ripe stage. During this stage, individual grain becomes mature, fully developed, hardened and turns yellow. As the grains ripe, the leaves become senescent and turn yellowish in ascending order. The rice grain collected at the end of the ripening stage is known as the harvesting phase.

2.3 Description and Global Significance of Aromatic Rice

Rice (*Oryza sativa* L.) is a monocot plant, belongs to the Gramineae family, an annual grass and one of the most important cereal crops. Based on the presence of aroma, rice cultivars are classified as aromatic and non-aromatic genotypes (Lang & Buu, 2008). The preference of different types of rice depends on different cultures and countries.

Aromatic rice which possesses pandan like flavor is highly desirable in many countries, especially in Asian countries. In Asian countries, sometimes pandan leaves extracts are added to several dishes to increase flavor. Aromatic rice perceives as premium quality in many rice consuming countries where consumer preferences are also different among countries. The consumers of Middle Eastern countries prefer long grain, well-milled rice with strong aroma while European consumers prefer long grain rice without aroma. Recent studies indicated that the European consumers, particularly in the U.K., started to choose Basmati-type aromatic rice which may spread throughout the Europe due to increasing number of immigrants from far-east countries and the rising interest as in ethnic cuisine (Ferrero & Nguyen, 2004). Among the Asian

consumer, Chinese consumers prefer semi-aromatic rice than pure aromatic rice and Chinese but Hong Kong consumers prefer Jasmine-type rice (Singh et al., 2000b). The Indonesian consumers prefer local aromatic rice (Damardjati & Oka, 1992) while the Philippines consumers do not have preferences to aroma (Abansi et al., 1992). The Indian consumers give highest preference to aroma followed by taste and elongation after cooking of aromatic rice. The consumers of rice eating countries demonstrated higher preferences for Jasmine-type rice than non-rice eating countries (Suwannaporn & Linnemann, 2008). The U.S. and the Canadian consumers have strong preferences for long grain and Jasmine-type rice while the Asian American consumers prefer imported Jasmine-type rice compared to American-grown aromatic rice (Suwansri et al., 2002). Recent studies indicated that the Indian, Pakistani and Turkish people live in Europe prefer aroma from Basmati-type rice while the Asian consumers live in North America addresses the sensory preferences of Jasmine-type rice (Suwansri et al., 2002).

The demand, production, and availability of rice affect the prices of the agricultural commodity as well as in rice trading but the prices of aromatic rice did not decrease after the peak in the spring of 2008. Still, it has remained the highest priced sector of the world rice market (FAO et al., 2012). The rice trading is low (only 7.13%) compared to the total rice production while aromatic rice (mainly Basmati-type and Jasmine-type) alone is 15-18% of the worldwide rice trading. Aromatic rice is considered marginal in global rice trade because of largely ignored in well-documented overviews of rice marketing (Baldwin & Childs, 2011; FAO et al., 2012; Young & Wailes, 2003). However, the Basmati rice trading increased from 5.2% to 8.3% in 2003 to 2008 respectively, in worldwide rice trading. In 2008, the India has a share of 84.9% and Pakistan has 68.5% of Basmati-type and Thailand has 51.7% of Jasmine-type rice trading worldwide. In 2010, India exported 1.8 Million Tonnes (MT), Pakistan 1.05

MT, Thailand 2.65 MT and Vietnam exported 0.24 MT of aromatic rice (Slayton & Muniroth, 2011). These total exports of aromatic rice represent 5.7 MT of 18.3% of the global rice trade. In 2011, globally the total rice production was 481.0 MT (Table 2.1) and total export was 34.3 MT which was 7.1% of the total rice trade where only aromatic rice was 16.6% (FAO et al., 2012).

Table 2.1: Information of total rice production and aromatic rice exports worldwide.

October 2011	Production (Million Ton)	Export (Million Ton)	Aromatic rice exports (Million Ton)
China	138.0	0.8	-
India	103.0	5.0	1.8
Pakistan	6.5	3.0	1.05
Thailand	21.2	8.5	2.65
USA	6.0	3.1	-
Vietnam	28.0	7.3	0.24
Others	178.3	6.6	-
World	481.0	34.3	5.7

Source: FAO et al. (2012)

Usually, the Basmati-type rice exported to Saudi Arabia, Kuwait, Union of the Arab Emirates and the USA whereas the Jasmine-type rice shipped to China, Hong-Kong, Singapore, USA and EU (Slayton & Muniroth, 2011). Thus, the aromatic rice plays a vital role in international rice trading.

2.4 Global Production of Aromatic Rice

Rice is the staple food, and more than half of the people on the globe depend on rice as their basic diet as well as a source of nutrition and extensively consumed in the rice producing countries (Jamal et al., 2009; Vaughan et al., 2003). The world population will increase about 2 billion over the next two decades and the demand for

rice will be a top priority (Gregory et al., 1999; Sasaki, 2002). To feed this increasing population about 35% more than the present level of rice production will be required globally (Duwayri et al., 2000). This phenomenon provides an avenue for an increased production of rice to keep pace with the growing population despite its productivity might affect by biotic and abiotic stresses (Zafar et al., 2004). However, recent rice breeding programs included the aim of improving traits to cope with both biotic stresses and abiotic stresses including heat tolerant that become increasingly prominent due to the global warming problems. Besides, both the rice producing and exporting countries are facing competition from stringent trade regulations and changes in consumer's preferences for higher quality rice. So, a new development of strategy in rice breeding program is to emphasize on improving grain quality due to high market price and increasing demand for aromatic, low amylose-containing and nutrient-enriched rice. Though, the market size of high-quality rice is smaller than regular rice but requires high value and more income for farmers. Among all quality attributes of rice, the aroma receives much more consideration in the breeding programs due to an increasing demand of aromatic rice in the importing countries (Napasintuwong, 2012).

The total rice production was estimated 721 Million Ton (MT) Worldwide while the milled rice was 481 MT and global rice trade was 34.3 MT on a milled basis during 2011 (FAO et al., 2012). Specific data related to aromatic rice production and trade is scarce though it comes mainly from three countries namely India, Pakistan and Thailand (Chaudhary et al., 2001). The USA started aromatic rice production in 1990 but no data available on the well-documented website of the United States Department of Agriculture (USDA). However, among the suppliers of the USA Rice Federation, 13 millers are providing aromatic rice globally. The rice trade experts stated that the aromatic rice from Vietnam and Cambodia exported to Thailand as coarse rice. The

same situation might occur during the export of aromatic rice from Nepal and Bangladesh to India.

The total aromatic rice production mainly depends on the production of Basmati rice in India and Pakistan along with Jasmine in Thailand. Jasmine-type rice known as Hom Mali or KDML 105 (Khao Dawk Mali) produced from the Isaan region in north-eastern Thailand (Rahman et al., 2009). The Hom Mali landrace released in 1959 but varietal development started during the 1980s through a governmental initiative for export purposes. As a result, Jasmine-type rice production increased by 74.0% from 1990 to 1998 and reached 28.3% of total rice production in Thailand (Rahman et al., 2009). In Pakistan, Basmati-type rice is produced mainly in the western Punjab region. A total of 91.2% of all Basmati-type rice produced in this area. An increase of 39.7% land and 32.8% yield regarding a total of 61.6% of land for rice and 50.3% of total rice production was observed in ten years in Pakistan on 2007. However, the yield of Basmati rice was lower with 1.7 Tonnes per Hectare (t/ha) in 2006 in western Punjab compared to 2.1 t/ha of all rice produced in Pakistan and 3.8 t/ha in the eastern Punjab region of India. In India, the total land used for Basmati-type rice production is unknown while production estimated from 4 to 7 MT based on different sources. Production of Basmati-type rice lead to 33.1% higher costs with 44.9% lower yields than non-Basmati rice in India (Uttaranchal) but the net price premium return was positive (+30.3%) for Basmati farmers (Singh et al., 2006a).

As the land areas for aromatic rice production become constant, so an increase in aromatic rice production depends on increasing yield and the substitution of aromatic rice instead of coarse rice. According to Mushtaq and Dawson (2002), the land area for Basmati rice production in Pakistan depends on environmental condition and irrigation

facilities, but not on price shocks (Singh, 2010). Though, the agricultural education helps farmers to use the best practices for rice production but the yield improvement mainly comes from genotypic selection and cross breeding. Hence, the agricultural development centers working on the improvement of aromatic rice yields by spreading crop areas and developing superior genotypes (Abedullah et al., 2007; Bashir et al., 2007; Rahman et al., 2009; Singh et al., 2006a).

2.5 Grain Quality of Aromatic Rice

Grain quality of rice is tough to define because the precision of preferences of rice grain quality varies from country to country and from region to region. The concept of grain quality of rice also varies based on the preparations for which the seeds will be used. Though, some of the grain quality traits of rice desired by the grower, miller, and consumer but each group may place different emphasis on various quality characteristics. The millers prefer a total recovery based on the proportion of head and broken rice upon milling while consumers consider the grain appearance, size, shape, cooking quality, taste, tenderness and flavor of cooked rice. The cooking quality preferences of rice also vary in different countries (Azeez & Shafi, 1966) and from one ethnic group to another ethnic group within a country or one geographical area to another as well as from country to country (Juliano et al., 1964). Kaosa-ard and Juliano (1991) stated that rice grain quality preferences also depend on the country and culture such as the Middle East consumers prefer long grain, well-milled rice with strong aroma while the European community prefers long grain but non-aromatic rice because the presence of aroma signals spoilage and contamination to them (Efferson, 1985). In West Africa, long grain aromatic rice used with sauces while short, and medium grain rice used in porridge mixed with sugar, salt and milk but the broken rice used as fried rice in

Senegal, Gambia and Mali. In general, the long grain aromatic rice required the greatest demand and the most expensive rice in local markets and international markets (Lang & Buu, 2008).

However, the grain quality of aromatic rice may be grouped as milling quality, cooking quality, grain size, shape, appearance, and aroma. Zhang and Yu (1999) mentioned that cooking and eating quality are the most important components of rice grain quality. So, the major components of cooking and eating quality of aromatic rice grains might be the aroma of cooked rice, grain size, and shape, grain appearance, cooked grain elongation, amylose content (AC), gelatinization temperature (GT) and gel consistency (GC) as described below:

Firstly, aroma is the most important quality trait of aromatic rice, and several chemical constituents are responsible for the aroma of cooked rice (Grosch & Schieberle, 1997). Previous researchers identified approximately 200 different types of volatile compounds in aromatic rice, but most of the researchers concluded that 2AP was the vital compound for aroma in aromatic rice. The volatile compound 2AP was observed to be associated with the flavor of a range of foods including popcorn (Schieberle, 1995), corn tortillas (Buttery & Ling, 1995), baguettes (Zehentbauer & Reineccius, 2002), ham (Carrapiso et al., 2002), cheese (Zehentbauer & Grosch, 1998) and green tea (Kumazawa & Masuda, 2002). The 2AP exhibited lower odor threshold level compared to other volatile compounds detected in aromatic rice varieties (Buttery & Ling, 1995). Buttery et al. (1988) was regarded as the first scientist who conducted a systematic study on the contribution of different volatiles to rice aroma in a long rice variety. Several other studies were also performed to assess the possible reason of aroma and to estimate the amount of 2AP in aromatic rice. They revealed that the field

location (Fushimi et al., 1996), the temperature during the ripening stage and drying process, the storage time and aging process affected the level of 2AP in aromatic rice (Laksanalamai & Ilangantileke, 1993; Widjaja et al., 1996). Weber et al. (2000) concluded that the pleasant odor of raw or cooked non-aromatic or aromatic rice controlled by a blend of various volatiles.

Secondly, preference of aromatic rice grain quality for grain size and shape of rice also varies from one group of consumers to another group (Cruz & Khush, 2000). Some ethnic groups prefer short bold grains while some prefer long medium grains and some other groups prefer long slender grains. Long grains preferred in the Indian subcontinent but medium to long rice favored in Southeast Asia. In temperate regions, short grain rice varieties are prevalent, but long grain rice had a high demand in the international market.

Thirdly, rice grain appearance depends on the size and shape of the kernel, translucency, and chalkiness of the grain. The physical dimensions of rice grains also play a vital for the consumers who work in the rice industry. Besides these quality traits, Sood et al. (1979) stated that linear elongation of the kernel on cooking is one of the major characteristics of aromatic rice. However, Rao et al. (1952) was identified a relation between amylose content and rice grain quality. The amylose content or amylose to amylopectin ratio was also observed to be the most important element that influences the cooking quality of rice (Bao et al., 2001). Besides, amylose content plays a crucial role to determine cooked rice texture. Seguchi et al. (2003) mentioned that though amylose, amylopectin, lipids, and proteins identified as the essential elements of starch granules but amylose plays an important role to maintain the structures of starch granules.

Finally, gelatinization temperature (GT) is a range of temperature where at least 90% of the starch granules swell irreversibly in hot water and loss crystallinity, and it also determines the cooking time (Cruz & Khush, 2000; Heda & Reddy, 1986). Ghosh and Govindaswamy (1972) declared that the cooking quality of rice greatly influenced by the gelatinization temperature. In another study, Tomar and Nanda (1985) stated that the GT played a significant role in the case of water uptake, volume expansion and kernel elongation of cooked rice. Several researchers (Heda & Reddy, 1984; Maningat & Juliano, 1978; McKenzie & Rutger, 1983) mentioned that the gelatinization temperature of the rice grain usually determined from a bulk sample and Golam (2004) stated that high air temperature after flowering stage increased gelatinization temperature (lowers grain quality) while low air temperature reduces it.

However, rice grain quality affects the nutritional and commercial value of aromatic rice. But the grain quality of aromatic rice is influenced by genotype, environment and their interaction (genotype and environment interaction) effects. Despite, rice grain quality improvement is the most important criterion for most of the rice breeding programs especially in aromatic rice variety selection and development program (Cruz & Khush, 2000).

2.6 Detection of Aroma in Rice

There are different techniques for detection of aroma in rice, and these techniques developed by several scientists around the world. Aroma detection by chewing technique of half of a single seed was developed by Berner and Hoff (1986) while chewing a few seeds was developed by Dhulappanavar (1976). Tasting individual

grain which was a preferred method for aroma assessment of aromatic rice varieties of Australian breeding program (Reinke et al., 1991) is still the principal technique for detecting aroma in many breeding programs worldwide. Nagaraju et al. (1975) were developed a simple technique to identify aroma from leaves where the leaves sample needs to boil in a water bath by keeping inside closed vials and smelling for aroma. This method was time-consuming, laborious in nature and unreliable for detection of aroma in green vegetative parts due to the strong chlorophyll smell. Sood and Siddiq (1978) developed a simple but rapid and reliable technique for determining the aroma from plant material by adding 1.7% KOH solution to plant samples in a petri-dish then assess the released aroma. Lorieux et al. (1996) and Widjaja et al. (1996) introduced a technique for identification of 2AP by using gas chromatography but it requires large samples and time consuming. Recently, the molecular markers such as single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs) which genetically linked to the aroma in rice, is being used to identify aromatic genotypes, is also an inexpensive, simple and rapid technique (Kibria et al., 2008). However, aroma of rice could be detected by organoleptic or sensory test, molecular marker analysis, and biochemical analysis as stated below:

2.6.1 Organoleptic or Sensory Test

The organoleptic or sensory test was first developed at IRRI (IRRI, 1971) to evaluate aroma in rice. According to this method, 1 g of freshly harvested milled rice was kept in a 50 ml centrifuge tube (round bottom). Then about 20 ml distilled water was added and the tubes covered with aluminum foil. The tubes placed in a boiling water bath for 10 minutes. The cooked samples were allowed to be cool, and the presence of aroma determined for every sample. The brown rice also used for aroma

detection but the cooking time increased to 30 minutes. Later on, Nagaraju et al. (1975) were tried to develop a simple technique to identify aroma from rice leaves. According to Nagaraju et al. (1975), 2 or 3 leaves were excised into 1 cm long pieces collected from individual plants and kept in corked vials. The vials then heated to 40 - 45°C for 5 minutes and aroma noted by smelling the contents of the vials. This procedure seems to be unreliable for the detection of aroma in green leaves due to the presence of potent chlorophyll content which reduced the precision of distinguishing aromatic from non-aromatic rice. Considering the drawbacks, a simple but rapid and reliable technique developed by Sood and Siddiq (1978) which had been found quite useful in detecting aroma in all plant parts except root. According to Sood and Siddiq (1978), approximately 2 g of sample (green leaf or stem) was cut into thin pieces and kept into small glass Petri-dishes containing 10 ml of 1.7% potassium hydroxide (KOH) solution. The Petri dishes were covered immediately after the addition of alkali and left at room temperature for about 10 minutes. The Petri dishes opened one by one, and the content in each petri dish smelt immediately. The samples scored as strongly aromatic, moderately aromatic, slightly aromatic and non-aromatic. All plant parts including leaf, stem (at all stages of development), ovary with stigma, anthers, husk and kernels (intact or ground) could be used for aroma determination by using this method. Though, organoleptic or sensory test have some limitations such as it might cause damage to the nasal passages, it might be not always reliable, it required trained panel during the processing of a vast number of samples (Reinke et al., 1991). However, it has been practiced by many researchers (Bounphanousay et al., 2008; Sarhadi et al., 2011; Yi et al., 2009) and until now it is being used as a standard protocol for aroma evaluation of rice samples (Golam et al., 2011; Yeap, 2012).

2.6.2 Molecular Analysis

The gene responsible for aroma in rice had been detected on chromosome 8 by both the qualitative and quantitative analysis of aroma (Lorieux et al., 1996) and the aroma gene was mapped at 4.5 cM away from RG28 marker (Ahn et al., 1992) but within 10 cM (Causse et al., 1994) or 12 cM from RG1 marker (Lorieux et al., 1996). Both the gene expression analysis and positional cloning experiments supported that the *fgr* or *badh2* or *osbadh2* gene was responsible for grain aroma and accumulation of 2AP (Vanavichit et al., 2008). Besides, the classical or Mendelian genetics and molecular genetics studies supported that the mutation in exon 2 or exon 7 was the basis of 2AP accumulation in aromatic rice (Vanavichit et al., 2008). The *badh2* gene identified as a member of the aldehyde dehydrogenase family and 8-bp deletion in exon 7 of this gene found in most of the aromatic rice. This deletion was observed to create a premature stop codon that leads to nonsense-mediated degradation of its mRNA leading to a loss of function of a complete gene but produced an aromatic phenotype. The RNA interference (RNAi) studies of aroma gene also showed that disruption of transcription of the mutated *badh2* gene led to an elevated level of 2AP in rice and increased aroma (Vanavichit et al., 2008). So, the molecular markers including SNPs and SSRs, which genetically linked to aroma could be used to select aromatic rice. The applications of molecular markers for aroma detection would be inexpensive, simple, rapid and required small amounts of tissue (Cordeiro et al., 2002). However, these markers were observed to be linked only with the aroma gene and could not discriminate aromatic with non-aromatic rice during recombinant stage (Bradbury et al., 2005b). In this situation, the availability of a complete rice genome sequence provided an opportunity to develop a specific molecular marker for the identification of aromatic genotype at recombinant condition by comparing the sequences of aromatic and non-aromatic

genotypes (Goff et al., 2002). Later on, Bradbury et al. (2005b) developed a perfect marker for aroma genotyping based on allele-specific amplification of *badh2* gene which encoding betaine aldehyde dehydrogenase 2 (BADH2) to distinguish aromatic with non-aromatic rice varieties. This method offers an additional advantage to select breeding population at an early stage compared to other methods which required seeds to produce for aroma analysis (Zeng et al., 2007). However, this multiplex marker system was observed to be complicated and tedious due to the use of more than one primer pair, weak amplification, inconsistencies and chances of non-specific amplification even in a slight difference in relative primer concentration. In addition, Amarawathi et al. (2008) reported that this multiplex marker system could not consistently discriminate the aroma allele present in the Pusa 1121 (Basmati type variety). Considering these inherent problems associated with the functional marker based on multiplex PCR, Sakthivel et al. (2009) developed a simple, co-dominant functional marker targeting the candidate gene for aroma. This functional co-dominant marker system could be used to discriminate aromatic and non-aromatic genotypes by using only 3.5% agarose gel system and be amenable to marker-assisted selection (MAS) used in case of large breeding materials (Rai et al., 2015).

2.6.3 Bio-Chemical Analysis

Biochemical analysis of a wide range of rice varieties represented that several compounds were different in aromatic and non-aromatic rice (Bradbury, 2009; Buttery et al., 1986; Widjaja et al., 1996). Buttery et al. (1983) mentioned that the 2AP was a primary chemical compound responsible for aroma in rice. They added that it was present in Jasmine and Basmati rice at low concentration which could be identified using a combination of sensory panels and gas chromatography techniques. The 2AP

was also detected in non-aromatic rice but at 10 to 100 time's lower concentration than that of aromatic rice (Buttery et al., 1986; Buttery et al., 1983; Widjaja et al., 1996). The detectable threshold concentration of 2AP was identified about 0.1 ppb in water (Buttery et al., 1983) while aromatic rice contained about 3000 times 2AP concentration and non-aromatic rice contained 30 times 2AP concentration (Buttery et al., 1986). However, a wide range of 2AP concentration was found in aromatic and non-aromatic rice due to the differences in extraction procedure, quantification method, and rice variety. The 2AP concentration was also influenced by cultivation practice and environmental factors such as temperature, salt and drought stress (Itani et al., 2004; Yoshihashi et al., 2004), harvest time and storage condition (Itani et al., 2004; Yoshihashi et al., 2005), milling property (Buttery et al., 1983), time and level of nitrogen fertilizer application (Wilkie et al., 2004) and ageing time (Faruq et al., 2015). The aroma of rice is very much similar to Pandanus flavor and in some Asian cultures dried Pandanus leaves are added to aromatic rice during cooking to get a higher aromatic flavor. Bryant and McClung (2011) characterized volatile profiles of nine rice cultivars before and after storage using solid phase microextraction (SPME) fibers in conjunction with the gas chromatography-mass spectrometry (GC-MS) and identified 93 volatile compounds among which 64 compounds had not been reported previously in rice. Laksanalamai and Ilangantileke (1993) detected 2AP at a low concentration in aged Khao Dawk Mali-105 rice and in pandan leaf using a steam distillation extraction method followed by gas chromatography but could not detect in non-aromatic rice. However, identification of 2-AP using gas chromatography is possible but requires large samples and is time-consuming when use steam distillation for extraction (Lorieux et al., 1996; Widjaja et al., 1996). Considering these problems, Mahatheeranont et al. (2001) developed indirect steam distillation technique where the volatile compound can be isolated from raw rice under reduced pressure and controlled temperature by

preventing cooking. They further simplified this technique by using a solvent extraction method to analyze the fresh extract by capillary gas chromatography-mass spectrometry (GC-MS) or capillary gas chromatographic system employing a flame ionization detector (GC-FID). Using solvent extraction procedure, the isolation of 2AP from uncooked brown rice samples without decomposition of 2AP by heating rice sample is possible (Mahatheeranont et al., 2001).

2.7 Factors Affecting Aromatic Rice Production

There was anticipation during the mid-1960s and in early 1970s that the technological changes and modern inputs might revolutionize rice yields. At the mid-1970s, it was clear that the environmental constraints were limiting rice yields and production (Yoshida et al., 1981). However, many factors have been identified that affects the aromatic rice production and are categorized into two broad factors i.e. biotic and abiotic factors. Among the abiotic factors the cultural factors (time and amount of nitrogen application, weeding, planting etc.) and environmental factors (temperature, humidity, Co_2 concentration, light etc.) are limiting aromatic rice production (Siebenmorgen et al., 2013). Studies on variability in environmental factors suggested that the environmental factors were the most important aspect for explaining the gap between yield potential of the modern rice and their average farm yields (Barker et al., 1979). Diversity and variability of the environment in different rice growing countries and at different rice growing regions within a country were the limitations for developing universal appropriate technology for a particular environmental factor (Herdt & Barker, 1977). Preparing a complete list and classify all the factors that limit rice production is difficult but Herdt and Barker (1977) reported a list of environmental factors that vary across sites, seasons and years (Table 2.2).

Table 2.2: Classification of environmental factors influences rice production.

Factor	Subfactor	Variable		
		Across Locations	Across Seasons	Across Years
Soil	Physical properties	√		
	Chemical properties	√		
	Biotic properties	√	√	
Geographic	Origin	√		
	Topography	√		
	Position	√		
	Photoperiod	√	√	
Water	Depth	√	√	√
	Rate of increase, decrease	√	√	√
Climatic	Temperature	√	√	
	Solar radiation	√	√	√
	Precipitation	√	√	√
	Air temperature	√	√	√
	Wind	√	√	√
Biotic	Competitive plants	√	√	√
	Dependent plants	√	√	√
	Insect predators	√	√	√
	Microbes	√	√	√

Source: Herdt and Barker (1977)

Among the factors listed in Table 2.2, the water temperature and air temperature are more important factors that affect aroma formation and retention in rice. Chakrabarti et al. (2010) stated that the effects of temperature were more pronounced in Basmati rice than non-Basmati rice. Besides, Yoshida et al. (1981) indicated that the extremely low or high temperatures normally below 20°C and above 30°C respectively, were destructive for plant growth and also vary from one growth stage to another (Table 2.3). These critical temperatures were observed to be different according to variety, duration of the critical temperature, diurnal changes and physiological status of the rice plant.

Table 2.3: Critical temperature for rice plant at different growth stages.

Growth stage	Critical temperature (°C)*		
	Low	High	Optimum
Germination	10	45	20-35
Seedling emergence and establishment	12-13	35	25-30
Rooting	16	35	25-28
Leaf elongation	7-12	45	31
Tillering	9-16	33	25-31
Initiation of panicle primordia	15	-	-
Panicle differentiation	15-20	38	-
Anthesis	22	35	30-33
Ripening	12-18	30	20-25

Adapted and modified from Yoshida and Hara (1977). * Refers to daily mean temperature except for germination.

Yoshida et al. (1981) observed that the pollen mother cells division decreased and the percentage of spikelet sterility increased below 20°C temperature. Besides, they noticed that the sterility rate decreased up to 12% when the temperature rose to 26°C. The optimum temperature which usually ranged from the critical low and high temperatures also affect grain yield and yield related traits such as tillering, spikelet formation and ripening. The effects of optimum temperature depend on physiological processes and rice variety. The temperature greatly influenced growth rate of rice plant just after germination and growth rate were increased almost linearly with increasing temperatures within a range of 22°–31°C. Similarly, respiration of rice plant increased with increasing temperatures up to 32°C above which it declined. At later stages (3–5 weeks after sowing) temperature slightly affected the tillering rate and the relative growth rate but during reproductive stage, the spikelet number per plant increased as the temperature dropped. Thus, the optimal temperature shifted from high to low as growth stage advanced from the vegetative to the reproductive stage (Yoshida et al., 1981). At the ripening stage, the temperature affects the weight per grain but the 1,000-grain weight almost constant under different environment and cultivation practice for the

same variety. An experiment under controlled temperature showed that the optimum daily mean temperature for grain filling stage range from 19°C to 25°C for *indica* rice and the length of the ripening stage inversely correlated with daily mean temperature.

The water temperature also found to affect the lowland rice which grown in flooded soils and varying water depths. If the rice plant remains under water before the initiation of panicle primordial, the growing points of leaves, tillers, panicles, growth and development of lowland rice will be affected by water temperature. After leaf elongation and plant height enlargement, both the air and water temperature affect the growth of rice plant presumably because of the existence of both underwater and aerial environment. As the growing panicles reached above the water surface, the effects of water temperature decrease and eventually air temperature becomes dominant in controlling panicle growth and maturity. Thus, the air and water temperature affects grain yield and yield components depending on the growth stages of rice (Yoshida et al., 1981).

Besides growth, development and yield, temperature also affects the grain quality traits of aromatic rice, particularly by being present at the time of flowering, grain filling, and maturity stage. The lower temperature during grain filling stage enhanced aroma formation. Similarly, a lower temperature (25°C and 21°C) during day and night at the ripening stage was observed optimum for better aroma retention (Mann, 1987).

However, aromatic rice cultivation is being practiced in two types of ecosystems i. e. upland or hills ecosystem and lowland or plain land ecosystem. Upland ecosystem is a low input system, use less amount of fertilizer, irrigation solely depends on natural

rain where higher production cost and prevalence of disease are the major problems for aromatic rice production. Lowland ecosystem also uses water from rainfall but use the necessary amount of fertilizer, control diseases, and pests, have the potentiality to produce more yields than the upland ecosystem (Adesina & Gaye, 1993). Sufficient rainfall during growth stages, adequate sunshine hours, and suitable temperature during grain filling periods provide favorable climatic conditions for the maximum development of grain quality traits in both ecosystems. These types of climatic conditions are also suitable for the development of pathogens, diseases prevalence, and insect pest infestation (Singh et al., 2006b). So, unfavorable biotic factor along with the adverse abiotic factors are the causes of a large yield gap and low-quality aromatic rice production. Thus, the main challenge for aromatic rice production worldwide is to find appropriate solutions for the major issues such as low temperature in the temperate region, high temperature in tropics, problems of water use efficiency, land constraints, major abiotic and biotic stresses, improvement of yield, reduction of yield gaps, improved rice grain quality, high production costs etc. Recently, in the aromatic rice breeding program, the adapted plant mechanisms is being considered to overcome some of the basic climatic and edaphic constrained for a better production. These two factors significantly reduced rice grain yield per unit area of about 49% of the global average. However, both biotic and abiotic factors are the main constraints for the production of aromatic rice worldwide (Tran, 1997).

2.8 Potentials for the Future Aromatic Rice Production

Characterization of morpho-physiological traits and evaluation of morpho-agronomic performances represents the overall performance of a particular variety towards the economic contribution and possibility of successful production (Riley et al.,

1995). However, the data for agronomic traits of aromatic rice requires large-scale experimental trials, high costs and extensive distribution of rice genotypes. Complete evaluation of agronomic data will open opportunities to accumulate desired features in a selected suitable rice variety (IRRI, 2002). Moreover, the characterization which explains a trait of an individual might carry out using morphological characters for superior variety selection. The agro-morphological traits also can be used for preliminary evaluation of genetic variability among phenotypically distinguishable cultivars. The morpho-agronomic performance may also be involved in the assessment of the suitability of a variety of various environmental conditions. A large number of aromatic rice germplasm exists all over the world and need to be characterized for using in breeding programs. Several of them have been reported possessing one or more excellent features which can be used as donors. However, from the ancient time, the aromatic rice is being considered as the food of choice for some peoples and the modern information technology improved its palatability and nutritional quality. Due to the increasing demand of aromatic rice in the domestic and international market, several countries such as the USA, Australia, Philippines, Vietnam, Japan, China, Bangladesh, India, Pakistan, and Thailand includes aromatic rice especially the Basmati rice in their varietal improvement program.

2.9 Genetic and Molecular Basis of Aroma in Rice

The genetic and molecular studies on aroma in rice become more authentic and explanatory after the availability of appropriate rice whole genome sequence which also opened new windows for identification and mapping of QTLs conferring aroma trait (Goff et al., 2002; Sarhadi et al., 2011). Genetic analysis of aroma character was conducted by several researchers and revealed that it was controlled by a recessive gene

and demonstrated monogenic inheritance (Table 2.4) though some researchers mentioned it as a polygenic or dominant complementary gene controlled trait (Amarawathi et al., 2008; Nayak & Acharya, 2004).

Table 2.4: Genetic information of aroma in rice.

Gene action	No. of gene	Reference
Dominant Complimentary	3	Nagaraju et al. (1975)
Dominant Complimentary	4	Dhulappanavar (1976)
Recessive	1	Sood and Siddiq (1978)
Dominant Complimentary	2	Tripathi and Rao (1979)
Dominant Complimentary	3	Reddy and Sathyanarayanaiah (1980)
Recessive	1	Berner and Hoff (1986)
Recessive, Inhibitory	1	Tsuzuki and Shimokawa (1990)
Recessive	1	Ahn et al. (1992)
Recessive	1	Ali et al. (1993)
Recessive	1 or 2	Pinson (1994)
Recessive	2	Vivekanandan and Giridharan (1994)
Recessive	1	Tragoonrung et al. (1996)
Polygenic	3	Lorieux et al. (1996)
Recessive	1	Garland et al. (2000)
Recessive	1	Cordeiro et al. (2002)
Recessive or Dominant, Complimentary	1 or 1 to 2	Nayak and Acharya (2004)
Dominant	1	Kuo et al. (2005)
Recessive	1	Dartey et al. (2006)
Recessive	2	Hien et al. (2006)
Dominant, Duplicate	1 or 2	Sarawgi and Bisne (2006)
Recessive	1	Sun et al. (2008)
Polygenic	3 QTLs	Amarawathi et al. (2008)
Recessive	1	Lang and Buu (2008)
Recessive	1	Niu et al. (2008)
Recessive	1	Sarhadi et al. (2009)
Recessive	1	Asante et al. (2010)
Dominant Suppression Epistasis Interaction	2	Chaut et al. (2010)
Recessive	1	Vazirzanjani et al. (2011)

It was evident that the aroma gene was a recessive gene known as *fgr* gene and demonstrated monogenic inheritance. The molecular mapping of the *fgr* gene with different markers had been reported by various investigators (Table 2.5) and most of them stated that the *fgr* locus was present on chromosome 8 in rice.

Table 2.5: Molecular markers and chromosome location for aroma allele.

Gene or QTL	Marker type	Chr. location	Reference
1	RFLP	8	Ahn et al. (1992)
1	RFLP	8	Yano et al. (1991)
1	RAPD	-	Tragoonrung et al. (1996)
1 major gene and 2QTLs	RFLP, STS	8, 4 and 12	Lorieux et al. (1996)
1	SSR	8	Garland et al. (2000)
1	SSR	8	Cordeiro et al. (2002)
1	SNP	8	Jin et al. (2003)
1	SSR	8	Bradbury et al. (2005b)
1	SSR, EST	8	Wanchana et al. (2005)
1	SSR	8	Chen et al. (2006)
1	SSR	8	Li et al. (2006)
2	SSR	-	Hien et al. (2006)
3 QTLs	SSR	3, 4 and 8	Amarawathi et al. (2008)
1	SSR	8	Sun et al. (2008)
1	SSR, RFLP, STS	8	Lang and Buu (2008)
1	SSR	8	Bradbury (2009)
1	SSR	8	Yi et al. (2009)
2	-	-	Chaut et al. (2010)

However, molecular marker is a powerful tool for studying the genetic models underlying different agronomic traits and Ahn et al. (1992) were the first investigators who combine the utilization of molecular markers with near-isogenic lines (NILS) to locate a gene controlling aroma in rice. They found that the aroma gene located at the end of a linkage group positioned at 4.5 cM (centimorgans) away from the restriction fragment length polymorphism (RFLP) marker RG 28 on chromosome 8 of rice. The first QTL analysis for aroma gene was performed on the core map of the whole genome revealed that only one "QTL" located on chromosome 8 with a peak between the markers RG 28 (RFLP)/Y5 (RAPD) and RG 1 (RFLP). This fragment was present in 10 cM away from the RG 28 marker (map distances calculated with MAPMAKER) with the maximum LOD score of 14.5 which could explain about 69% of the variance of the character (Lorieux et al., 1996; Lorieux et al., 1997). Thus, aroma gene considered as a major gene and the mapping effort concentrated on this linkage group. They used

sixteen markers for mapping of the chromosome 8 (Fig. 2.3) and found minimum two-point map LOD was greater than 10, except in between RG20 and A5J560 (LOD was 3.45). They further observed that the probe RG 28 which was found to be close to aroma (4.5 cM) which was not polymorphic with the six enzymes used for the map construction (Ahn et al., 1992). Moreover, using segregation analysis they located 2AP on chromosome 8 unambiguously between RG 28/Y5 and RG 1 (at 6.4 ± 2.6 and 5.3 ± 2.7 cM, respectively). The total length of the linkage map was 161.3 cM (estimated with MAPMAKER) which was same as 117.5 cM long using two-point map distance.

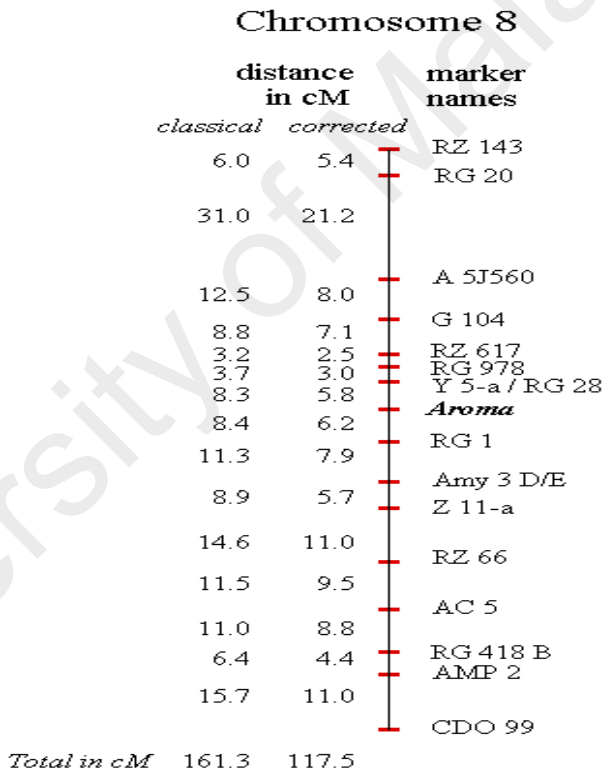

Figure 2.3: The genetic map of chromosome 8 representing the location of aroma gene. Source: Lorieux et al. (1997)

Therefore, the QTL mapping, fine mapping and complementation testing of aroma gene indicated that the *fgr* or *badh2* gene was a major gene for aroma and was present on chromosome 8 in rice (Bradbury et al., 2005a; Fitzgerald et al., 2010; Yi et al., 2009).

