CHAPTER V

DISCUSSION

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5.1 Methanol extracts of *Chakhao Poireiton* and *Chakhao Amubi*

Extraction of anthocyanins is commonly conducted with methanol or ethanol containing a small amount of acid (15% 1.0 mol/L HCL) which obtained the flavylium cation form, which is stable in a highly acidic medium and there is no significant difference in absorbance readings or extraction efficiency between ethanol or methanol (Shipp & Abdel-Aal, 2010). In the present study acidified methanol extraction at 60ºC method was used and the extracts were used for the measurement of total anthocyanin, total phenolics, antioxidant activity test and the identification of the anthocyanin compositions in the two black scented rice cultivars (*Chakhao Poireiton and Chahao Amubi*) of Manipur. But acid may cause partial hydrolysis of the acyl moieties in acylated anthocyanins, especially those with dicarboxylic acids such as malonic acid and addition of an organic solvent such as hexane to the extract can eliminate any unwanted lipid-containing substances (Shipp & Abdel-Aal, 2010). The pH level has also been shown to have a significant influence on the color of the anthocyanin extracts, the absorbance readings and the extractability of the extract, at lower pH (pH < 2), blue and purple wheat extracts exhibited a red to dark red color after extraction, while at a higher pH (pH > 4) extracts displayed a yellow color and absorbance readings increased with declining pH levels of the solvents tested (water, ethanol and methanol) (Shipp & Abdel-Aal, 2010). There is no significant effect on the absorbance readings when there is changed with the temperature between 25-60ºC during extraction of anthocyanin and also the storage of the anthocyanin extracts at 4ºC but the increased in the temperature from 25ºC to 60ºC during anthocyanin extraction from purple and blue wheat increased the absorbance readings by 15% (Shipp & Abdel-Aal, 2010). The extracts become cloudy due to precipitation of the

soluble proteins when the blue and purple wheat anthocyanin extracts were stored under cold conditions (4ºC) but has no significant effect on the absorbance readings (Shipp & Abdel-Aal, 2010).

5.2 Anthocyanin compositions in *Chakhao Poireiton* and *Chakhao Amubi*

High performance liquid chromatography (HPLC) was used in the present study to identify the anthocyanin compositions in the methanol extracts. Separated anthocyanins were detected and measured at 517 nm and the identification of anthocyanins were based on corresponding retention times and ultraviolet-visible (UV-vis) spectra with those of pure authentic standards such as delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside, cyanidin-3- galactoside, cyanidin-3-rutinoside, peonidin-3-glucoside, petunidin-3-glucoside, pelargonidin-3-glucoside and cyanidin chloride that are commercially available. The nature of the anthocyanidin, glucosylation pattern and possibility of acylation can be provided by UV-vis absorbance spectrum of an anthocyanin (Abdel-Aal et al. 2006). The most important factor affecting the retention times is the polarity of the anthocyanidin, anthocyanidins become more polar as the number of the hydroxyl group in the B-ring increases, thereby decreasing retention and become more polar as the number of methoxyl group increases and thus, the observed order of elution of the six most common anthocyanidins is delphindin→ cyanidin→ petunidin → pelargonidin→ peonidin → malvidin (Shipp & Abdel-Aal, 2010).

Considering the potential of colored cereals such as purple wheat as nutraceutical ingredients and functional foods, the interest in the anthocyanin content has increased (Choia et al. 2007). As such, there is also an increased in the interest of supplementing colored rice in the diet. Anthocyanins can play as antioxidants and

provide other health benefits and are regarded as safe and effective food colorants (Manach et al. 2004; Prior & Wu, 2006).

Identification and peak assignment of anthocyanins in all foods were based on comparison of their retention times (RT) with those of standards and published data (Jing et al. 2007; Hosseinian et al. 2008; Jia et al. 2008; Lee et al. 2009). Four main anthocyanins were identified in *Chakhao Poireiton* (Fig. 7: Table 4) i.e. peak no. 3 as Dp-3-gal (RTmin. ranging from 3 to 3.9), peak no. 5 as Dp 3-ara (RTmin. ranging from 5.2 to 5.7), peak no. 6 as Cy-3-gal (RTmin. ranging from 14.6 to 16.2) and peak no. 7 as Cy-3-glc (RTmin. ranging from 16.4 to 18.4). Three main anthocyanins, peak no. 2 as Dp-3-gal (RTmin. ranging from 3.0 to 3.9), peak no. 3 as Dp 3-ara (RTmin. ranging from 5.2 to 5.7) and peak no. 4 as Cy-3-gal (RTmin. ranging from 14.6 to 16.2) were identified in *Chakhao Amubi* (Fig. 8: Table 4). There were also some unidentified peaks (Fig. 7 & 8) observed in both the black scented rice cultivars that showed UV absorption at 517 nm at shorter retention time. This is due to the lack of standards and published data. Recently, the studies on purple wheat and purple corncob, observed some unidentified peaks showing that the anthocyanins could be acylated accounting for 35.6–54.0% of total anthocyanins and the unknown anthocyanins in the purple wheat samples could be due to the presence of acylated anthocyanins, thus, the unknown anthocyanins in the purple wheat samples could be due to the presence of acylated anthocyanins (Jing et al. 2007; Hosseinian et al. 2008). Furthermore, it was also reported that the environmental factors such as growing location likely affects the anthocyanin levels and antioxidant activities (Mpofu et al. 2006; Jing et al. 2007; Hosseinian et al. 2008).