2.10 Rice Genome Sequencing

The International Rice Genome Sequencing Project (Project, 2005) has presented a map-based, finished quality sequence that covered 95% of the rice genome that contained 389 Mb genome size included all of the euchromatin and two complete centromeres. They also identified a total of 37,544 non-transposable element-related protein coding genes and found that the number and classes of transposable elements in the rice genome were consistent with the expansion of syntenic regions in the maize and sorghum genomes. Thay further added that the map-based sequence has proven useful for the identification of genes underlying agronomic traits. Moreover, the single-nucleotide polymorphisms (SNPs) along with simple sequence repeats (SSR) would be an added advantage to accelerate grain quality improvement in rice breeding program. The International Rice Genome Sequencing Project (Project, 2005), established in 1998 and pooled the resources of sequencing groups in ten Nations to obtain a finished quality sequence of the rice genome (*Oryza sativa* L. ssp. *Japonica* cv. Nipponbare). They declared that quality finished sequence might contain less than one error in 10,000 nucleotides, resolved ambiguities and made all state-of-the-art attempts to close gaps. They released a high-quality map-based draft sequence in December 2002 and as an immediate-release policy, the high-grade map-based sequence has been public for some time. The rice genome sequence has permitted rice geneticists to identify several genes underlying traits and revealed vast and previously unknown segmental duplications that comprise 60% of the genome. Goff et al. (2002) mentioned that the genome of the *japonica* subspecies of rice has been sequenced and assembled by whole-genome shotgun sequencing method. They also predicted that the number of genes on the assembled sequence might be 32,000 to 50,000 genes that can produce different protein or gene product.

2.11 Aroma Gene Discovery

There were conflicting observations regarding aroma gene in respect of the number of genes, nature of inheritance and genetic loci involved (Rani et al., 2006; Sakthivel et al., 2009). The conflicting reports suggested the possibility of several genes (either dominant or recessive) being responsible for aroma trait. However, several researchers (Bradbury, 2009; Cordeiro et al., 2002; Sakthivel et al., 2009) stated that contradictions between results might be due to variation in examined rice varieties, incorrect evaluation of the endospermic trait, presence of segregation distortion in backcross or in double haploid populations, and various approaches to aroma determination. Nevertheless, many researchers (Amarawathi et al., 2008; Jewel et al., 2011; Siddiq et al., 2012) stated that aroma gene was located on the long arm of rice chromosome 8 and codes for BADH enzyme. Kovach et al. (2009) and Shi et al. (2008) mentioned that two recessive alleles present in rice, one with an 8-bp deletion in exon 7 and three single nucleotide polymorphisms (SNPs) known as *badh2E7* and another with a 7-bp deletion in exon 2 of the *badh2* allele named as *badh2E2* (Fig. 2.4). They mentioned that both null *badh2* alleles contributed to rice flavor. However, they did not find any distinction between these two null *badh2* alleles about producing rice aroma and influencing its yield. Hence, it is possible that both can be employed in breeding for aromatic rice varieties.

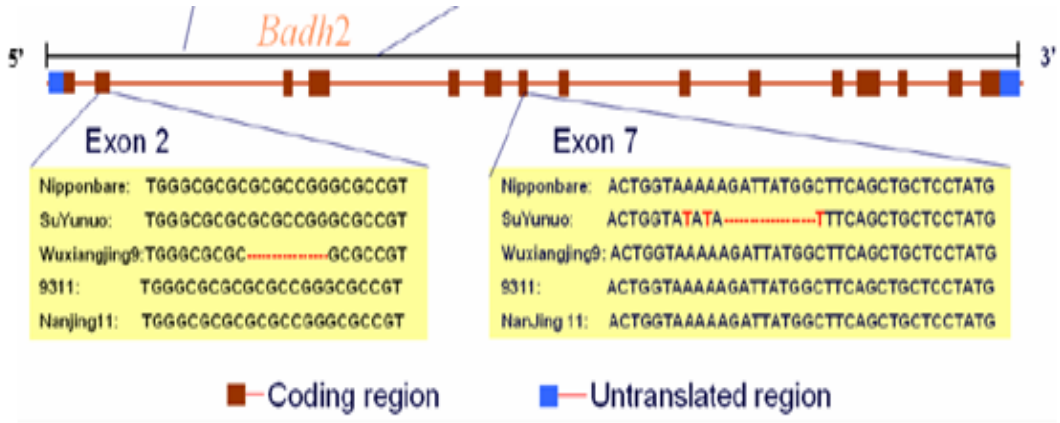

Figure 2.4: Characterization of nucleotide acid sequences of the *Badh2*, *badh2E2* and *badh2E7* alleles. Nucleotide sequence variations among the *Badh2*, *badh2E2*, and *badh2E7* alleles representing the functional *Badh2* allele which consists of 15 exons (brown boxes) and 14 introns (black lines) as in three non-fragrant varieties: Nipponbare, 93-11, and Nanjing11. Source: Chen et al. (2008)

The both deletions in *badh2* gene suggested that the event occurred after the divergence of aromatic and non-aromatic varieties from the common ancestor (Fig. 2.5). The functional *Badh2* converts AB-ald (presumed 2AP precursor) into GABA (4-amynobutyraldehyde) in non-aromatic rice and the non-functional *badh2* causes accumulation of AB-ald and thereby enhances 2AP biosynthesis in aromatic rice (Chen et al., 2008). A recent study by Kovach et al. (2009) suggested that Basmati cultivars were nearly identical to the ancestral *japonica* haplotype across 5.3 Mb regions. The flanking of the *badh2* gene region indicated that Basmati cultivars had a close evolutionary relationship with *japonica* varietal group.

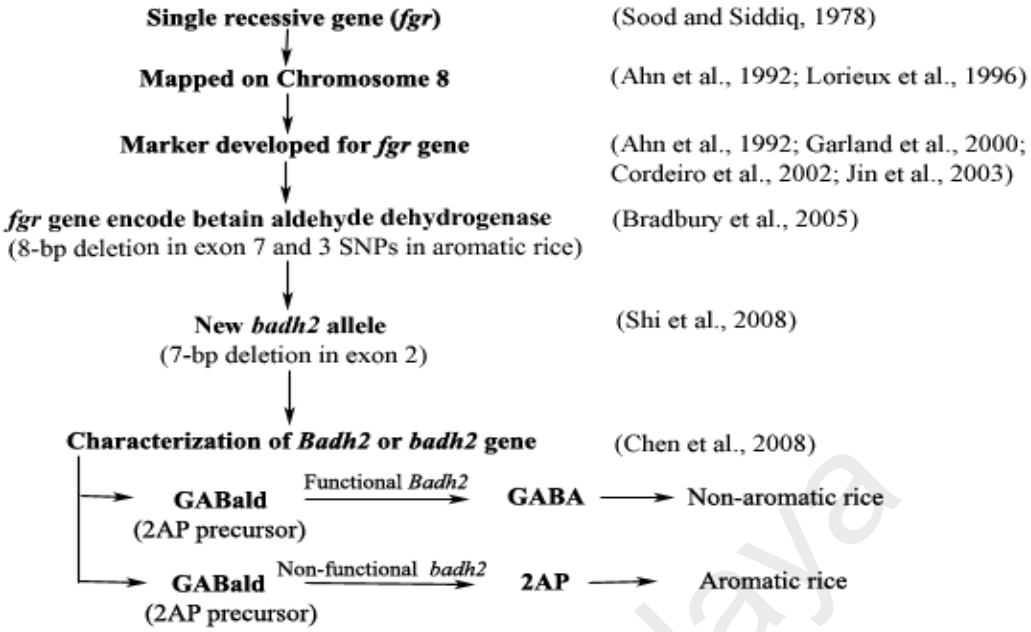

Figure 2.5: Genetics and mapping of gene governing aroma in rice.

Source: Siddiq et al. (2012)

However, a close relation among aromatic genotypes and sequence divergence of aroma gene makes it as an important aspect for in detail investigation. Moreover, aroma is an important grain quality trait but its expression, genetic reason, production pathway, and quality improvement are still under exploration. The sequence analysis of aroma gene in aromatic genotypes can provide some fundamental and possible reasons for the expression of aroma in rice.

2.12 Polypeptide Sequence of *badh2* Gene Product

The complete functional *Badh2* gene produced an intact 55-kD long BADH2 protein consisted of 503 amino acids in the non-aromatic rice varieties. The intact 503–amino acid containing BADH2 protein (encoded by the complete *Badh2* gene) inhibits 2AP synthesis and rice become non-aromatic. The *in vitro* expression of the

Badh2/*badh2E7* cDNAs resulted in an intact 503-amino acid polypeptide encoded by the complete *Badh2* cDNA, a partial 393-aminoacid polypeptide encoded by the partial *Badh2* cDNA, a truncated 251-amino acid polypeptide encoded by the complete *badh2E7* cDNA, and a truncated 141-amino acid polypeptide encoded by the partial *badh2E7* cDNA, respectively. The fusion proteins were 116 kD, 104 kD, 88 kD, and 76 kD, respectively which confirmed the production of appropriate proteins (Chen et al., 2008).

However, the complete *Badh2* gene inhibits 2AP synthesis in non-aromatic rice while partial recessive *badh2* allele accumulates 2AP in aromatic rice (Bradbury et al., 2005a). The aroma of a range of foods, including, popcorn (Schieberle, 1995), corn tortillas (Buttery & Ling, 1995), baguettes (Zehentbauer & Grosch, 1998), ham (Carrapiso et al., 2002), cheese (Zehentbauer & Reineccius, 2002), mung bean (Brahmachary & Ghosh, 2002), green tea (Kumazawa & Masuda, 2002), and wine (Herderich et al., 1995) also associated with the presence of 2AP. This compound is most closely associated with the aroma of Basmati rice (Buttery et al., 1983; Lorieux et al., 1996; Widjaja et al., 1996; Yoshihashi et al., 2002). Although many other compounds found in the headspace of aromatic rice varieties (Widjaja et al., 1996) possibly due to secondary effects related to the genetic background of the rice variety. The 2AP is widely known to be the primary cause of the distinctive Basmati and Jasmine type aroma. The desirability of aroma would result in strong human preference and selection for this trait. Non-aromatic rice varieties contain very low levels of 2AP while the levels in aromatic genotypes are much higher (Widjaja et al., 1996). A recessive gene, on chromosome 8 of rice, primarily controlling the level of 2AP, has been identified in genetic studies. Genetic markers for this gene have been developed to allow selection for this trait in rice breeding. The chromosomal location of the gene

defined by mapping in segregating populations using simple sequence repeat (SSR) or microsatellite (Cordeiro et al., 2002) and single nucleotide polymorphism (SNP) markers (Jin et al., 2003).

2.13 Biosynthetic Pathway of Aromatic Compound

The volatile compounds which express a unique flavor of aromatic rice could be present as a single predominant-compound or a complex mixture of both the volatile and semi-volatile compounds. Among over 300 volatile compounds identified in aromatic and non-aromatic rice, 2AP was considered as one of the most important aroma compounds in rice (Buttery et al., 1982). Though, L-proline was identified as a precursor of 2AP in rice (Vanavichit et al., 2005; Yoshihashi et al., 2002) but there were different views regarding the origin of pyrroline (Sakthivel et al., 2009). However, during the elucidation of the biosynthetic pathway of 2AP in rice Vanavichit et al. (2005) proposed that it might be synthesized through the polyamine pathway. They found 1-pyrroline (1P) which produced from 4-aminobutyraldehyde (AB-ald; the immediate precursor of 4-aminobutyric acid, GABA) was the immediate precursor of 2AP. Chen et al. (2008) mentioned AB-ald and its cyclic form Δ^1-pyrroline appeared to be an important factor for regulating the rate of 2AP biosynthesis. They suggested that the functional BADH2 enzyme (coded by the dominant aroma gene *Fgr*) inhibits 2AP biosynthesis in non-aromatic rice by converting AB-ald to GABA while the non-functional badh2 enzyme (coded by recessive aroma gene *fgr*) resulted in AB-ald accumulation leading to the formation of 2AP in aromatic rice. Bradbury et al. (2008) also suggested that accumulation and spontaneous cyclisation of γ-aminobutyraldehyde (GAB-ald) to form Δ^1-pyrroline due to a non-functional badh2 enzyme might be the cause of 2AP accumulation in aromatic rice. In another study, Huang et al. (2008)

concluded that the Δ^1-pyrroline-5-carboxylate, an immediate precursor of proline synthesized from glutamate was reacted directly with methylglyoxal to form 2AP and no direct role of BADH2 formation was observed. Until now, two pathways of 2AP biosynthesis in rice have been proposed i.e. *BADH2*-dependent 2AP synthesis pathway (Bradbury et al., 2008; Chen et al., 2008) and *BADH2*-independent 2AP synthesis pathway (Huang et al., 2008) presented in Figure 2.6.

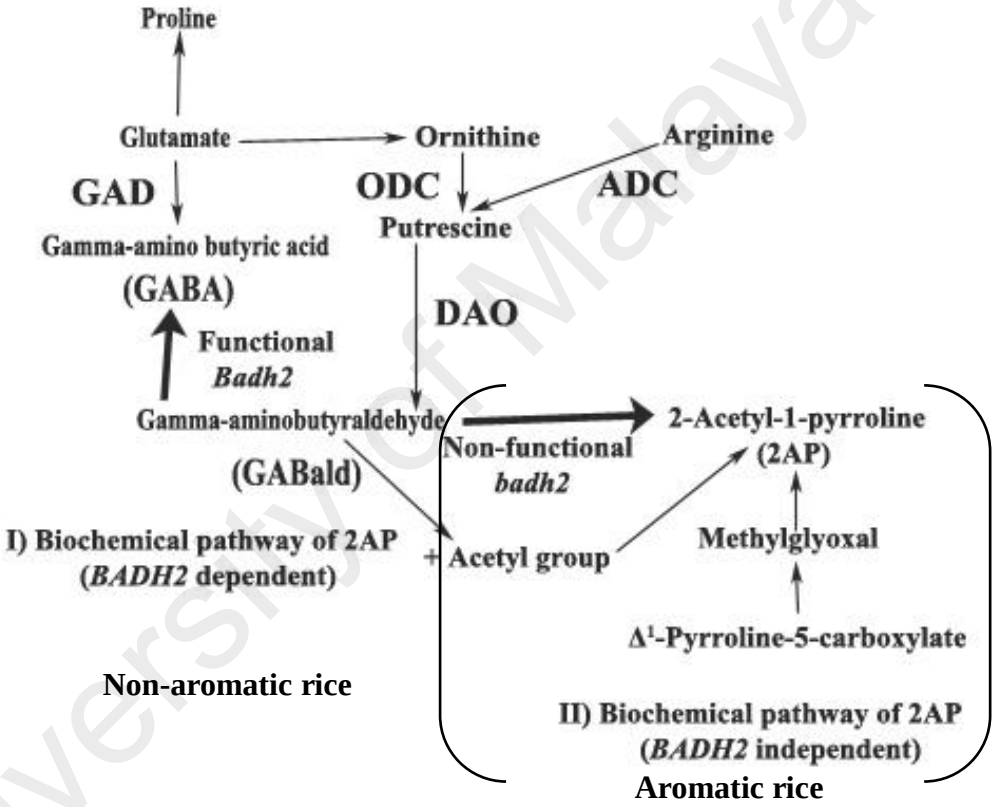

Figure 2.6: Biosynthetic pathway of 2AP in rice. Here, (I) Biochemical pathway of *BADH2* gene dependent (Bradbury et al., 2008; Chen et al., 2008), (II) Biochemical pathway of *BADH2* gene independent (Huang et al., 2008). Source: (Hashemi et al., 2013).

The *BADH2*-dependent pathway model suggested that functional *Badh2* inhibits the biosynthesis of 2AP in non-aromatic rice by converting GAB-ald to GABA whereas in aromatic rice truncated *badh2* results in the accumulation of GAB-ald, which then leads to the formation of 2AP. The *BADH2*-independent pathway models depend on Δ^1-pyrroline-5-carboxylate synthetase which catalyses the formation of Δ^1-pyrroline-5-

carboxylate. The Δ^1-pyrroline-5-carboxylate then reacts with methylglyoxal compound to form 2AP in the aromatic rice. Further investigation is required to reveal the 2AP formation pathway and the involvement of responsible enzyme which will connect the genetic, molecular and chemical aspect of aromatic rice.

2.14 Betaine Aldehyde Dehydrogenase and Plant Abiotic Stress Tolerance

There are several aromatic cultivars worldwide, but only a few of them have made it prestigious in the world market. The primary cause of this situation is that the aromatic rice varieties are susceptible to biotic and abiotic stress and produce significantly less grain yield than non-aromatic varieties. For example, the Basmati rice is susceptible to blast, bacterial leaf blight, stem borer, and white backed plant hopper. Jasmine rice is also susceptible to brown plant hopper, blast, and bacterial leaf blight. Both traditional Basmati rice and Jasmine rice are photosensitive and require short day length during flowering stage thus the harvest season limited to only one crop per annum. Another important reason is that the market for aromatic rice is highly competitive. Moreover, import regulations and technical trade barriers have made it difficult for newly developed aromatic rice (Fitzgerald et al., 2010; Singh et al., 2000a).

The abiotic stress tolerance of rice genotypes seems to be related to the both *Badh* gene homologues (*Badh1* and *Badh2*). The *Badh1* gene transcript exhibits a consistent increase in response to salt treatment in both aromatic and non-aromatic rice varieties (Fitzgerald et al., 2008). But the *badh2* gene transcript levels did not increase in salt treatment. RNAi analysis of the transgenic non-aromatic rice with inhibited expression of the *Badh2* gene confirms that the *badh2* gene expresses only in aromatic rice (Niu et al., 2008). The transgenic non-aromatic plants with inhibited *Badh2* gene

expression were shown to have reduced ability to tolerate salt stress, and they concluded that *Badh2* contributes to salt tolerance in rice. Recently, Fitzgerald et al. (2010) revealed that the non-aromatic rice lines with inhibited *Badh2* gene expression are more susceptible to salt stress than wild type *Badh2* gene expression. They also found that the aromatic rice lines produce a few mature seeds compared to non-aromatic rice lines during salt stress. When the aromatic rice lines exposed to 17 mM and 22 mM NaCl stress, the mature seed production decreased by 92% and 96.5%, respectively compared to non-aromatic rice lines. These results suggest that the *Badh2* gene has a role in salt tolerance and producing mature seeds.

Rice is a non-accumulator of glycine betaine and the BADH function correlated to the synthesis of GABA from GABald (Bradbury et al., 2008). Since then it was thought that the decrease in salt tolerance, low yield, and lacking of functional *Badh2* gene could be due to a reduced ability of aromatic plants to accumulate GABA. However, no significant difference in the concentration of GABA levels in aromatic and non-aromatic rice was reported by Fitzgerald et al. (2010). It is a challenge for the researchers worldwide to understand the mechanism of 2AP synthesis in aromatic rice and its correlation with reduced yield and susceptible nature to biotic and abiotic stress.

2.15 Betaine Aldehyde Dehydrogenase and Rice Aroma

The *fgr* locus of aromatic rice constitutes the *fgr* gene which responsible for the aroma of rice. The *Fgr* locus also possesses three candidate genes i.e. *Cah*, *Mccc2*, and *Badh2*, encoding putative eukaryotic-type carbonic anhydrase, 3-methylcrotonyl-CoA carboxylase β-chain, and betaine aldehyde dehydrogenase, respectively. Among these three genes, only *Badh2* gene significantly reduced 2AP content and rice become non-

aromatic. Previously, the *Fgr* gene corresponds to the non-aromatic character was mapped in the rice genomic region. So, the *badh2* represents the *fgr* and a non-functional allele either *badh2E7* or *badh2E2* required for aroma expression. The dominant allele (*Fgr* or *Badh2*) determine the non-aromatic status and the recessive allele (*fgr* or *badh2*) corresponds to the aromatic condition of rice. In aromatic rice, both non-functional *badh2E2* and *badh2E7* alleles demonstrated low transcription levels compared to the functional *Badh2* allele by quantitative real-time PCR (RTqPCR) and RNA gel blot analysis. The lower transcription level indicates that a loss of function due to mutations in *Badh2* gene suppress the mRNA transcription level. Hypothetically, the deletions in the full-length *Badh2* gene sequence causes frameshifts mutation and result in truncated BADH2 proteins. The 8-bp deletion in exon 7 produced a truncated 251 amino acid containing BADH protein while the complete *Badh2* cDNA resulted in an intact 503-amino acid containing BADH protein by *in vitro* expression. No truncated BADH2 proteins detected in the Wuxiangjing9 and Suyunuo aromatic rice cultivar using protein gel blot hybridization. However, the presence of truncated BADH2 protein suggests that the *badh2* alleles non-functional for transcription and translation rather than truncation of the BADH2 protein. The RACE analysis for determination of transcription start point represented that both the *Badh2* and *badh2* alleles produced shorter RACE products (59-RACE products) than expected. The quantitative real-time PCR (RTqPCR), real-time PCR (RT-PCR), and RNA gel blot analyses demonstrated that the complete *Badh2* transcript less abundant compared to partial *badh2* transcripts. The protein gel blots analysis showed that the mass of BADH2 protein is about 55 KD which encoded by the longest cDNA. The longest *Badh2* transcript which produced 503-amino acid containing longest BADH2 protein was present in non-aromatic rice. This longest protein demonstrated potent aldehyde dehydrogenase activity and broad substrate specificities in protein gel blot analysis. Another cereal crops such as wheat

(*Triticum aestivum*) and barley (*Hordeum vulgare*) also observed to encode full-length BADH proteins by the *Badh2* gene.

In an experiment, the abundant of partial *Badh2* transcripts and its role in the expression of the intact BADH2 protein assessed by transforming aromatic rice with different *Badh2* cDNAs and their genomic DNA segments. The overexpression of the complete *Badh2* gene resulted in low levels of BADH2 protein compared to the native *Badh2* gene. These analyses indicated that the full-length *Badh2* transcript did not result in more BADH2 protein, and the partial *Badh2* transcript itself cannot be translated into protein. The presence of abundant partial *Badh2* transcripts leads to high-efficiency translation of the complete *Badh2* transcript. The absence of intact BADH2 protein results in aroma which suggests that the *Badh2* is not directly involved in 2AP biosynthesis. Another possibility for explaining the effect of complete BADH2 protein is that the BADH2 enzyme involves in a competing pathway or participates in 2AP catabolism (Bradbury et al., 2005a). An alternative investigation showed that the BADH oxidization catalyzes not only the Bet-ald but also other substrates structurally similar to Bet-ald (3-dimethylsulfoniopropionaldehyde, AP-ald, and AB-ald) in sugar beet. The AB-ald maintained an equimolar ratio of D-1-pyrroline (immediate 2AP precursor) and AB-ald converted into 4-aminobutyric acid (GABA). The GABA found in the leaves of the aromatic isogenic line (in lower amounts) than the non-aromatic lines. So, consumption of AB-ald by converting it into GABA inhibits 2AP synthesis and the accumulation of AB-ald results in increased 2AP synthesis (Trossat et al., 1997).

In non-aromatic rice, the *Badh2* gene encodes intact BADH2 protein which possesses substantial AB-ald dehydrogenase activity to convert the AB-ald into GABA and to inhibit 2AP biosynthesis. A low level of BADH2 protein also detected in some

transgenic lines which explained that the *Badh2* might not completely inhibit the consumption of AB-ald and resulted in a small quantity of 2AP in non-aromatic rice line. Conversely, in aromatic rice, due to the absence of BADH2 enzymatic activity results in AB-ald accumulation which activates 2AP biosynthesis. Nakamura et al. (2001) stated that the Betaine is nontoxic, and protective cytoplasmic osmolyte allowed the normal growth of plants in a saline or arid environment. The Betaine synthesized in two-step oxidation of choline, and the BADH catalyzes the second step (from Bet-ald to betaine). In barley, two BADH isozymes (BBD1 and BBD2) reported to induced higher levels by salt, drought, and abscisic acid treatments. Similarly, in rice the BADH2 protein showed high betaine aldehyde dehydrogenase activity and the *Badh2* play a vital role in osmoregulation in non-aromatic rice. Nevertheless, the absence of intact BADH2 protein in aromatic rice did not negatively affect the normal growth of rice plant, such as the aromatic rice variety of Thailand named Khao Dawk Mali 105 grows well in the arid region of the Tung Kula Rong Hai (Bradbury et al., 2005a; Yoshihashi et al., 2002). This phenomenon suggests that another *Badh* genes present in the rice genome might compensate the defective null *badh2* alleles as well as allow tolerance to salinity and drought stresses. The *Badh1* gene present on chromosome 4 showed high homology with the *Badh1* genes in barley (*Hordeum vulgare*) and sorghum (*Sorghum bicolor*) and thought to play a significant role in stress tolerance (Bradbury et al., 2005a). So, the intact and 503–amino acid containing BADH2 protein encoded by the complete *Badh2* gene inhibits 2AP biosynthesis by converting AB-ald to GABA, while the absence of full BADH2 protein due to the non-functional *badh2* allele results in AB-ald accumulation and stimulate the 2AP biosynthesis.

2.16 *BADH* Gene Expression

The RNA interference (RNAi) technique and molecular analysis demonstrate that the down-regulation of the *badh2* transcripts in the transgenic plants results in significant elevation of 2AP production. Moreover, the extensive sequence analysis indicates that both the traditional and modern aromatic rice varieties with diverse origins possess the same mutant allele. The presence of same mutant allele reveals that the donor of the mutant allele in aromatic rice contains a single evolutionary origin (Bradbury et al., 2005a). The presence of the spontaneous mutant allele in all aromatic rice represents the capacity of rice plants to evolve phenotypic modifications in response to cultural preferences. The mutation might cause even before the rice domestication and disperse worldwide (Perozich et al., 1999). Several studies stated that the glycine betaine accumulation in rice plants was undetectable which indicated the possibility of functional defect resulted from an unusual post-transcriptional processing during choline mono-oxygenase or betaine aldehyde dehydrogenase activity for glycine betaine biosynthesis (Niu et al., 2007; Sophos & Vasiliou, 2003). In a study, Chen et al. (2008) stated that the down-regulated *Osbadh2* leads to reduce productivity which indicated the influences of this gene in crop performance. However, the recessive nature of aroma allele suggests that a loss of function of complete *Badh2* allele is responsible for the accumulation of aroma compound (Huang et al., 1995; Ren et al., 2004). They also mentioned that the beneficial effects of *osbadh2* gene suppression (homozygous condition) in aromatic rice production without adverse effects on crop performance could inhibit the accumulation of the functional *Osbadh2* mRNA by RNA interference (RNAi) specifically in rice grain. The accumulation of 2AP also observed during incorporation of the loss of function spontaneous mutant aromatic allele (*osbadh2*) into any one of the parental lines in hybrid rice. Besides, the vegetative growth of the

heterozygous F_1 plants from the hybrid rice varieties was not adversely affected and the endosperm homozygous for the mutant allele produced fertile grain with the accumulation of aroma compound as its aromatic parent.

2.17 Selection of Housekeeping Gene

The gene expression analysis is an important tool for functional genomic and proteomic studies of an organism. It is essential to normalize the target gene expression data with a suitable internal control gene which demonstrate uniform expression in that experimental condition for accurate and reliable gene expression results. Several housekeeping genes such as *actin*, *tubulin*, *glyceraldehyde- 3-phosphate dehydrogenase* (*GAPDH*), *ubiquitin*, *cyclophilin*, *tubulin*, and *18S rRNA* frequently used as an internal control gene for gene expression analysis because of their uniform expression in an experimental treatment. In some cases, the transcript levels of these genes are also regulated by the experimental treatment and make them unsuitable for normalization of gene expression in that condition (Bustin, 2002; Bustin & Nolan, 2004; Suzuki et al., 2000). Numerous studies performed to screen suitable reference gene by evaluating the uniform expression of housekeeping genes under different experimental conditions (Dheda et al., 2004; Jain et al., 2006; Radonic et al., 2004; Vandesompele et al., 2002). Recently, some novel reference genes identified for normalization of gene expression in *Arabidopsis* by using whole-genome gene chip data from genome-wide analysis (Czechowski et al., 2005).

The gene expression analysis also becomes essential in rice because it is a model monocot crop for genetic and molecular studies. Moreover, the availability of complete genome sequence and annotation made it suitable for molecular analysis (Project,

2005). Jain et al. (2006) validated the stability of expression of the most commonly used internal control genes in various tissues of rice using real-time PCR analysis and determined the uniform expression of *eEF-1a* and *UBQ5* gene. However, due to the investigation on a limited number of genes, it is unclear that the commonly used gene should use for normalization of gene expression data in rice or other internal control genes need to identify. Jain (2009) studied the whole genome gene expression data of 45 arrays which represented 15 different developmental stages to identify novel internal control genes with uniform expression in a wide range of developmental stages of rice. They identified about 100 genes as superior internal control genes and the expression from low to very high level makes these genes suitable for normalization of the gene expression over a wide range of transcript levels. They also evaluated the expression stability of the genes and validated by geNORM and NormFinder software. The geNORM is a statistical algorithm which can be used to determine the average expression stability of different genes based on the geometric average of multiple reference genes (Vandesompele et al., 2002). The NormFinder is also an algorithm which ranks a set of candidate genes according to their expression stability under different experimental conditions (Andersen et al., 2004). So, the gene identified by their investigation can be used for accurate normalization of transcript levels during various developmental stages in rice (Jain, 2009).

Thus, validation of the expression of a control gene and stability analysis under a particular experimental condition is essential for proper normalization. Previously, Kim et al. (2003) observed *18S rRNA* as the most reliable reference gene within four genes for normalization of real-time PCR data in rice. However, the *18S rRNA* gene expresses at very high levels and requires greater template dilutions for measurement in cDNA samples within the dynamic range of real-time PCR or with weakly expressed genes.

Moreover, the *18S rRNA* is not suitable as a reference gene when the real-time reaction carried out using mRNA as a template or an oligo-dT primer. Brunner et al. (2004) observed high expression variability in *UBQ10*, *ACT11*, and *β-TUB* gene while highly stable expression of *UBQ5*, *eEF-1a*, *UBQ*, and *TUA* gene in the tissue samples of poplar (genus *Populus*). Furthermore, the GAPDH found as a suitable reference gene for sugarcane. The *ef1a* gene was the most stably expressed gene during biotic and abiotic stresses in potato. Hance, the housekeeping genes regulated differently in different plant species and might exhibit differential expression patterns. A housekeeping gene with stable expression in an organism might not be suitable for another organism. However, in rice, under various environmental conditions of stress and hormone treatments the *18S rRNA*, *25S rRNA*, *UBC*, *UBQ5*, and *eEF-1a* demonstrated the most stable expression (Jain et al., 2006).

The *β-actin* is also the most frequently used control in gene expression analysis but need to assess its modulation for better results. The *β-actin* was used as a reference gene in previous studies, but its expression level was not as stable as had been expected and the poor performance was observed in potato, rice and soybean, while it was rather variable in *Arabidopsis* (Tenea et al., 2011). The *β-actin* mRNA levels increase following hypoxia and decrease in human uroepithelial cell lines in response to bacterial infection (Zhong & Simons, 1999). The *β-actin* mRNA transcription level also reduced during ionizing radiation in Syrian hamster embryo cells (Woloschak et al., 1990). During the *in vivo* experiments, the *β-actin* mRNA increases significantly in the pancreas following a supramaximal dose of cerulein, an agent that leads to an acute interstitial pancreatitis and increases in liver from rats with vitamin B6 deficiency (Yuan et al., 1999). Moreover, *β-actin* mRNA levels found to increase in the adrenal glands of hypophysectomized rats (Suzuki et al., 2000).

So, gene expression analysis of the *badh2* gene using a suitable internal reference gene could be used to assess the changes due to the influence of environmental components as well as the response of the genotype against the environmental condition.

2.18 Rice Aroma Compound and Extraction Method

Aroma or flavor of rice is not only associated with the Basmati and Jasmine-type rice but also reported to be present in non-Basmati/Jasmine type aromatic rice and consumers are willing to pay a premium price for this character. Though, the aroma quality of Basmati and Jasmine rice is better than the non-Basmati/Jasmine type rice but their aroma quality is highly dependent on environmental condition and the chemical composition of the grain (Rohilla et al., 2000b). Till to date, rice aroma studies have mainly focused on identification and quantification of volatile compounds emanating from cooked and uncooked rice. Some volatile compounds have been detected and identified using GC-MS while Buttery et al. (1988) identified 64 volatile compounds including seven alcohols, fifteen aldehydes, nine ketones, four ester, eight acids, ten aromatics, ten nitrogen compounds, etc. as emanating from a California long-grain rice cultivar. However, more than 300 volatile compounds identified by previous researchers and a remarkable variation were observed in the identified volatile compounds (Yang, 2007). The variation appears to be mainly due to differences in isolation method and type of rice analyzed. Different isolation methods developed by various researchers used for the separation of rice aroma compounds such as distillation methods (modified Likens-Nickerson simultaneous distillation extraction method) as mentioned by Widjaja et al. (1996), steam distillation (Tava & Bocchi, 1999), headspace methods (solid-phase

microextraction, SPME) developed by Grimm et al. (2001), Tenax trapping (Buttery et al., 1988), solvent extraction method (Bergman et al., 2000).

However, distillation methods or simultaneous distillation extraction (SDE) methods are known to be a rapid isolation method that gives good recoveries of volatile compounds with much polar and water soluble compounds. Formation of artifacts and decomposition of labile components due to heat induction was a major drawback of this method (Yang, 2007). The headspace method is of two types i.e. static and dynamic methods while the static headspace method with solid phase microextraction widely used in environmental, petrochemical, botanical, forensic and clinical analyses due to its simplicity and speed. It does not require organic solvents for either sample preparation or cleanup hence reduced a possibility of forming artifacts. The major disadvantage of this method is that it is mainly limited to non-polar or semi-polar volatiles (Kolb, 1999). Tenax trapping is a well-known method which used to absorb volatile compounds from the headspace that are subsequently desorbed thermally or using a solvent before analysis. Tenax is widely used method due to its ability to adsorb and desorb a wide-range of organic volatiles. Tenax degrades if react with O_2 at high temperature and abundance of phenolic compounds and oligomers observed which considered as a drawback of this method. However, solvent extraction is one of the simplest methods where the variables depend on the solvent used and method of concentrating. Though this technique has some limitations but subsequently proved to be convenient, inexpensive and an efficient way to extract and quantify rice aroma (Liyanaarachchi et al., 2014; Mahatheeranont et al., 2001). Hence, this method could be used to extract volatile compounds from uncooked brown rice (Liyanaarachchi et al., 2014).

2.19 Volatile Compounds in Rice

The primary chemical components which identified from different rice were grouped mainly as lipids, starch, and proteins. These chemical compounds were also known to be different with varying production environment, soil, cultural method and rice variety. Kennedy and Burlingame (2003) mentioned that the lipid content in rice ranged from 1% to 4%, protein from 6% to 8%, and starch from 60% to 80% depending on different rice varieties.

(a) Lipid and lipid derivative volatiles

The lipid in rice classified as starch lipids and non-starch lipids. The starch lipids associated with the starch granules and non-starch lipids obtained from other cellular components. The non-starch lipids primarily located near the surface and significantly reduced during milling. Volatile compounds derived from lipids due to lipolysis, lipid oxidation, and decomposition. During lipolysis, lipase produced free fatty acids that undergo oxidation. The free fatty acids found in rice were mainly palmitic, stearic, oleic and linoleic acid (Zhou et al., 2002b). Volatile compounds obtained by decomposition of lipid included aldehydes, ketones, alcohols, furanone, acids, lactones, and hydrocarbons. During storage condition, the activity of lipase and lipoxygenase increases resulted in enhanced production of the volatile compounds, particularly hexanal (Zhou et al., 2002a). The 2-alkanone produced from saturated fatty acids in substantially larger quantities during thermal oxidation. Lipids are also essential to the flavor of foods because they increase the binding of lipophilic flavor compounds. Lipids, therefore, can moderate both the flavor release and perception of rice (Reineccius, 2006).

(b) ***Protein and protein derivative volatiles***

The protein content of brown rice was observed to be in between 6.6 to 7.3% and after milling it become 6.2 to 6.9% indicated that a significant portion of protein was inside the aleurone layer of the rice grain. The proteins which present in rice observed to be heat stable, insoluble in water and the primary protein in rice is glutenin. Albumin and globulin are water soluble, prevalent in the rice bran and also observed to be present in rice grain (Zhou et al., 2002b). The concentration of volatile sulfur compounds (e.g., hydrogen sulfide, dimethyl sulfide) formed from protein decreased by protein oxidation (Zhou et al., 2002a). Volatile sulfur compounds are not significant contributors to the aroma for several reasons. Carbonyl compounds formed by lipid oxidation can react with sulfhydryl groups on cysteine or methionine decreasing the formation of volatile sulfur compounds. Likewise, protein oxidation reduces the level of volatile sulfur compounds (e.g., hydrogen sulfide, methyl mercaptan, dimethyl sulfide, dimethyl disulfide and sulfur dioxide) formed during cooking (Zhou et al., 2002a). The Maillard reaction is the primary route for aroma formation in foods. A diverse range of products is formed such as nitrogen-containing heterocyclic compounds (pyrazine, methoxypyrazine, pyrrole, pyridine, pyrroline, pyrrolidine, pyrrolizine and piperine), oxygen-containing heterocyclic compounds (maltol, furaneol, cyclotene, oxazole, and oxazoline), and sulfur-containing heterocyclic compounds (thiazole and thiophenes). Chemical interaction between flavor compounds and proteins involves reversible, weak, hydrophobic interactions, stronger ionic effects, and irreversible covalent bonds (Reineccius, 2006).