In the present study, Dp 3-glc showed the shortest RT (Table 4). Whereas cyanidin showed the longest RT i.e. Cy-3-glc and Cy-3-gal (Table 4) in *Chakhao*

Poireiton and *Chakhao Amubi*, respectively. Dp-3-gal, Dp-3-ara, Cy-3-ara, Pt (Petunidin)-3-glc, Cy-3-gal, Cy-3-glc, Pg (pelargonidin)-3-glc, Pn-3-glc, Mv (malvidin)-3-glc, Cy-cl (chloride), Pg-3-gal , Pg-3-ara and Pn-3-ara were identified in purple wheat (Hosseinian et al. 2008). In their study, Cy-3-glc was the most abundant anthocyanin in purple wheat; however, this was followed by Cy-3-gal and Mv-3-glc. Cy-3-glc and peonidin 3- glucoside (Pn-3-glc) are the two major anthocyanins present in the Korean black rice (*Heugjinjubjeo*) (Park et al. 2008). Lee (2010) also showed Cy-3-glc exhibited a markedly higher content in the black rice. They isolated and elucidated, the anthocyanins, cyanidin-3-O-glucoside and peonidin-3- O-glucoside, using reverse phase C18 chromatography, nuclear magnetic resonance (NMR) spectroscopy and high performance liquid chromatography (HPLC) with diode array detection and electrospray ionization/mass spectrometry (DAD-ESI/MS) showing the anthocyanin have significant differences and cyanidin-3-O-glucoside exhibited a markedly higher content than peonidin-3-O-glucoside. Their results suggested that anthocyanin can be a key factor in functional property of black rice and an important source concerning nutritional value. Cy 3- glc, Pg 3-glc, Pn 3-glc, Cy-3-(malonyl)glc , Cy 3-(malonyl)glc, Pg 3-(malonyl)glc , Pn 3-(malonyl)glc and Cy-3- dimalonylglc were the major anthocyanins identified in the purple corncob (Jing et al. 2007). Cyanidin-3-glucoside and cyanidin-3 maloylglucoside were the two major anthocyanins present in the purple corn cob. Kim et al. (2008) reported that the acidic methanol extracts of the black and wild rice showed three different types of pigments by HPLC whereas red rice variety did not show any anthocyanins. Out of three pigments detected, one was characterized as cyanidin-3-glucoside (C3G) by comparison of spectroscopic and chromatographic properties with an authentic standard and another was tentatively identified as cyanidin-fructoside on the basis of

spectroscopic properties with λmax of aglycone in 1% HCl methanol at 537 nm. They found that the most abundant anthocyanin in black and wild rice was C3G. Yawadio et al. (2007) reported the HPLC profile of anthocyanins extracted from black rice identified as cyanidin-3-O-glucoside and peonidin 3-O-b-D-glucoside while that of pigmented brown rice showed ferulic acid and tocols. They isolated and identified several tocols from the unsaponifiable fractions of both the rice showing some difference on their structures and amounts and also reported the aldose reductase inhibitory activity of isolated compounds in the decreasing order: cyanidin-3-glucoside > quercetin > ferulic acid > peonidin-3-glucoside > tocopherol. Two main anthocynains, i.e. Cy 3-rut (rutinoside) and Cy 3-glu were identified in mulberry (*M. rubra*) and four main anthocyanins, Pt (petunidin) 3-rut, Dp 3-glu, Dp 3-rut and Mv 3-rut were identified in *Liriope platyphylla* fruits (Wang et al. 2014). Six main anthocyanins, including Pn-3,5-glc, Pn-3-glc-5-ara, Pn-3-glc, Pg-3,5-glc, Cy-35-glc and Cy-3-glc were detected among nine wild herbaceous peony (Jia et al. 2008). Dp-3-gal, Dp-3-glc, Cy-3-gal, Cy-3-glc, Pt-3-glc, Pl-3-glc, Cy, catechin-cy-3-glucoside and Pn-3-glc were identified in black soybean (Lee et al. 2009). The studies of many researchers commonly showed cyanidin and delphidin are obtained as major groups of anthocyanin, which was the case in the present study also. The most abundant anthocyanin in coloured cereal grains are reported to be Cy 3-glu, Pg-3-gal, Pn-3-glu (Naczk & Shahidi, 2006; Prior & Wu, 2006). Cy-3-glc is the most predominant anthocyanin on purple corncob, purple wheat, black soybean and black rice. In mulberry and *Liriope platyphylla* fruit Pt-3-rut is the most predominant anthocyanin followed by Cy-3-glc (Wang, 2014). However, in *Chakhao Poireiton* and *Chakhao Amubi*, Dl-3-gal is the most predominant anthocyanin. As reported by Park et al.

(2008) reported delphinidin could inhibit cell invasion of human fibrosarcoma HT-1080 cell *in vitro*.

Jing et al. (2007) reported anthocyanins are relatively unstable and often undergo degradation during processing and storage and this was again proved by the work of Wu et al. (2013) during the black rice tea production, they assessed the changes in nutritional composition, anthocyanins and volatile compounds in 3 successive processes (soaking, steaming and roasting) and they found out that the cyanidin-3-glucoside and peonidin- 3-glucoside total content were decreased. They also reported that only steaming and roasting caused significant decreased in the contents of total starch and crude, however a stable level of total starch during all the processes was observed. Many other factors such as genetics, light, temperature, and agronomic conditions also affect the total anthocyanin content in plants (Majoul et al. 2003; Jing et al. 2007). The nutritional and functionality of crops is also adversely altered by environmental stresses such as drought, heat, salt and UV (Jing et al. 2007). Stress due to drought can cause rupturing of the cell, thereby damaging the cell membrane and this can cause lipid peroxidation as well as osmolyte leakage (Hoisseinian et al. 2008). Heat and UV exposure may be associated with free radical formation and singlet oxygen production, the elevated levels of free radicals and singlet oxygen may increase the oxidative stress in the cereal crop such as wheat and as a result, specific activities of antioxidant enzymes can increase to protect the plant against environmental stress (Keles & O¨ ncel, 2004, Hoisseinian et al. 2008).

The results suggest that black scented rice (*Chakhao Poireiton and Chakhao Amubi*) contains high amounts of anthocyanins, which have strong antioxidant activities *in vitro* system. ROSs plays a crucial role in a wide range of common diseases and age-related degenerative conditions including cardiovascular disease,

inflammatory conditions and neurodegenerative diseases such as Alzheimer's disease and cancer (Han et al. 2004). So, antioxidant capacity is widely used as a parameter for characterizing the food or medicinal plants and their bioactive components and in the present study also reported the antioxidant activities of the anthocyanin extracts of the two black scented rice were evaluated and they showed very strong antioxidant activity. A high level of anthocyanins and a high percentage of anthocyanins over the total phenolics present would be desirable for the rice colorant processing and application.

5.3 Total monomeric anthocyanin content in *Chakhao Poireiton* and *Chakhao Amubi*

The pH differential method is a rapid and easy procedure for the quantification of monomeric anthocyanin (Giusti & Wrolstad, 2001). Anthocyanin pigments undergo reversible structural transformations with a change in pH manifested by strikingly different absorbance spectra. The colored oxonium form predominates at pH 1.0 and the hemiketal (colourless) form at pH 4.5. The pH differential method based on this reaction permits accurate and rapid measurements of the total amount of anthocyanin, even in the presence of polymerized degraded pigments and other interfering compounds (Giusti & Wrolstad, 2001). The total monomeric anthocyanins, present in the two black scented rice cultivars (*Chakhao Poireiton* and *Chakhao Amubi*) were measured. The acidified methanol extract was used for the measurement of the total anthocyanin in both the cultivars. The levels of monomeric anthocyanins were found to be 740 mg/kg in *Chakhao Poireiton* and 692 mg/kg in *Chakhao Amubi*. The results were consistent with the results reported for purple wheat (526.0 mg/kg and 500.6 mg/kg) (Hosseinian et al. 2008) and 1214.85 mg /kg of black rice (Park et al. 2008). Hosseinian et al. (2008) studied the total anthocyanin content of heat

stressed purple wheat and normal purple wheat. The total anthocyanin content of heat stressed purple wheat was 526.0 mg/kg, which was significantly higher than that of normal purple wheat, 500.6 mg/kg. According to Tananuwong and Tewaruth, 2010, the total monomeric anthocyanin content ranged from 275- 352 µg/g of the black rice flour. The monomeric anthocyanins content of purple corncob ranged from 290 to 1333 mg cyanidin 3-glucoside equivalents/100 g of dry matter (Jing et al. 2007). Recently, Moko et al. (2014) reported that the colored varieties from Minahasa, North Sulawasi, Indonesia had more higher anthocyanin content than the non colored varieties and the highest anthocyanin content dissolved in semi polar solvent, ethyl acetate. They measured the total anthocyanin content of colored varieties, red variety was 68.61 mg/g and non colored varieties Cigeulis and Superwin, were 54.45 mg/g and 43.30 mg/g, respectively. In the study of Sompong et al. (2011) on the anthocyanin of colored rice in Thailand, China and Srilanka reported that all rice varieties with black colored pigments had the highest amount of total anthocyanin compared to red pigment varieties. In their study, black pigmented varieties have 109.52- 256.61 mg/100 g anthocyanin, while the total anthocyanin contents of red varieties vary between 0.33-1.38 mg/100 g. Yodmanee et al. (2011) studied 8 different pigmented varieties in Thailand and reported that rice varieties with dark purple color contained a higher amount of anthocyanin ranging between 208.42-329.24 mg/100 g, compared to the red pigmented varieties ranging between 58.89-84.43 mg/100 g. A previous study on three rice varieties in Thailand by Sutharut and Sudarat (2012), also reported that the non pigmented variety contained anthocyanin at a range between 1.09-10.83 mg/100 g and a range of 17.89-99.53 mg/100 g was reported for the two colored varieties.

5.4 Total phenol content in *Chakhao Poireiton* and *Chakhao Amubi*

The total phenol content was determined according to the Follin-Ciocalteu method with acidified methanol extract and the results were expressed as gallic acid equivalents. The total phenol content in *Chakhao Poireiton* was 577 and in *Chakhao Amubi* was 500 mg/100g of the powdered sample as gallic acid equivalents. The total phenol content of the purple corncob ranged from 950 to 3516 mg/100 g of dry matter as gallic acid equivalents (Jing et al. 2007). According to the study reported by Tananuwong and Tewaruth, (2010) the total phenol content was 1815- 1992 µg gallic acid/ g of the black rice flour. Moko et al. (2014) reported the highest of total phenol content was Cigeulis 261.96 ± 6.52 mg/g gallic acid, red variety 258.23 ± 2.83 mg/g gallic acid and the lowest was Superwin variety 239.04 ± 8.16 mg/g gallic acid. Muntana and Prasong, (2010) observed the total penol content of white, red and black rice bran extract were in the range 0.8931-0.9884, 1.0103-1.0484 and 1.0810-1.2239 mg gallic acid equivalent, respectively. A similar study on antioxidant activity of colored and non colored Thai rice cultivars with various solvents reported that the total phenol content of methanol extract had the higher value than distilled water, hexane and ethyl acetate extract (Chakuton et al. 2012). A previous study was reported by Lai et al. (2009) and they noted that the ethyl acetate extract of rice bran had a highest total phenol content (19.7 ± 0.8 g GAE/kg) than the methanol and hexane extracts, 15.7 ± 0.6 g GAE/kg and 14.7 ± 1.2 g GAE/kg, respectively. In a previous study of Arab et al. 2011, the antioxidant activity of Iranian rice bran varieties, extracted with three different solvents (methanol, ethanol and ethyl acetate) observed that the methanol extract of Fajr variety had a higher total phenolic content (3.31 ± 0.03 mg GAE/g) than those of ethanol and ethyl acetate extracts, 1.67 ± 0.01 mg GAE/g and 1.29 ± 0.03 mg GAE/g, respectively. While a study by Lum and

Chong (2012), reported the antioxidant properties of pigmented rice from Sabah, Malaysia and they observed that red rice variety contained the highest quantity of phenolic acids (329.93 ± 19.17 mg/100 g) than the black rice (290.77 ± 13.72 mg/100 g), brown rice (69.63 ± 5.58 mg/100 g) and the white rice variety (22.59 ± 1.31 mg/100 g). Similar result was obtained by Saenkod et al. (2013) the total phenolics content in the Chinese black rice (Brown Himi variety) was 634.83 mg/Kg. Lum and Chong (2012) reported the quantity of total phenol content in Malaysia rice ranged from 22.59 to 329.53 mg/kg. Parr and Bolwell, (2000) reported that the phenolic compounds are secondary metabolites of plants, with different activities such as protection against pathogens and predators, mechanical support, attraction of pollinating animals and protection against ultraviolet radiation. The phenolic compounds contain a chemically heterogeneous group, having a phenol group (a functional hydroxyl group in an aromatic ring) in its basic structure and they differ structurally from simple molecules, such as phenolic acids, to highly polymerized compounds, such as tannins, comprising different classes (Walter and Marchesan, 2011). However, the phenolic acids, flavonoids and tannins are the main phenolics in the diet (King and Young, 1999).