(c) ***Carbohydrate and carbohydrate derivative volatiles***

Rice is classified as waxy or non-waxy based on the amylose content of the grain. Waxy rice has very low levels of amylose (0 to 5 %) content. Amylose content is

a key indicator for predicting the behavior of rice during cooking and processing because it influences the texture, water absorption ability, and hardness of cooked rice. Waxy rice has higher free fatty acid content than non-waxy rice. Therefore, the increased concentration of free fatty acids in waxy rice may lead to enhance the formation of volatile carbonyl compounds through lipid oxidation (Zhou et al., 2002b). The polysaccharide may also influence the release of flavor through vapor pressure reduction due to chemical bonding (ionic, hydrophobic, covalent, hydrogen bonding, and Van der Waals forces) or by influencing mass transfer rate due to enhanced resistance. For example, free amylose forms a helical structure with hydrophobic areas which contain individual aroma compounds (Reineccius, 2006).

However, volatile compounds produced by rice usually determined by the gas chromatography-mass spectrometry (GC-MS) which is being used by several researchers. The flavor chemistry of rice grain reveals the existence of numerous volatiles in aromatic rice, but the relationships between volatiles and aroma not established yet (Yoshihashi et al., 2002). Bryant and McClung (2011) reported that the hydrocarbon compounds not significantly different between aromatic and non-aromatic rice varieties, but the aromatic rice possesses higher levels of alcohol (n-pentanol, l-octen-3-ol, menthol, and estragole), aldehydes and ketones (n-pentanal, n-heptanal, and n-nonanal), acids and other compounds. Moreover, aromatic rice retains 15 times more 2AP than non-aromatic rice. The other important compounds implicated with aroma were the aldehyde, alk-2-enals, alka-2,4-dienals, 2-pentylfuran, and 2-phenyl ethanol, etc. (Widjaja et al., 1996). Jezussek et al. (2002) identified the 2-amino acetophenone and 3-hydroxy-4,5-dimethyl-2(5H)-furanone at a high level in Basmati 370. Recently, Yang et al. (2008) observed that the guaiacol, indole, and p-xylene along with 2AP predominantly responsible for the unique flavor of Black rice. The 2AP associated with

pleasant, popcorn-like aroma of aromatic rice while the hexanal develops from lipid oxidation correlated with off-odors (Bergman et al., 2000). The non-aromatic rice possesses high levels of n-hexanal, (E)-2-heptenal, 1-octen-3-ol, n-nonanal, (E)-2-octenal, (E)-2,(E)-4-decadienal, 2-pentylfuran, 4-vinylguaiacol, and 4-vinyl phenol than the aromatic rice (Widjaja et al., 1996). Although, both the Basmati and Jasmine rice considered as the world class premium aromatic rice but significant differences exist in concentrations of various flavor or off-flavor compounds between them such as methyl salicylate, deca-2, 4-dienal, hexanal, hept-2-enal, 2-butenal, and 2-pentylfuran. The differences in concentration of different volatile compounds may contribute to their respective flavors. Thus, each variety has a unique aroma resulted from a mixture of some volatile compounds which may vary from the well characterized popcorn-like aroma (Champagne, 2008; Kirstin & Michael, 2004).

2.20 Odor-Active Compounds in Rice

Among the identified volatile compounds in cooked rice (more than 320 compounds), only a small number (Table 2.6) was reported to be the most important for the rice aroma (Buttery et al., 1988; Jezussek et al., 2002; Widjaja et al., 1996). These essential compounds characterized using odor units, charm analysis or aroma extract dilution analysis (AEDA) using gas chromatography-olfactometry (Acree, 1997). An odor unit or odor activity value (OAV) obtained by dividing the concentration of the individual compound by its odor threshold (the lowest detectable level). Compounds with high odor units contribute more to the aroma and are important for flavor differences among foods (Reineccius, 2006).

Table 2.6: Odor-active compounds present in cooked rice.

Volatile compounds	Mol. Wt	Odor description
2- Acetyl-1-pyrroline	111.14	Popcorn-like
Lipid degradation products		
Hexanal	100.16	Green
Octanal	128.21	Citrus-like
Nonanal	142.24	Floral, fruity
Decanal	156.27	Soapy
(*E*)-2-Nonenal	140.22	Fatty, tallowy
(*E*,*E*)-2,4-Decadienal	152.23	Fatty
4,5-Epoxy-(*E*)-dec-2-enal	168.23	Metallic
2-Pentylfuran	138.21	Beany
Vanillin	152.15	Vanilla-like
Maillard reaction products		
2-Phenylethanol	122.16	Rose-like
Phenylacetic acid	136.15	Rose-like
2-Aminoacetophenone	135.16	Naphthalene, floor polish
Thermally induced products		
3-Hydroxy-4,5-dimethyl-2(5H)-furanone	128.13	Seasoning-like
Bis-(2-methyl-3-furyl)-disulfide	226.32	Meaty
4-Vinylguaiacol	150.17	Phenolic, medicinal, spicy
4-Vinylphenol	120.15	Phenolic, medicinal

Source: Buttery et al. (1988)

Several odor-active compounds determined using odor units (Buttery et al., 1988) and AEDA (Jezussek et al., 2002) in cooked rice and can be divided into different groups based on their origin (2AP, Maillard reaction, lipid degradation, thermally induced products, etc.). In cooked and processed foods, these avenues of synthesis often play a significant role in the formation of either pleasant or unpleasant flavors (Whitfield & Mottram, 1992). However, the flavor can not be explained by just odor units since the aroma is a complex feature and made up of a mixture of compounds.

(a) ***2-Acetyl-1-Pyrroline***

The 2AP is the most crucial aroma compound of aromatic rice, emit popcorn or butter-like odor (Buttery et al., 1983). During 2AP synthesis, the 1-Pyrroline and 2-oxopropanal identified as prime intermediates (Hofmann & Schieberle, 1998). However, 2AP synthesized from L-proline and L-ornithine in the aerial parts of aromatic rice enzymatically during growth and development but not during cooking

(Yoshihashi et al., 2002). Its formation in rice plants varies with genetic factors and production conditions such as location, cultivation method, harvest time, and temperature (Hien et al., 2006; Itani et al., 2004; Yoshihashi et al., 2004). For getting higher concentrations of 2-AP, the aromatic rice needs to grow in a cold climate with relatively low levels of nitrogen fertilization and harvest earlier than ordinary cultivars (Itani et al., 2004).

(b) ***Maillard Reaction Products***

The Maillard reaction involves a chemical reaction between the carbonyl group of the open-chain form of a reducing sugar and the primary amino group of an amino acid, peptide, or similar compound. It occurs during storage, however, the rate of which is greatly facilitated by heat. The Maillard reaction contributes significantly to flavor formation in foods resulting in a diverse range of distinctive odors (BeMiller, 2007). Several Maillard reaction products in cooked rice are thought to be important in the rice flavor (Table 2.6). The 2-Phenylethanol and phenylacetic acid are Strecker degradation products of the amino acid L-phenylalanine and 2-aminoacetophenone which is also a Strecker degradation product of tryptophan (Etschmann et al., 2005; Hofmann & Schieberle, 2000). Strecker degradation considered as a part of the overall Maillard reaction (Bemiller & Whistler, 1996). The 2-Aminoacetophenone is an off-odor compound present in brown rice because it has a naphthalene or floor polish odor. The 2-Phenylethanol and phenylacetic acid contribute a rose-like odor in aromatic (Jasmine, Basmati, Goolarah, and YRF9) and non-aromatic (Pelde) rice (Jezussek et al., 2002; Widjaja et al., 1996).

(c) Lipid Degradation Products

Both oxidative and thermally induced degradation of lipids results in the formation of volatiles. For example, the oxidation of unsaturated lipid acyl chains is a major route for volatile synthesis during cooking. The lipid oxidation products yield not only rancid odors but also induce various deteriorative reactions with proteins, amino acids, and other components. Cooked rice formed odor-active compounds during the degradation of the principal unsaturated fatty acids such as oleic, linoleic and linolenic acid (Zhou et al., 2002b). The degradation of oleic acid formed octanal, heptanal, nonanal, (E)-2-nonenal, decanal, and 2-heptanone, whereas the linoleic acid formed hexanal, pentanol, pentanal, (E)-2-octenal, (E, E)-2,4-decadienal and 2-pentylfuran (Monsoor & Proctor, 2004). The vanillin in cooked brown rice cultivars also contributed to the aroma (Jezussek et al., 2002). Many consumers prefer the presence of vanillin which formed via the β-oxidation pathway because of its positive impact on flavor quality. Conversely, the hexanal contributes to consumer rejection due to its rancid odor (Bergman et al., 2000). The hexanal formed considerable amount in partially milled rice than in wholly milled rice. During storage, the (E)-2-nonenal (rancid), octanal (fatty), and hexanal (green) increase significantly to contribute off-flavors formation with aging time (Lam & Proctor, 2003). The aldehydes such as octanal, nonanal, (E)-2-nonenal, decanal, (E)-2-decanal, and (E, E)-2,4-decadienal also regular components of other foods, have low odor thresholds and contributed to rice aroma (Buttery et al., 1988).

However, there is no individual compound found to have the characteristic aroma of raw and cooked rice. Therefore, Bullard and Holguin (1977) concluded that the aroma in rice formed by a blend of various volatiles.

2.21 Concentration of 2-Acetyl-1-pyrroline in Rice

The concentration of 2AP quantified in several aromatic rice varieties such as Della (76.2 ppb), Basmati 370 (87.4 ppb), and Jasmine (156.1 ppb) to estimate the ranges of 2AP concentration (ppb) present as the essential aroma compound of aromatic rice (Tanchotikul & Hsieh, 1991). Buttery et al. (1983) determined the concentration of 2AP in 10 rice varieties (Table 2.7) and found it ranges from 6 ppb to 90 ppb in milled rice while 100 ppb to 200 ppb in unmilled rice (brown rice).

Table 2.7: Concentration of 2AP in cooked rice varieties in terms of dry weight of rice.

Variety	2-Acetyl-1-pyrroline (2AP) conc. (ppm)	
	Milled rice	Brown rice
Malagkit Sungsong	0.09	0.2
IR841-76-1	0.07	0.2
Khao Dawk Mali105	0.07	0.2
Milagross	0.07	-
Basmati	0.06	0.17
Seratus Malam	0.06	-
Azucena	0.04	0.16
Hieri	0.04	0.1
Texas Long Grain	<0.008	-
Calrose	<0.006	-

Source: Buttery et al. (1983)

The analysis of 2AP concentration showed that the 2AP present in a slight amount (0.0001 ppm) for contributing the odor of aromatic rice. The panel members sniffed the effluent of peaks separated by gas chromatography and observed that the 2AP contained peak highly correlated with the odor of aromatic rice. Conversely, the hexanal was negatively associated with the odor of rice.

2.22 Factors Affecting Volatile Profile

2.22.1 Genetic Factors

The chemical composition and characteristic aroma vary widely among cultivars such as the waxy rice contained lower levels of amylose (0-5%) than non-waxy rice (Chaudhary, 2003). The black and red pigmented rice contained anthocyanin pigments (cyanidin 3-glucoside, peonidin 3-glucoside, cyanidin 3, 5-diglucoside, and cyanidin 3-rutinoside) which also influence their flavor. The red rice contains up to 50 times higher tannin content than the brown rice which increases its astringency (Goffman & Bergman, 2004). Besides, there is a variation of 2AP content among aromatic cultivars such as Makagkit (760 ppb), Goolarah (691 ppb), YRF 9 (670 ppb), Basmati 370 (610 ppb), IR 841-76-1 (560 ppb), Jasmine (156 ppb), to Della (76 ppb) (Grosch & Schieberle, 1997). Thus, the genetic factor plays a vital role for rice aroma.

2.22.2 Effect of Storage Condition

Freshness is an important quality attribute for rice and aging leads to some physicochemical changes such as pasting properties, chemical composition, texture, color, and flavor. Postharvest changes in rice flavor can partly control with storage conditions. The modified atmosphere during storage conditions greatly helps to maintain rice flavor through the reduction of oxidation. The optimum storage conditions are moisture content below 15%, the temperature below 15°C, relative humidity 70%, and a gas atmosphere of 2-7% O_2 and 3-5% CO_2. Therefore, both the storage duration and storage temperature affects aroma of storage rice.

(a) ***Storage Duration***

Many studies have reported that the rancidity increases during storage concurrently with increasing levels of hexanal, a lipid oxidative product. Besides, octanal (fatty acid) and 2-nonenal (rancid) increase during storage and contribute to the reduction of flavor quality (Lam & Proctor, 2003). Aging of Jasmine rice results in a decreased concentration of 2AP and increased the off-flavor compounds such as hexanal and 2-pentylfuran (Wongpornchai et al., 2004). As storage duration increases the activities of lipase and lipoxygenase increases leading to progressively increasing the amount of hexanal (Suzuki et al., 1999).

(b) ***Storage Temperature***

Storage temperature affects the change of both desirable and undesirable compounds in rice grain. The concentration of 2AP also varies with temperature during storage. The 2AP concentration dramatically decreased at 30°C compared to 5°C and 25°C temperature, due to its high volatility and lipophilic properties. Moreover, higher extraction temperatures (75°C) resulted in a lower recovery of 2AP than at 40°C (Yoshihashi et al., 2005). High storage temperature (35°C) increases the lipase and lipoxygenase activity along with the concentration of hexanal, pentanal, and pentanol (Suzuki et al., 1999). The levels and combinations of various chemicals also differ with the flavors and fragrances associated with long-term rice storage. Masumoto et al. (2004) determined that the 1-butanal, 1-hexanal, 1-heptanal, methyl ethyl ketone, 1-pentanal, and propanal responsible for the old or stale aroma of stored rice while the 1-butanal and 1-heptanal involved in the fresh aroma of rice. Many of these old flavors or fragrances components particularly hexanal reported by other researchers as presented in Table 2.8 (Lam & Proctor, 2003; Masumoto et al., 2004; Zhou et al., 2002b).

Table 2.8: Concentration, thresholds, odor unit and odor descriptions of significant volatile aroma compounds in aromatic and non-aromatic rice.

Aroma compound	Threshold (ppb)	Odour description	Jasmine	Basmati	Non-aromatic
			Conc. (ppb)	Conc. (ppb)	Conc. (ppb)
Hexanal	5	Green, grass	853	751	1960
Butanol	500	Medicinal	5	1	9
Heptan-2-one	140	Fruit, spicy	23	22	40
Heptanal	3	Fruit, fatty	25	34	26
(E)hex-2-enal	17	Green, fruity	7	5	15
2-Pentyl furan	-	Nutty, beany	35	21	78
Pentan-1-ol	4000	Sweet, strong	84	139	104
Octanal	0.7	Citrus, fatty	26	40	29
(E)hept-2-enal	13	Fatty, green	45	22	80
2-acetyl-1-	0.1	Sweet, popcorn	49	7	3
pyrroline	50	Herby, green	11	3	3
6-methylhept-	2500	Sweet, green	51	45	59
5-en-2-one	1	Floral, fatty	28	25	42
Hexan-1-ol	3	Green, herby	47	27	95
Nonanol	1	Herby, earthy	34	25	58
(E)oct-2-enal	-	Oily, sweet	0	-	44
Oct-1-en-3-ol	350	Nutty, sweet	36	27	49
2-ethyl hexanol	0.08	Fatty, waxy	14	6	28
Benzaldehyde	0.4	Fatty, green	11	9	15
(E)non-2-enal	0.07	Fatty, citrus,	13	8	31
(E)dec-2-enal	3	Powerful	15	23	42
(E,E)deca-2,4-	140	Spicy, fruity	12	3	17
dienal	5	Faecal, floral	853	751	1960
4-vinylguaicol	500	Green, grass	5	1	9
Indole	140	Medicinal	23	22	40

Source: Lam and Proctor (2003)

Due to an accumulation of off odor flavors in long term storage with high-temperature rice requires some modification before use. The addition of fresh aromatic rice to old rice developed as an effort to mask or dilute old flavors of rice (Fukai & Ishitani, 2004). The protease treatment followed by washing in water is another method of reduction of old flavor in stored rice (Arai & Watanabe, 1994).

2.22.3 Degree of Milling

The white rice produced through an abrasive removal of the brown surface layer from the individual rice grains. The rice pericarp includes the seed coat, testa, and aleurone layer. The pericarp removed during milling which also reduced the

concentration of lipids, protein, fibre, reducing and total sugars, ash and some minor components such as vitamins, and free amino and fatty acids. At the same time, milling improves the sensory quality of stored rice by reducing oxidation products (Piggott et al., 1991; Zhou et al., 2002a; Zhou et al., 2002b). The reduction in the hexanal levels between brown and milled rice indicated that the compound mainly found in the rice bran. However, the 2AP concentration did not decline with milling, indicating an endosperm origin of this compound (Bergman et al., 2000).

2.22.4 Environmental Factors

Production conditions such as temperature, location, harvest time, and soil type influence the aroma of the harvested grain product (Rohilla et al., 2000a). The day/night temperatures of 25ºC/15ºC during ripening resulted in a better aroma of Basmati rice (Bhattacharjee et al., 2002; Juliano, 1972) while higher temperature and early transplanting diminish the aroma (Ali et al., 1991). The production location of Khao Dawk Mali 105 affects the 2AP concentration ranging from 87 ppb to 532 ppb (Yoshihashi et al., 2004). The 2AP concentrations in Japanese aromatic cultivars such as Hieri, Miyakaori, and Sari Queen were higher in brown rice harvested early and ripened at a low temperature compared to timely harvest at ambient temperature (Itani et al., 2004). The Basmati and Jasmine types had a stronger aroma when harvested at the beginning of winter than the late winter (Lorieux et al., 1996). High altitude and low soil moisture content also suitable for higher 2AP concentration in aromatic rice (Nakamura, 1998; Yang et al., 2007). The lower soil moisture content also helps to increase proline concentration, a substrate in 2AP biosynthesis. For superior aroma quality, Itani et al. (2004) recommended rice cultivation at a cold temperature at high altitude, low levels of nitrogen application, early harvesting, and drying at a low air

temperature. Rohilla et al. (2000a) also indicated some environmental factors suitable for aroma formation in aromatic rice such as cool weather during flowering and grain development, fertile soil, direct sowing, production on lighter soils in upland conditions, low soil moisture during grain filling, and manual dehulling. Drought stress also is known to increase the concentration of 2AP through an increased accumulation of proline (Yang & Kao, 1999). After grain harvest, there is a decline in 2AP concentration with increasing storage duration that is acerbated by higher temperatures (Yoshihashi et al., 2005). A research by Golam et al. (2011) reported that aroma in rice also affected by high temperature during grain filling and ripening stages. The globally renowned aromatic rice cultivars such as Rato Basmati and Ranbir Basmati demonstrated distinct aroma in their cultivated country but exhibited moderate aroma when grown in a tropical environment. This situation further confirms the findings of Juliano (1972) and Mann (1987) that the Basmati rice cultivars require relatively cooler temperature (25ºC/ 21ºC- day/ night temperature) during crop maturity for better retention of aroma.

Hence, it is essential to investigate the volatile profile, 2AP concentration, *badh2* gene expression, and morphoagronomic performance of rice using an integrated approach of biochemistry, molecular biology and plant breeding for evaluating the effects of environmental components on aroma as well as to get a perception of the aroma status of a genotype.

CHAPTER 3: EFFECTS OF TEMPERATURE ON MORPHO-AGRONOMIC PERFORMANCE OF AROMATIC RICE

3.1 INTRODUCTION

The phenotypic expression of morphological characters and agronomic performance of a crop is the reflection of the cumulative effect of genotype, environment and their interaction (genotype and environment). The phenotypic responses of all genotypes are not same for the changes in environmental conditions and the consequences of the environment also vary on the genotype (Uphoff et al., 2015). Thus, the nature of the genotype and the environmental factor is an important determinant of morpho-agronomic performance of a particular rice genotype.

Among all the environmental factors that affect agricultural productivity, the most powerful and least modifiable factor is the climate. According to the regional climate projections reports (the Fourth Assessment Report) of the Intergovernmental Panel on Climate Change (Solomon et al., 2007), the climate of the earth is changing, and the air temperature is rising due to increasing concentration of CO_2 and greenhouse gasses. They also predicted that the average temperature of the earth surface will increase at the end of the 21st century in between 2-4°C. In a study, Ziska et al. (1997) observed that an increase of 4°C temperature during growing season resulted in the 5 to 6 days earlier maturation of the crop. The temperature greatly influences not only the growth duration but also the growth pattern and the productivity of crops. However, the rising temperature may cause detrimental effects on growth parameters, yield components and quality traits of rice which will reduce total yield and qualitative performance (Peng et al., 2004).

Aromatic rice is a small group of rice but plays a vital role in global rice trading due to its aromatic flavor and good quality traits. The quality characteristics of aromatic rice especially Basmati type rice is highly influenced by temperature particularly at the time of flowering, grain filling, and maturity stage. The aroma formation and retention in grain is to be enhanced at low temperature during grain filling periods (Akhter et al., 2007). However, the unusual rise in atmospheric temperature during ripening periods also may hamper aroma in the kernel (Shahidullah et al., 2009).

The effects of temperature on aromatic rice are very much divergent, complex, and differ from different physiological aspects even in different organs of a plant (Nishiyama, 1976). Both the extremely high (above 30°C) and low (below 20°C) temperatures were observed to be unfavorable for aromatic rice production (Yoshida & Parao, 1976). Yoshida and Hara (1977) mentioned that the daily mean temperature was found to be the most meaningful for describing the effects of temperature on grain filling as well as grain yield. Baker (2004) investigated the yield responses of four southern US rice cultivars in outdoor, natural sunlight, controlled environmental chambers with constant day–night air temperature regimens of 24, 28, 32, 36 and 40°C and found that all cultivars died during early vegetative stage at 40°C treatment, all varieties survive and develop panicle but failed to produce seeds at 36°C and all the cultivars demonstrated increasing yield about 46-71% at 28°C. Oh-e et al. (2007) investigated the effects of temperature on growth, yield and dry matter production of rice which was grown in the paddy field but inside temperature gradient chamber with the temperature range of 26.8 to 28.8°C and found that the brown rice yield declined above 28°C mean air temperature. Rani and Maragatham (2013) studied the effects of elevated (2 and 4°C) and ambient temperature on rice phenology and yield where they observed the highest yield under ambient temperature and yield loss under elevated

temperature due to sterile florets and lesser crop duration. Previously Ziska et al. (1997) found that increase in temperature by 4°C during growing season resulted in an earlier maturation of the crop by five and six days for the wet and dry season, respectively. Later on, Xu et al. (2012) observed the effects of four different daily mean temperature (21, 23, 26 and 30°C) during grain filling stage on endosperm structure and appearance quality of aromatic rice where they found the starch granule changed from regular shape to various shaped with gaps due to increasing temperature.

The aromatic rice frequently linked with undesirable agronomic characters, such as low yield, seed shattering and lodging (Ahmad et al., 2005). But the agronomic potentiality of a rice variety depends on several characteristics namely high yielding capability, resistance to adverse environmental factors and high grain quality. So, the ultimate aim of aromatic rice production is to increase grain yield with superior grain quality. Rice grain yield also depends on several agronomic characters such as the number of fertile tillers, days to flowering, days to maturity, grain filling period, plant height, the number of fertile grain per panicle, panicle length, 1000 grain weight and grain yield per plant (Halil & Necmi, 2005). The number of fertile tillers and number of grains per panicle are measured at vegetative and reproductive stage but the 1000 grain weight and grain yield per plant need to be evaluated at ripening stage.

The agronomic character of rice was observed to be influenced by the environmental temperature. The tiller numbers per plant determine the panicle number per plants which is an essential component of grain yield, increased at a higher temperature (Yoshida, 1973; Yoshida, 1981). The number of days to flowering not linearly related to temperature during a daily mean temperature range of 21°C to 30°C but when the temperature dropped from 24°C to 21°C a sharp increase of days to

flowering occurred. However, high temperature accelerates and low-temperature delays the flowering days of rice plant (Ahn & Vergara, 1969; Hosoi & Takahashi, 1973) but Azmi (1969) reported that high temperature delayed flowering. Plant height increased with the rise of temperature within the range of 30°C to 35°C (Oh-e et al., 2007). High temperatures seemed to decrease the number of panicles per plant and spikelet per panicle, especially at maturity stage. Though rising temperature during ripening stage affects the weight per grain, the 1000-grain weight of a particular genotype almost remains constant under different environmental conditions. Nevertheless, the temperature influences growth rate, duration, productivity and yield of aromatic rice by affecting the morpho-physiological processes involved in grain production (Krishnan et al., 2011). Information on morpho-agronomic characters and genotypic expression of aromatic rice genotypes is essential to identify promising aromatic rice cultivar with high-quality traits and high yield potential (Ashrafuzzaman et al., 2009). So, characterization of morphological and agronomic traits and evaluation of superior genotypes will enable rice breeders to exploit good performer aromatic rice genotype in the global warming condition.

However, previous researchers studied morphological traits or agronomic trait under cold stress or high-temperature stress but did not identify suitable temperature for superior morpho-agronomic performance which is the most important part for good performer aromatic rice production in various rice growing regions as well as in the traditional aromatic rice growing areas.

The aim of this study was to characterize and evaluate the morpho-agronomic parameters of some aromatic rice genotypes under different temperature to select

promising genotypes with a suitable temperature which could be used in crop improvement program in diverse climatic conditions.

The objectives of the experiment were

- To evaluate vegetative growth performance of the selected rice genotypes at different temperature condition.
- To estimate and compare yield performance of rice genotypes under different environmental temperature.
- To assess the phenotypic aroma performance of rice genotypes at different temperature and growth stages.

The obtained information will help to select superior aromatic genotypes with better agronomic performance and superior phenotypic aroma expression for persistent aromatic rice production under a particular temperature condition.

This experiment was designed to evaluate morpho-agronomic performance (number of tiller per hill, number of fertile tiller per hill, flowering days, grain filling periods, days to maturity, plant height, panicle length, grain per panicle, fertile grain per panicle, 1000 grain weight and grain yield per plant) and phenotypic aroma (organoleptic test) of five aromatic and a non-aromatic rice genotype under three different temperature condition (ambient or 28°C, 25°C and 20°C) to observe the effects of temperature on yield components at different growth stages of aromatic rice genotypes.

3.2 MATERIALS AND METHODS

3.2.1 Plant Materials

A total of 6 rice genotypes (Table 3.1) which were collected from the International Rice Research Institute (IRRI) and the Malaysian Agricultural Research Development Institute (MARDI) were used in this investigation. Among these six genotypes, five are aromatic including MRQ 50 (Malaysian local aromatic rice variety) which was used as the local check genotype and MR 219 (Malaysian non-aromatic genotype) which was used as a control.

Table 3.1: Description of 6 rice genotypes used as plant materials

Genotypes	Designation	Cross	Origin
MRQ 50	MRQ 50	Variety	Malaysia
Ranbir Basmati	Ranbir Basmati	Land race	India and Pakistan
Rato Basmati	Rato Basmati	Land race	Nepal
E 7	IR 77734-93-2-3-2	NSIC RC 148/PSB RC 18//NSIC RC 148	IRRI
E 13	IR 77512-2-1-2-2	IR 68726-3-3-1-2/IR 71730-51-2	IRRI
MR 219	MR 219	Variety	Malaysia

3.2.2 Experimental Site

Two experimental sites were used for morpho-agronomic trait evaluation of the collected genotypes. First, one known as “net house” situated at the experimental field of the Genetic and Molecular Biology Division of the Institute of Biological Science, University of Malaya, used for the benchmark study. The experiment field located at Latitude 3°07' N and Longitude 101°39' E with an elevation of 104 m from the sea level and the experiment was conducted from October 2013 to February 2014. The

environment weather data was collected from the Meteorological Department of Malaysia (Meteorologi, 2014) and presented in Fig. 3.1.

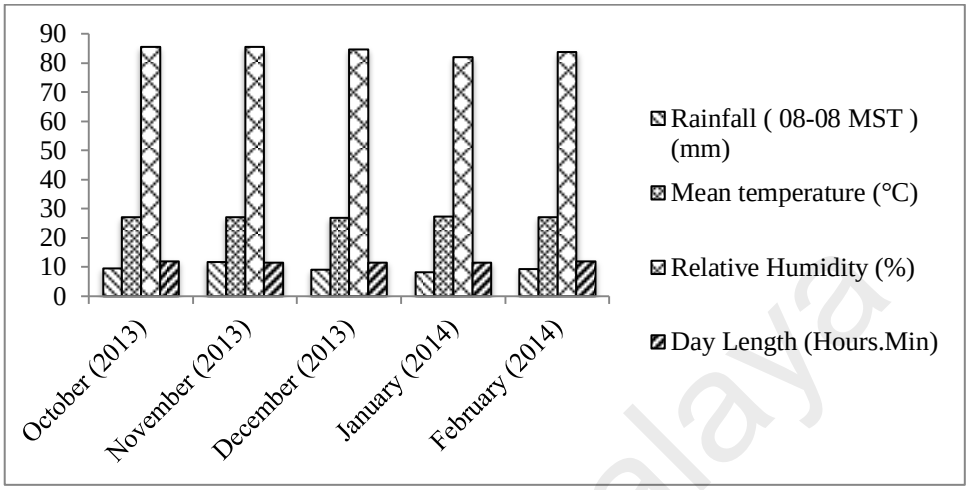

Figure 3.1: Meteorological information of the experimental site (net house). Average ambient temperature recorded 27.09 ± 0.17°C during the study periods. Source: Meteorological Department of Malaysia (Meteorologi, 2014)

The second experimental site is known as "Glasshouse" is located at the botanical garden (Rimba Ilmu) of the Institute of Biological Sciences, University of Malaya, Malaysia, at Latitude 3°8' N and Longitude 101°40' E along with the elevation of 104 m from the sea level. The experiment was conducted from April 2014 to August 2014, and the environment data was collected from Meteorological Department of Malaysia (Meteorologi, 2014) as presented in Fig. 3.2.

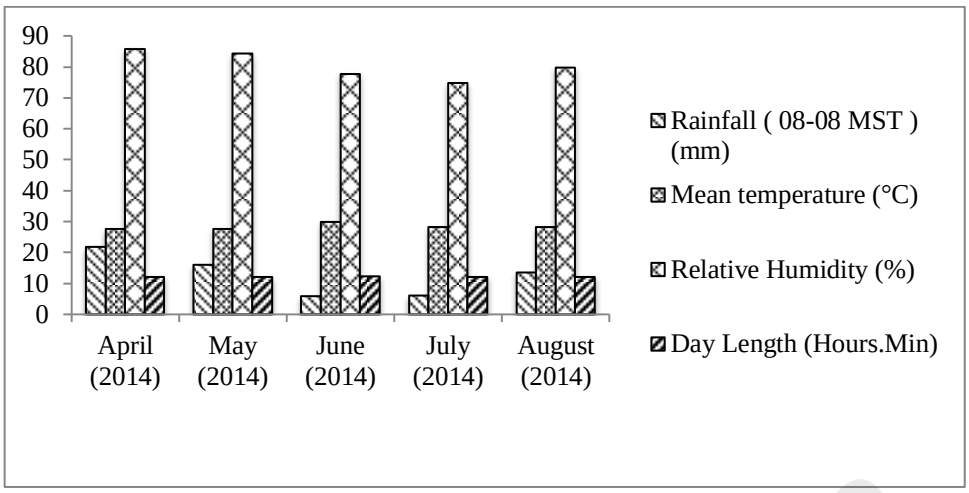

Figure 3.2: Meteorological information of the experimental site (glasshouse). The average ambient temperature was 28.29 ± 0.91°C during the study period.

Source: Meteorological Department of Malaysia (Meteorologi, 2014)

The environmental data was collected from Meteorological Department (University of Malaya station) which was compared to the experimental location data (net house and glasshouse).

3.2.3 Experimental Design

A Completely Randomized Design (CRD) with three replications was used as the experimental layout for both experimental sites. The experimental tanks (75 × 100 × 30 cm) were used for the first experiment at the net house while experimental medium sized pail (30 × 90 × 30 cm) was used for the second experiment at glasshouse. Both the tanks and buckets were filled with sandy loam topsoil, collected from Institute of Biological Sciences and were mixed with 100 g NPKS as 15:15:15:2 before transplantation.

3.2.4 Growth Chambers

The net house contained only one growth chamber with an ambient condition (ensured natural rain, humidity, sunlight and temperature) suitable for aromatic rice production. Moreover, to protect the rice plant from insect infestation and damage, the net house was surrounded by a net. Conversely, at the glasshouse three growth chambers (ambient or 28°C, 25°C and 20°C temperature containing chamber) were made under transparent plastic tin shade room, orientated to east–west direction and surrounded by the net. The growth chambers were 4.57×1.83×2.13 m in length, width and height, respectively while each chamber was 1.83 m away from another chamber to avoid mutual shading. The framework of growth chamber consisted of thick and transparent plastic with steel structure. Each chamber was equipped with two air conditioners (Wall Mounted 1.5HP, 12,000 BTU/h, A5WM 15N/A5LC, Acson Malaysia Sales & Service Sdn. Bhd., Malaysia) except the ambient chamber. Two inlet and two outlet fans were placed at an upper portion of the chamber to adjust relative humidity. Four tube lights placed at the top for light supply and four doors kept for cultural practice in each chamber. The chambers remained close, and the air conditioner was turned on alternatively 4 hours to maintain 25°C and 20°C temperature while the ambient chambers did not contain air conditioner and depended on natural air temperature.

3.2.5 Crop Husbandry

The rice seeds were sown in small plastic pots that contained 500 g black organic soil. Three weeks later the seedlings were transplanted into the experimental tanks inside the net house and in pails inside the glasshouse.

In the net house, 2-3 cm deep transplantation was done using two seedlings per hill with a hill spacing of 20×20 cm. In the glasshouse, two seedlings per hill were transplanted in a pail with a spacing of 25×25 cm. Urea fertilizer was manually broadcasted as 20 kg per ha during the tillering stage, 30 kg per ha during panicle initiation stage and 20 kg per ha at flowering stage. Moreover, the recommended intercultural operations (weeding, water management, and plant protection measures) were followed for normal growth of rice plant (Razak et al., 2012).

3.2.6 Data Collection for Morpho-agronomic Traits

The morpho-agronomic data of quantitative and qualitative characters were collected at different growth stages of the rice plant. The vernier caliper, 5-meter tape, 18 inches ruler and balance were used to measure some quantitative parameters. The parameters were measured in 5 plants randomly from each replication according to the methods of Chang and Bardenas (1965) and Committee and Resources (1980) as stated below:

3.2.6.1 Number of tillers per hill

The number of tillers per hill was physically counted at maturity stage. The total number of tillers per hill was the average number of tiller from randomly selected five hills in each replication.

3.2.6.2 Number of fertile tillers per hill

The number of fertile tillers per hill was also physically counted at maturity stage. The total number of fertile tillers per hill was the average number of fertile tiller from randomly selected five hills in each replication.

3.2.6.3 Flowering days

The data for flowering days was recorded as soon as 50% of the flowers appeared on a hill from the date of seed sowing.

3.2.6.4 Days to maturity

The data for days to maturity was recorded when 80% of the grains on the panicle were fully mature and ripened from the date of sowing.

3.2.6.5 Grain filling periods

The length of the grain filling periods was measured from the day of 50% flowering to the day of 80% maturity of a sample. The average duration of grain filling periods of five samples from the top upper primary branches was considered as the final grain filling periods.

3.2.6.6 Plant height

The height of rice plant was measured from the soil surface to tip of the panicle at maturity stage in centimeter (cm) scale. The average height of randomly selected five rice plants in each replication was considered as the final plant height.

3.2.6.7 Panicle length

The panicle length was measured from the base to tip of a panicle. The average length of five panicles from each replication was considered as the final panicle length (cm).

3.2.6.8 Grain per panicle

The number of grain per panicle was counted at maturity stage where the total number of grain per panicle was the average number of grain per panicle from randomly selected five panicles in each replication.

3.2.6.9 Fertile grain per panicle

The number of fertile grain per panicle was counted at maturity stage. The total number of fertile grain per panicle was the average number of fertile grain per panicle from randomly selected five panicles in each replication.

3.2.6.10 1000 grain-weight

A total of 1000 well-developed rice seeds were counted and weighted from each replication after harvest and sun dried to 13% moisture content. The average weight of randomly selected 1000 rice grain from each replication was considered as the final 1000 grain weight (g).

3.2.6.11 Grain yield per plant

The grain yield per plant was measured after harvested and sun-dried rice seed to 13% moisture content. The mean weight of five plants in each replication was considered as the grain yield per plant (g).