The concentration of total phenol in the rice grains has been positively correlated with the antioxidant activity (Itani et al. 2002; Goffman and Bergman, 2004; Zhang et al. 2006). In red pericarp grains, a high correlation was observed between this activity and the content of proanthocyanidins, but in the case of black pericarp grains the correlation depended on the content of anthocyanins (Oki et al. 2002). The results suggest that the phenolic compounds were among the main responsible ones for the antioxidant activity of rice grains (Goffman and Bergman, 2004). The presence of phenolics show antioxidant activity as phenolic compounds

are a class of antioxidant agents which act as free radical terminators and their bioactivities may be related to their abilities to chelate metals, inhibit lipoxygenase and scavenge free radicals (Roya et al. 2013).

5.5 The antioxidant activity of the methanol extracts of *Chakhao Poireiton* and *Chakhao Amubi*

The antioxidant activity of the black scented rice has been determined using the DPPH assay and showed very strong antioxidant activity. The maximum DPPH free radical scavenging activity of *Chakhao Poireiton* and *Chakhao Amubi* were 70.28% and 60.84%, respectively. Although, scavenging activity is a little lower than the standard ascorbic acid, the anthocyanin extract showed strong antioxidant activity. The result has been consistent with work on Korean black rice (*Heugjunjubyeo),* where the DPPH radical scavenging capacity of anthocyanin extract exhibited 17.65%, 40.39% and 55.20% scavenging activity at 10 μg/mL, 50 μg/mL and 100 μg/mL, respectively (Park et al. 2008). They reported that the DPPH free radical scavenging activities of anthocyanin extract, BHA, BHT, and α-tocopherol decreased in the order: BHA (73.80%), BHT (62.43%), α-tocopherol (58.80%) and the anthocyanin extract (55.20%) at 100 μg/ mL concentration and total antioxidant activities of the anthocyanin extract, BHA, BHT, α-tocopehrol showed more than 90% inhibition on linoleic acid emulsion peroxidation. In their study on superoxide anion free radical scavenging activities of the anthocyanin extract, BHA, BHT, α-tocopherol, the anthocyanin extract had higher scavenging activity than α-tocopherol (32.68%) and lower scavenging activity than BHA (69.67%), and BHT (64.61%) at 100 μg/mL concentration. They also reported that the anthocyanin extract showed higher reducing power than α-tocopherol and lower reducing power than BHT and BHA and the hydrogen peroxide scavenging activities of the anthocyanin extract from

the Korean black rice (*Heugjunjubyeo)* which was measured at 230 nm had higher hydrogen peroxide scavenging activity than BHA (52.27%), BHT (64.79%), and α-tocopherol (56.99%). Their results suggested that anthocyanin extract from black rice can be used as antioxidant material, food addictives. Their results suggested that the use of anthocyanin extract from black rice may offer an attractive new antioxidant agent against ROS.

In a very recent study by Moko et al. (2014) the antioxidant activity was determined with DPPH free radical scavenging for each fraction of the crude extract of rice bran and the highest percentage of radical scavenging inhibition were of non colored and colored varieties, respectively, Cigeulis 73.81 ± 2.32% (non polar fraction), Superwin 76.10 ± 1.20% (semi polar fraction) and red variety 88.29 ± 5.62% (polar fraction) which showed that the highest radical scavenging activity was obtained by extraction with polar solvents, n-butanol. A similar study was performed by Arab et al. (2011) and Chakuton et al. (2012) that the DPPH scavenging activity of colored rice extract was better than that of non colored extracts, while the methanol extracts of Fajr rice bran had a higher inhibition (93.91%) than ethanol and ethyl acetate extracts.

In a previous study by Lum and Chong (2012), reported that the antioxidant properties of pigmented rice from Sabah, Malaysia and showed that the red rice variety had the highest DPPH radical scavenging activity (65.54 ± 0.57%) than the black and brown rice varieties, 37.66 ± 3.85% and 13.74 ± 11.77%, respectively and the scavenging activity of white variety cannot be determined which could be due to the low content of phytochemical compounds. Lai et al. (2009) reported the methanol extract of Japonica rice bran had a 93% inhibition of DPPH radical scavenging which showed a higher scavenging activity than the ethyl acetate and hexane extracts, this

may be due to the possibility of more polar phenolic compounds and lipids eluted in the methanol extract than in the ethyl acetate extract. Zubair et al. (2012) observed that the DPPH radical scavenging activity of the 80% isopropanol extract had a higher activity than the 100% methanol and the 100% ethanol extract. Muntana and Prasong, (2010) performed the DPPH assay on the methanol extract of rice and showed that the colored rice have more antioxidant activity than the white rice. The study of Saenkod et al. (2013) also showed the Chinese black rice (Brown Himi variety) has strong antioxidant activity which 70.82 % DPPH scavenging activity.

Antioxidant capacity is becoming a parameter to characterize food or medicinal plants and their bioactive components. Higher percentage of DPPH scavenging shows higher antioxidant capacity (Sultana et al. 2009). In the human body, dietary antioxidants protect against reactive oxygen species (Saenkod et al. 2013). Thus, if the anti-oxidants intake is increased, there may have a number of health effects, such as reducing the incidence of cancer and cardio-vascular diseases (Diplock et al. 1998). It has been also reported that anthocyanins may reduce the risks of cardiovascular diseases and cancer with anti-inflammatory, antioxidant and chemoprotective properties (Park et al. 2008). In mice, the supplementation of black rice pigment in the diet reduced oxidative stress (Xia et al. 2003) and its pigment fraction may have antiatherogenic activity (Xia et al. 2006). The black rice is a good source of fiber, minerals and several important amino acids (Zhang et al. 2005) and there is an increased interest in the alternative sources of anthocyanins due to a rising demand for economical sources of natural and stable pigments (Hu et al. 2003). Many new bio-active compounds have been detected in many source of food with possible antioxidant activity and the increased interest in the relationship between antioxidants and disease risk mechanisms, there is an urgent need to establish the antioxidant

capacity in different foods, especially the rice crop which constitutes the main food for populations in different countries (Saenkod et al. 2013).

The result of this study indicated that the antioxidant properties of black scented rice from Manipur, India were broadly comparable with previous studies. Studies on antioxidant properties of colored rice and non colored rice showed a significant positive correlation between pigmented varieties and their antioxidant activity, antioxidant properties of colored rice bran were better than that of non colored rice bran (Moko et al. 2014). The antioxidant properties of colored rice bran varieties are because of their pigment compounds of anthocyanin. Pigmented rice variety had a better scavenging activity than non pigmented rice variety because pigmented variety had a higher anthocyanin content which is potent reducing agents and possesses strong radical scavenging activity (Nam et al. 2006). There are many studies which reported that the colored rice variety contains rich anthocyanin and other polyphenolic compounds much more abundantly than non colored rice variety (Moko et al. 2014).

Anthocyanins have shown to be potent antioxidants which are superior to well-known antioxidants such as butylated hydroxyanisole (BHA), alpha-tocopherol, 6-hydroxy-2,5,7,8-tetramethychromane-2-carboxylic acid (Trolox), catechin and quercetin (Kahkone & Heinonen, 2003). There are numerous methods to evaluate antioxidant properties which include radical scavenging capacity such as free radical, radical cations and radical ions and the inhibition of lipid oxidation such as human low density lipoprotein (LDL) cholesterol, methyl linoleate (MeLo) and oils (Shipp). One of the most common and frequently employed method to check the ability of anthocyanin compounds is the ability to act as free radical scavengers against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. In the presence of an antioxidant, the

colored stable DPPH free radical is reduced into a nonradical DPPH-H resulting in a change of the violet color to pale yellow and the discoloration is proportional to the number of electrons taken up by DPPH-H once the odd electron becomes paired off by a free radical scavenger, resulting in a decrease of absorption (Shipp & Abdel-Aal, 2010). During an electron transfer reaction, the change occurs in which the substrate added acts as a hydrogen atom donor and the percentage of radicals scavenged over the period of reaction time determines the degree of radical scavenging capacity. Shipp & Abdel-Aal, 2010, reported that the anthocyanidins/ anthocyanins with the highest antioxidant activity are delphinidin, cyanidin and cyanidin-3-glucoside which are also significantly higher than the standards alpha-tocopherol, BHA, butylated hydroxytoluene (BHT) and ascorbic acid. They also reported that the compounds with the lowest antioxidant activity are malvidin 3,5-diglucoside and pelargonidin 3,5-diglucoside and monoglucosides generally have a higher scavenging capacity than the diglucosides. The results of the present study are consistent to the findings obtained by Kahkonen and Heinonen (2003) and Astadi et al.(2009) who also showed that anthocyanins possessed higher radical scavenging activity compared to the reference antioxidants (ascorbic acid, alpha-tocopherol, Trolox, BHT and rutin) in the DPPH test.). In their study, the monoglucosides were comparable to their corresponding anthocyanidin but peonidin-3-glucoside and pelargonidin-3-glucoside were lower than their corresponding anthocyanidin. They observed that cyanidin, peonidin and malvidin galactosides were weaker scavengers than their corresponding glucosides by 15-23% and they also found that cyanidin and delphindin rutinosides were also less active than their glucosides. The radical scavenging capacity has been significantly improved by the acylation of sugar residues with aromatic hydroxyl acids (Fukumoto & Mazza 2000; Seeram et al. 2002). But the findings of Abdel-Aal et al. (2008) were

in contrast to the previous two studies, they found cyanidin-containing anthocyanins to have a higher DPPH scavenging capacity than the delphinidin-based anthocyanins. Abdel-Aal et al. (2008) and Astadi et al. (2009) found that the higher the concentration of anthocyanins, the higher reducing power which in turn showed the DPPH scavenging capacity of the aqueous plant extracts to be dose-dependent. Therefore, overall, all of the studies have consistently shown that anthocyanin compounds tested had a significantly high DPPH scavenging capacity comparable to the reference antioxidants.

Determination of the antioxidant activity of the extracts from different rice varieties were done using total phenolic compounds, total flavonoids component and antioxidant activity (DPPH scavenging, reducing power and inhibition of lipid peroxidation). Due to their different actions and the complexity of natural antioxidants from plant materials, an integration of different methods is necessary. Therefore, Saenkod et al. (2013), reported the correlation coefficient between total phenolic content or total flavanoid content and antioxidant activities (DPPH scavenging, reducing power and inhibition of lipid peroxidation in each rice variety extract was determined and they showed that the antioxidative activities (DPPH scavenging activity, reducing power, and inhibit on lipid peroxidation) as a function of total phenolic compounds and total flavoniod contents has a positive correlation coefficient. Similar results were also observed by Butsat and Siriamornpun (2010) that the antioxidant activity of different Thai Rice varieties was positively correlated with total phenolic contents.

There are many reports that various new bio-active compounds in food with possible antioxidant activity were detected with the increased interest in the relationship between antioxidants and disease risks and mechanisms, thus, there is an

urgent need to establish the antioxidant capacity in different foods, especially the rice crop which constitute the main food for populations in different countries.

5.6 Volatile oils profiling of *Chakhao Poireiton* and *Chakhao Amubi*

A complex mixture of volatile compounds comprised the aroma of black scented rice. GC-MS analysis of the *Chakhao Poireiton* identified 26 volatile compounds (Table 7: Fig. 12). They are in the decreasing order of n-Hexadecanoic acid (22.92%), Octadec-9-enoic acid (11.66%), 4-Beta-H-Pregna (8.78%), 9, 12-Octadecad ienoic acid (Z.Z) (8.11%), Benzene methyl (5.61%), 2-Furancarboxaldehyde 5- methyl (3.92%), g-Hexadecenoic acid Octadecyl ester. (Z)- (3.88%), 9-Hexadecenoic acid, eicosyl ester, (Z)- (3.75%), 2-Furancarboxaldehyde (3.41%), Pentadecane (2.90%), I 7-Pentatriacontene z (2.59%), Stigmasts 5-EN-3-OL. (3.Beta.24S)- (2.00%), Tetradecanoic acid (1.91%), Stigmast-5-EN.3-OL. Oleat (1.68 %), 9-Octadecenoic acrd (Z), 9-octadecenyl ester, (Z)- (1.67%), Dodecane (1.59%), Furan, 2,2'-methylenebis[5-methyl (1.59%), 9-Hexadecenoic acid. 9-octadecenyl ester. (Z.Z)- (1.58%), Furan 2-(2-furanylmethyl)-5-methyl (1.51%), Benzene butyl- (1.44%), Benzofuran 4, 7-dimethyl (1.20%), Benzofuran. 2-methvl-3(0.78%), Undecane(0.71%), Butanenitrile. 3-methyl (0.25%).