3.2.7 Sensory Aroma Evaluation (Organoleptic test)

The aroma score of the studied genotypes was estimated from leaf and grain using the organoleptic test (Golam et al., 2011). For the aromatic test of leaf, leaves were collected from different growth stages of rice plant and 0.2 g leaf samples were cut into tiny pieces (1-2 mm) and kept in Petri dishes containing 10 ml of 1.7% Potassium Hydroxide (KOH) solution. The Petri dish cover was opened after 10 minutes then the aroma was smelled and scored on a 1-4 scale where 1, 2, 3 and 4 corresponds to an absence of aroma, mild aroma, moderate aroma and strong aroma, respectively by three well-trained panelists.

For grain aroma test, forty grains from each genotype were soaked in a covered Petri dish containing 10 ml 1.7% KOH solution for an hour at room temperature. The samples were scored on a 1- 4 scale where 1, 2, 3 and 4 corresponds to an absence of aroma, mild aroma, moderate aroma and strong aroma, respectively. All the samples were sniffed by three well-trained panelists.

3.2.8 Statistical Analysis

The SAS 9.3 Software (SAS Institute, Cary, NC) was used for Duncan Multiple Range Test (DMRT) while the Minitab software (Minitab 16 Statistical Software, Sydney, Australia) was used for normality test (Anderson-Darling test), Analysis of Variance (ANOVA), traits correlation study and descriptive statistics of agronomic traits.

3.3 RESULTS

The obtained results of morpho-agronomic performance of the studied genotypes grown under ambient condition (27°C temperature) at the net house and in ambient condition (28°C) along with two controlled temperatures (25°C and 20°C) at glasshouse were presented in two steps. In the first step, the difference between different group mean was tested by DMRT (Table 3.2), the correlation was estimated (Table 3.3) and ANOVA was performed (Table 3.4) for agronomic traits of rice plant grown at the ambient condition inside the net house. Then the agronomic performance of different traits obtained from the ambient condition at the net house was compared with the agronomic performance of ambient condition at glasshouse. The differences of agronomic traits performance between these two ambient conditions were evaluated using t-test (Table 3.5) while the data were normaly distribute in Anderson-Darling test. At the second step, the differences between different group mean were evaluated using DMRT (Table 3.6), the correlation coefficient was estimated (Table 3.7, 3.8 and 3.9) and ANOVA test was performed (Table 3.10) for the traits collected from three different temperatures (ambient or 28°C, 25°C and 20°C) inside glasshouse to assess the effects of temperature on morpho-agronomic traits of aromatic rice. The aroma of the rice genotypes was also evaluated to observe the effects of temperature on the aroma trait (Table 3.11).

3.3.1 Performance of Morpho-agronomic Traits at Net House

The Duncan Multiple Range Test (DMRT) of agronomic traits represented that rice genotypes grown at ambient condition inside the net house belong to different

groups and different traits of the same genotype also fall into the various groups (Table 3.2).

Table 3.2: Duncan Multiple Range Test (DMRT) for agronomic performance in ambient condition (27°C) at the net house.

Genotype	NTH	NFTH	FD	GFP	DM	PH	PL	GP	FGP	TGW	GYP
MRQ 50	41.00a	35.80a	107.20c	18.60d	125.80d	67.20e	19.20c	93.00b	77.80d	22.80a	64.68a
Ranbir	44.20a	38.20a	99.20d	18.80d	118.00e	96.60b	26.40b	95.80b	87.00c	18.40b	34.50c
Rato	19.80b	16.60b	114.00b	31.00ab	145.00b	113.20a	30.40a	92.20b	83.20cd	22.90a	32.74c
E 7	41.80a	37.40a	106.00c	27.80b	133.80c	88.40c	25.00b	150.60a	142.00a	23.71a	41.27b
E 13	42.69a	39.40a	94.20e	34.00a	128.20d	73.80d	26.40b	143.00a	133.00b	25.46a	59.34a
MR 219	21.80b	18.60b	125.20a	22.60c	147.80a	73.60d	18.60c	144.40a	131.20b	25.45a	61.77a

Means with the same letter are not significantly different at 5% level. NTH = Number of tiller per hill, NFTH = Number of fertile tiller per hill, FD = Flowering days, GFP = Grain filling periods, DM = Days to maturity, PH = Plant height (cm), PL = Panicle length (cm), GP = Grain per panicle, FGP = Fertile grain per panicle, TGW = Thousand Grain weight (g), GYP = Grain yield per plant (g).

The maximum variation was observed in the case of plant height where Rato Basmati demonstrated the highest plant height (113.20 cm) and MRQ 50 exhibited the lowest plant height (67.20 cm). Conversely, the least variation was found in the number of tiller per hill, the number of fertile tiller per hill, grain per panicle and in 1000 grain weight. The grain yield per plant was observed to be higher in local aromatic MRQ 50 (64.68 g) and local non-aromatic MR 219 (61.77 g) as compared to other rice genotypes.

The correlation studies among the agronomic traits obtained from ambient condition at net house (Table 3.3) represented that the number of tiller per hill had significant positive correlation with the number of fertile tiller per hill (0.984) while significant negative correlation with flowering days (-0.823) and days to maturity (-0.875) at 1% level (p<0.01).

Table 3.3: Correlation of agronomic traits in ambient condition (27°C) at the net house.

	NTH	NFTH	FD	GFP	DM	PH	PL	GP	FGP	TGW
NFTH	0.984**									
FD	-0.823**	-0.834**								
GFP	-0.154	-0.096	-0.223							
DM	-0.875**	-0.853**	0.826**	0.364*						
PH	-0.332	-0.353	0.035	0.251	0.179					
PL	-0.008	-0.020	-0.404*	0.583**	-0.050	0.776**				
GP	0.056	0.120	0.068	0.407*	0.300	-0.400*	-0.203			
FGP	0.075	0.133	0.032	0.443*	0.287	-0.326	-0.123	0.987**		
TGW	-0.323	-0.250	0.286	0.464*	0.541**	-0.416*	-0.217	0.613**	0.575**	
GYP	0.082	0.105	0.133	-0.133	0.051	-0.925**	-0.727**	0.324	0.245	0.513**

* $p<0.05$, ** $p<0.01$, NTH = Number of tiller per hill, NFTH = Number of fertile tiller per hill, FD = Flowering days, GFP = Grain filling periods, DM = Days to maturity, PH = Plant height (cm), PL = Panicle length (cm), GP = Grain per panicle, FGP = Fertile grain per panicle, TGW = Thousand Grain weight (g), GYP = Grain yield per plant (g).

The significant positive correlation was observed in the case of flowering days (0.826) and 1000 grain weight (0.541) with days to maturity, plant height with panicle length (0.776), Grain per panicle with fertile grain per panicle (0.987) and 1000 grain weight (0.613). The significant negative correlation was observed in the case of the flowering days with the number of tiller per hill (-0.823), the number of fertile tiller per hill (-0.834) and panicle length (-0.404). Panicle length also demonstrated significant negative correlation with grain yield per plant (-0.727) and negative correlation with most of the studied traits except grain filling periods (0.583) and plant height (0.776).

The experimental genotypes demonstrated significant differences among each other at 1% level (Table 3.4) for all studied traits under the ambient condition at the net house.

Table 3.4: ANOVA (F-distribution value) for agronomic traits in the net house.

Source	df	NTH	NFTH	FD	GFP	DM	PH	PL	GP	FGP	TGW	GYP
Genotype	5	61.33**	72.04**	282.92**	64.21**	352.30**	1296.25**	36.21**	247.64**	240.95**	15.92**	101.65**

* $p<0.05$, ** $p<0.01$, df = Degrees of freedom, NTH = Number of tiller per hill, NFTH = Number of fertile tiller per hill, FD = Flowering days, GFP = Grain filling periods, DM = Days to maturity, PH = Plant height (cm), PL = Panicle length (cm), GP = Grain per panicle, FGP = Fertile grain per panicle, TGW = Thousand Grain weight (g), GYP = Grain yield per plant (g).

The ANOVA test result represented that the maximum variation was in the case of plant height (1296.25) while the minimum difference was in the 1000 grain weight (15.92).

The agronomic performances of experimental genotypes which grew in the net house and glasshouse under ambient condition were compared using t-test (Table 3.5).

Table 3.5: Students t-test for agronomic performance in ambient condition at the net house and glasshouse.

	Estimate for difference	**T value**	**P value**	**95% CI for difference**
NTH	0.60	0.22	0.82	-4.90, 6.10
NFTH	0.77	0.31	0.76	-4.26, 5.80
FD	1.17	0.44	0.66	-4.19, 6.53
GFP	-0.10	-0.06	0.95	-3.28, 3.08
DM	1.07	0.38	0.70	-4.55, 6.69
PH	0.60	0.14	0.88	-7.70, 8.90
PL	0.80	0.67	0.50	-1.60, 3.20
GP	0.87	0.12	0.90	-13.53, 15.27
FGP	1.20	0.17	0.86	-13.29, 15.69
TGW	0.49	0.69	0.49	-0.93, 1.92
GYP	0.59	0.17	0.86	-6.48, 7.66

NTH = Number of tiller per hill, NFTH = Number of fertile tiller per hill, FD = Flowering days, GFP = Grain filling periods, DM = Days to maturity, PH = Plant height (cm), PL = Panicle length (cm), GP = Grain per panicle, FGP = Fertile grain per panicle, TGW = Thousand Grain weight (g), GYP = Grain yield per plant (g).

All studied traits demonstrated the non-significant difference between two environments (net house and glasshouse) under ambient condition. Hence, further investigation was continued within glasshouse samples and compared agronomic performance of ambient condition (28°C) with 25°C and 20°C temperature.

3.3.2 Performance of Morpho-agronomic Traits at Glasshouse

Agronomic performance of studied genotypes was analyzed using Duncan Multiple Range Test (DMRT) which represented that different trait of a genotype fall into the various groups (Table 3.6) and different genotypes also fall into different groups for a single trait due to the temperature condition.

Table 3.6: Duncan Multiple Range Test (DMRT) for agronomic performance at different temperature in the glasshouse.

Temperature	Genotype	NTH	NFTH	FD	GFP	DM	PH	PL	GP	FGP	TGW	GYP
Ambient	MRQ 50	39.80a	33.40b	106.00e	18.40k	124.40h	66.20h	18.20g	89.80d	75.00fg	23.33cde	63.35b
	Ranbir	43.60a	38.60ab	97.40f	19.60jk	117.00i	96.40c	25.40cdef	93.00d	84.40ef	18.25fg	33.97hij
	Rato	18.80b	16.60c	112.80cd	31.20cde	144.00b	112.00b	30.40b	91.40d	82.00ef	22.27cdef	32.67hij
	E 7	41.40a	35.80ab	105.60e	26.80fgh	132.40ef	86.80d	23.60ef	149.40ab	140.80abc	22.57cdef	40.72fgh
	E 13	41.40a	37.80ab	92.80g	34.20c	127.00gh	72.80fg	26.00cdef	147.60ab	134.60bc	23.26cde	58.65bcd
	MR 219	22.60b	19.20c	124.20b	23.20hij	147.40b	75.00f	17.60g	142.60b	130.20c	26.06bcd	61.41bc
25°C	MRQ 50	45.80a	37.00ab	110.80d	19.20jk	130.00fg	69.80gh	23.00f	121.40c	108.40d	21.45def	74.19a
	Ranbir	46.20a	38.00ab	104.00e	22.20ijk	126.20h	117.80a	27.80bcd	109.40c	91.60e	18.98efg	38.23ghi
	Rato	22.40b	17.60c	121.60b	29.60def	151.20a	119.80a	34.80a	108.40c	87.40ef	26.31bcd	33.04hij
	E 7	42.20a	39.60ab	111.40d	24.80ghi	136.20d	93.00c	29.00bc	159.00a	142.00abc	28.83ab	52.21cde
	E 13	46.80a	43.00a	104.00e	27.80defg	131.80ef	75.40ef	28.00bcd	161.40a	151.40a	32.67a	76.73a
	MR 219	19.80b	13.80c	129.00a	23.00hij	152.00a	72.60fg	24.00ef	156.60ab	147.80ab	14.78g	76.82a
20°C	MRQ 50	40.60a	36.60ab	96.20f	27.40efg	123.60h	60.00i	18.30g	72.20e	60.00hi	18.01fg	45.09efg
	Ranbir	42.20a	39.20ab	82.20h	42.80b	125.00h	88.60d	25.00def	47.20f	38.20j	17.78fg	28.01j
	Rato	18.40b	17.40c	112.40cd	27.80defg	140.20c	85.20d	27.20bcde	41.00f	33.20j	16.43g	24.97j
	E 7	38.00a	33.60b	91.80g	43.40ab	135.20de	78.80e	16.60g	54.80f	45.80ij	22.57cdef	30.63ij
	E 13	42.80a	42.80a	79.20i	47.40a	126.60gh	69.20gh	24.80def	73.60e	65.40gh	15.19g	48.98def
	MR 219	18.40b	17.00c	115.00c	31.80cd	146.80b	66.40h	16.40g	49.00f	39.80j	26.45bc	51.98cde

Means with the same letter are not significantly different at 5% level. NTH = Number of tiller per hill, NFTH = Number of fertile tiller per hill, FD = Flowering days, GFP = Grain filling periods, DM = Days to maturity, PH = Plant height (cm), PL = Panicle length (cm), GP = Grain per panicle, FGP = Fertile grain per panicle, TGW = Thousand Grain weight (g), GYP = Grain yield per plant (g).

The DMRT results (Table 3.6) signify that the maximum variation was in the grain filling periods where genotype E 13 demonstrated longer grain filling periods (47.40 days) at 20°C compared to ambient (34.20 days) and 25°C (27.80 days)

temperature while shorter grain filling periods was in genotype MRQ 50 (18.40 days) under ambient condition.

At ambient temperature, the lowest values were observed for the number of fertile tiller per hill (16.60) in Rato Basmati, and days to maturity (117.00 days) in Ranbir Basmati genotype.

At 25°C temperature, the maximum value was observed for the number of fertile tiller (43.00), grain per panicle (161.40), fertile grain per panicle (151.40), 1000 grain weight (32.67 g) and grain yield per plant (76.73 g) in E 13 genotype. Similarly, the highest value was for the flowering days (121.60 days), days to maturity (151.20 days), plant height (119.80 cm) and panicle length (34.80 cm) in Rato Basmati genotype.

At 20°C temperature, the lowest value was observed for flowering days (79.20 days), panicle length (16.60 cm) and 1000 grain weight (15.19 g) in E 13 genotype, for plant height (60.00 cm) in MRQ 50 while for fertile grain per panicle (33.20) and grain yield per plant (24.97 g) in Rato Basmati genotype.

So, the temperature influenced the agronomic performance of rice genotypes which were distributed into different groups.

The Pearson's correlation coefficients of morpho-agronomic traits obtained from ambient, 25°C and 20°C condition inside glasshouse determined at $p < 0.05$ and $p < 0.01$ levels (Table 3.7, 3.8 and Table 3.9, respectively). The result represented that grain filling period had a negative correlation with the number of tiller per hill, the number of fertile tiller per hill, flowering days and grain yield per plant while the significant

positive correlation with panicle length, grain per panicle and fertile grain per panicle at ambient condition (Table 3.7).

Table 3.7: Correlation of agronomic traits in ambient conditions at glasshouse.

	NTH	**NFTH**	**FD**	**GFP**	**DM**	**PH**	**PL**	**GP**	**FGP**	**TGW**
NFTH	0.982**									
FD	-0.795**	-0.833**								
GFP	-0.202	-0.133	-0.205							
DM	-0.870**	-0.867**	0.839**	0.361						
PH	-0.381*	-0.344	0.068	0.242	0.199					
PL	-0.086	-0.038	-0.383*	0.607**	-0.027	0.766**				
GP	0.095	0.128	0.046	0.429*	0.282	-0.384*	-0.194			
FGP	0.095	0.128	0.043	0.440*	0.285	-0.316	-0.134	0.991**		
TGW	-0.407*	-0.411*	0.529**	0.190	0.610**	-0.465*	-0.431*	0.452*	0.404*	
GYP	0.071	0.056	0.141	-0.124	0.065	-0.907**	-0.725**	0.324	0.250	0.578**

* $p<0.05$, ** $p<0.01$, NTH = Number of tiller per hill, NFTH = Number of fertile tiller per hill, FD = Flowering days, GFP = Grain filling periods, DM = Days to maturity, PH = Plant height (cm), PL = Panicle length (cm), GP = Grain per panicle, FGP = Fertile grain per panicle, TGW = Thousand Grain weight (g), GYP = Grain yield per plant (g).

At 25°C temperature, grain filling period was also negatively correlated with the number of tiller per hill, the number of fertile tiller per hill and grain yield per plant while significantly positively correlated with days to maturity, panicle length, and 1000 grain weight (Table 3.8).

Table 3.8: Correlation of agronomic traits at 25°C temperature in the glasshouse.

	NTH	NFTH	FD	GFP	DM	PH	PL	GP	FGP	TGW
NFTH	0.958**									
FD	-0.880**	-0.897**								
GFP	-0.235	-0.106	0.081							
DM	-0.876**	-0.841**	0.923**	0.458*						
PH	-0.124	-0.121	-0.091	0.355	0.056					
PL	-0.248	-0.182	0.037	0.645**	0.282	0.706**				
GP	0.005	0.124	0.082	0.097	0.111	-0.628**	-0.243			
FGP	0.008	0.117	0.097	0.038	0.101	-0.696**	-0.325	0.982**		
TGW	0.314	0.457*	-0.424*	0.477**	-0.194	0.040	0.448*	0.284	0.247	
GYP	0.100	0.103	0.074	-0.308	-0.053	-0.944**	-0.665**	0.630**	0.694**	-0.059

* $p<0.05$, ** $p<0.01$, NTH = Number of tiller per hill, NFTH = Number of fertile tiller per hill, FD = Flowering days, GFP = Grain filling periods, DM = Days to maturity, PH = Plant height (cm), PL = Panicle length (cm), GP = Grain per panicle, FGP = Fertile grain per panicle, TGW = Thousand Grain weight (g), GYP = Grain yield per plant (g).

At 20°C temperature, grain filling periods demonstrated significant positive correlation with the number of tiller per hill and the number of fertile tiller per hill while negative correlation with days to maturity, 1000 grain weight and grain per panicle whilst significant negative correlation with flowering days (Table 3.9).

Table 3.9: Correlation of agronomic traits at 20°C temperature in the glasshouse.

	NTH	NFTH	FD	GFP	DM	PH	PL	GP	FGP	TGW
NFTH	0.987**									
FD	-0.880**	-0.907**								
GFP	0.611**	0.651**	-0.805**							
DM	-0.821**	-0.826**	0.829**	-0.336						
PH	-0.064	-0.071	-0.122	0.270	0.061					
PL	0.013	0.088	-0.236	0.128	-0.254	0.566**				
GP	0.582**	0.605**	-0.508**	0.221	-0.598**	-0.686**	-0.169			
FGP	0.576**	0.610**	-0.543**	0.282	-0.596**	-0.658**	-0.123	0.972**		
TGW	-0.388*	-0.449*	0.489**	-0.157	0.628**	-0.200	-0.736**	-0.282	-0.311	
GYP	0.061	0.107	0.038	-0.007	0.053	-0.857**	-0.452*	0.562**	0.559**	0.231

* P<0.05, ** p<0.01, NTH = Number of tiller per hill, NFTH = Number of fertile tiller per hill, FD = Flowering days, GFP = Grain filling periods, DM = Days to maturity, PH = Plant height (cm), PL = Panicle length (cm), GP = Grain per panicle, FGP = Fertile grain per panicle, TGW = Thousand Grain weight (g), GYP = Grain yield per plant (g).

The grain yield per plant exhibited significant positive correlation with grain per panicle and fertile grain per panicle at 25°C and 20°C temperature while with the 1000 grain weight at ambient condition. So, the correlation of agronomic traits observed to be influenced by the temperature.

The temperature also affects the variance components of morpho-agronomic performance of all studied traits except the number of fertile tiller per hill and 1000 grain weight (Table 3.10) while all genotypes were significantly different for all studied traits except grain filling periods and 1000 grain weight.

Table 3.10: ANOVA (F-distribution value) for agronomic traits in the glasshouse.

Source	df	NTH	NFTH	FD	GFP	DM	PH	PL	GP	FGP	TGW	GYP
Genotype	5	142.76**	66.84**	37.08**	2.71	59.07**	17.70**	15.02**	4.86*	4.81*	0.55	28.99**
Temperature	2	7.89**	0.46	39.75**	9.97**	10.53**	8.94**	16.62**	41.25**	33.15**	1.17	25.68**
MRQ 50	2	1.28	0.63	244.29**	64.17**	44.49**	68.26**	17.63**	89.47**	85.81**	15.60**	118.60**
Ranbir Basmati	2	1.38	0.16	749.84**	263.41**	56.00**	177.65**	8.60**	375.63**	290.87**	0.99	10.48**
Rato Basmati	2	6.74*	0.37	55.56**	2.32	24.77**	1122.86**	20.41**	471.35**	361.37**	20.92**	11.33**
E 7	2	1.96	4.64*	543.07**	161.22**	8.69**	90.50**	54.70**	168.64**	189.32**	16.68**	10.53**
E 13	2	2.28	3.48	525.82**	356.76**	27.91**	30.94**	4.51*	138.52**	141.41**	35.60**	58.29**
MR 219	2	0.80	1.77	113.31**	33.21**	24.28**	72.05**	49.10**	712.02**	679.69**	68.56**	77.30**

* $p<0.05$, ** $p<0.01$, df = Degrees of freedom, NTH = Number of tiller per hill, NFTH = Number of fertile tiller per hill, FD = Flowering days, GFP = Grain filling periods, DM = Days to maturity, PH = Plant height (cm), PL = Panicle length (cm), GP = Grain per panicle, FGP = Fertile grain per panicle, TGW = Thousand Grain weight (g), GYP = Grain yield per plant (g).

The ANOVA test result for agronomic traits (Table 3.10) for six genotypes revealed that the temperature significantly affected most of the agronomic traits. So, both the temperature and genotype exhibited significant influences for variance component of morpho-agronomic traits.

3.3.3 Performance of Phenotypic Aroma

At the maximum tillering stage, all aromatic genotypes demonstrated highest aroma score (score 4) while non-aromatic (MR 219) genotype showed the lowest score (score 1) at 25°C temperature. At the flowering stage, the highest aroma score (score 4) was observed in all aromatic genotypes at 25°C temperature. At the maturity stage, highest aroma score (score 4) was found in MRQ 50 and E 13 genotypes at 25°C while Ranbir Basmati demonstrated moderate aroma score (score 3) at all temperature conditions (Table 3.11).

Table 3.11: Phonotypic expression of aroma in leaf and grain of rice.

Genotype	Leaf aroma									Grain aroma		
	Maximum tillering stage			Flowering stage			Maturity stage					
	Ambient	25°C	20°C	Ambient	25°C	20°C	Ambient	25°C	20°C	Ambient	25°C	20°C
MRQ 50	3	4	2	3	4	3	2	4	2	3	4	2
Ranbir Basmati	3	4	3	3	4	3	3	3	3	3	4	2
Rato Basmati	3	4	2	3	4	3	2	3	2	3	4	2
E7	3	4	3	3	4	3	3	3	2	3	4	2
E13	4	4	3	3	4	3	3	4	3	3	4	2
MR 219	1	1	1	1	1	1	1	1	1	1	1	1

1 = Absence of aroma, 2 = mild aroma, 3= Moderate aroma and 4 = strong aroma.

The aroma evaluated by the organoleptic test (Table 3.11) representing the leaf aroma score of three temperature conditions at three growth stages while the grain aroma score of three temperature at maturity stage of rice. The phenotypic aroma varied at different growth stages under different temperature.

3.4 DISCUSSION

The present study evaluated the morpho-agronomic performance of rice genotypes under three temperature conditions to assess the effects of temperature on these traits as well as to identify a suitable temperature for the superior agronomic performance of aromatic rice.

The results of this investigation represented that genotypes or temperature or their interactions influenced morpho-agronomic traits performance of studied genotypes at different growth stages. The morpho-agronomic traits such as the number of tiller per hill, the number of fertile tiller per hill, flowering days, grain filling periods, days to maturity, plant height, panicle length, grain per panicle, fertile grain per panicle, 1000 grain weight and grain yield per plant were significantly different at 5% level (Table 3.6) as in DMRT test. Previous researchers also observed significant changes in agronomic performance of aromatic and non-aromatic rice due to heat stress, elevated temperature, cold stress etc. The detail discussion is as below:

3.4.1 Number of Tiller per Hill

The maximum number of tiller per hill (46.80) obtained from E 13 genotype grown at 25°C while the minimum number of tiller per hill (18.40) from Rato Basmati genotype grown at 20°C (Table 3.6). All genotypes produced their maximum number of tiller per hill at 25°C temperature. After 25°C temperature, the greater number of tiller per hill was observed at ambient condition compared to 20°C in Ranbir Basmati, Rato Basmati, and E 7 genotypes. However, a total number of tillers per hill differed significantly in experimental rice genotypes due to genetic nature and influence of

environmental temperature. Previously, Yoshida (1973) and Aghamolki et al. (2014) stated that tiller number increased at higher temperatures. They also found that the only temperature affected the tillering rate after 3–5 weeks of sowing. Yoshida (1981) mentioned that the tiller number per plant determined the panicle number of rice plant which is an essential component of grain yield.

3.4.2 Number of Fertile Tiller per Hill

The number of fertile tiller per hill was observed as the maximum (43.00) in E 13 genotype grown at 25°C while the minimum number of fertile tiller per hill (16.60) found at 25°C temperature (Table 3.6) in Rato Basmati genotype. All genotypes produced their highest number of fertile tiller per hill at 25°C except Ranbir Basmati genotype which produced at 20°C. A total number of fertile tillers per hill varied on genotypic condition and temperatures while the slight difference observed for ambient condition and 20°C temperature. Yoshida (1973) observed that higher temperature favorable for faster tillering and fertile tiller number increased at 28 to 31°C temperature but decreased at 22°C temperature. However, Golam et al. (2011) stated that the genetic differences were observed to be non-significant among rice genotypes for fertile tiller number per plants.

3.4.3 Flowering Days

Flowering days were observed to be different for genotypes and temperatures. The maximum time for flowering days noticed in Rato Basmati genotype (121.60 days) grown at 25°C while minimum days to flowering was found in E 13 genotype (79.20 days) at 20°C temperature (Table 3.6). The flowering days found to be affected by

growing temperature where longest duration to flowering days was at 25°C than at ambient condition and 20°C in all studied genotypes. So, flowering days which is a genetic character was highly affected by environmental temperature. In an earlier study using analysis of variance (ANOVA) Newmah (2010) observed highly significant genetic variations among genotypes for days of flowering. They assumed that this type of variability might be due to the genetic makeup of rice genotype and their interaction with environmental factors. Xue et al. (2008) also found variations in flowering days of their studied genotypes and identified a regulatory gene responsible for this type of differences. Later on, Golam et al. (2011) also observed significant genetic variation for flowering days among genotypes even in different replications.

3.4.4 Grain Filling Period

Grain filling period of rice genotypes was observed to be influenced by the temperature condition where a prolong grain filling period was in E 13 genotype (47.40 days) grown at 20°C and short grain filling period was in MRQ 50 genotype (18.40 days) at ambient condition (Table 3.6). The longest grain filling period was found at 20°C in all studied genotypes except Rato Basmati, which exhibited it at ambient condition. However, in most of the studied genotypes, a prolong grain filling period was observed at a lower temperature. Golam et al. (2011) also observed significant difference for grain filling period in their studied genotypes. Previously, Yoshida and Hara (1977) found a linear association between temperature and grain filling period within a daily mean temperature range of 16° to 28°C, they explained it as the higher the temperature, the faster the grains filled. However, they stated that the daily mean temperature might be the most meaningful expression for describing the effect of temperature on grain filling in rice.

3.4.5 Days to Maturity

The temperature affected the days to maturity, the maximum days to maturity needed for Rato Basmati genotype (151.20 days) grown at 25°C while the minimum days to maturity taken by Ranbir Basmati genotype (117.00 days) at ambient condition (Table 3.6). Most of the rice genotypes demonstrated their maximum days to maturity at 25°C but a fluctuation in days to maturity was exhibited within 20°C and ambient condition. However, days to maturity was observed to be controlled by genetic nature of the genotypes and influence of temperature. Though, Golam et al. (2011) did not found significant differences in days to maturity of their studied genotypes but Rani and Maragatham (2013) observed that the days taken to attain maturity was less at an elevated temperature of 4°C and 2°C compared to the ambient temperature.

3.4.6 Plant Height

The highest plant height observed in Rato Basmati genotype (119.80 cm) grown at 25°C while lowest plant height was in MRQ 50 genotype (60.00 cm) at 20°C temperature (Table 3.6). All genotypes demonstrated their highest plant height at 25°C compared to the ambient condition and 20°C temperature. However, both the genetic factors and environmental components seem to influence the plant height of the studied genotypes. Aghamolki et al. (2014) stated that plant height was a genetic character and effect of heat stress was non-significant. On the other hand, Oh-e et al. (2007) reported that the increase in plant height was steeper under high temperature (30-35°C) than under ambient temperature condition. Shrivastava et al. (2012) stated that plant height exhibited differential performance under different temperature conditions.

3.4.7 Panicle Length

All studied genotypes demonstrated their highest panicle length at 25°C followed by the ambient condition and 20°C temperature. The panicle length was maximum in Rato Basmati genotype (34.80 cm) grown at 25°C while minimum panicle length was in E 7 genotype (16.60 cm) at 20°C temperature (Table 3.6). However, Shabir et al. (2013) stated that the panicle length had a significant positive association with yield, but it is not necessary the maximum panicle length is responsible for higher yield. Machunde (2013) observed a significant difference in the panicle length due to genotype, environment and their interaction effects (genotype x environment) on this trait. Yoshida et al. (1981) observed that the seasonal climatic variables such as high fluctuations of day and night temperatures, changing rainfall patterns and day-length affected the panicle length negatively.

3.4.8 Grain per Panicle

The maximum number of grain per panicle was in E 13 genotype (161.40) grown at 25°C while the minimum number of grain per panicle was in Rato Basmati genotype (41.00) at 20°C temperature (Table 3.6). There were uniform performances of the number of grain per panicle in all genotypes regarding the temperature condition, at 25°C all genotypes demonstrated their maximum number of grain per panicle followed by the ambient condition and 20°C. Shabir et al. (2013) found wide ranges of variability in different genotypes for the number of grain per panicle. They also stated that all genotypes were significantly different from each other by the number of grains per panicle. Zhang et al. (2013) observed significant differences in the number of grain per

panicle between low and high night temperature. Yoshida (1973) stated that grain number per plant inversely correlated with temperature and within a temperature range of 22 to 31°C, grain number increased as temperature decreased. Jamal et al. (2009) observed non-significant variability among their tested genotypes and other factors such as soil fertility, plant nutrients, and weather condition might be responsible for little variation in the number of grain per panicle.

3.4.9 Fertile Grain per Panicle

The number of fertile grain per panicle was maximum in E 13 genotype (151.40) grown at 25°C while the minimum number of fertile grain per panicle was found in Rato Basmati genotype (33.20) at 20°C temperature (Table 3.6). Like grain per panicle, a uniformity of performances for the number of fertile grain per panicle was observed in all genotypes regarding the temperature condition, at 25°C, all genotypes demonstrated their maximum number of fertile grain per panicle followed by the ambient condition and 20°C. Previously, Machunde (2013) observed a broad range of genetic variability and significant genotype x environment interaction for the number of fertile grain per panicle. They also noticed that plants with many panicles per plant tend to compensate for too few seeds per panicle might be due to competition within a panicle. However, they found most of their studied genotypes with many panicles per plant had been a moderate number of grains per panicle and they concluded that the number of filled grains per panicle contributed positively to the grain yield. Patel et al. (2012) also reported significant variation in the number of fertile grains per panicle in rice. Golam et al. (2011) observed significant genetic variation among genotypes within replications but non-significant difference among all varieties for the fertile grain per panicle.

3.4.10 1000 Grain Weight

The maximum 1000 grain weight (32.67 g) and the minimum 1000 gain weight (15.19 g) were observed in E 13 genotype grown at 25°C and 20°C temperature, respectively (Table 3.6). All other genotypes demonstrated their maximum 1000 grain weight at 25°C temperature. The reduction of 1000 grain weight of E 13 genotype at 20°C indicated that well adapted aromatic genotypes subjected to reduce temperature during life cycle might affect 1000 grain weight but not have an effect on final grain yield per plant. Previously, Oh-e et al. (2007) observed that the reduction in 1000 grain weight under high-temperature conditions caused by the reduced activity of sink for starch synthesis. Though, Golam et al. (2011) did not found significant variations among all tested genotypes, but Machunde (2013) observed genetic variation for the 1000 grain weight among genotypes. Machunde (2013) assumed that the revealed genetic variability might be due to the genetically diverse origin of the genotypes and the presence of inherent genetic differences among the genotypes. They also pointed that the genotype maintained high grain yield through a compensatory effect of having a large number of panicles per plant, more filled grains per panicle and high percent filled grains per panicle but with lowered 1000 grain weight. Jamal et al. (2009) observed a non-significant variation of 1000 grain weight among tested genotypes of rice. Newmah (2010) considered 100-grain weight instead of 1000 grain weight and concluded that the varieties possessed longer, and slender grains have lower grain weight.

3.4.11 Grain Yield per Plant

Grain yield per plant of the aromatic rice genotypes demonstrated the suitability of 25°C temperature for better yield in controlled condition. The highest grain yield per plant observed in E 13 genotype (76.73 g) grown at 25°C while lowest grain yield per plant was in Rato Basmati genotype (41.00 g) at 20°C temperature (Table 3.6). In this investigation, all genotypes exhibited their maximum grain yield at 25°C including indigenous non-aromatic MR 219 genotype. The studied genotypes also demonstrated uniformity of the grain yield per plant which reduced from ambient condition to 20°C in all genotypes. This phenomenon indicated the influences of both genetic and environmental component for grain yield performance. Previously, Aghamolki et al. (2014) observed that the indigenous MR 219 genotype produced higher grain yield in all growing environment and growth stages, but its grain yield reduced when heat stress imposed during booting and flowering stage. They also found a similar situation in other exotic cultivars and concluded that greater yield reduction was due to a decrease in all important yield components. Newmah (2010) found significant variability in the number of grain yield per plant and assumed that this variation might be due to the environment and genetic constitution or the correlation of grain yield per plant with various yield contributing characteristics. Zhang et al. (2013) found significant differences (about 11% on an average of 4 seasons) in grain yield due to low and high night temperature differences (about 4°C temperature differences) in all experimental genotypes. Oh-e et al. (2007) noticed that the grain yield declined steeply when the daily mean temperature exceeded 28°C. Baker (2004) also stated that the rice grain yield progressively reduced in both *indica* and *japonica* cultivars when daily mean temperature increase above 26°C. Rani and Maragatham (2013) indicated that the grain yield declined significantly at the elevated temperature than the ambient temperature.

Machunde (2013) identified that the genotype x environment interaction played a significant role on rice grain yield and grain yield per plant. Xing and Zhang (2010) reported that rice varieties display tremendous levels of variation in grain yield owing to the diversity of genetic constitution. Therefore, breeding strategy to improve grain yield per plant should also focus on developing dense panicles or plant with a large number of panicles. The grain yield per plant and the number of panicles per plant attributed to the major genetic variability among genotypes for grain yield. Moreover, many characters interacted with each other to give the final grain yield.

3.4.12 Phenotypic Aroma Expression

Aroma of the experimental genotypes was observed to be divergent at different at growth stages depend on the temperature conditions. Only the leaf sample remained common and could be collected from different growth stages under different temperature. So, the leaf aromatic test played a vital role to discriminate the effects of temperature on different growth stages which also demonstrated differential expression of aroma within the growth stages (Table 3.11). On the other hand, uniformity and stability of phenotypic aroma were observed in harvested grain. The grain aroma represented a stable aroma score under a particular temperature which leads to continuing extraction of volatile compounds from rice grain for GC-MS and GC-FID analysis. However, in this experiment, a fluctuation of aroma score was found in all growth stages while it becomes stable in matured harvested grain. Previously, Vazirzanjani et al. (2011) stated that the sensory test using 1.7% KOH seemed to be cheaper and simpler method for differentiating the aromatic to non-aromatic rice. Moreover, several scientists (Golam et al., 2011; Hossain et al., 2008; Sarhadi et al., 2011) used leaf and grain sensory test for evaluating aromatic and non-aromatic rice.

Golam et al. (2011) mentioned that the aroma score of Basmati type rice demonstrated strong aroma (score 4) in Indian sub-continent (day-night average 22-23°C) and moderate aroma (score 3) in Malaysian tropical environment (day-night average 28-30°C) which was the similar observation of Golam et al. (2010). On the other hand, Nagarajan et al. (2010) did not found the significant interaction of aroma with the environment.

3.4.13 Correlation among Morpho-Agronomic Traits

Correlation analysis of agronomic traits is an important tool for indirect selection which helps the plant breeder during a breeding program and provides a clear indication of yield components. However, in the present investigation, the number of tiller per hill demonstrated significant positive correlation with the number of fertile tiller per hill and significant negative correlation with flowering day and days to maturity at 1% level in all temperature conditions (Table 3.7, 3.8 and 3.9). It exhibited positive correlation with the number of grain per panicle in all three temperature conditions while the negative correlation with grain filling period at ambient and 25°C but positive correlation at 20°C temperature. Previously, Shabir et al. (2013) stated that the number of tillers per plant had exposed significant negative correlation with the number of grains per panicle, positive association with panicle length and negative correlation with the 1000 grain weight. They also declared that the number of tiller per plant had a significant positive relation with grain yield.