Similarly, the GC-MS analysis of the *Chakhao Amubi* identified 11 volatile compounds (Table 8: Fig.13). The decreasing order of volatile compounds comprising the aroma of *Chakhao Amubi* analysed by GC-MS are as follow:- 17-Pentatriacontene (40.60%), l3-Octadecenal, (Z)- (12.03%), 9-Hexadecenoic acid. eicosvl ester- (Z) (11.98%), Tetracosamethvl-cvclododecasiloxane (10.27%), Z-9-Pentadecenol (9.79%), 9-Octadecenoic acid (Z)- tetradecvl ester (0. 4.29%), Toluene (3.38%), l-(+)-Ascorbic acid 2,6-dihexadecanoate (2.21%), Z.Z-3 -13 -O ctadecedien- I -o I

(1.96%), 9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E) (1.93%), Cyclononasiloxane octadecamethvl- (1.56%).

All these compounds together contributed to the unique aroma of their respective cultivars and their aroma increase during cooking, this may due the interaction of the compounds with the protein and lipids.

In black rice, 2-acetyl-1-pyrroline and guaiacol confer a unique aroma affecting the overall flavor (Yang et al. 2008a). Black Rice Flavor Chemistry was also studied by Yang et al. 2008a. They reported thirty-five volatile compounds emanating from cooked black rice, collected using a dynamic headspace system with a Tenax trap, were identified and quantified by GC-MS. 10 aromatic, 4 nitrogen-containing, 6 alcohol, 10 aldehyde, 3 ketone and 2 terpenoid compounds were characterized and the relative proportion of the main classes of volatiles in 100% black rice was significantly different from that in 100% white rice. The highest percentage of the total volatiles emanating from black rice was represented quantitatively by aldehydes (51.7%) and aromatics (28.4%) and the aldehydes, identified in decreasing order of their relative proportion, were hexanal, nonanal, octanal, heptanal, (*E*)-2-octenal, decanal, (*E*)-2-nonenal,(*E*,*E*)-2,4-decadienal, (*E*)-2-hexenal and (*E*)-2-decenaland all the aldehydes were considered odor-active compounds in cooked black rice. Yang et al. (2008a) also reported that the aldehydes, products derived predominantly via lipid oxidation, had low odor thresholds and were considered to be important contributors to the overall aroma of black rice and aromatics in black rice comprised of two phenols, one furan, five benzenes and two naphthalene derivatives. 2-pentylfuran and 4-vinylguaiacol were reported as odor-active compounds in California long-grain rice and brown rice cultivars, respectively (Jezussek et al. 2002).

2-AP which confers a popcorn-like odor has a very low odor threshold in water and often reported as a key aroma compound in many food products (e.g., aromatic rice, black rice, bread, green tea, and popcorn (Yang et al. 2008a). 2-Acetyl-1-pyrroline, guaiacol, 1-nonanol, 3-octen-2-one, 1,2-dimethoxybenzene, pyridine and pyrrole were found in Heugjinjubyeo (black rice) which confer the aroma to the flavor and in Jeogjinjubyeo (red rice) only phenylacetaldehyde was found (Yang et al. 2008c).

Yang et al. (2008b) reported the flavour active compounds of five aromatic rice cultivars viz. Royal (basmati, a long-grained Indian indica), Golden Elephant (jasmine, a long-grained Thai indica), Hyangmibyeo 1 (a medium-grained Korean japonica), Hyangmibyeo 2 (a medium-grained Korean japonica), Goemjeongssal (a medium-grained japonica black rice from Korea) and one nonaromatic rice cultivar, Jeongilpum (a traditional medium-grained, nonaromatic japonica from Korea) and detected 30, 24, 17, 21, 27, and 24 odorants, respectively. They reported thirteen odor-active compounds 2-AP, hexanal, (*E*)-2-nonenal, octanal, heptanal, nonanal, 1-octen-3-ol, (*E*)-2-octenal, (*E*,*E*)-2,4-nonadienal, 2-heptanone, (*E*,*E*)-2,4-decadienal, decanal and guaiacol were the primary compounds explaining the differences in aroma among the six flavor types. Yang et al. (2010) studied ten cultivars including six premium-quality, *Oryza sativa* L. *japonica* cv. Hwaseongbyeo, Ilpumbyeo, Gopumbyeo, Taebongbyeo, Chucheongbyeo, and Samkwangbyeo, two waxy cultivars, *O. sativa* L. *japonica* cv. Hwaseonchalbyeo and *O. sativa* L. *indica* cv. Hangangchalbyeo and two black-pigmented, *O. sativa* L. *japonica* cv. Heugjinjubyeo and Heugkwangbyeo for the odour active coumpounds. They identified 21 odorants, among the six premium-quality cultivars that included 12 aldehydes, two alcohols, three ketones and four aromatic compounds. Based on the OAV, they detected

hexanal was the dominant odorant, followed by (*E*)-2-nonenal, octanal, heptanal, (*E,E*)-2,4-nonadienal and nonanal in all six cultivars and all these compounds collectively accounted for over 95% of the total odor activity value and were key aroma compounds in premium rice. However, their results showed that, the aroma of the premium cultivars was less than the waxy and black-pigmented rice cultivars. They identified twenty-one odorants in the waxy rice cultivar Hwaseonchalbyeo and 20 in Hangangchalbyeo that included 12 aldehydes, one alcohol, three ketones, and four aromatic compounds. They also reported that 1-Pentanol was present only in Hwaseonchalbyeo, Hexanal in Hwaseonchalbyeo and Hangangchalbyeo had the highest odor activity value. The very high hexanal concentration is because of the higher lipid content in waxy rice and its subsequent degradation resulting in the formation of hexanal, it is a product of linoleic acid oxidation which has a higher concentration in broken rice than in head rice due to greater surface lipids and free fatty acids (Yang et al. 2010).

While 2AP is associated with pleasant, popcorn-like aroma of fragrant rice, hexanal has been correlated with off-odors that developed from lipidoxidation (Bergman et al. 2000). In the Basmati and Jasmine fragrant rice, significant differences exist in concentrations of various flavor/off-flavor compounds such as methyl salicylate, deca-2,4-dienal, hexanal, hept-2-enal; 2-butenal and 2-pentylfuran which may contribute to their respective flavors (Kirstin and Wootton, 2004). Champagne (2008) reported that each variety has a unique fragrance resulting from a number of volatile compounds which may vary from well characterized popcorn-like aroma/2AP associated aroma although little is known about their relationships with aroma/flavor.

2-Acetyl-1-pyrroline has been found as one of the odor active compounds which give aroma to the black rice, however, in the present study of *Chakhao Poireiton* and *Chakhao Amubi*, no 2-AP has been detected. Similarly in the study of Wu et al. (2013) 2-AP was not detected in the black rice, they identified 94 volatile compounds of which nonanal, butylated hydroxytoluene, 1-hexanol, naphthalene and 1-octen-3-ol were the main volatile compounds. Several other compounds other than 2-AP were also reported to be associated with unique flavor and strength of aroma of diverse fragrant rice, alkanals, alk-2-enals, alka-2,4-dienals, 2-pentylfuran and 2-phenylethanol (Widjaja et al. 1996), 2-aminoacetophenone and 3-hydroxy-4,5-dimethyl-2(5H)-furanone (Jezussek et al.2002), 2AP, guaiacol, indole, and p-xylene (Yang et al. 2008a).

Previouly, the concentration of 2-AP has been used as an indicator of aroma in the selection of aromatic lines due to its significant importance in aromatic rice, several techniques have been developed for the identification and quantification of 2-AP. However, aromatic rice cultivars with distinctively different flavors cannot be adequately characterized just by 2-AP concentration (poporn like odor) because other distinct odors (e.g., earthy, nutty, roasty, and green) are present, indicating that the overall aroma is made up of a cross section of compounds. The formation of volatiles during cooking is relatively complex, with qualitative and quantitative differences occurring due to differences in chemical composition among cultivars and with location in the grain. In particular, dissociation of the starch lipid complex during cooking appears to enhance the formation of a cross-section of lipid-derived volatiles even though removal of the bran significantly decreases the amount of the total lipids. Therefore, characteristic compounds in rice bran and chemical components such as

lipids and proteins associated with starch in the endosperm contribute to the overall aroma.

5.7 The *Pb* gene of *Chakhao Poireiton*

Extensive researches on anthocyanin biosynthesis in other plants were reported in the past years, little is known for the molecular genetic mechanism of pigmentation in grain pericarp of purple rice. In the present study, cloning and sequencing of the *Pb* gene using the gene specific primer *Pb* forward primer 5' GGGAGAAGCTCAACGAGATG 3' and *Pb* reverse primer 5' GGGTGGCAGATTCATCACTT 3' obtained 1122 bp fragment (Genebank accession numbers KP830117). Sequence analysis (www.ncbi.nlm.nih.gov) of the 1122 bp of the *Pb* gene fragment was found to be identical with four candidate genes encoding respective anthocyanin regulatory *Lc* protein (*Ra*) gene, anthocyanin regulatory *Lc* protein (*Ra*) gene mRNA for R-type basic helix-loop-helix protein and transcriptional anctivator (*Ra*) mRNA.

The BLAST/BLAT type BLASTN (www.gramene.org) against *Oryza sativa Indica* ASM465v1 (Genomic sequence) mapped the 1122 bp *Pb* gene fragment on chromosome 4 (Genomic location Chromosome 4: 26781084 to 26782210 (+)), E-value 1.5E- 243 and 98 % identity (Table 9: Fig. 19 & 20) and found to be overlapping with gene BGIOSGA014565 (1662 bp, 553 aa) (Fig. 21). This gene has protein that correspond to the Protein domain transcriptional activators Myc/Myb N-terminal and Myc typr Basic-Helix-Loop- Helix Domains (Fig. 21). And it was found to present on the locus LOC_Os08g32870.1 against the Rice Genome Annotation Project Rice Genome Browser Release 7: Chr4:27949384 -27955640 (http://www.tigr.org). The *Pb* gene fragment obtained from the *Chakhao Poireiton* showed GT deletion on the exon 7 (Fig. 17). The same result was also shown by

Wang and Shu, (2007) on purple rice *Oryza sativa* L. *indica* (Yunanheixiannuo) and *Oryza sativa* L. *japonica* purple rice (Chuanheinuo) and by Rahman et al. (2013) also on black rice, *Oryza sativa* L. *japonica* var. Kewha and *O. sativa* L. *japonica* var. Heugnambyeo when compared with the white rice. They observed that the DNA sequence analysis of the PCR fragments of all the purple/black rice showed the 2 bp (GT) deletion but that those of white rice showed the 2bp (GT) insertion, confirming that the rice *Ra* and *Pb* genes are the same gene. According to the TIGR Rice Genome, R.5, there are two annotated genes within about 25 kb region. Both are Myc transcription factor genes, one is the *bhlh16* gene (LOC_Os04g47059, 27696505-27720731) and the other is the *Ra* gene (LOC_Os04g47080, 27730292- 27736548). The *bhlh16* gene putatively encodes the protein homologue of *TRANSPARENT TESTA 8* (*TT8*), which regulates biosynthesis of flavonoid in the pericarp of *Arabidopsis* (Nesi et al. 2000). The *Ra* gene encodes a protein homologue of *Lc*, which is involved in the biosynthesis of anthocyanin in maize (Ludwig et al. 1989). Wang and Shu (2007) delimited the *Pb* gene within a 25 kb region (TIGR, pseudomolecule v4: chr4: 27712299—27737750) by the upstream marker RID3 and the downstream marker RID4, where only two genes were annotated; they further reported that the *bhlh16* gene is involved in proanthocyanidin synthesis while the *Ra* gene in anthocynanin synthesis, so they found out that the *Ra* gene is more likely to be the same gene as the *Ra1* gene (a homologue of the maize *Lc* gene) (Hu et al. 2000) and the *OSB1* gene which is located in the *Plw* locus (Sakamoto et al. 2001). They sequenced the *Ra* gene of Yunanheixiannuo and Chuanheinuo (GenBank accession number: Yunanheixiannuo, EU095985; Chuanheinuo, EU095986) and compared with those of white rice lines, Pei'ai 64S, 9311 and Nipponbare and obtained the 2 bp (GT) insert/deletion within the exon 7 coincided the white/purple

difference. The GT was absent in both purple rice lines (Yunanheixiannuo and Chuanheinuo) but present in all three white rice lines (Pei'ai 64S, 9311 and Nipponbare) which is similar to that of the present study *Chakhao Poireiton*, *Pb* gene fragment.