The correlation study of plant height demonstrated a significant positive association with panicle length in all three temperature condition which indicated that this trait was genetically controlled, and the environment has little influence. Plant

height of the studied genotypes also demonstrated negative correlation with the number of fertile grain per panicle. These findings were similar as previous researchers who stated significant positive correlation of plant height with panicle length but negative correlation with the number of fertile grain per panicle and the fertile grain percentage (Mathure et al., 2011; Samal et al., 2014; Shabir et al., 2013). A negative correlation of plant height with the number of tiller per hill and the number of grains per panicle observed at all three temperatures. These results were opposite of Shabir et al. (2013) who found highly significant and positive association of plant height with panicle length, the number of tiller per plant and the number of grain per panicle. In the present experiment, plant height also displayed a negative correlation with 1000 grain weight at ambient and 20°C temperature but positive correlation at 25°C, indicated the influence of temperature on this trait. Previously, Shabir et al. (2013) observed significant negative interactions between plant height and 1000 grain weight though Golam et al. (2011) stated that plant height has no significant correlation with grain yield.

Panicle length showed the negative correlation between the numbers of grain per panicle at all temperatures which were opposite findings of Shabir et al. (2013) who observed highly significant positive association of panicle length with the number of grains per panicle.

Correlation coefficient analysis of 1000 grain weight had represented a positive association with grain per panicle at ambient and 25°C temperature while negative correlation at 20°C temperature. Whereas, Shabir et al. (2013) observed highly significant positive correlation of the 1000 grain weight with the number of grains per panicle. At all temperatures, the fertile tiller per hill exhibited positive correlation with

grain yield per plant and grain per panicle also demonstrated a positive relation with grain yield per plant which was similar as mentioned by Golam et al. (2011).

In this study, the grain yield per plant indicated a positive correlation with the number of tiller per hill, the number of fertile tiller per hill, flowering days, grain per panicle, and fertile grain per panicle in all temperatures. The grain yield per plant also demonstrated a positive correlation with days to maturity at ambient and 20°C while a negative correlation at 25°C temperature. It also exhibited a negative correlation with grain filling period, plant height and panicle length in all three temperatures. Positive correlations of the grain yield per plant with 1000 grain weight notified at ambient and 20°C while negative correlation was only at 25°C temperature. Previously, Samal et al. (2014) stated that grain yield had a positive correlation with panicle length and fertile grain per panicle but negative correlation with plant height. They also added that total grain per panicle had a positive association with grain yield per plant. Besides, Golam et al. (2011) indicated that the number of fertile tiller per hill, grain per panicle and fertile grain per panicle had a positive contribution to grain yield. Kibria et al. (2008) found a positive correlation between grain yield with panicle length and productive tillers. Shabir et al. (2013) found a significant positive association of grain yield with all other parameters such as plant height, the number of tiller per plant, panicle length, 1000 grain weight and the number of grain per panicle. Moreover, Golam et al. (2011) assumed that the number of tiller per hill, panicle length and 1000 grain weight would show a positive correlation with yield but they could not found a significant association of yield with the characters as they mentioned.

Based on the correlation analysis of morpho-agronomic traits, it was observed that some character were directly associated with other characters regardless of

genotypic and temperature conditions. Some character displayed positive or negative correlation based on the temperature condition and represented the influences of temperature on the correlation among agronomic characters. However, the number of tiller per hill, the number of fertile tiller per hill, days to flowering, days to maturity, grain filling periods, panicle length, 1000 grain weight, the number of grain per panicle, and the number of fertile grain per panicle were the most important traits for contributing grain yield and represented the effects of temperature.

3.5 CONCLUSION

This study characterized and evaluated the morpho-agronomic parameters of some aromatic rice genotypes under different temperature to select promising genotypes for crop improvement program and to observe the effects of temperature on these parameters.

In this regards, the agronomic performance of six rice genotypes was compared between two growing seasons at two different ambient temperature i.e. net house (ambient temperature 27°C) and glasshouse (ambient temperature 28°C) and found that the traits were non-significantly different within these two temperatures and locations. This finding concluded that the season and location did not affect the morpho-agronomic performance of aromatic rice genotype within the same temperature condition. Moreover, it justified the uses of any one ambient condition (net house or glasshouse condition) to compare agronomic performance of rice with the controlled temperature (25°C and 20°C).

The DMRT results represented that the maximum variation was in the grain filling periods and the minimum variation was in the number of tiller per hill of the studied genotypes under different temperature. This result concluded that the performance of a trait depends on temperature condition.

The Pearson's correlation analysis exhibited that the grain yield per plant positively correlated with days to maturity and 1000 grain weight at ambient and 20°C temperature but negatively correlated with these traits at 25°C temperature. Besides, the grain yield per plant was negatively correlated with grain filling period at all three temperatures. So, the correlations among morpho-agronomic traits were influenced by the specific trait and the temperature condition.

The ANOVA results for agronomic traits revealed that the 1000 grain weight of rice genotypes remained uniform and did not differ regarding temperature condition and genotypes. On the other hand, all other morpho-agronomic traits were highly affected ($p \leq 0.01$) by the environmental temperature in all studied genotypes. So, the temperature had significant effects on morpho-agronomic trait performance of aromatic rice.

The organoleptic test for aroma showed that all aromatic rice genotypes possessed highest aroma score (score 4) at 25°C temperature while a fluctuation of aroma score (score 2 to 4) was in ambient and 20°C temperature at different growth stages. Therefore, both the growth stages and temperature influenced aroma score of aromatic rice and it become stable at the matured grain stage.

However, the performance of grain yield and yield related traits of E 13 genotype (collected from IRRI) were better and aroma score was higher at 25°C

temperature compared to other genotypes and another two temperature condition. So, the E 13 genotype can be used for further aromatic rice development program.

This experiment was conducted in the Malaysian tropical environment where controlling temperature at 25°C allowed producing good performer aromatic rice and conducting such type of experiment in other regions of the world will help to establish a suitable strategy for future aromatic rice production. Moreover, searching the places with available 25°C temperature during rice growing season will widen the high-quality aromatic rice growing area. Though, the cost and benefit of aromatic rice production under controlled temperature was not considered but the obtained information will help to the development of an appropriately controlled environment (where 25°C can be ensured) and to assess the possibility of superior aromatic rice production in the diverse temperate region.

CHAPTER 4: SEQUENCE ANALYSIS AND ALIGNMENT OF *Badh2* GENE FRAGMENTS IN AROMATIC RICE

4.1 INTRODUCTION

Rice is an important cereal crop which consumed significantly for nutrition and caloric intake that made it be the obvious choice for the first whole genome sequencing of a cereal crop. The International Rice Genome Sequencing Project (IRGSP) has analyzed and generated a highly accurate finished sequence of the rice genome (Project, 2005). The completion of genome sequences for both *indica* and *japonica* rice subspecies let to develop unlimited molecular markers depend on remarkable sequence divergences between these two rice subspecies for contrasting traits. Molecular markers linked to an important trait and sequence analysis of a specific gene has opened a new era to study individuals with favorite grain quality trait. Aroma is an important grain quality trait of high-quality rice that inspires researchers to investigate in details about the sequence divergence as well as genetic analysis of this trait (Chen et al., 2006; Shi et al., 2008).

Genetic analysis of aroma in rice suggests that two or three recessive or dominant genes determine the aroma trait (Reddy & Reddy, 1987). On the other hand, most of the researchers agreed that aroma of rice controlled by one single recessive gene (Jin et al., 2003; Sood & Siddiq, 1978). However, Ahn et al. (1992) mentioned that aroma governed by the *fgr* gene which was present on chromosome 8 and at the genetic distance of 4.5 cM from the RFLP marker RG28. The *fgr* gene then mapped to a chromosomal segment flanked by the RFLP markers RG1 and RG28 with the genetic distance ranged from 10 cM (Causse et al., 1994) and 12 cM (Lorieux et al., 1996) to

25.5 cM (Cho et al., 1998). Additionally, Cordeiro et al. (2002) and Jin et al. (2003) developed an SSR marker and an SNP marker linked to the *fgr* locus for distinguishing the aroma allele from the non-aroma allele. All these markers facilitated to restrict the *fgr* locus with an interval of 69 kb and sequence analysis of this (*fgr*) region revealed the presence of three candidate genes *Cah*, *Mccc2* and *Badh2* encoding carbonic anhydrase, 3-methylcrotonyl-CoA carboxylase β-chain, and betaine aldehyde dehydrogenase, respectively (Chen et al., 2006).

Sequencing analysis of 17 genes present in the *fgr* regions in 64 non-aromatic and 14 aromatic rice indicated that a gene encoded betaine aldehyde dehydrogenase isoform 2 (*Badh2*) is the *fgr* gene, due to its sequence divergence between aromatic and non-aromatic rice (Bradbury et al., 2005a). The *badh2* allele from aromatic rice cultivars had common insertions and deletions with single nucleotide polymorphisms (SNPs) compared to non-aromatic genotypes. Gene structure analysis of *Badh2* gene represented that it contains 15 exons and 14 introns. Chen et al. (2008) reported that the *Badh2* of Chinese aromatic rice had 8-bp deletion (5´-GATTATGG-3´) and 3 SNPs at exon 7 which not found in non-aromatic rice. This 8-bp deletion and 3 SNPs at exon 7 of *Badh2* gene resulted in truncated BADH2 protein of 251 residues which also indicated loss of the function of this exon segment in aromatic rice (Chen et al., 2008). However, the loss of function of *Badh2* segment did not seem to limit the growth of aromatic rice genotypes. Moreover, sequence analysis of another 23 aromatic rice varieties at their *badh2* loci, a null *badh2* allele (named *badh2E2*) with a 7-bp deletion (5´-CGGGCGC-3´) in exon 2 was identified (Shi et al., 2008). This 7-bp deletion in exon 2 also resulted in a truncated BADH2 protein consisted of a shorter peptide with 82 residues (Chen et al., 2008; Shi et al., 2008). However, based on the above information it is clear that the deletion of 7-bp in exon 2 (*badh2E2*) or 8-bp in exon 7

(*badh2E7*) or in both exons (*badh2E2* and *badh2E7*) of the *badh2* gene can represent aromatic condition while absent of deletion (presence of all bases) can represent the non-aromatic condition in rice.

Aroma quality of rice is naturally quantitative trait and the gene controlling this trait is recessive or minor genes that demonstrate Mendelian inheritance but highly affected by the environmental component (Hashemi et al., 2015). However, aromatic rice production is currently facing a lot of problems including environmental degradation, pollution, an increase in temperature due to global warming, reductions of cultivable land, water deficiency, increasing labor cost and uses of energy-dependent fertilizer. To reduce the effects of these factors on yield potential and gaining good quality aromatic rice, a combination of molecular biological technique, biotechnological approach, morphological approach, metabolomics analysis, and improved conventional breeding strategy are necessary where a high-quality aroma gene sequence might be an essential tool for integrative investigation.

The aim of this experiment was to analyze the DNA sequences of selected rice genotypes for assessing the possible genetic cause of aroma expression.

The objectives were:

- To observe the DNA sequence of exon 2 and exon 7 for the identification of possible reason of aroma in the selected rice genotypes.
- To detect the specific deleted bases in aromatic rice genotypes by comparing their DNA sequences with non-aromatic rice.

- To identify mutations or changes in the DNA sequences of aromatic rice contrast to non-aromatic rice.

The genetic background and sequences analysis of exon segments will help to determine the deletion and mutations of the specific base for phenotypic aroma expression in studied genotypes.

This experiment will explain details about the DNA extraction procedure, PCR conditions, Gel Electrophoresis system, DNA sequencing, alignments and comparison of sequences among the selected rice samples for identification of genetic condition for aroma expression.

4.2 MATERIALS AND METHODS

4.2.1 Plant Materials

The leaf samples from all studied genotypes (Table 3.1) were collected at the maximum tillering stage, wrapped in aluminum foil, kept in ice box then transfer to -80°C freezer until DNA extraction preparation. The samples were taken out from -80°C freezer and kept into -20°C freezer then stored in ice for DNA extraction. The samples were ground by mortar and pestle using liquid nitrogen for DNA extraction and then followed the manufacturer protocol.

4.2.2 DNA Extraction

The genomic DNA from leaves of all studied genotypes was extracted using Qiagen DNeasy plant mini kit (Qiagen GMbH, Germany) according to the manufacturer protocol as stated below:

The DNeasy Plant Mini Kit was stored at room temperature (15–25°C), all centrifugation steps were performed at room temperature (15–25°C), and the water bath was preheated to 65°C. The leaf samples (100 mg) were disrupted using mortar and pestle in liquid nitrogen. The ground samples were then transferred into 1.5 ml microcentrifuge tubes. The AP1 buffer (400 µl) and 4 µl RNase A were added and then mixed by vortex. The mixture was incubated for 10 min at 65°C and inverted 2–3 times during incubation. Buffer P3 (130 µl) was added to the mixture and incubated for 5 min on ice. The mixture was centrifuged at 14,000 rpm for 5 min and lysate was pipet into a QIAshredder spin column which was placed in a 2 ml collection tube and centrifuged at 14,000 rpm for 2 min. The flow-through was transferred into a new tube without disturbing the pellet. Buffer AW1 (1.5 volumes) was mixed by pipetting and about 650 µl of the mixture was transferred into a DNeasy Mini spin column placed in a 2 ml collection tube, centrifuged at 8000 rpm for 1 min and discarded the flow-through. This step was repeated for the remaining sample. The spin column was placed into a new 2 ml collection tube, and buffer AW2 (500 µl) was added then centrifuged for 1 min at 8000 rpm. The flow through was discarded and another 500 µl of Buffer AW2 was added and centrifuged at 8000 rpm for 2 min. Care was taken to remove the spin column from the collection tube so that the column did not come into contact with the flow-through. The spin column was transferred to a new 1.5 ml micro-centrifuge tube. A 100 µl buffer AE was added for elution and incubated at room temperature (15–25°C)

for 5 min then centrifuged at 8000 rpm for 1 min. This step was repeated to get a total of 200 µl DNA solution.

The quality and concentration of extracted DNA were estimated by NanoDrop 2000 (Thermo Scientific, USA) as mentioned in Table 4.1.

Table 4.1: Quality and concentration of extracted DNA.

Sample	A260	A280	260/280	260/230	Concentration (ng/µl)
MRQ 50	0.76	0.38	2.00	2.62	41.3
Ranbir Basmati	0.27	0.13	2.07	3.09	14.7
Rato Basmati	0.32	0.15	2.13	3.53	18.4
E 7	0.61	0.27	2.25	2.52	37.5
E 13	0.54	0.27	2.00	2.75	30.0
MR 219	0.21	0.10	2.10	2.76	25.9

A = absorbance.

4.2.3 Primer Design

A total of 8 primers containing reverse and forward sequences of different exon segments were considered based on the complete nucleotide sequences of *Badh2* gene obtained from NCBI (National Center for Biotechnology Information, USA) with Gene Bank accession number of EU770319. The primers were designed using the Primer Quest tools (Integrated DNA Technology Inc., USA) as stated in Table 4.2, and were synthesized by My TACG Bioscience (Malaysia).

Table 4.2: List of Primers with expected PCR product sizes.

Primer name	Accession No.	Available Primer sequence	Sizes (bp)
badh2E1-3	EU770319	Forward: 5C'-AGCGGCAGCTCTTCGTC-3C' Reverse: 5C'- CCCACAATCAAGCGTCTCTAGT -3C'	1173
badh2E6-8	EU770319	Forward: 5C'-CAGGTGTGCAAACATGTGC-3C' Reverse: 5C'- CATCATCAAACACCACTATAGGAC -3C'	530
badh2E2	EU770319	Forward: 5C'-GAGGCGCGAAGAGGAAC-3C' Reverse: 5C'-ATTGCGCGGAGGTACTTG-3C'	76
badh2E7	EU770319	Forward: 5C'-TAGGTTGCATTTACTGGGAGTT-3C' Reverse: 5C'- ACAAACCTTAACCATAGGAGCA-3C'	75/67

4.2.4 PCR Amplification

The extracted DNA was used for PCR amplification where 5 µl of each primer, 25 µl of Gotaq Green master mix (Promega, USA) and 100 ng DNA were added to nuclease free water (Table 4.3) to get a total of 50 µl reaction mixtures. The MultiGene™ OptiMax Thermal Cycler (Labnet International Inc, USA) was used, and the PCR cycling conditions were 95°C for 2:00 min followed by 30 cycles of 95°C for 1:00 min, 52°C for 30 sec, 72°C for 30 sec and final extension at 72°C for 5:00 min. The PCR products were then run on agarose gel electrophoresis system.

Table 4.3: DNA concentration, DNA amount and water for PCR amplification.

Sample	DNA Concentration (ng/µl)	DNA (µl)	Water (µl)
MRQ 50	41.3	2.42	12.58
Ranbir Basmati	14.7	6.80	8.20
Rato Basmati	18.4	5.43	9.57
E 7	37.5	2.67	12.33
E 13	30.0	3.33	11.67
MR 219	25.9	3.86	11.14

4.2.5 Agarose Gel Preparation

Agarose gel was prepared with a mixture of 0.70 g of agarose powder and 35 ml of 1x TBE buffer according to Table 4.4 to get 2% gel. The mixture of agarose powder

and 1x TBE buffer (Table 4.5) was heated in an oven until disappearance of crystal granule. The mixture containing flask were taken out from oven and poured into the tray which previously set up with cassette and comb. The mixture was kept at room temperature for 30 minutes to become rigid.

Table 4.4: Agarose gel mixture condition.

TBE buffer 1X (ml)	Agarose powder (g)			
	0.8 %	**1 %**	**2 %**	**4 %**
20	0.16	0.20	0.40	0.80
25	0.20	0.25	0.50	1.00
30	0.24	0.30	0.60	1.20
35	0.28	0.35	0.70	1.40
40	0.32	0.40	0.80	1.60

Table 4.5: Components for the preparation of 10X TBE buffer.

Component	Volume
Tris Base	108 g
Boric Acid	55 g
EDTA	40 ml
dH_2O	Top up to 1 Liter

The 10X TBE buffer was diluted to get the 1X solution (100 ml 10X TBE was added to 900 ml of distilled water).

The comb was taken out and solid agarose gel was transferred into gel electrophoresis system and submerged by 1X TBE buffer. The PCR product was loaded with 1.5Kb DNA ladder (Roche, Castle Hill, NSW, Australia) and was stained by ethidium bromide to observe in gel documentation system.

4.2.6 Sequencing

The PCR product of total volumes (50 µl) was run on 2% agarose gels and stained with ethidium bromide. The bands were excised and purified with universal gel extraction kit (Tiangen Biotech, Beijing Co. Ltd. China). The PCR products were

sequenced for both strands (product obtained by forward and reverse primer) using the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the same primers (forward and reverse primer) as in the PCR. The reaction mixture included 100 ng of the PCR-template, 50 ng primers, 1 µl BigDye mix, 1 µl 5 × reaction buffers in a 10 µl of total volume. The PCR reactions were performed in a GeneAmp PCR System 2700 Thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following cycling parameters: 96°C initial de-naturation for 1:00 min followed by 35 cycles of 96°C for 10 sec (denaturation), 50°C for 10 sec (annealing), 60°C for 4:00 min (elongation). The sequencing products were analyzed by an ABI 3730xl DNA analyzer (Applied Biosystems, USA). The chromatogram and alignments of the sequence were evaluated with chromas lite (Technelysium Pty Ltd, Australia) and MEGA software (Tamura et al., 2013).

4.3 RESULTS

The results related to sequence analysis of four different fragments of the *badh2* gene has been categorized into three types. At first, the PCR amplified products were run on gel electrophoresis system and presented in Fig. 4.1, 4.2, 4.3 and Fig. 4.4. Secondly, the sequence analysis results were presented in Appendix (A, B), Table 4.7, and Table 4.10. Finally, the primary protein sequences from the functional gene fragments (exon fragments) were presented in Table 4.6, 4.8, 4.9 and Table 4.11.

The fragment *badh2E1-3* contained a partial fragment of exon 1 and exon 3 while the complete fragment of intron 2 and exon 2 with a total length of 1173 bp. In the agarose gel system, all genotypes produced 1173 bp bands except Rato Basmati, which did not demonstrate band with *badh2E1-3* primer. Moreover, non-aromatic rice

genotype MR 219 demonstrated more distinct band than aromatic rice genotype (Fig. 4.1).

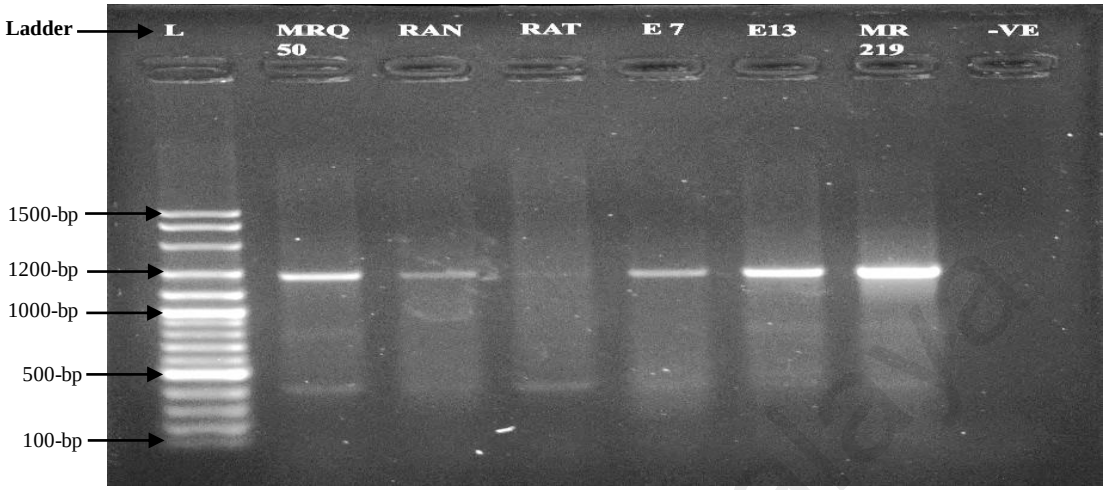

Figure 4.1: Agarose gel electrophoresis of *badh2E1-3* fragment amplified by PCR. Here, all genotypes demonstrate 1173-bp bands except Rato Basmati.

The sequences of *badh2E1-3* segments were presented in Appendix A and the protein sequence is in Table 4.6.

Table 4.6: Protein sequence from the *badh2E1-3* segment.

Genotype	Protein Sequence (amino acid)
EU770319	SGSSSSPASGAPPRSAAASPSSTPPPSPPSARSRRARRRT WTRRWRRRGRREEEPGPRLGARAGRRPGQVPPRNRGQIIE RKSELARLETLDCG
MRQ 50	SGSSSSRSAAASPSSTPPPSPPSARSRRARRRTWTRRWR RRGRREEEPGPRLGARAGRRPGQVPPRNRGQLKKLETGP NWIRGAFLG
Ranbir Basmati	SGGGGPRSAPPPRRHPATESPIARSQRARRMMWTRPW PRPGRREEEPGPRLGARAGRRPGQVPPRNRGQLKNVETGP KWYTDVVLR
E 7	SGSSSSGGGTRARPPPPVVTPPPNPPSARSQRARRMTW TRRRRRRRRREEEPGPRLGARAGRRPGQVPPRNRGQWKK LETGGMVYGSCFLG
E 13	NDGSSWRRLPVVNPATESPIARSRRARRRTWTRRWRR RGRREEEPGPRLGARAGRRPGQVPPRNRGQPTPPGGGLP WRGGNPPG
MR 219	SGSSSSCSDAASPSSTPPPSPPSARSRRARRRTWTRRWR RRGRREEEPGPRLGARAGRRPGQVPPRNRGQWNKLEAW LIAYLDAVFV

The PCR amplified products from *badh2E2* demonstrated 76-bp bands in agarose gel (Fig. 4.2).

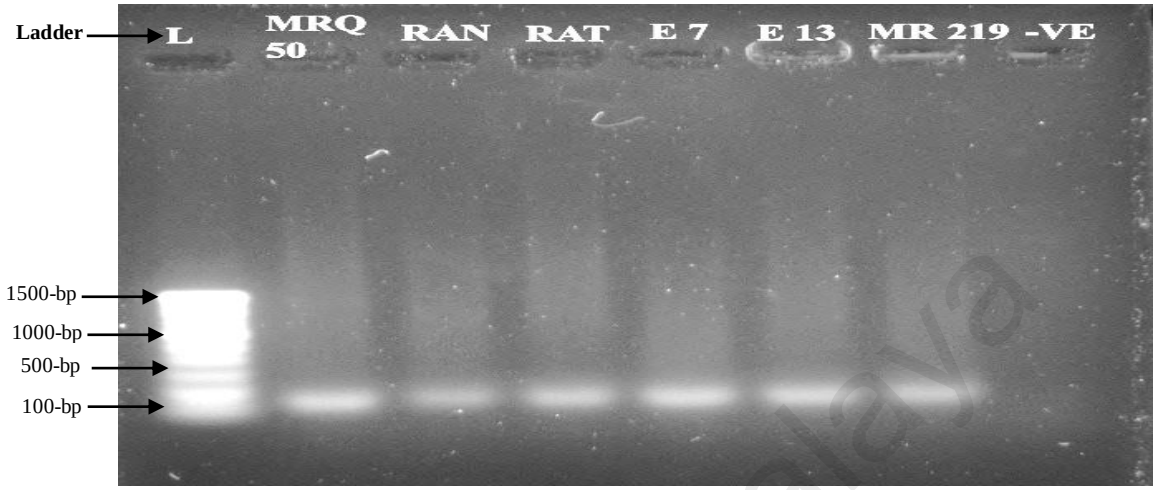

Figure 4.2: Agarose gel electrophoresis of *badh2E2* fragment amplified by PCR. Here, all genotypes demonstrate 76-bp bands.

The *badh2E2* primer which amplified exon 2 (partially) of *badh2* gene demonstrated uniform band with 76-bp amplicon length (Fig. 4.2) for all studied genotypes. The sequence analysis of exon 2 exhibited intact exon sequence (Table 4.7) without any deletion in all aromatic and non-aromatic genotypes.

Table 4.7: Sequence alignments for the *badh2E2* segment.

Genotype	Start (bp)	Sequence 5´ - 3´ (76-bp)	End (bp)
EU770319	1508	GAGGCGCGAAGAGGAACCGGGGCCGCGACTGGGCGCGCGCGCCGG GCGCCGTCCGGGCCAAGTACCTCCGCGCAAT	1584
MRQ 50	224	GAGGCGCGAAGAGGAACCGGGGCCGCGACTGGGCGCGCGCGCCGG GCGCCGTCCGGGCCAAGTACCTCCGCGCAAT	300
Ranbir Basmati	222	GAGGCGCGAAGAGGAACCGGGGCCGCGACTGGGCGCGCGCGCCGG GCGCCGTCCGGGCCAAGTACCTCCGCGCAAT	298
E 7	236	GAGGCGCGAAGAGGAACCGGGGCCACGACTGGGCGCGCGCGCCGG GCGCCGTCCGGGCCAAGTACCTCCGCGCAAT	312
E 13	216	GAGGCGCGAAGAGGAACCGGGGCCGCGACTGGGCGCGCGCGCCGG GCGCCGTCCGGGCCAAGTACCTCCGCGCAAT	292
MR 219	224	GAGGCGCGAAGAGGAACCGGGGCCGCGACTGGGCGCGCGCGCCGG GCGCCGTCCGGGCCAAGTACCTCCGCGCAAT	300

The protein sequence of exon 2 also demonstrated intact protein sequence (Table 4.8) which indicated the absence of a deletion in exon 2 of studied genotypes.

Table 4.8: Protein sequence from the *badh2E2* segment.

Genotype	Protein Sequence (25 amino acid)
EU770319	EARRGTGAATGRARRAPSGPSTSAQ
MRQ 50	EARRGTGAATGRARRAPSGPSTSAQ
Ranbir Basmati	EARRGTGAATGRARRAPSGPSTSAQ
E 7	EARRGTGATTGRARRAPSGPSTSAQ
E 13	EARRGTGAATGRARRAPSGPSTSAQ
MR 219	EARRGTGAATGRARRAPSGPSTSAQ

The amplification of exon 6-8 fragments which possessed exon 6 and exon 8 partially but exon 7 and intron 7 completely of the *badh2* gene. The *badh2E6-8* primer represented 530-bp amplicon length (Fig. 4.3) for all studied genotypes except non-aromatic MR 219 genotype. Moreover, aromatic genotype MRQ 50 demonstrated more distinct band than other genotypes.

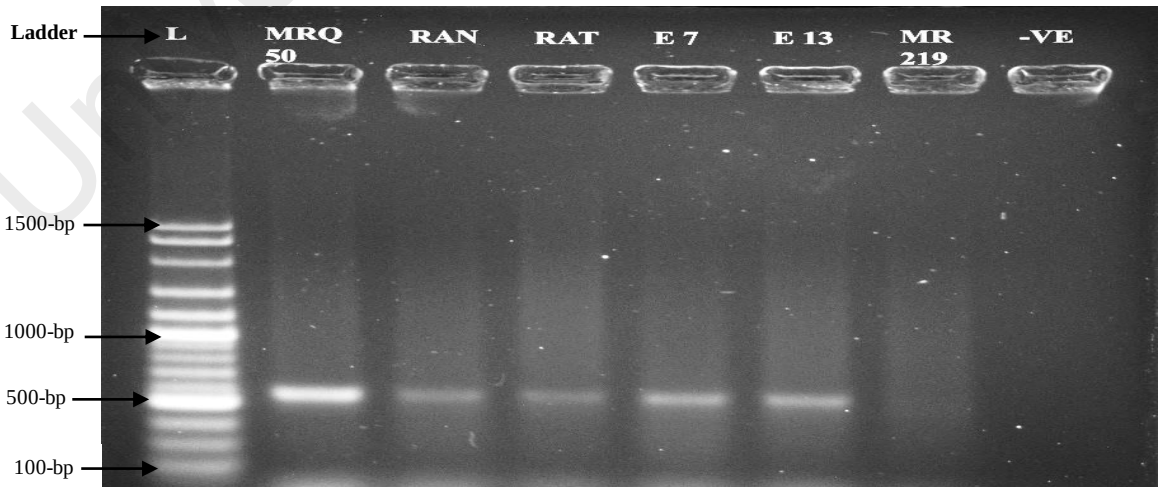

Figure 4.3: Agarose gel electrophoresis of *badh2E6-8* fragment amplified by PCR. Here, all genotypes demonstrate 530-bp bands except MR 219.

The sequences of *badh2E6-8* fragments are presented in Appendix B (NCBI accession number KX932086-91) while the protein sequences are presented in Table 4.9.

Table 4.9: Protein sequence from the *badh2E6-8* segment.

Genotype	Protein Sequence (amino acid)
EU770319	QVAKHSDDVLKPVLLCHHTLVTRLHLLGVMKLVKRL WLQLLLWLSLFHWNLVEKVLWFDD
MRQ 50	QVAKHSDGSLVLCHTLVTRLHLLGVMKLVYFQLLLW LSLFHWNLVEKVLWFDD
Ranbir Basmati	QVWAHSDDVLKPVLLCHHTLVTRLHLLGVMKLVYFQ LLLWLSLFHWNLVEKVLW
E 7	QVAKHSDLDLVLCHTLVTRLHLLGVMKLVYFQLLLW LSLFHWNLVEKVLW?FDE
E 13	QVAKHSDDLVLCHTLVTRLHLLGVMKLVYFQLLLWL SLFHWNLVEKVLW
MR 219	QVAKHSDKRVLLCHHTLVTRLHLLGVMKLVYFQLLL WLSLFHWNLVEKVLWCLDDE

The amplified products of exon 7 fragments (partial segment) demonstrated uniform band (Fig. 4.4) with 75-bp (without 8-bp deletion) or 67-bp (due to 8-bp deletion) amplicon lengths for studied genotypes using the *badh2E7* primer. Though comprehensible differences between 75-bp and 67-bp amplicon length could not observe in agarose gel documentation but the sequence analysis of exon 7 (Table 4.10) exhibited distinct differences between aromatic and non-aromatic genotypes.

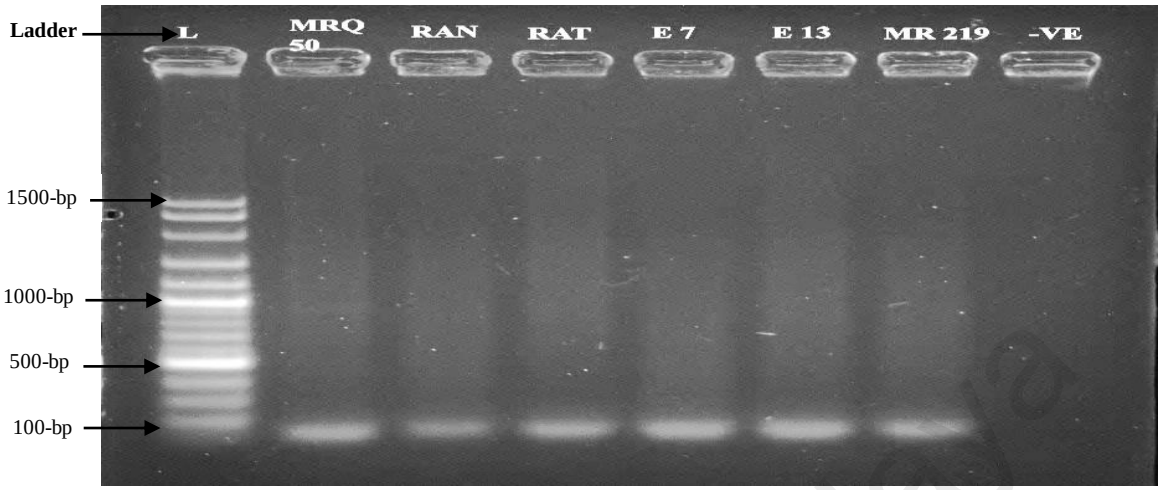

Figure 4.4: Agarose gel electrophoresis of *badh2E7* fragment amplified by PCR. Here, all aromatic genotypes demonstrate 67-bp bands and non-aromatic MR 219 exhibits 75-bp band.

However, aromatic genotypes demonstrated 8-bp deletions in exon 7, but non-aromatic MR 219 genotype did not contain the deletion.

Table 4.10: Sequence alignments for the *badh2E7* segment.

Genotype	Start	Sequence 5´ - 3´ (75/67bp)	End
EU770319	2500	TAGGTTGCATTTACTGGGAGTTATGAAACTGGTAAAAAGATTATGGC TTCAGCTGCTCCTATGGTTAAGGTTTGT	2575
MRQ 50	146	TAGGTTGCATTTACTGGGAGTTATGAAACTGGTATATA-------- TTTCAGCTGCTCCTATGGTTAAGGTTTGT	213
Ranbir Basmati	154	TAGGTTGCATTTACTGGGAGTTATGAAACTGGTATATA-------- TTTCAGCTGCTCCTATGGTTAAGGTTTGT	221
Rato Basmati	146	TAGGTTGCATTTACTGGGAGTTATGAAACTGGTATATA-------- TTTCAGCTGCTCCTATGGTTAAGGTTTGT	213
E 7	145	TAGGTTGCATTTACTGGGAGTTATGAAACTGGTATATA-------- TTTCAGCTGCTCCTATGGTTAAGGTTTGT	212
E 13	144	TAGGTTGCATTTACTGGGAGTTATGAAACTGGTATATA-------- TTTCAGCTGCTCCTATGGTTAAGGTTTGT	211
MR 219	145	TAGGTTGCATTTACTGGGAGTTATGAAACTGGTAAAAAGATTATGGC TTCAGCTGCTCCTATGGTTAAGGTTTGT	220

Moreover, protein sequence analysis of exon 7 of the *badh2* gene (Table 4.11) exhibited intact protein with 24 amino acid in non-aromatic genotype (MR 219) while a

deletion of 4 amino acid (20 amino acids) was observed in all aromatic genotypes which might be due to an 8-bp deletion in exon 7 of studied aromatic genotypes.

Table 4.11: Protein sequence from the *badh2E7* segment.

Genotype	Protein Sequence (24/20 amino acid)
EU770319	VAFTGSYETGKKIMASAAPMVKVC
MRQ 50	VAFTGSYETGIYFSCSYG*GL---
Ranbir Basmati	VAFTGSYETGIYFSCSYG*GL---
Rato Basmati	VAFTGSYETGIYFSCSYG*GL---
E 7	VAFTGSYETGIYFSCSYG*GL---
E 13	VAFTGSYETGIYFSCSYG*GL---
MR 219	VAFTGSYETGKKIMASAAPMVKVC

* = premature protein or incomplete protein.

Therefore, an 8-bp deletion in exon 7 was the cause of aromatic flavor of studied aromatic genotypes while such deletion was absence in non-aromatic of genotypes.

4.4 DISCUSSION

Aroma is an utmost important grain quality of aromatic rice and is controlled by a recessive gene which contained a 7-bp deletion in exon 2 or 8-bp deletion in exon 7 of *Badh2* gene (Shi et al., 2008). Hence, this study aimed to investigate the cause of aroma either for 7-bp deletion in exon 2 or 8-bp deletion in exon 7 of the *badh2* gene of the experimental genotypes. This information was considered to design appropriate primer for gene expression study and to explain the genetic state of the genotype.

This experiment was designed to amplify exon 1 and exon 3 partly while intron 2 and exon 2 completely to get more clear information about the presence of a 7-bp

deletion in exon 2. Moreover, to get smaller DNA sequence and protein sequence, a short fragment of exon 2 was also amplified by the *badh2E2* primer. So, by the final evaluation of the sequence of exon 2 amplified by *badh2E1-3* primer and partial sequence of exon 2 amplified by *badh2E2* primer it was clear that there was no deletion in exon 2 and its sequence was similar in all studied genotypes (Table 4.7).

Similarly, a portion of exon 6 and exon 8 along with a complete portion of exon 7 and intron 7 was amplified by the *badh2E6-8* primer. A small portion of exon 7 was also amplified by the *badh2E7* primer to get shorter DNA sequence and polypeptide sequence. This sequence analysis confirmed the presence of an 8-bp deletion in exon 7 of aromatic genotypes while non-aromatic genotype did not possess the deletion (Table 4.10).

The sequence analysis of exon 7 of the *badh2* gene also demonstrated three single nucleotide sequence polymorphism (TTT) in aromatic rice genotypes (MRQ 50, Ranbir Basmati, Rato Basmati, E 7 and E 13 genotype) compared to non-aromatic rice genotype (MR 219). In a previous study, similar polymorphism (3 SNP) was detected by Bradbury (2009). He also found 8-bp deletions within a 25-bp region and assumed that this mutation would render the protein non-functional which explained aroma being a recessive trait. Sequence analysis of this region in different aromatic and 64 non-aromatic rice varieties showed that 14 aromatic varieties contained identical sequence polymorphism as in Kyeema genotype while 64 non-aromatic varieties exhibited sequence identical to the published Nipponbare sequence (Bradbury, 2009).

In the present investigation, a truncated protein encoded in aromatic rice varieties observed to be shorter of 5 amino acid residues (KKIMA) compared to non-

aromatic rice genotype (Table 4.11). Previously, Bradbury (2009) mentioned similar result when analyzed the nucleotide sequence of exon 7 of both non-aromatic and aromatic rice varieties. He stated that aromatic rice variety showed a large deletion and three SNPs which terminates protein prematurely. The truncated protein encoded by the mutated *badh2* gene lack of the highly conserved sequences encoded by exons 8, 9 and 10 in aromatic rice varieties. The conserved sequence is believed necessary for the production of correct and functional protein. Bradbury (2009) also stated that aroma is a recessive trait and a loss of function of complete *Badh2* gene is responsible for aroma expression in aromatic rice. The truncated protein encoded by the experimental aroma genotypes was a nonfunctional protein that also supported this hypothesis. The mutation in the *Badh2* gene does not seem to associate with any loss of plant performance, besides, have a positive effect under some environmental conditions, such as the aromatic rice variety Khao Dawk Mali 105 demonstrated higher concentration of 2AP in response to drought stress and increased salt concentrations (Yoshihashi et al., 2004). The presence of the same allele of the *badh2* gene in all aromatic rice genotypes was consistent which indicated that the aroma trait inherited from a common aromatic ancestor. Thus, the modern aromatic rice varieties which possessed the 8-bp deletion in exon 7 of the *badh2* gene might derive from the same aromatic parent. However, aroma is a complicated trait because of the recessive nature of the gene and the difficulty of assessing aroma of individual rice grain. Hence, the inheritance of *badh2* gene in aromatic rice is likely to be the product of human selection for aroma in aromatic rice breeding.

During this investigation, only Rato Basmati did not perform satisfactory amplification using *badh2E1-3* primer but demonstrated successful amplification with the *badh2E2* primer which might be due to non-specific primer or alteration of the

bases. The similar incident also observed in the case of MR 219 genotype which did not perform successful amplification with *badh2E6-8* primer but exhibited successful amplification with *badh2E7* primers. However, this study only aimed to identify deletion in either exon 2 or exon 7 which is responsible for aroma status of the studied genotypes. This study confirmed the presence of an 8-bp deletion in exon 7 and identified it as the cause of aroma trait of the studied genotypes.

4.5 CONCLUSION

Aroma of rice is controlled by a recessive gene which possesses a 7-bp deletion in exon 2 or 8-bp deletion in exon 7 of *Badh2* gene. For getting a complete understanding of the genotypic condition of aroma gene it is important to know the position of deletion as well as the cause of aroma in rice genotypes.

Sequence analysis of aroma gene exhibited that all aromatic genotypes possessed 8-bp deletion in exon 7 but no deletion in exon 2 of the *badh2* gene. The sequence analysis also showed three single nucleotide sequence polymorphisms (TTT) in aromatic rice genotypes. This study concluded that an 8-bp deletion in exon 7 is responsible for aroma expression of the studied genotypes.

CHAPTER 5: EFFECTS OF TEMPERATURE ON AROMA GENE EXPRESSION AT DIFFERENT GROWTH STAGES IN AROMATIC RICE

5.1 INTRODUCTION

The aroma of aromatic rice which makes it eminent worldwide, considered as an important determination of rice grain quality resulted in strong human preference and determines its market price (Hori et al., 1994). The investigations on aromatic rice varieties at molecular level identified an aroma related locus on chromosome 8 and specified the locus for the *fgr* gene (Sakthivel et al., 2009). The *fgr* gene is also known as *osbadh2* or *badh2* gene was proposed to be responsible for aroma expression in aromatic rice (Niu et al., 2008). The *badh2* gene which encodes betaine aldehyde dehydrogenase (BADH) possessed either 7-bp deletion in exon 2 or 8-bp deletion in exon 7 or both deletions in aromatic rice. The presence of deletions in *badh2* gene creates a premature stop codon leading to a truncated *badh2* gene product. The truncated *badh2* gene or partial loss of function of complete *Badh2* gene was also proposed to account for the accumulation of 2AP, a potent aromatic compound for aroma in rice. Conversely, the functional *Badh2* gene that produced complete and mature protein was found to be responsible for the reduction of the 2AP level and make rice non-aromatic (Fitzgerald et al., 2009).

However, it was evident that *Badh2* gene is responsible for aroma traits, and it produces non-aromatic rice when present as homozygous dominant (*Badh2/Badh2*) or heterozygous (*Badh2/badh2*) condition, but it produces aromatic rice only in the homozygous recessive (*badh2/badh2*) condition. So, *Badh2* express non-aromatic trait by decreasing 2AP accumulation while *badh2* express aroma attribute by increasing

2AP accumulation. Therefore, the 2AP accumulation is associated with *badh2* gene expression at transcriptional level. Moreover, RNA interference (RNAi) technique, molecular analyses, panel sensory evaluation and gas chromatography-mass spectrometry demonstrated down-regulation of *badh2* transcripts in the transgenic plants resulted in the significant elevation of 2AP concentration (Niu et al., 2008). Conversely, the reduction of 2AP level was detected in a transgenic aromatic rice line when it transformed with functional *Badh2* gene (Chen et al., 2008). The *Badh2* transcripts which corresponded to the accumulation of 2AP in rice found in all tissues except roots tissues (Buttery et al., 1983; Srivong et al., 2008) and the transcripts were more abundant in young and healthy leaves (Chen et al., 2008). Moreover, the levels of partial *Badh2* transcripts in non-aromatic rice and its mutants form in aromatic rice were different in the RT-qPCR analysis where the lower aroma containing mutants demonstrated a higher level of complete *Badh2* gene transcript and vice-versa (Srivong et al., 2008). The *Badh2* gene expression analysis at translation level and encoded protein analysis using Western blot technique has explained that intact 503-amino acid containing BADH2 protein inhibited 2AP synthesis and made rice non-aromatic (Chen et al., 2008). The genes encoding BADH1 and BADH2 protein were studied at transcription levels using quantitative real-time PCR (RTqPCR) where the sample collected from leaf and seed of rice plant at different developmental time points with response to salt stress. The results represented very similar levels of *Badh1* and *Badh2* transcript in developing seed of non-aromatic rice varieties. On the contrary, in aromatic rice, the *badh2* transcript levels were significantly higher than those of *Badh1* in leaf and mature seed. The results indicated that the deletion present in aroma gene of aromatic rice generate a very significant reduction of the total functional BADH transcript (*Badh1* and *Badh2* transcript). The *Badh2* transcripts in non-aromatic rice varieties were significantly more abundant than the *badh2* transcripts in aromatic rice

varieties in leaf and seed samples collected from all developmental stages. The less copy number of the *badh2* transcript in aromatic rice might be associated with the loss of function of *Badh2* gene in aromatic rice genotypes (Fitzgerald et al., 2008).

Aroma of rice is significantly affected by the genetic and environmental factors along with abiotic stress such as cold, salinity, heat and others (Golam et al., 2010). Among the environmental factors, increasing temperature due to global warming is the utmost concern of aromatic rice production. However, the gene expression analysis of *badh2* gene using reverse transcription quantitative PCR (RTqPCR) can explore genetic conditions of aromatic rice by analyzing biochemical and physiological changes for *badh2* gene expression (Kim et al., 2003). Moreover, the relative quantification method (relative expression of a target gene normalized to a reference gene) compared with the control sample can explain the fold changes of the gene of interest (Livak & Schmittgen, 2001) due to the changes of environmental condition. Besides, the *badh2* gene expression analysis can correlate its expression with phenotypic aroma status of an aromatic genotype which can be used to identify possible changes happened by the environmental components (Fitzgerald et al., 2008).

Based on the above information, the aim of this study was to evaluate an appropriate growth stage and a suitable temperature for optimum expression of aroma gene which eventually ensure high-quality aromatic rice production in changing climatic condition.

To achieve these aims the following objectives have considered:

- To evaluate the effects of different temperature on aroma gene expression at different growth stages.
- To identify a suitable temperature for the superior expression of the *badh2* gene in aromatic rice.
- To compare the levels of aroma gene expression of studied genotypes grown under different temperature conditions.

The acquired information will help to identify superior rice genotypes, suitable temperature and appropriate growth stages for high-quality aromatic rice production with the maximum aroma gene expression in diverse temperature condition.

In the present investigation, five aromatic rice genotypes (MRQ 50, Ranbir Basmati, Rato Basmati, E 7, and E 13) were studied using RTqPCR to evaluate the effects of temperature on aroma gene expression (*Badh2*/*badh2*).

5.2 MATERIALS AND METHODS

5.2.1 Plant Materials

The leaves and grains collected from six studied rice genotypes (Table 3.1) grown in a glasshouse at different temperatures were used to analyze the gene expression level of the *badh2* gene.

5.2.2 RNA Extraction

5.2.2.1 RNA extraction from leaf sample

The total RNA from the leaves of different growth stages was extracted using RNA extraction kit (SV Total RNA Isolation System, Promega Corporation, USA) following manufacturer instruction. The rice leaves were grind using mortar and pestle in liquid nitrogen. About 30 mg of ground tissue were transferred to autoclaved tube containing 175µl RNA Lysis Buffer and mixed thoroughly by inversion. RNA Dilution Buffer (350µl) was added and mixed by inverting 3-4 times then centrifuged at 14000 × g for 10 minutes. The clear lysate was transferred to a fresh tube by pipetting without disturbing the pellet. A total of 200 µl of 95% ethanol was added to cleared lysate and mixed by pipetting 3-4 times then transferred the mixture to Spin Basket Assembly, and centrifuged at 14000 × g for 1 minute. The spin basket was takeout from the spin column assembly, and the liquid was discarded from collection tube. RNA Wash Solution (600µl) was added to spin column assembly, and centrifuged at 14000 × g for 1 minute then discarded the liquid from collection tube. The DNase incubation mix using 40µl of yellow core buffer, 5µl of 0.09 M $MnCl_2$ and 5µl of DNase I for each sample was prepared, mixed by pipetting and transferred to the membrane inside the spin basket. The membrane was incubated for 15 minutes at room temperature then 200 µl of DNase Stop Solution was added to spin basket and centrifuged for 1 minute. The RNA Wash Solution (600 µl) was added and centrifuged at 14000 × g for 1 minute. The flow through was discarded then 250 µl of RNA Wash Solution was added and centrifuged at 14000 × g for 2 minutes. The cap was removed from the spin basket by twisting motion and transferred the spin basket to 1.5 ml elution tube. Nuclease-free water (100 µl) was added to the membrane of the spin basket then centrifuged at 14000

× g for 1 minute. The spin basket was removed and discarded then collected elution tube containing purified RNA for further investigation.

5.2.2.2 RNA extraction from grain sample

The rice grains contain a high level of polysaccharide, so a modified CTAB method was followed for total RNA extraction from seeds using Transzol^{TM} plant RNA extraction kit (Transzol^{TM} plant, Beijing Transgen Biotech Co. Ltd, China) following manufacturer's instruction. The rice grains were grind using mortar and pestle in liquid nitrogen then 1 ml of TPI was added for every 80-100 mg grain sample, mixed thoroughly by pipetting 3 - 4 times. The lysate was transferred to an RNase-free microcentrifuge tube then centrifuged at 14,000 × g for 5 minutes. The supernatant was transferred into two microcentrifuge tubes. An equal volume of TP II solution (pink) was added to each tube then mixed thoroughly by pipetting 3 -4 times. Chloroform (equal to 1/4 volume of the supernatant from step B, about 100 µl) was mixed thoroughly by pipetting several times then incubated at room temperature for 5 minutes. The lysates were centrifuged at 14,000 × g for 5 minutes, and the lysates were divided into three layers: clear layer (colorless), interphase (colorless transparent oil) and the organic layer (pink). The RNA containing transparent layer from two tubes was transferred to a new 1.5 ml RNase-free tube, an equal volume of isopropanol was added then mixed by inverting 4 - 6 times and incubated 10 minutes at room temperature. After incubation, centrifuged at 14,000 × g for 10 minutes then discarded the supernatant leaving RNA precipitate (white) at the bottom of the tube. A total of 500 µl of 75% ethanol was added and centrifuged at 14,000 × g for 2 minutes then discarded the supernatant. This step was repeated, spin down the tubes and suck out remaining 75% ethanol. The pellet was dried inside laminar flow for 10 minutes. The RNA

dissolving solution (100 µl) was added and dissolved the pellet by pipetting several times then purified RNA was kept for further investigation.

The quality and concentration of extracted total RNA were determined spectrophotometrically (Nano Drop 2000, Thermo Scientific, USA) at 260, 280, 260/280 and 260/230 nm. The integrity of extracted RNA sample was also observed in agarose gel (1% gel) electrophoresis system. The gel was prepared as stated at agarose gel preparation section (4.3.5) in chapter 4, loaded with 0.1–2 Kb RNA ladder (Thermo Fisher Scientific, USA) using 2.2 M formaldehyde, stained with ethidium bromide visualized under gel documentation system.

5.2.3 Primer Design

The Primer quest tools (Integrated DNA Technology Inc., USA, http://sg.idtdna.com/Primerquest/Home/Index) was used to design the primers (Table 5.1) and were synthesized by My TACG Bioscience (Malaysia). Complete nucleotide sequences of *Actin*, *18s rRNA*, *eEF-1β* and *Badh2* gene were obtained from NCBI (National Center for Biotechnology Information, USA) with accession number of X16280, AF069218, CK738248, and EU770321 respectively.

Table 5.1: List of Primers with expected RTqPCR product sizes.

Primer name	Accession No.	Selected Primer Sequence	Sizes (bp)
Actin	X16280	Forward: 5C'- CTTCATAGGAATGGAAGCTGCGGGTA-3C' Reverse: 5C'-CGACCACCTTGATCTTCATGCTGCTA-3C'	196
18s rRNA	AF069218	Forward: 5C'- AGCGGCAGCTCTTCGTC -3C' Reverse: 5C'- CCCACAATCAAGCGTCTCTAGT -3C'	284
eEF-1β	CK738248	Forward: 5C'- CAGGTGTGCTAAACATAGTGAC -3C' Reverse: 5C'- CATCATCAAACACCACTATAGGAC -3C'	195
badh2E7	EU770319	Forward: 5C'- TAGGTTGCATTTACTGGGAGTT-3C' Reverse: 5C'- ACAAACCTTAACCATAGGAGCA-3C'	75/67

5.2.4 Standard Curve Preparation

The PCR efficiency for the target gene (*badh2* gene) and selected reference gene (*Actin* gene) were estimated as the equation: PCR efficiency (%) = (1 – 1) X 100
The standard curve was prepared from 1:10 time's serial dilution of total RNA (Table 5.2).

Table 5.2: Preparation of the RNA for standard curve.

Dilution No	Source of RNA	Initial concentration (ng/µl)	Final concentration (ng/µl)
1	Stock	100	20.75
2	Dilution 1	10	2.075
3	Dilution 2	1	0.2075
4	Dilution 3	0.1	0.02075
5	Dilution 4	0.01	0.002075

5.2.5 Selection of Housekeeping Gene

The housekeeping genes (*Actin, 18s rRNA* and *eEF-1β*) were initially selected from published articles and their transcription levels (C_T values) were compared as stated by Radonic et al. (2004). The box-plot was prepared by using Minitab 16 Software (Minitab Pty Ltd, Sydney, NSW, Australia).

5.2.6 Real-Time Quantitative PCR

Real-time quantitative PCR amplification was conducted using RTqPCR (CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad, USA). A total of 20 μl reaction volume containing SYBR Green mix (GoTaq 1-step RTqPCR reaction mix, Promega Corporation, USA), primer (forward and reverse), nuclease-free water, and

RNA template (100 ng) were used for amplification. Negative control or no reverse transcriptase control (without reverse transcriptase) and no template control (without RNA) were included with each reaction set which was also assayed triplicate for three biological replicates. The thermal cycling condition was 48°C for 15 min (reverse transcription), 95°C for 10 min (reverse transcription inactivation) followed by 40 cycles of 95°C for 10 sec (denaturation), 60°C/52°C (*Actin*/*badh2*, respectively) for 30 sec (annealing), 72°C for 30 sec (extension). After amplification, the samples were kept at 95°C for 10 sec and 65°C for 5 sec then raised gradually by 0.5°C every 5 sec to obtain a melting curve.

5.2.7 Gene Expression Analysis

The amplification results from CFX Manager Software (included with CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad, USA) were exported to Excel file and the quantification of gene expression was conducted according to the relative quantification methods of Livak and Schmittgen (2001). The C_T values of *Actin* gene was used as internal control (endogenous reference), and C_T values of non-aromatic rice genotype (MR 219) were used as calibrator (control). The effect of temperature on internal control gene and aroma gene was estimated using $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) where the same amount of RNA was used for external normalization.

5.2.8 Statistical Analysis

The Minitab 16 Software (Minitab Pty Ltd, Sydney, NSW, Australia) was used for ANOVA and descriptive statistics of the *badh2* gene expression.

5.3 RESULTS

This study represented results of relative expression of the *badh2* gene compared to housekeeping genes in different growth stages of rice which were grown and harvested from three different temperatures (ambient or 28°C, 25°C and 20°C).

5.3.1 Selection of Housekeeping Gene

The RNA transcription levels of selected three housekeeping genes were compared directly by the comparisons of C_T values. The housekeeping gene e*EF-1β* did not perform satisfactory amplification while *Actin* and *18s rRNA* represented successful amplification. The comparison of the expression of housekeeping genes at transcriptional level expressed a uniform expression of *Actin* gene (Fig. 5.1).

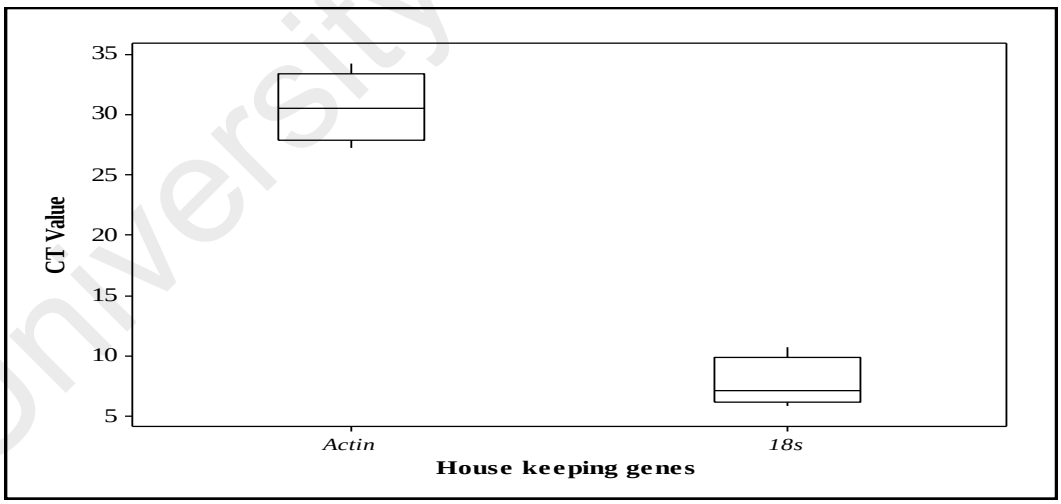

Figure 5.1: RNA transcription levels of housekeeping genes.

The expression values of the *Actin* gene (25 Percentile, 75 percentile, and median value) is more uniform than the *18s rRNA* gene. The lower C_T values also represented a higher abundance of *18s rRNA* in the total RNA. Therefore, in this study,

the *Actin* gene was used as housekeeping gene for normalization and expression analysis of *badh2* gene.

5.3.2 Standard Curve for PCR Efficiency Estimation

The standard curve represented almost similar efficiency for the amplification of targeted *badh2* gene (103%) and *Actin* gene (107%). The standard curve also corresponds to the optimum amount of RNA for targeted *badh2* gene (Fig. 5.2 and 5.3) and housekeeping *Actin* gene (Fig. 5.4 and 5.5).

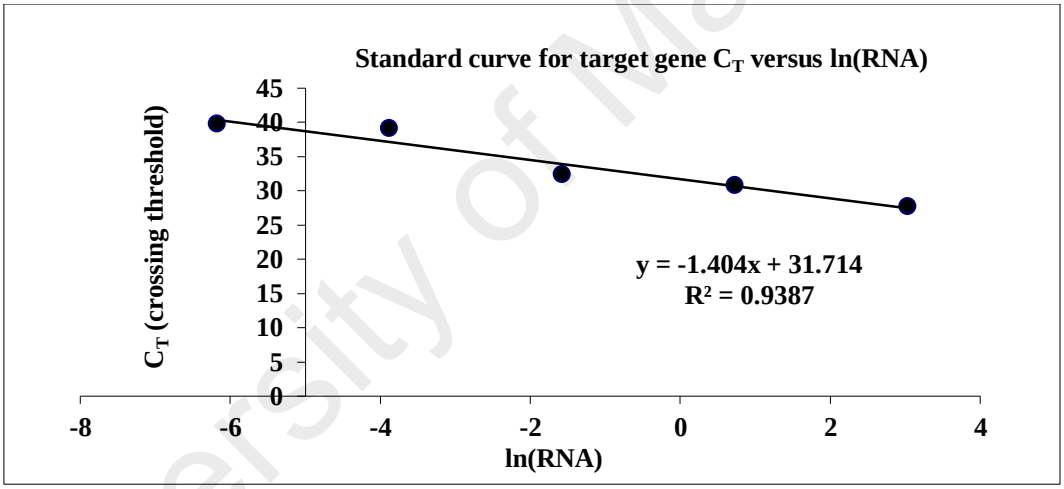

Figure 5.2: Standard curve of targeted *badh2* gene.

The slope (1.404) and Pearson Coefficient of Determination (R^2 = 0.938) representing optimum efficiency (103%) for *badh2* gene.

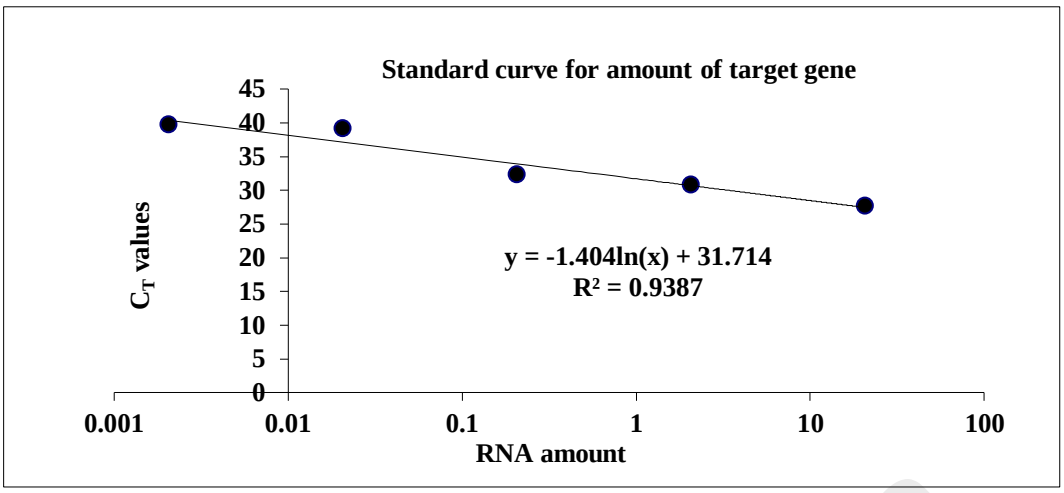

Figure 5.3: Standard curve for RNA amount for targeted *badh2* gene.

The serial dilution of 100 ng total RNA is representing the C_T values for log RNA concentration for 103% PCR efficiency for targeted *badh2* gene.

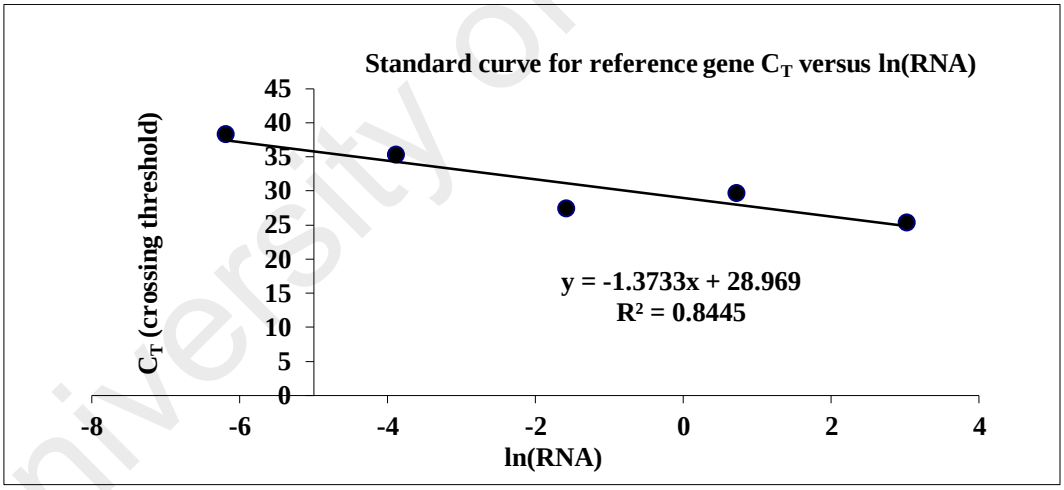

Figure 5.4: Standard curve of housekeeping *Actin* gene.

The slope (1.373) and Pearson Coefficient of Determination (R^2 = 0.844) representing optimum efficiency (107%) for *Actin* gene.

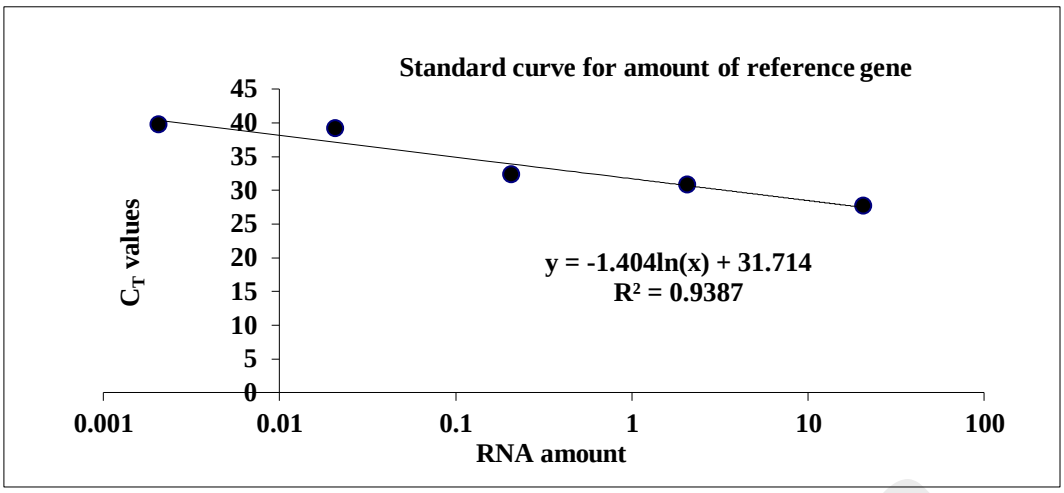

Figure 5.5: Standard curve of RNA amount for housekeeping *Actin* gene.

The serial dilution of 100 ng total RNA is representing the C_T values for log RNA concentration for 107% PCR efficiency for reference *Actin* gene.

The PCR efficiency estimation by standard curve methods represented the optimum condition for relative gene expression analysis by $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) where both target and reference genes need to be amplified with similar efficiency (near 100% and difference within 5% from each other). So, relative expression of the *badh2* gene was estimated by normalizing against *Actin* gene as the similar procedure of Livak and Schmittgen (2001).

5.3.3 Relative Expression of Aroma Gene

The relative expression of the *badh2* gene compared to the *Actin* gene (internal control) in aromatic rice was evaluated where non-aromatic rice genotype (MR 219) was used as control. The non-aromatic rice genotype demonstrated a unique fold measurement (almost 1.00) and aromatic genotypes showed different levels of *badh2* gene expression (Fig. 5.6, 5.7, 5.8 and 5.9).

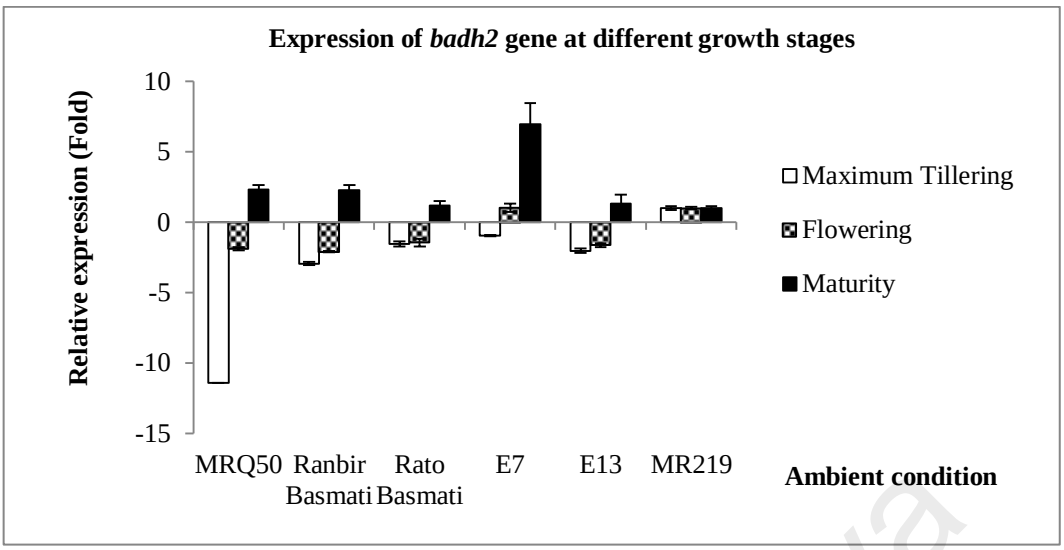

Figure 5.6: Relative expression of *badh2* at different growth stages of rice in ambient condition.

Figure 5.6 exhibited relative expression of the *badh2* gene at different growth stages under ambient temperature condition. The MRQ 50 genotype demonstrated higher down-regulation of the *badh2* gene (about -12 fold) at maximum tillering stage and up-regulation of the *badh2* gene at maturity stage compared to non-aromatic rice genotype MR 219. At flowering stage, all genotypes demonstrated down-regulation of the *badh2* gene except E 7 genotype which exhibited up-regulation of the *badh2* gene. During maturity stage, all studied genotypes showed up-regulation of the *badh2* gene at ambient condition.

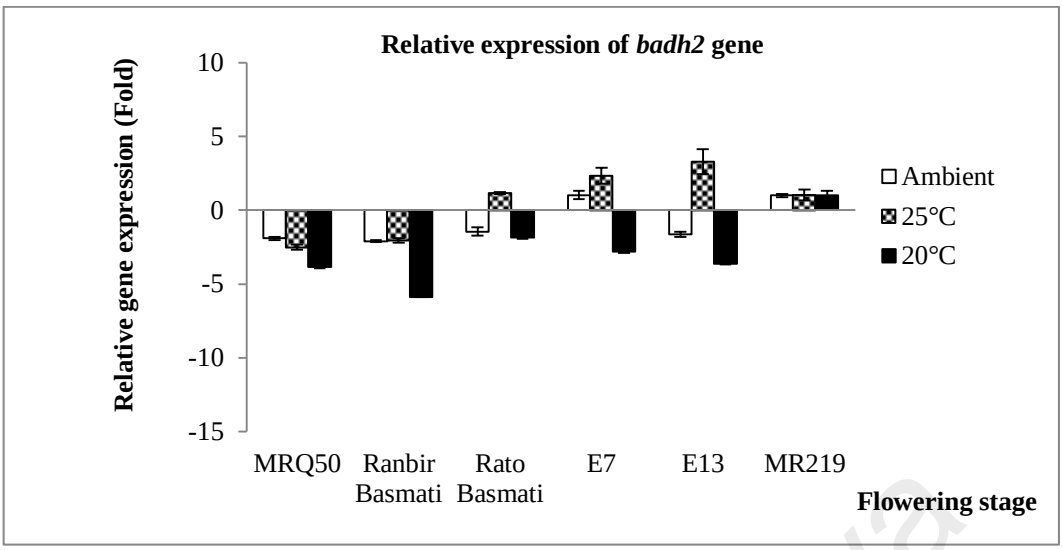

Figure 5.7: Relative expression of *badh2* at different temperature during flowering stage.

During flowering stage, the *badh2* gene was down-regulated in all aromatic genotypes at 20°C temperature while Rato Basmati, E 7, and E 13 genotypes demonstrated up-regulation at 25°C (Fig. 5.7). The down-regulation of the *badh2* gene was also observed at ambient condition during the flowering stage.

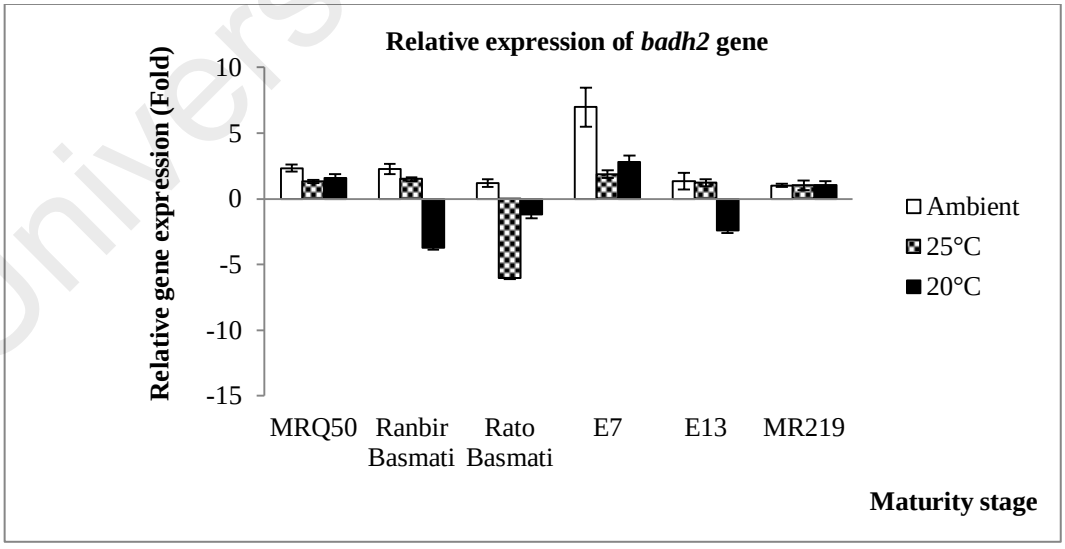

Figure 5.8: Relative expression of *badh2* at different temperature during maturity stage.

At maturity stage (Fig. 5.8), all aromatic genotypes demonstrated up-regulation of the *badh2* gene (aroma gene) compared to *Badh2* gene (non-aromatic gene) except Ranbir Basmati, Rato Basmati, and E 13 genotypes. Ranbir Basmati and E 13 genotype demonstrated down-regulation (-3.75 and -2.41 fold) at 20°C while Rato Basmati showed down-regulation (-6.07 fold) of the *badh2* gene at 25°C temperature.

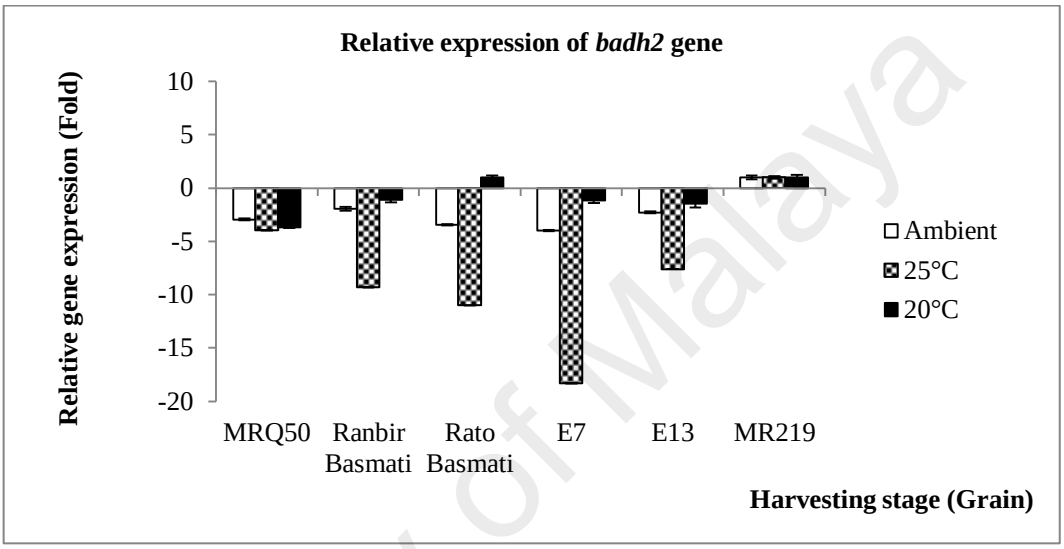

Figure 5.9: Expression of *badh2* at different temperature in harvested grains.

In the case of harvested grain, all the varieties demonstrated a lower abundance of aroma gene at all temperature conditions except Rato Basmati at 20°C (1.00 fold) expressed same as MR 219 genotype (Fig. 5.9). The down-regulation of recessive *badh2* gene confirmed the expression of aroma trait in all aromatic rice genotypes.

5.3.4 Effect of Temperature on Aroma Gene

The effect of temperature on the expression of aroma gene was analyzed using ANOVA test where differences observed at 5% level (p<0.05) and 1% level (p<0.01) as presented in Table 5.3, 5.4, 5.5 and Table 5.6.

Table 5.3: ANOVA for *badh2* gene for growth stages and genotypes at ambient condition.

Source	DF	SS	MS	F	P
Genotype	5	61.69	12.33	1.81^{NS}	0.19
Growth stage	2	92.33	46.16	6.76*	0.01

* p<0.05, NS = Non significant, DF = Degrees of freedom, SS = Sum of Squre, MS = Mean Sum of Squre, F = F value and p = Probability.

Table 5.3 is representing the significant differences of growth stages within genotypes at 5% level (p< 0.05) while non-significant differences among studied genotypes. So, growth stages of rice plant influenced the expression of aroma gene.

Table 5.4: ANOVA for *badh2* gene for genotypes and temperatures at flowering stage.

Source	DF	SS	MS	F	P
Genotype	5	42.63	8.52	3.87^{*}	0.03
Temperature	2	34.53	17.26	7.84^{**}	0.00

* p<0.05, ** p<0.01, DF = Degrees of freedom, SS = Sum of Squre, MS = Mean Sum of Squre, F = F value and p = Probability.

At flowering stage, both the genotypes and temperatures affected the expression of the *badh2* gene significantly ($p\leq$ 0.05) at 5% level and ($p\leq$ 0.01) at 1% level, respectively as presented in Table 5.4.

Table 5.5: ANOVA for *badh2* gene for genotypes and temperatures at maturity stage.

Source	DF	SS	MS	F	P
Genotype	5	59.04	11.80	2.60^{NS}	0.09
Temperature	2	27.83	13.91	3.06^{NS}	0.09

NS = Non significant, DF = Degrees of freedom, SS = Sum of Squre, MS = Mean Sum of Squre, F = F value and p = Probability.

The above Table 5.5 demonstrated that both the genotypes and temperature did not affect the expression of aroma gene at maturity stage.

Table 5.6: ANOVA for *badh2* gene for genotypes and temperatures at harvesting stage.

Source	DF	SS	MS	F	P
Genotype	5	119.93	23.98	1.91^{NS}	0.17
Temperature	2	180.51	90.25	7.19^{*}	0.01

* $p<0.05$, NS = Non significant, DF = Degrees of freedom, SS = Sum of Squre, MS = Mean Sum of Squre, F = F value and p = Probability.

At harvested grain, though the expression of the *badh2* gene was non-significantly different among the genotypes, the temperatures affected the expression significantly ($p< 0.05$) at 5% level.

Hence, the temperature significantly affected the expression of the *badh2* gene at different growth stages of aromatic rice. Moreover, all aromatic genotypes demonstrated higher down-regulation of the *badh2*/*badh2* allele at 25°C temperature (harvested grain) compared to the expression of the *badh2*/*badh2* allele in the same genotype at 20°C and ambient condition. Thus, molecular analysis of the *badh2* gene expression revealed that down-regulation of the recessive *badh2* allele was responsible for aroma status of genotypes and the environmental temperature regulated the expression of the *badh2* gene.

5.4 DISCUSSION

In this study, serial dilution (1:10) of total RNA in one-step method was used to estimate the PCR efficiency for target gene *badh2* (103%) and reference gene *Actin* (107%). The similar amplification efficiency of RTqPCR for the target gene and reference gene is the prerequisite for $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The most significant pitfalls for relative quantification are to select appropriate reference gene (Bustin & Nolan, 2004). Several housekeeping genes such as *18s rRNA*, *25s rRNA*, *Actin*, *GAPDH*, *eEF-1β* was used by researchers (Jain et al., 2006; Radonic et al., 2004; Vandesompele et al., 2002).

The structure related gene (*Actin* gene), the translation related gene (*18s rRNA*) and elongation-related gene (*eEF-1β*) were used as standard in this experiment. Selection of an ideal reference gene depends on the stability of that gene on experimental treatments (Radonic et al., 2004). The stability of the reference genes in experimental treatment can be assessed by geometric mean of multiple housekeeping genes (Vandesompele et al., 2002), statistical algorithms (geNORM and BestKeeper), calibration curve based quantification model (Pfaffl, 2001) and direct comparison of C_T values with standard deviation (Kim et al., 2003; Radonic et al., 2004).

The direct comparison of C_T values from housekeeping genes (*Actin* and *18s rRNA*) were used to select reference gene for the present study. The housekeeping gene *eEF-1β* did not produce amplification and due to uniform amplification, the *Actin* gene was chosen for normalization of the *badh2* gene. Previously, many researchers (Caldana et al., 2007; Jain et al., 2006; Kim et al., 2003; Tong et al., 2009) were used *Actin* as an

internal control and validated for rice gene expression analysis. Jain et al. (2006) stated that expression of housekeeping gene can vary with the experimental condition and Schmittgen and Zakrajsek (2000) also observed treatment effects on housekeeping genes. In this investigation, the *Actin* gene showed uniformity in expression level at different temperatures and was selected as an internal control (reference gene).

In this study, the *badh2* gene which was produced 75-bp or 67-bp products were more down-regulated at 25°C during harvesting stage (-18.31 fold) and at ambient temperature during maximum tillering stage (-12 fold). The relative expression of the *badh2* gene in aromatic rice genotypes was observed more down-regulated (Fig. 5.6) in maximum tillering stage compared to the non-aromatic *Badh2* allele. The MRQ 50 genotype demonstrated higher down-regulation of the *badh2* gene (about -12 fold) at the maximum tillering stage and up-regulation of the *badh2* gene at maturity stage compared to non-aromatic rice genotype MR 219. At flowering stage, all genotypes demonstrated down-regulation of the *badh2* gene compared to non-aromatic genotype except E 7 genotype which exhibited up-regulation of the *badh2* gene. During maturity stage, all studied genotypes showed up-regulation of the *badh2* gene at ambient condition. In the case of harvested grain, all the varieties demonstrated a lower abundance of aroma gene at all temperature conditions except Rato Basmati at 20°C (1.00 fold) expressed same as MR 219 genotype. Formerly, Fitzgerald et al. (2008) observed more abundant of *Badh2* gene transcripts in non-aromatic rice varieties compared to aromatic rice varieties during salt stress and concluded that *Badh2* gene has no role in the response to salt stress. Chen et al. (2008) observed less abundance of full-length *Badh2* transcript than partial *badh2* transcripts. They also observed low transcriptional levels of non-functional *badh2E2* and *badh2E7* gene compare to functional *Badh2* gene. However, in this study while investigated the effects of three

different temperatures on the expression of the *badh2* gene, the highest down-regulation of mRNA transcript of the *badh2* gene observed at 20°C during flowering stage and 25°C at harvested grain stage compared to functional *Badh2* transcripts in non-aromatic rice genotypes.

Gene expression analysis of *badh2* gene represented that down-regulation of the recessive *badh2/badh2* allele was responsible for the phenotypic aroma expression in the experimental aromatic rice genotypes. This result clearly indicated by Chen et al. (2008) who stated that the presence of dominant *Badh2* allele encoding betaine aldehyde dehydrogenase (BADH2) inhibited synthesis of 2AP while its recessive alleles induced 2AP formation and rice become aromatic. They also revealed that dominant *Badh2* was more abundant in non-aromatic rice compared to aromatic rice. Besides, they declared that over-expression of complete *Badh2* rendered transgenic lines non-aromatic and caused a reduction in 2AP levels. Conversely, Niu et al. (2008) observed that down-regulation of dominant *Badh2* transcripts in the transgenic rice plants resulted in higher 2AP concentration. Bradbury et al. (2005a) stated that aroma is a recessive trait, and a loss of function of *Badh2* allele is responsible for aroma expression.

Perozich et al. (1999) studied the nature and character of the *Badh2/badh2* allele and stated that it belongs to the aldehyde dehydrogenases (ALDH) superfamily. The ALDH family is composed of such a group which produces divergently related enzymes that catalyze the irreversible NAD (P) + dependent oxidation of a wide variety of aliphatic and aromatic aldehydes to their corresponding carboxylic acids. This gene family is also involved in environmental stresses responses and tolerance (Sophos & Vasiliou, 2003). It is clear that two closely related *betaine aldehyde dehydrogenase* (*BADH*) homologs *Badh1* and *Badh2* present in the rice genome and Fitzgerald et al.

(2008) mentioned that *Badh1* is responsible for salt tolerance, and *Badh2* associated with aroma production. The biochemical pathway lead to aroma production in rice has not established yet. However, the recessive nature of aroma allele (*badh2/badh2*) and expression of aroma due to loss of function (Bradbury et al., 2005a; Chen et al., 2008) of *Badh2* gene well explained by the present experiment where down-regulation of recessive *badh2* mRNA transcripts helped to express aromatic flavor. Further elucidation of the biochemical pathway of 2AP accumulation is necessary to conclude the molecular and biochemical reason for aroma in rice. So, the molecular analysis of this experiment represented that the expression of the *badh2* gene, as well as aroma status of a genotype depended on the environmental temperature. The effects of environmental temperature on the expression of the *badh2* gene also depended on the growth stages of rice which could be considered for aromatic rice improvement and production in different climatic condition.

5.5 CONCLUSION

The present investigation concluded that the growth stages of rice represented the different level of aroma expression, and temperature affects the level of *badh2* gene transcripts at different growth stages. The less abundance of *badh2* gene transcripts compared to non-aromatic *Badh2* gene expressed more aroma in relative quantification methods (Up to -18.31 fold) normalized with a housekeeping gene (*Actin* gene). The relative expression of the *badh2* gene depends on the environmental temperature and the rice variety. The 25°C temperature was an ideal temperature for *badh2* gene expression, and the expression reduced subsequently from the maximum tillering stage, flowering stage and maturity stage. So, controlling temperature at 25°C from flowering stage to

maturity stage might be suitable for aromatic rice production which will eventually ensure high-quality aroma performance a particular aromatic rice variety.

This information will help to develop high yielding aromatic rice genotypes by controlling suitable temperature (25°C temperature) during growth stages or searching the places where this suitable temperature (25°C temperature) remains during different growth stages. This information will also be helpful for high-quality aromatic rice production.

CHAPTER 6: EFFECTS OF TEMPERATURE ON VOLATILE PROFILE AND 2-ACETYL-1-PYRROLINE CONCENTRATION IN AROMATIC RICE

6.1 INTRODUCTION

Aromatic rice is one of the most popular groups of rice which demand higher price compared to non-aromatic rice in the local and global markets due to its popcorn-like flavor. In the world markets, most of the trading of aromatic rice is from Pakistan, India, and Thailand. Though, several countries of the world produce local aromatic rice varieties such as Iran (Sadri), Nepal (Rato Basmati), Bangladesh (Kathari Bhog), USA (Della) but Basmati from Pakistan and India, Jasmine from Thailand are the leading source of high-quality aromatic rice for worldwide trading. Some other countries are also trying to develop aromatic rice varieties by backcrossing (Myanmar, Manawthukha), adoption of fragrant rice farming (Malaysia, MRQ 74) and optimization of harvest time and temperature (Japan, Hieri). The aroma quality of available aromatic rice varieties in other countries is not superior as Basmati and Jasmin rice produced in India, Pakistan, and Thailand. In earlier studies, Oad et al. (2006) stated that the Basmati rice grows well in the places where the temperature remains cooler during maturity stage and demonstrate high-quality aroma performance. If it is grown outside Panjab region as well as in temperate regions it will become non-aromatic. They mentioned that the effects of environmental components, genotypic constitution and their interactions (genotype × environment interaction) were the primary cause of such phenomenon. Among the environmental factors, the atmospheric temperature could cause significant effects on growth, yield and grain quality of rice crop (Peng et al., 2004). Hence, the temperature of some tropical, subtropical and desert environments where the air temperature is above the optimum temperature range is the adverse

environmental condition for high-quality aromatic rice production (Yoshida, 1973). Aroma quality of aromatic rice also could be influenced by the air temperature within the optimum temperature range (20°C to 30°C) during the ripening stage and at harvest time. High temperatures and late harvesting time may reduce aroma quality while cold climatic conditions and early harvest are favorable for aromatic rice production with a strong aroma (Itani et al., 2004). They also mentioned that the 2AP concentration was higher at a low temperature (25°C/20°C day/night) than the high temperature (35°C/30°C day/night).

The aromatic rice grains correspond to the presence of numerous volatile compounds which can explain the aromatic status of a rice variety (Sakthivel et al., 2009). Among the volatile compounds present in aromatic rice, the 2AP is primarily responsible for the phenotypic aroma (Buttery et al., 1986; Jezussek et al., 2002; Widjaja et al., 1996). A very low odor threshold value (within a minimum detectable level) of 2AP (0.00002 ppb) indicated that it can play a vital role in rice aroma if present in a small amount (Harrison & Dake, 2005; Yang et al., 2008). In aromatic rice genotypes, the 2AP was detected in all parts of the plant except the roots (Buttery et al., 1983; Maraval et al., 2010) whereas the 2AP also detected in non-aromatic rice at a much lower concentration (0.0015 ppm) (Widjaja et al., 1996). Moreover, an interaction between 2AP concentration and stress responses were observed, for instance, drought stress during grain formation increased 2AP content (Yoshihashi et al., 2002) and salt stress increased 2AP accumulation (Gay et al., 2010). Though, the elite aromatic rice varieties observed to be susceptible to abiotic and biotic stresses (Niu et al., 2008). Recently, an association of aroma phenotype with salt susceptibility reported which indicated a small probable effect of salt on the 2AP concentration (Fitzgerald et al., 2010; Wijerathna et al., 2014).

However, earlier researchers, estimated and quantified the 2AP concentration of several aromatic rice genotypes to study the influences of salinity stress (Fitzgerald et al., 2008; Gay et al., 2010), accelerated aging treatments (Pisithkul et al., 2010) and shading during grain filling periods on its concentration (Mo et al., 2015). Some researchers identified and characterized 2AP from cooked rice (Buttery et al., 1988; Laksanalamai & Ilangantileke, 1993; Maraval et al., 2008; Tanchotikul & Hsieh, 1991; Yang et al., 2007) and some from raw rice (Itani et al., 2004; Mahatheeranont et al., 2001; Vercellotti et al., 1988). Furthermore, previous researchers mentioned that aroma of rice was not expressed only for 2AP but also for a mixture of different odor-active volatile compounds (Widjaja et al., 1996; Yang et al., 2008). From rice grain, more than 200 volatile compounds has been identified (Buttery et al., 1988; Champagne, 2008; Tsugita, 1985), and Buttery et al. (1988) stated that among the detected compounds 2AP, (E, E)-2,4-decadienal, nonanal, hexanal, (E)-2-nonenal, octanal, decanal, 4-vinyl-guaiacol, and 4-vinylphenol were the probable key contributors to cooked rice aroma. Jezussek et al. (2002) mentioned the presence of two previously unknown chemical compounds i.e. 2-amino acetophenone and 4, 5-epoxy-(E)-2-decenal which were also observed to be a major rice aroma compounds. Maraval et al. (2008) observed similar aroma profiles in aromatic genotypes, but different levels of volatile compounds and Yang et al. (2008) mentioned that a total of 13 volatile compounds may contribute to differences in the aroma. Liyanaarachchi et al. (2014) stated that volatile profile of a rice genotype was necessary not only for using in rice breeding programs but also to assure the quality of rice grain or grain products in the market. They also added that most of the volatile compounds identified depended on the variety, agronomic practices, storage conditions, post harvest operation and growing condition of the rice variety.

It is important to investigate the volatile profile as well as the 2AP concentration of a rice genotype which grow at different temperature for identifying the suitable temperature responsible for the superior phenotypic aroma of a particular rice variety.

Former researchers studied the influences of salinity stress, accelerated aging treatments and shading during grain filling periods on 2AP concentration to investigate the effects of these components on aroma quality (Fitzgerald et al., 2008; Gay et al., 2010; Mo et al., 2015; Pisithkul et al., 2010). But none of the experiments were conducted to observe the effects of temperature (within the optimum temperature range) on volatile components and 2AP concentration of rice samples.

Thus, the aim of this study was to identify volatile compounds and quantify 2AP concentration of studied rice samples which were grown under different temperature conditions to observe the effects of temperature on volatile compounds as well as on 2AP concentration.

The specific and the most important objectives were:

- To identify volatile aroma compounds present in rice genotypes for assessing the contribution of volatile compounds to aroma characteristics of aromatic rice.
- To quantify the concentration of 2AP in aromatic and non-aromatic rice genotypes for analyzing the aroma status of a rice genotype grows under different temperature condition.

The obtained information from this experiment will guide to assess the involvement of volatile compounds and 2AP concentration for high-quality aromatic

rice production under diverse temperature condition. Moreover, the assessment results will point out the presence of biochemical products due to the genetic and environmental interaction between aroma gene and environmental temperature.

In this study, the volatile profiles and 2AP concentration of selected rice genotypes were analyzed using GC-MS and quantified using GC-FID to observe the effects of temperature on volatile compounds and 2AP concentration in rice as well as to identify the suitable temperature for the high-quality aromatic rice production under diverse temperature condition.

6.2 MATERIALS AND METHODS

6.2.1 Plant Materials

The grains from experimental six rice genotypes (Table 3.1) grown at three temperatures (ambient or 28°C, 25°C and 20°C) in glasshouse were used to extract volatile compounds for GC-MS and GC-FID analysis. The description of experimental site (3.2.2), experimental design (3.2.3), growth chambers (3.2.4) and crop husbandry (3.2.5) were stated in chapter 3 at Materials and Methods section.

6.2.2 Solvent Extraction of Volatile Compound

For isolation of chemical components from collected samples using solvent extraction method, the rice grains were hulled and kept at -20°C. One gram of rice grains was ground into mortar and pastle using liquid nitrogen. The grind seeds were transferred into a 125 ml conical flask containing 40 ml of 20 ppm 2, 4, 6-tri methyl

pyridine (TMP, Sigma-Aldrich Chemical Co., Germany) which was used as an internal standard (Mahatheeranont et al., 2001; Tanchotikul & Hsieh, 1991). Supplied TMP was dissolved in a precisely measured volume of 0.1 M HCl to give an internal standard solution of 20.00 ppm concentration of TMP (0.218 ml TMP was mixed in 999.782 ml of 0.1M HCl). The mixture (40 ml TMP and 1 g rice) was stirred for 30 min and filtered into 50 ml centrifuge tubes. A total of 3 ml of 1.0 M NaOH was added to 25 ml filtrate to make the solution slightly basic then centrifuged at 6000 rpm for 10 min. The supernatant liquor was transferred to a 250 ml pear-shaped separatory funnel then 50 ml of dichloromethane was immediately added as an organic solvent. The extraction was conducted twice, resulting in 100 ml of dichloromethane solution. After drying over anhydrous sodium sulfate, the extract was concentrated to 1 ml using a rotary evaporator (Eyela N-2100, Tokyo Rikakikai Co., LTD, Japan) under reduced pressure (300 hPa) and a temperature of 26°C. The concentrated extract was transferred to a vial and 1 µl was taken for qualitative analysis by GC-MS and quantitative analysis by GC-FID.

6.2.3 Gas Chromatography-Mass Spectrometry (GC-MS)

The samples were analyzed on a GC/MS system (GCMS-QP2010W, Shimadzu, Japan) where Helium gas (purity 99.99%) at a pressure of 80 Kpa was used as the GC carrier gas. The injector and the GC/MS interface temperatures were set at 250°C and 220°C, respectively. The temperature of the DB5 capillary column (30 m × 0.25 mm id, film thickness 0.25 µm, J & W Scientific, Folsom, CA) was programmed by starting at 30°C after splitless injection of samples. The initial temperature of 30°C held for 1 min, it was ramped to 185°C at 5°C/min. After hold for 2 min at 185°C, it increased to 220°C at a rate of 7°C/min and held there for 20 min. The effluent from the capillary column

went directly into the mass spectrometer, operated in the electron impact (EI) mode with an ionization voltage of 70 eV, and the ion source temperature was 220°C. The volatile compounds from rice samples were then identified.

6.2.4 Gas Chromatography- Flame Ionization Detector (GC-FID)

The similar method as stated for GC-MS (6.2.3) was also used for quantitative analysis of 2AP using GC-FID (GC-FID QP2010W, Shimadzu, Japan). The synthetic standard of 2AP was obtained from BOC science (Shirley, NY, USA) and a 30 ppm standard solution of collected 2AP was made by dissolving in 0.1 M HCl. Similar solvent extraction protocol which was used to extract volatile compounds from rice samples were followed to estimate 2AP recovery and peak area. The concentrations of 2AP present in samples were calculated using following formula (Laohakunjit & Kerdchoechuen, 2006):

$$2AP\ conc.\ (ppm) = \frac{\left\{Area\ of\ 2AP\ \times Conc.\ of\ TMP\ \left(\frac{ng}{\mu l}\right) \times Vol.\ of\ injection\ (\mu l)\right\}}{Area\ of\ TMP\ \times Wt.\ of\ sample\ (g)} \times 0.001$$

6.2.5 Authentic Standard Compounds and Compound Identification

The standard chemical compounds were collected from the organic chemistry laboratory of the University of Malaya. The laboratory collected the analytical reagent grade with 99% purity of the standard from different companies (Appendix C).

The volatile compounds were identified primarily by comparing their corresponding mass spectra with the reference compounds compiled in both the Wiley and NIST mass spectral libraries. The volatile compounds were then finally identified by comparing their corresponding mass spectra with those of the standard compounds.

The retention time and retention indexes of identified compounds were also compared with those compounds reported in the literature. The data of three biological replications and three technical replications were compared to finalize the volatile profiles.

6.3 RESULTS

The consequences of temperature on volatile profiles and 2AP concentration of investigated rice genotypes presented as bellow:

6.3.1 Effect of Temperature on Volatile Profile

The volatile profile of the extracted compound from six rice genotypes collected from ambient or 28°C, 25°C, and 20°C temperature were analyzed and the chromatogram were presented in appendix D. The identified compounds and their retention time with retention index were indicated in Table 6.1, 6.2, 6.3, 6.4, 6.5, and Table 6.6.

In ambient condition, the Malaysian local aromatic rice genotype (MRQ 50 genotype) exhibited the presence of 12 compound with more abundance of 1-benzylindole while Ranbir Basmati demonstrated 13 compound (more abundant Nonadec-1-ene), Rato Basmati 4 compound (more abundant Methyl pentadecanoate), E 7 genotype 5 compound (more abundant Methyl 14-methylpentadecanoate), E 13 genotype 5 compound (more abundant Methyl 14-methylpentadecanoate) and the Malaysian local non-aromatic rice genotype (MR 219 genotype) produced 10 compounds (more abundance of Heptadec-1-ene).

Table 6.1: Volatile compound of MRQ 50 rice grain obtained from different temperature.

Temp.	R.Time	Area %	Height %	A/H	IUPAC/Chemical name	Formula	Mol weight
(Ambient)	8.84	0.69	0.92	6.07	Bicyclo[4.2.0]octa-1,3,5-triene	C_8H_8	104
	11.95	20.02	13.1	12.34	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	12.42	0.09	0.24	3.00	2-acetyl-1-pyrroline	C_6H_9NO	111
	21.28	1.04	3.06	2.73	Dodecamethylcyclohexasiloxane[a]	$C_{12}H_{36}O_6Si_6$	444
	25.63	1.42	3.77	3.04	Tetradecamethyl-cycloheptasiloxane[b]	$C_{14}H_{42}O_7Si_7$	518
	32.28	0.61	1.79	2.73	(2-phenylcyclobutyl)benzene	$C_{16}H_{16}$	208
	44.64	0.88	1.26	5.63	Hexadecamethylheptasiloxane[c]	$C_{16}H_{48}O_6Si_7$	532
	48.34	1.16	1.55	6.03	N,N-dibenzylhydroxylamine	$C_{14}H_{15}NO$	213
	50.74	1.05	1.02	8.31	Hexadecamethyl-cyclooctasioxane[d]	$C_{16}H_{48}O_8Si_8$	592
	53.36	3.01	2.57	9.47	1-benzylindole	$C_{15}H_{13}N$	207
	54.42	1.11	1.00	9.02	[(1E,3E)-4-phenylbuta-1,3-dienyl]benzene	$C_{16}H_{14}$	206
	60.23	1.10	0.68	13.15	Tetradecamethylhexasiloxane	$C_{14}H_{42}O_5Si_6$	458
(25°C)	11.67	10.04	7.10	10.24	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	12.08	0.04	0.11	2.52	2-acetyl-1-pyrroline	C_6H_9NO	111
	17.88	1.18	2.76	3.09	Dodec-1-ene	$C_{12}H_{24}$	168
	18.12	0.46	0.93	3.60	Tridecane	$C_{13}H_{28}$	184
	23.47	2.47	5.45	3.28	Tridec-1-ene	$C_{13}H_{26}$	182
	23.65	0.69	1.59	3.13	Tetradecane	$C_{14}H_{30}$	198
	26.39	2.84	4.81	4.27	2,4-ditert-butylphenol	$C_{14}H_{22}O$	206
	28.46	3.77	7.06	3.87	Pentadec-1-ene	$C_{15}H_{30}$	210
	33.16	1.08	2.28	3.43	Octadecane	$C_{18}H_{38}$	254
	37.78	1.20	3.30	2.63	Nonadecane	$C_{19}H_{40}$	268
	41.62	5.85	6.98	6.07	Nonadec-1-ene	$C_{19}H_{38}$	266
	41.71	1.18	2.47	3.46	Triacontane	$C_{30}H_{62}$	422
	47.77	6.15	5.08	8.76	Octacosan-1-ol	$C_{28}H_{58}O$	410
	47.90	0.99	1.29	5.59	Tetracosane	$C_{24}H_{50}$	338
	58.82	4.21	2.28	13.41	Heptacosan-1-ol	$C_{27}H_{56}O$	396
(20°C)	11.83	29.88	25.66	2.74	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	31.67	2.98	1.79	3.91	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242
	35.58	22.00	19.07	2.71	Methyl hexadecanoate	$C_{17}H_{34}O_2$	270
	38.36	1.00	0.92	2.55	Methyl (9Z,12Z)-octadeca-9,12-dienoate	$C_{19}H_{34}O_2$	294
	38.49	8.05	6.65	2.84	Methyl (E)-octadec-7-enoate	$C_{19}H_{36}O_2$	296
	39.01	2.04	1.75	2.74	Methyl 16-methyl-heptadecanoate	$C_{19}H_{38}O_2$	298

[a]Dodecamethylcyclohexasiloxane: 2,2,4,4,6,6,8,8,10,10,12,12-dodecamethyl-1,3,5,7,9,11-hexaoxa-2,4,6,8,10,12-hexasilacyclododecane. [b]Tetradecamethylhexasiloxane: [dimethyl(trimethylsilyloxy)silyl]oxy-[[dimethyl(trimethylsilyloxy)silyl]oxy-dimethylsilyl]oxy-dimethylsilane. [c]Hexadecamethylheptasiloxane: bis[[[dimethyl(trimethylsilyloxy)silyl]oxy-dimethylsilyl]oxy]-dimethylsilane; Tetradecamethyl-cycloheptasiloxane: 2,2,4,4,6,6,8,8,10,10,12,12,14,14-tetradecamethyl-1,3,5,7,9,11,13-heptaoxa-2,4,6,8,10,12,14-heptasilacyclotetradecane. [d]Hexadecamethyl-cyclooctasioxane: 2,2,4,4,6,6,8,8,10,10,12,12,14,14,16,16-hexadecamethyl-1,3,5,7,9,11,13,15-octaoxa-2,4,6,8,10,12,14,16-octasilacyclohexadecane.
IS: Internal standard

Table 6.2: Volatile compound of Ranbir Basmati rice grain obtained from different temperature.

Temp.	R.Time	Area%	Height %	A/H	Compound (IUPAC/Chemical name)	Formula	Mol weight
(Ambient)	08.38	19.66	17.78	3.80	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	13.43	0.01	0.02	2.36	2-acetyl-1-pyrroline	C_6H_9NO	111
	13.52	1.64	2.05	2.76	Dodec-1-ene	$C_{12}H_{24}$	168
	15.98	1.30	1.98	2.26	Dodecamethylcyclohexasiloxane[a]	$C_{12}H_{36}O_6Si_6$	444
	18.18	3.88	4.86	2.74	Tridec-1-ene	$C_{13}H_{26}$	182
	19.61	1.08	1.62	2.30	Tetradecamethyl-cycloheptasiloxane[b]	$C_{14}H_{42}O_7Si_7$	518
	20.62	5.44	7.20	2.60	2,4-ditert-butylphenol	$C_{14}H_{22}O$	206
	22.36	5.58	6.69	2.86	Pentadec-1-ene	$C_{15}H_{30}$	210
	28.28	5.81	3.82	5.23	Nonadec-1-ene	$C_{19}H_{38}$	266
	36.83	5.13	4.45	3.96	Tetracosan-1-ol	$C_{24}H_{50}O$	354
	37.01	0.81	0.70	4.00	Heptadecane	$C_{17}H_{36}$	240
	38.41	1.54	0.46	11.60	Heptacosan-1-ol	$C_{27}H_{56}O$	396
	44.63	4.30	2.13	6.93	Docosan-1-ol	$C_{22}H_{46}O$	326
(25°C)	11.69	10.09	6.04	11.56	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	12.09	0.05	0.13	2.48	2-acetyl-1-pyrroline	C_6H_9NO	111
	17.90	1.41	3.35	2.92	Dodecan-1-ol	$C_{12}H_{26}O$	186
	18.13	0.48	1.19	2.77	Tridecane	$C_{13}H_{28}$	184
	23.67	0.76	1.85	2.86	Hexadecane	$C_{16}H_{34}$	226
	26.41	2.88	4.19	4.75	2,4-ditert-butylphenol	$C_{14}H_{22}O$	206
	28.49	3.85	6.21	4.29	Pentadec-1-ene	$C_{15}H_{30}$	210
	33.06	5.06	6.18	5.67	Heptadec-1-ene	$C_{17}H_{34}$	238
	37.72	5.15	6.22	5.73	Nonadecan-1-ol	$C_{19}H_{40}O$	284
	37.81	1.11	3.03	2.54	Nonadecane	$C_{19}H_{40}$	268
	41.65	5.70	5.89	6.69	Nonadec-1-ene	$C_{19}H_{38}$	266
	41.73	1.02	2.10	3.38	Heptadecane	$C_{17}H_{36}$	240
	47.94	0.89	1.14	5.43	Docosane	$C_{22}H_{46}$	310
	56.19	1.98	1.04	13.14	Octacosan-1-ol	$C_{28}H_{58}O$	410
	58.91	4.97	2.35	14.60	Heptacosan-1-ol	$C_{27}H_{56}O$	396
(20°C)	11.71	9.99	11.25	6.98	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	12.08	0.02	0.06	2.32	2-acetyl-1-pyrroline	C_6H_9NO	111
	17.89	0.76	1.84	3.25	Dodecan-1-ol	$C_{12}H_{26}O$	186
	23.47	1.88	4.68	3.16	Pentadec-1-ene	$C_{15}H_{30}$	210
	23.66	0.51	1.34	3.01	Tetradecane	$C_{14}H_{30}$	198
	26.38	2.59	5.80	3.52	2,4-ditert-butylphenol	$C_{14}H_{22}O$	206
	28.46	2.89	7.04	3.22	Nonadec-1-ene	$C_{19}H_{38}$	266
	28.60	0.61	1.67	2.86	Hexadecane	$C_{16}H_{34}$	226
	33.15	0.61	1.53	3.15	Heptadecane	$C_{17}H_{36}$	240
	37.68	3.82	8.77	3.42	Nonadecan-1-ol	$C_{19}H_{40}O$	284
	37.77	0.63	1.80	2.75	Docosane	$C_{22}H_{46}$	310
	41.59	3.75	6.84	4.32	Octacosan-1-ol	$C_{28}H_{58}O$	410
	46.02	0.68	0.51	10.53	Heptacosan-1-ol	$C_{27}H_{56}O$	396

[a]Dodecamethylcyclohexasiloxane: 2,2,4,4,6,6,8,8,10,10,12,12-dodecamethyl-1,3,5,7,9,11-hexaoxa-2,4,6,8,10,12-hexasilacyclododecane. [b]Tetradecamethyl-cycloheptasiloxane: 2,2,4,4,6,6,8,8,10,10,12,12,14,14-tetradecamethyl-1,3,5,7,9,11,13-heptaoxa-2,4,6,8,10,12,14-heptasilacyclotetradecane.
IS: Internal standard

Table 6.3: Volatile compound of Rato Basmati rice grain obtained from different temperature.

Temp.	R.Time	Area %	Height %	A/H	Compound (IUPAC/Chemical name)	Formula	Mol weight
(Ambient)	11.85	61.29	44.22	3.27	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	31.72	0.48	0.47	2.41	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242
	35.60	2.09	2.27	2.18	Methyl pentadecanoate	$C_{16}H_{32}O_2$	256
	38.52	0.42	0.43	2.33	Methyl (E)-hexadec-5-enoate	$C_{17}H_{32}O_2$	268
(25°C)	11.71	11.95	7.10	12.6	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	12.10	0.08	0.16	3.52	2-acetyl-1-pyrroline	C_6H_9NO	111
	17.90	1.45	3.70	2.94	Dodecan-1-ol	$C_{12}H_{26}O$	186
	23.50	3.12	6.31	3.69	Tetradec-1-ene	$C_{14}H_{28}$	196
	23.67	0.89	2.34	2.84	Pentadecane	$C_{15}H_{32}$	212
	26.41	3.26	4.94	4.94	2,4-ditert-butylphenol	$C_{14}H_{22}O$	206
	28.49	4.44	7.33	4.53	Hexadec-1-ene	$C_{16}H_{32}$	224
	28.62	1.12	3.06	2.73	Octadecane	$C_{18}H_{38}$	254
	33.06	5.68	7.31	5.81	Nonadec-1-ene	$C_{19}H_{38}$	266
	37.72	5.37	7.40	5.43	Docosan-1-ol	$C_{22}H_{46}O$	326
	37.80	1.14	3.35	2.53	Heptadecane	$C_{17}H_{36}$	240
	41.71	1.02	2.30	3.32	Docosane	$C_{22}H_{46}$	310
	47.78	5.66	4.87	8.70	Octacosan-1-ol	$C_{28}H_{58}O$	410
	54.02	0.53	0.42	9.46	2-(2-ethylhexoxycarbonyl)benzoic acid	$C_{16}H_{22}O_4$	278
	59.12	0.54	0.45	9.09	Tetracosane	$C_{24}H_{50}$	338
(20°C)	11.88	7.22	39.28	4.34	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	35.60	0.54	5.79	2.22	Methyl 14-methylpentadecanoate	$C_{17}H_{34}O_2$	270
	36.74	0.04	0.40	2.15	Nonadec-1-ene	$C_{19}H_{38}$	266
	38.52	0.23	2.24	2.45	Methyl (E)-octadec-7-enoate	$C_{19}H_{36}O_2$	296

Table 6.4: Volatile compound of E 7 rice grain obtained from different temperature.

Temp.	R.Time	Area %	Height %	A/H	Compound (IUPAC/Chemical name)	Formula	Mol weight
(Ambient)	6.07	0.34	0.34	1.93	(E)-hex-3-en-1-ol	$C_6H_{12}O$	100
	11.85	45.21	32.75	2.67	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	31.74	0.76	0.61	2.40	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242
	35.61	3.12	2.73	2.21	Methyl 14-methylpentadecanoate	$C_{17}H_{34}O_2$	270
	38.53	0.69	0.55	2.44	Methyl (E)-hexadec-5-enoate	$C_{17}H_{32}O_2$	268
(25°C)	11.73	12.84	8.9	11.05	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	12.10	0.06	0.14	3.25	2-acetyl-1-pyrroline	C_6H_9NO	111
	17.90	1.15	3.00	2.94	Dodecan-1-ol	$C_{12}H_{26}O$	186
	18.13	0.39	1.07	2.77	Dodecane	$C_{12}H_{26}$	170
	23.49	2.36	5.68	3.18	Pentadec-1-ene	$C_{15}H_{30}$	210
	23.67	0.65	1.65	2.99	Tetradecane	$C_{14}H_{30}$	198
	26.40	2.88	5.61	3.93	2,4-ditert-butylphenol	$C_{14}H_{22}O$	206
	28.62	0.79	2.18	2.76	Hexadecane	$C_{16}H_{34}$	226
	33.04	4.43	7.85	4.32	Docosan-1-ol	$C_{22}H_{46}O$	326
	37.71	4.56	8.54	4.09	Nonadec-1-ene	$C_{19}H_{38}$	266
	37.79	0.83	2.48	2.56	Heptadecane	$C_{17}H_{36}$	240
	41.62	4.73	7.37	4.92	Heptacosan-1-ol	$C_{27}H_{56}O$	396
	41.71	0.74	1.68	3.38	Docosane	$C_{22}H_{46}$	310
	46.05	1.24	0.87	10.91	Octacosan-1-ol	$C_{28}H_{58}O$	410
	47.76	4.45	4.41	7.73	Tetracosan-1-ol	$C_{24}H_{50}O$	354
	47.91	0.61	0.79	5.88	Tetracontane	$C_{40}H_{82}$	562
(20°C)	11.88	5.03	21.32	4.08	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	21.26	0.08	0.59	2.31	Dodecamethylcyclohexasiloxane[a]	$C_{12}H_{36}O_6Si_6$	444
	25.62	0.09	0.66	2.32	Tetradecamethyl-cycloheptasiloxane[b]	$C_{14}H_{42}O_7Si_7$	518
	35.61	0.57	4.51	2.19	Methyl 14-methylpentadecanoate	$C_{17}H_{34}O_2$	270
	38.53	0.3	2.06	2.52	Methyl (E)-octadec-7-enoate	$C_{19}H_{36}O_2$	296
	39.04	0.1	0.65	2.59	Methyl 16-methylheptadecanoate	$C_{19}H_{38}O_2$	298

[a]Dodecamethylcyclohexasiloxane: 2,2,4,4,6,6,8,8,10,10,12,12-dodecamethyl-1,3,5,7,9,11-hexaoxa-2,4,6,8,10,12-hexasilacyclododecane. [b]Tetradecamethyl-cycloheptasiloxane: 2,2,4,4,6,6,8,8,10,10,12,12,14,14-tetradecamethyl-1,3,5,7,9,11,13-heptaoxa-2,4,6,8,10,12,14-heptasilacyclotetradecane.
IS: Internal standard

Table 6.5: Volatile compound of E 13 rice grain obtained from different temperature.

Temp.	R.Time	Area %	Height %	A/H	Compound (IUPAC/Chemical name)	Formula	Mol weight
(Ambient)	6.07	0.31	0.33	1.89	(E)-hex-3-en-1-ol	$C_6H_{12}O$	100
	11.85	43.7	31.95	2.70	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	31.72	0.33	0.26	2.48	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242
	35.60	1.51	1.36	2.18	Methyl 14-methylpentadecanoate	$C_{17}H_{34}O_2$	270
	38.52	0.29	0.24	2.37	Methyl (E)-octadec-6-enoate	$C_{19}H_{36}O_2$	296
(25°C)	11.70	11.2	6.67	12.56	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	12.10	0.08	0.17	3.47	2-acetyl-1-pyrroline	C_6H_9NO	111
	17.91	1.44	3.73	2.89	Dodec-1-ene	$C_{12}H_{24}$	168
	18.14	0.49	1.35	2.72	Dodecane	$C_{12}H_{26}$	170
	23.50	2.98	6.00	3.72	Hexadec-1-ene	$C_{16}H_{32}$	224
	23.68	0.80	2.19	2.75	Tetradecane	$C_{14}H_{30}$	198
	26.43	3.03	4.66	4.86	2,4-ditert-butylphenol	$C_{14}H_{22}O$	206
	28.63	1.01	2.74	2.74	Heptadecane	$C_{17}H_{36}$	240
	33.07	5.27	6.95	5.68	Heptadec-1-ene	$C_{17}H_{34}$	238
	33.19	1.11	2.61	3.17	Nonadecane	$C_{19}H_{40}$	268
	37.73	5.07	6.90	5.50	Tetracosan-1-ol	$C_{24}H_{50}O$	354
	37.81	1.07	3.10	2.57	Henicosane	$C_{21}H_{44}$	296
	41.65	5.57	6.43	6.48	(Z)-tricos-9-ene	$C_{23}H_{46}$	322
	41.73	0.97	2.24	3.23	Icosane	$C_{20}H_{42}$	282
	46.10	2.39	0.94	18.93	Triacontan-1-ol	$C_{30}H_{62}O$	438
	47.95	0.88	1.29	5.11	Tetracosane	$C_{24}H_{50}$	338
	58.90	4.67	2.41	14.51	Octacosan-1-ol	$C_{28}H_{58}O$	410
	62.16	1.00	0.26	29.00	Heptacosan-1-ol	$C_{27}H_{56}O$	396
(20°C)	11.58	1.64	6.23	3.17	1H-pyrazole	$C_3H_4N_2$	68
	11.91	10.42	25.66	4.89	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	31.73	0.38	1.96	2.35	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242
	35.61	2.16	11.22	2.32	Methyl 14-methylpentadecanoate	$C_{17}H_{34}O_2$	270
	38.52	0.93	4.39	2.56	Methyl (E)-octadec-6-enoate	$C_{19}H_{36}O_2$	296
	39.03	0.31	1.37	2.70	Methyl 16-methylheptadecanoate	$C_{19}H_{38}O_2$	298

IS: Internal standard

Table 6.6: Volatile compound of MR 219 rice grain obtained from different temperature.

Temp.	R.Time	Area %	Height %	A/H	Compound (IUPAC/Chemical name)	Formula	Mol weight
(Ambient)	9.50	49.63	64.78	6.03	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	17.10	0.91	0.62	11.52	Dodec-1-ene	$C_{12}H_{24}$	168
	17.29	1.02	0.65	12.45	3,7-dimethylnonane	$C_{11}H_{24}$	156
	23.84	2.84	2.18	10.25	Tridec-1-ene	$C_{13}H_{26}$	182
	24.03	3.03	1.78	13.43	4,7-dimethylundecane	$C_{13}H_{28}$	184
	27.29	7.66	8.87	6.80	3,5-ditert-butylphenol	$C_{14}H_{22}O$	206
	29.99	5.13	4.53	8.90	Pentadec-1-ene	$C_{15}H_{30}$	210
	30.15	4.56	3.10	11.58	2-methyl-5-propylnonane	$C_{13}H_{28}$	184
	35.55	10.63	5.90	14.20	Heptadec-1-ene	$C_{17}H_{34}$	238
	40.69	9.18	4.93	14.68	Nonadec-1-ene	$C_{19}H_{38}$	266
(25°C)	11.87	20.45	25.88	2.72	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	20.68	6.38	3.63	6.05	Tetradecamethylcycloheptasiloxane[a]	$C_{14}H_{42}O_7Si_7$	518
	27.97	4.83	5.26	3.16	Hexadecamethyl-cyclooctasioxane[b]	$C_{16}H_{48}O_8Si_8$	592
	32.28	3.49	4.08	2.94	Hexadecamethylheptasiloxane[c]	$C_{16}H_{48}O_6Si_7$	532
	37.74	3.21	4.29	2.58	Tetradecamethylhexasiloxane[d]	$C_{14}H_{42}O_5Si_6$	458
	40.43	1.08	1.21	3.09	Nonadec-1-ene	$C_{19}H_{38}$	266
	46.85	1.06	0.64	5.73	Docosan-1-ol	$C_{22}H_{46}O$	326
(20°C)	11.57	4.14	4.99	23.33	1H-pyrazole	$C_3H_4N_2$	68
	11.91	2.07	13.14	4.45	2,4,6-trimethylpyridine (IS)	$C_8H_{11}N$	121
	35.61	0.33	4.24	2.17	Methyl 14-methylpentadecanoate	$C_{17}H_{34}O_2$	270
	38.52	0.11	1.19	2.53	Methyl (E)-octadec-7-enoate	$C_{19}H_{36}O_2$	296

[a]Tetradecamethylcycloheptasiloxane: 2,2,4,4,6,6,8,8,10,10,12,12,14,14-tetradecamethyl-1,3,5,7,9,11,13-heptaoxa-2,4,6,8,10,12,14-heptasilacyclotetradecane; [b]Hexadecamethyl-cyclooctasioxane: 2,2,4,4,6,6,8,8,10,10,12,12,14,14,16,16-hexadecamethyl-1,3,5,7,9,11,13,15-octaoxa-2,4,6,8,10,12,14,16-octasilacyclohexadecane; [c]Hexadecamethylheptasiloxane: bis[[[dimethyl(trimethylsilyloxy)silyl]oxy-dimethylsilyl]oxy]-dimethylsilane; [d]Tetradecamethylhexasiloxane: [dimethyl(trimethylsilyloxy)silyl]oxy-[[dimethyl(trimethylsilyloxy)silyl]oxy-dimethylsilyl]oxy-dimethylsilane.
IS: Internal standard

At 25°C temperature, MRQ 50 genotype exhibited the presence of 15 compounds (abundance of Octacosan-1-ol), Ranbir Basmati 15 compound with the higher abundance of Nonadec-1-ene, Rato Basmati genotype produced 15 compound (abundance of Nonadec-1-ene), E 7 genotype 16 compound (abundance of Nonadec-1-ene) and E 13 genotype produced 18 compounds with higher abundance of (z)-tricos-9-ene.

At 20°C temperature, MRQ 50 genotype represented 6 compound (abundant of methyl hexadecanoate), Ranbir Basmati 13 compound (abundance of Nonadecan-1-ol), Rato Basmati genotype produced 4 compound (more abundant Methyl 14-methylpentadecanoate), E 7 genotype 6 compound (abundant Methyl 14-methylpentadecanoate) and E 13 genotype produced 6 compounds (more abundant Methyl 14-methylpentadecanoate).

The MR 219 genotype exhibited presence of 10 compounds at the ambient condition, 7 compounds at 25°C (abundance of Tetradecamethylcycloheptasiloxane) and 4 compounds at 20°C temperature with a higher abundance of 1H-pyrazole.

The 2AP was identified in MRQ 50 and Ranbir Basmati genotype at ambient condition and only in Ranbir Basmati genotype at 20°C temperature. Besides, all aromatic rice genotypes exhibited the presence of 2AP at 25°C temperature.

The volatile profile using GC-MS analysis represented that all genotypes produced their maximum number of volatile compound at 25°C temperature which might be considered as a suitable temperature for better volatile profile as well as for discriminating the volatile profile of aromatic with non-aromatic rice genotype.

6.3.2 Effect of Temperature on 2AP Concentration

In ambient condition, the numbers of identified compound were 13 and the Malaysian aromatic genotype MRQ 50 demonstrated 20.02% peak area for TMP and 0.09% for 2AP. At 25°C temperature, the numbers of identified compound were 18 and the Malaysian aromatic genotype MRQ 50 demonstrated 10.04% peak area for TMP and 0.04% for 2AP. In the case of 20°C temperature, the numbers of identified

compound were also 13 as in ambient conditions and the Malaysian aromatic genotype MRQ 50 demonstrated 29.88% area for TMP while 2AP was not detected.

During quantitative analysis, genotype Ranbir Basmati produced 2AP in all three conditions, and MRQ 50 genotype demonstrated 2AP at ambient and 25°C temperature while all other aromatic genotypes produced 2AP at 25°C only (Table 6.7). The non-aromatic genotype MR 219 did not produce 2AP in any of the three conditions.

Table 6.7: Duncan Multiple Range Test (DMRT) for 2AP concentration identified in three temperatures.

Genotype	2AP Conc. (ppm)		
	Ambient	**25°C**	**20°C**
MRQ 50	0.09a	0.08b	nd
Ranbir Basmati	0.01b	0.10ab	0.04a
Rato Basmati	nd	0.13ab	nd
E 7	nd	0.09b	nd
E 13	nd	0.14a	nd
MR 219	nd	nd	nd

Means with the same letter are not significantly different at 5% level. nd = not detectable

Table 6.7 shows that only Ranbir Basmati genotype produced a quantifiable amount of 2AP in all temperature conditions and the quantity reduced gradually from 25°C (0.10 ± 0.01 ppm) to 20°C (0.04 ± 0.02 ppm) and ambient condition (0.01 ± 0.01 ppm). The Malaysian aromatic genotype MRQ 50 produced a quantifiable amount of 2AP in ambient condition (0.09 ± 0.01 ppm) and at 25°C temperature (0.08 ± 0.02 ppm) but did not produce 2AP at 20°C temperature. Only at 25°C temperature, all aromatic genotypes produced a quantifiable amount of 2AP which indicated the suitability of 25°C temperature for 2AP production in aromatic rice genotypes.

6.4 DISCUSSION

The volatile profile which shows metabolomic profile of a genotype can explain the quality of whole grain or grain products. The volatile compounds which are produced through metabolic pathways are also dependent on the genotype, agronomic practices and environmental condition (Liyanaarachchi et al., 2014). So, the volatile profile of a genotype can be used to identify the genotype, to interpret its quality and to assess the changes due to environmental condition.

In the present investigation, a profile of volatile compounds identified by the previous researcher was constructed to compare the number of compounds and possible variation among the identified compounds. About 332 volatile compounds (Table 6.8 and Table 6.9) were identified by former scientists (Bryant & McClung, 2011; Liyanaarachchi et al., 2014; Mahatheeranont et al., 1995; Mahatheeranont et al., 2001; Mahattanatawee & Rouseff, 2010; Maraval et al., 2008; Park et al., 2010; Pisithkul et al., 2010; Sukhonthara et al., 2009; Yang et al., 2007) while 60 compounds were detected in this experiment (Table 6.1, 6.2, 6.3, 6.4, 6.5, and Table 6.6).

Table 6.8: Qualitative analysis of volatile compounds of previous researchers.

Liyanaarachchi et al. (2014)	**Pisithkul et al. (2010)**	**Mahattanatawee and Rouseff (2010)**	**Park et al., (2010)**	**Yang et al. (2008)**	**Mahatheeranont et al. (1995)**	
α-terpeneol	Benzaldehyde	Decanal	Decanal	Benzaldehyde	Benzene ethanol	Octanoic acid
Benzaldehyde	Benzyl alcohol	Ethyl hexanoate	Hexanal	Benzothiazole	Benzothiazole	Pentadecane
Benzyl alcohol	n-decanal	Hexanal	Methional	Decanal	Butyl acetate	Pentylcyclopropane
Hexanal	n-dodecane	Linalool	Nonanal	*d*-limonene	Decanal	Phenol
Hexanol	*n*-heptanal	Methional		Guaiacol	Diethyl carbonate	Tetracosane
Indole	*n*-hexanal	Neral		Heptanal	Dodecane	Tetradecane
Limonene	n-nonanal	Octanal		Hexanal	Ethyl benzene	Tricosane
Linalool	n-tetradecane	β-Damascenone		Indole	Hexadecane	Undecane
n-octanol	n-tridecane			Naphthalene	Hexanal	
Octanal				Nonanal	Isocyanato methylbenze	
Phenol				Octanal	Methyl benzene	
				Phenylacetaldehyde	N,N-dimethyl formamide	
				p-xylene	Nonanal	
				Toluene	Octadecane	

Table 6.9: Qualitative analysis of volatile compounds of previous researchers.

Bryant and McClung (2011)	Sukhonthara et al. (2009)		Maraval et al. (2008)	Mahatheeranont et al. (2001)
Benzothiazole	Benzaldehyde	n-hexadecane	Acetophenone	
Butylated hydroxytoulene	Benzoic acid	n-hexanol	Benzaldehyde	Benzothiazole
Cyclodecanol	Benzothiazole	n-nonadecane	Benzothiazole	Benzyl carbinol
Decyl benzene	Biphenyl	n-nonanol	Butan-1-ol	Butyl acetate
Diethyl phthalate	Cadina-1,4-diene	n-octadecane	Butanoic acid	Decanal
Dotriacontane	Capric acid	n-octanol	Butylbenzene	Diethyl carbonate
Eicosanol	Caproic acid	Nonanal	Decanal	Ethylbenzene
Heptadecane	Caprylic acid	n-pentadecane	Ethanoic acid	Heptadecane
Heptanal	Decanal	n-pentanol	Ethylbenzene	Hexadecane
Heptylcyclohexane	Dihydroactinidiolide	n-tetradecane	Hept-2-enal (isomer)	Hexanal
Hexadecyl ester, 2,6-difluro-3-methyl benzoic acid	Dodecanal	n-tridecane	Heptanoic acid	Hexanoic acid
Hexanal	Enantic acid	n-undecanal	Hexan-1-ol	Isocyanatomethylbenzene
Hexanol	Epi-α-muurolol	n-undecane	Hexanal	Methylbenzene
Hexylpentadecyl ester-sulphurous acid	Ethyl hexadecanoate	o-cresol	Hexanoic acid	*N*,*N*-dimethylformamide
Indole	Ethyl tetradecanoate	Octanal	Indole	Naphthalene
Isobutyl hexadecyl ester oxalic acid	Furfural	Oleic acid	Longifolene	Nonanal
Isobutyl nonyl ester oxalic acid	Furfurylalcohol	Palmitic acid	Methional	Octanoic acid
Methoxy-phenyl-oxime	Geranylacetone	p-cresol	*N* ,*N*-diethylformamide	Pentadecane
N,N-dimethyl chloestan-7-amine	Guaiacol	*p*-cymene	Non-2-enal	Pentylcyclopropane
N,N-dinonyl-2-phenylthio ethylamine	Heptanal	Pelargonic acid	Nonan-2-one	Phenol
Naphthalene	Hexanal	Pentadecanal	Nonanal	Tetracosane
n-Heptadecylcyclohexane	Isovaleric acid	Pentadecylic acid	Nonanoic acid	Tetradecane
n-Nonadecanol	Lauric acid	Phenethyl alcohol	Oct-1-en-3-ol	Tricosane
Nonadecane	Limonene	Phenol	Oct-1-en-3-one	Undecane
Nonene	Linalool	p-menthan-3-one	Oct-2-enal	
Octadecyne	Linoleic acid	Stearic acid	Oct-3-en-2-one	
Octanal	Linolenic acid	Tetradecanal	Octa-3,5-dien-2-one	
O-decyl hydroxamine	Longifolene	Tridecanal	Octan-1-ol	
Pentacontonal	Methyl hexadecanoate	Tridecylic acid	Octanal	
Pentadecanal	Methyl linoleate	Valericacid	Pent-3-en-2-ol	
Pentanal	Methyl oleate	α-cadinol	Pentan-1-ol	
Propiolonitrile	Methyl tetradecanoate	α-muurolene	Pentanoic acid	
Pyrolo[3,2-d]pyrimidin-2,4(1H,3H)-dione	Myristic acid	α-terpineol	Phenol	
Tetrahydro-2,2,4,4-tetramethyl furan	Naphthalene	β-cyclocitral	Phenylacetaldehyde	
Tritetracontane	n-butanol	β-ionone	Propanoic acid	
Z-10-pentadecen-1-ol	n-dodecane	β-myrcene	Vanillin	
	n-heptadecane	δ-cadinene	*γ*-decalactone	
	n-heptanol		*γ*-nonalactone	
	n-hexadecanal		*δ*-decalactone	

In this study, the quantifiable concentration of 2AP was detected up to 0.14 ± 0.02 ppm which also varied depend on rice genotype and temperature conditions (Table 6.7). At 25°C temperature, all aromatic rice genotypes produced 2AP and the maximum concentration observed in E 13 genotype (0.14 ± 0.02 ppm) while only, Ranbir Basmati produced a quantifiable amount of 2AP at all three conditions. The higher concentration

of 2AP obtained from Ranbir Basmati genotype was 0.10 ± 0.01 ppm at 25°C, and the lower concentration was 0.01 ± 0.01 ppm at ambient condition. So, the temperature influenced the amount of 2AP in a rice genotype. Previous researchers (Bryant & McClung, 2011; Itani et al., 2004; Liyanaarachchi et al., 2014; Mahatheeranont et al., 2001; Maraval et al., 2008; Pisithkul et al., 2010) have also performed characterization and quantitative analysis of volatile compounds present in rice. The available information for quantitative estimation of 2AP in different types of rice varieties (Bergman et al., 2000; Buttery et al., 1988; Laksanalamai & Ilangantileke, 1993; Mahatheeranont et al., 2001; Wongpornchai et al., 2003) was listed in Table 6.10 to compare the obtained results of the present study.

Table 6.10: Quantitative analysis results for 2AP of previous researchers.

Researchers	Experimental observation	2AP concentration (ppm)
Wongpornchai et al. (2003)	KDML 105 brown rice seeds	3.000
Mahatheeranont et al. (2001)	Fresh brown rice	0.340
	Brown rice stored for 12 months	0.120
	Brown rice from local market (no brand)	0.320
	Milled rice (Surintip brand)	0.250
	Milled rice (Matusorn brand)	0.120
	Milled rice (changchuroungkhao brand)	0.050
Bergman et al. (2000)	Basmati easy cook (Tilde) Milled	0.019
	Jasmin (Fantastic foods) Brown	0.550
	Amber aromatic (Lundberg) Brown	0.345
Laksanalamai and Ilangantileke (1993)	Fresh aromatic	0.100 (Peak area ratio)
	Aged aromatic	0.050 (Peak area ratio)
	Non-aromatic	0.000 (Peak area ratio)
Buttery et al. (1988)	Cooked rice	0.0006

The obtained concentrations of 2AP in six rice genotypes were in between the range of 2AP concentration estimated by the previous researchers. The temperature

seems to influence both the numbers of volatile compound and the amount of 2AP of studied genotypes. The 25℃ temperature was identified as a suitable temperature for the maximum number of volatile compounds as well as the highest concentration of 2AP, which might be considered during high-quality aromatic rice production.

6.5 CONCLUSION

Aromatic rice grains correspond to the presence of numerous volatile compounds and the composition of volatile compounds, especially the concentration of 2Acetyl-1-pyrroline (2AP) determine the aroma status of a rice variety. Moreover, the aroma of aromatic rice is affected by the environmental components and genotypic constitution of the variety. This study investigated the volatile profile and the 2AP concentration of six rice genotypes to observe the effects of temperature on these components. Based on the results obtained from volatile profile analysis and 2AP quantification of rice samples, it can be concluded that the numbers and variation of volatile constituents along with the 2AP concentration of rice was influenced by the temperature condition. The differences of relative contents of the volatile components among rice genotypes were also depended on the genotype and temperature condition. Moreover, the 25℃ temperature was observed to be suitable for better aroma quality in terms of the higher amount of 2AP as well as the presence of other volatile compounds in the studied aromatic rice cultivars.

CHAPTER 7: DISCUSSIONS

Aroma is controlled by a recessive gene which possesses a 7-bp deletion in exon 2 or 8-bp deletion in exon 7 for its expression. Hence, determination and identification of the position of deletion of bases and variation in the expression level of the *badh2* gene fragments might be used to explain the genetic cause of aroma as well as the status of aroma quality in rice. Previous researchers identified the deletions in different rice genotypes (Bradbury et al., 2005b; Chen et al., 2006; Shi et al., 2008) and studied the effects of salt stress, shading, and aging on *badh2* gene expression (Fitzgerald et al., 2008; Golam et al., 2010; Zhang et al., 2012).

In the present investigation, sequence analysis of exon 7 of the *badh2* gene of studied genotypes demonstrated three single nucleotide sequence polymorphism (TTT) and 8-bp deletion (5´-GATTATGG-3´) in aromatic genotypes (Table 4.10) while the non-aromatic genotype (MR 219) showed similarity to the base sequence of the *badh2* gene cds of *Oryza sativa indica* group cultivar Nanjing 11 as mentioned in GenBank accession number EU770319 on the NCBI website. In a previous study, similar polymorphism (3 SNP) was detected by Bradbury (2009). They also found 8-bp deletion within a 25-bp region and assumed that this mutation would render the protein non-functional which explained aroma being a recessive trait.

In this experiment, a truncated protein encoded in aromatic rice varieties observed to be shorter of 4 amino acid residues (KKIM) compared to non-aromatic rice genotype (Table 4.11). Previously, Bradbury (2009) mentioned similar incidence when analyzed the nucleotide sequence of exon 7 of both non-aromatic and aromatic rice varieties. They stated that aromatic rice variety showed a large deletion and three SNPs which terminates protein prematurely.

In this research, no deletion was found in exon 2 (Table 4.7) but an 8-bp deletion in exon 7 was found in all aromatic rice genotypes which did not influence the normal growth of studied genotypes. Moreover, aromatic rice performed better at 25°C temperature and temperature did not alter nucleotide sequences. Previously, Bradbury (2009) stated that aroma is a recessive trait and a loss of function of complete *Badh2* gene is responsible for aroma in aromatic rice. The mutation in the *Badh2* gene does not seem to associate with any loss of plant performance, besides, have a positive effect under some environmental conditions, such as drought stress and salinity stress (Yoshihashi et al., 2004).

The relative quantification methods ($2^{-\Delta\Delta C}{}_T$ method) were used to investigate the relative expression of the targeted exon segments where the *Actin* gene was used as an internal control and the non-aromatic variety (MR 219) was used as calibrator. Previously, many researchers (Caldana et al., 2007; Jain et al., 2006; Kim et al., 2003; Tong et al., 2009) have used *Actin* as an internal control to validate rice gene expression analysis. Chen et al. (2008) reported that the full-length *Badh2* transcript was less abundant than the partial *badh2* transcript which possessed multiple transcription starting points. In the present study, less abundance of *badh2* transcripts and several transcript start points were observed in the different exon fragments of the *Badh2* gene.

Fitzgerald et al. (2008) observed the effects of salt on *BADH2* gene transcripts which were more abundant in non-aromatic rice varieties compared to aromatic rice varieties and concluded that the *BADH2* gene played no role in the response to salt stress. Meanwhile, Chen et al. (2008) reported less abundance of full-length *Badh2* transcript compared to partial *badh2* transcripts. They also observed low transcriptional

levels of non-functional *badh2E2* and *badh2E7* compared to functional *Badh2* gene. However, this study investigated the effects of three different temperatures on the expression of *Badh2* gene where more down-regulation of mRNA transcripts of *badh2E7* fragment was observed at 25℃ compared to the functional *Badh2E7* transcripts in non-aromatic rice genotypes.

Experimental evidences shows that aroma of rice is controlled by a major gene which also influence aroma quality, volatile compound composition, 2AP concentration, and agronomic performance (Hashemi et al., 2013). Previous researchers (Bergman et al., 2000; Buttery et al., 1988; Laksanalamai & Ilangantileke, 1993; Mahatheeranont et al., 2001; Wongpornchai et al., 2003) performed characterization, qualitative analysis and quantitative analysis of volatile compounds present in rice (Table 6.8, 6.9 and 6.10). Similarly, characterization, qualitative analysis and quantitative analysis of volatile compounds in the studied genotypes were performed through this research (Table 6.1, 6.2, 6.3, 6.4, 6.5, 6.6 and Table 6.7).

The phenotypic aroma score of studied genotypes were also depends on temperature and variety (Table 3.11). The aroma score indicated that 25℃ temperature was favorable for optimum aroma expression. Though, different phenotypic aroma score was observed at different temperatures, but the uniform aroma expression was found within same temperature condition. Moreover, fluctuation of aroma score was found in all growth stages while it becomes stable in matured harvested grain. Several researchers (Golam et al., 2011; Hossain et al., 2008; Sarhadi et al., 2011) used leaf and grain sensory test for evaluating aromatic and non-aromatic rice. Golam et al. (2011) mentioned that the aroma score of Basmati type rice demonstrated strong aroma (score

4) in Indian sub-continent and moderate aroma (score 3) in Malaysian tropical environment.

The morphological and agronomic characters of all studied genotypes were varied on temperature condition (Table 3.6). The ambient temperature influenced the number of fertile tiller per hill, grain filling period and days to maturity while 20°C temperature reduced days to flowering, plant height, panicle length, fertile grain per panicle, 1000 grain weight and finally the grain yield. On the other hand, 25°C facilitated better vegetative growth as well as grain yield which were as similar observation of Golam et al. (2011) for aromatic rice varieties. Previously, Aghamolki et al. (2014) studied the influences of high temperature on different growth stages of rice and observed that heat stress reduced yield when it imposed during booting and flowering stage. They also mentioned that heat stress did not affect later growth stages (ripening stage) of rice. Zhang et al. (2013) stated that a mild increase of night-time temperature during reproductive growth stage reduced yield and performances of yield-related traits. Similar observation was also pointed out by Shrivastava et al. (2012) and Islam (Islam, 2011) who mentioned that high temperature at booting and grain filling stage reduced growth rate and grain yield of aromatic rice.

Hence, the molecular, biochemical and organoleptic analysis of this experiment represented that the expression of the *badh2* gene, as well as aroma status of a genotype, depended on the growing temperature. The agronomic performance of aromatic rice genotypes was also regulated by environmental temperature which should consider during aromatic rice breeding and improvement program for high-quality aromatic rice production.

CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS

Aromatic rice is a small but an important sub-group of rice which is highly regarded for grain quality and aromatic flavor. The aroma quality of rice is influenced by the cultural practice, genotypic condition, and environmental factors. Among the environmental factors, increasing temperature is the most important factor which may affect the growth, development, production, and aroma performance of aromatic rice. Moreover, the aroma trait is controlled by a recessive gene which contains a 7-bp deletion in exon 2 or 8-bp deletion in exon 7 and can express only at homozygous recessive condition (*badh2*/*badh2*). Besides, the presence of some volatile compounds influences aroma quality as well as a unique flavor of a rice variety. More than 300 volatile compounds have been identified in different aromatic rice variety and 2-Acetyl-1-pyrroline (2AP) is the essential component responsible for aromatic flavor. However, most of the aromatic rice demonstrate lower agronomic performance and associated with undesirable agronomic characters. So, it is important to investigate the effects of temperature on the production and performance of aromatic rice to determine a suitable temperature for better agronomic performance, complete expression of aroma gene and superior phenotypic aroma which eventually ensure high-quality aromatic rice production in changing climatic condition.

In this investigation, the agronomic performance of studied genotypes did not differ when grown at similar temperature condition (ambient condition) under two different environments (net house and glasshouse). The performance of agronomic traits was significantly different at 5% level for different traits of a genotype or the same trait of different genotypes grown in different temperature when analyzed using Duncan Multiple Range Test (DMRT). The DMRT results also signified that the maximum

variation was in the grain filling periods. The genotype E 13 demonstrated longer grain filling periods (47.40 days) at 20°C compared to ambient (34.20 days) and 25°C temperature (27.80 days).

The Pearson's correlation coefficients of morpho-agronomic traits represented that the grain filling period was negatively correlated with the number of tiller per hill, the number of fertile tiller per hill, flowering days and grain yield per plant while significantly positively correlated with panicle length, grain per panicle and fertile grain per panicle at ambient condition. The grain yield per plant exhibited significantly positive correlation with grain per panicle and fertile grain per panicle at 25°C and 20°C temperature but with the 1000 grain weight at ambient condition.

The phenotypic aroma was different at different growth stages under different temperature and all aromatic rice genotypes demonstrated strong aroma (score 4) at 25°C temperature.

The sequence analysis of exon 2 and exon 7 of the *badh2* gene represented no deletion in exon 2 while 8-bp deletion in exon 7 in aromatic genotypes. The sequence analysis of exon 7 also demonstrated three single nucleotide sequence polymorphism (TTT) in aromatic rice genotypes (MRQ 50, Ranbir Basmati, Rato Basmati, E 7 and E 13 genotype) compared to non-aromatic rice genotype (MR 219).

The relative expression of the *badh2* gene compared to the *Actin* gene in aromatic and non-aromatic rice genotypes showed different levels of *badh2* gene expression at different growth stages under different temperature. The temperature significantly affected the expression of the *badh2* gene at different growth stages of

aromatic rice. Moreover, all aromatic genotypes demonstrated higher down-regulation of the *badh2*/*badh2* allele at 25°C temperature compared to the expression of the *badh2*/*badh2* allele in the same genotype at 20°C and ambient condition.

Volatile profile of the studied genotypes showed that all genotypes produced more volatile compounds at 25°C compared to ambient and 20°C temperature. During quantitative analysis, genotype Ranbir Basmati produced 2AP in all three conditions and MRQ 50 genotype produce 2AP at ambient and at 25°C temperature while all aromatic genotypes produced a quantifiable amount of 2AP only at 25°C temperature, which indicated the suitability of 25°C temperature for 2AP production in aromatic rice genotypes. The non-aromatic genotype MR 219 did not produce 2AP in all three conditions.

Gene expression analysis of the *badh2* gene, gas chromatography-mass spectrometry for qualitative analysis of volatile compounds and quantitative analysis of 2AP combined with organoleptic analysis of phenotypic aroma represented that down-regulation of the recessive *badh2/badh2* allele was responsible for the significant elevation of 2AP concentration as well as phenotypic aroma expression in rice. Moreover, the agronomic performance of agronomic rice genotypes was better at 25°C compared to 20°C and ambient condition.

However, this study did not perform cost and benefit analysis of aromatic rice production under a controlled environment. Therefore, in future, the overall probability of aromatic rice production under control environment should be studied in detail. The biochemical pathway of volatile compounds should be analyzed to improve the quality of rice for better aroma. Other grain quality traits should also be studied.

Temperature affects the agronomic performance, expression of aroma gene, and the level of volatile compounds, as well as the production of 2AP. This information will help to develop as a platform for production of high-quality aromatic rice under different temperature. Additionally, development of new technologies, availability of numerous molecular biological tools and designing of novel markers for aroma gene will enhance the production and development of superior aromatic rice. A combined approach of plant breeding, functional genomic analysis, molecular biological analysis and biochemical analysis are essential to overcome the limitation of aromatic rice production. Besides, an investigation for the expression levels of differentially regulated genes present in aromatic and non-aromatic lines are necessary to clarify the evolution and molecular basis of aroma in rice. For a deeper understanding, both genetic and environmental factors that affect this important trait should be considered.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications

1. **Zakaria, H. P.**, Golam, F., Rosna, M. T., & Kamaludin, A. R. (2016). Agronomic, transcriptomic and metabolomic expression analysis of aroma gene under different temperature regimes in rice. *International Journal of Agriculture and Biology* (Accepted) (Q2-ISI-Cited Publication)
2. Faruq, G., **Prodhan, Z. H.**, & Nezhadahmadi, A. (2015). Effects of ageing on selected cooking quality parameters of rice. *International Journal of Food Properties*, *18*(4), 922-933. (Q3-ISI-Cited Publication)
3. Faruq, G., Taha, R. M., & **Prodhan, Z. H.** (2014). Rice ratoon crop: A sustainable rice production system for tropical hill agriculture. *Sustainability*, *6*(9), 5785-5800. (Q3-ISI-Cited Publication)
4. Golam, F., & **Prodhan, Z. H.** (2013). Kernel elongation in rice. *Journal of the Science of Food and Agriculture*, *93*(3), 449-456. (Q1- ISI-Cited Publication)
5. Yeap, H. Y., Faruq, G., **Zakaria, H. P.**, & Harikrishna, J. A. (2013). The efficacy of molecular markers analysis with integration of sensory methods in detection of aroma in rice. *The Scientific World Journal*, 2013, 1-6. (Q1-ISI-Cited Publication)
6. **Zakaria, H. P.**, Golam, F., Subha, B., Rosna, M. T., & Kamaludin, A. R. The effect of temperature on the expression of *badh2* gene at different growth stages of aromatic rice (*Oryza sativa* L.). *PeerJ* (under review)
7. **Zakaria, H. P.**, Golam, F., Subha, B., Rosna, M. T., & Kamaludin, A. R. Expression of *badh2* gene, characterization of volatile compound and evaluation of aroma in aromatic rice at different temperature. *Journal of the Science of Food and Agriculture* (under review)

Proceedings

1. **Zakaria, H. P.**, & Faruq, G. (2014). *A promising aromatic rice genotype for better cooked kernel elongation with excellent ratoon performance in Malaysian Agro-climatic condition*. Paper presented at the 19th Biological Sciences Graduate Congress (BSGC), 12-14th December 2014, National University of Singapore, Singapore.
2. Faruq, G., & **Prodhan, Z. H.** (2014). *Ratooning analysis of few aromatic rice genotypes in Malaysian agro-climatic condition for extended food security.* Paper presented at the Rice International Conference 2014, 24-27 November 2014, Pingtung University of Science and Technology, Pingtung, Taiwan.