With the completion of genome sequencing of the *indic*a rice 9311 and the *japonica* rice Nipponbare has made easier to develop a great number of SNP markers through sequence comparison and used for mapping and eventually cloning of the genes (Shen et al. 2004; Ji et al. 2005; Chen et al. 2006). Zhuang et al. (1996) mapped the *Pb* gene controlling pericarp pigmentation in the purple rice, Heizhenmi to a position at a distance of 18.9 cM to the RFLP marker RG329 where RG329 is now known to be at the physical position of 30819286-30819457 bp on chromosome 4. Extensive studies on anthocyanin metabolism have been performed on maize, pentunia and snapdragon. Three types of regulatory gene, *Myb*, *Myc* and *WD40* are involved in regulation of anthocyanin biosynthesis in plants. The Myc proteins contain a basic helix-loophelix (bHLH) domain, which interacts with Myb protein in anthocynanin biosynthesis (Chandler, 1989; Ludwig and Wessler, 1990). The present findings also showed that *Pb* gene fragment of *Chakhao Poireiton* has similarity with that of anthocyanin regulatory Lc protein (*Ra*) gene, anthocyanin regulatory Lc protein (*Ra*) gene mRNA for R-type basic helix-loop-helix protein and transcriptional activator *(Ra*) mRNA. Thus, the present study showed that the *Pb* gene sequence characterized is involved in regulation of anthocyanin biosynthesis in the black scented rice causing the purple pigmentation. The completion of genome sequence makes the study of gene and homologues gene easier, hence, once a gene is cloned in one plant species, using the comparative genomics tools or other techniques can identify their homologue genes in other plant species.

Hu et al. (1996; 2000) identified three rice genes, *Ra1*, *Ra2* and *Rb* in rice which are homologous to the *Lc* gene in maize and involved in rice anthocynanin biosynthesis using maize *Lc* cDNA. In the present study, *Pb* gene fragment overlapped with gene BGIOSGA014565 (Chromosome 4: 26,781,48026,786,213) which codes for the protein that correspond to the Protein domain transcriptional activators Myc/Myb N-terminal, and Myc typr Basic-Helix-Loop- Helix Domains and present on the locus LOC_Os08g32870.1 which has the anthocyanin regulatory Lc protein. The *Ra* gene which is homologue to the *Pb* gene is known to be involved in anthocynanin biosynthesis in maize and can activate the biosynthesis of anthocynanin in rice (Hu et al. 1996; 2000; Sakamoto et al. 2001). In *Arabidopsis,* the homologoue gene of *bhlh16, TT8,* is involved in the biosynthesis of proanthocyanidins through cell specific accumulation of flavonoids (Baudry et al. 2006). Sakamoto et al. (2001) also found two rice genes, *OSB1* and *OSB2* homologous to the maize *B-Peru* gene of which both the *Lc* and *B-Peru* are members of the *R*/*B* gene family and belong to Myc type of transcription factors in maize. Similarly, Saitoh et al. (2004) isolated the rice gene homologous to maize *C1* gene, *OsC1*, which belongs to Myb transcription factor. Wang and Shu, (2007) inclined to assume that the *Pb*, *Ra* and *OSB1* genes are likely to be indeed identical because one of the two candidate genes of the *Pb* gene (the *Ra* gene) is identical to one of the two genes at the Pl^{w} locus, the *OSB1* gene.

Hence, in the present study the *Ra/Pb* gene was sequenced and found a 2-bp (GT) difference between white rice and purple rice and the purple characteristic of rice pericarp may be resulted from a GT deletion within exon 7 of the *Ra/Pb* gene. However, the complete correlation of the 2 bp insertion/ deletion with the non-purple/purple pericarp color in present and other several studies reports strongly supported that the *Ra* gene is more likely than the *bhlh16* gene to be the gene

controlling purple pericarp characteristic in rice. Therefore, the present study is of importance to the understanding of anthocyanin biosynthesis in plants and especially of the molecular and genetic mechanism of pericarp pigmentation in rice.

5.8 The *fgr* gene of *Chakhao Poireiton*

Great contradictory genetics are reported with the genetic model for fragrance as monogenic to polygenic with a dominant or recessive character. However, the availability of high-density molecular marker maps and genome sequences in rice has allowed mapping, fine mapping and positional cloning of gene for fragrance. As that of the *Pb* gene, cloning and sequencing of the *fgr* gene using the gene specific primer (*fgr* forward primer 5' GCAAGTGACGGAGTACGCCT 3' *fgr* reverse primer 5' GCTAACTTCCGCTCACGCAA 3') was performed and isolated a 396 bp *fgr* gene fragment (Genebank accession no. KP830118). Sequence analysis (www.ncbi.nlm.nih.gov) of the 396 bp of *fgr* observed to be identical with two candidate genes encoding respective 3-methylcrotonyl-CoA carboxylase beta chain and betaine aldehyde dehydrogenase. Using the latest version (www.gramene.org) the BLAST/BLAT type BLASTN against *Oryza sativa Indica* ASM465v1 (Genomic sequence) obtained the 396 bases *fgr* gene fragment was mapped on chromosome 8 (Table 10: Fig. 22 & 23) (Genomic location 8 21706850 to 21707134 (+) forward strand), E-value 1.1E-57 and 100 % identity and found to be overlapping with gene BGIOSGA028697 (1512 bp, 503aa) (Fig. 24). This gene encodes the protein that corresponds to the following Uniprot identifiers: B3VMC0 - BADH2_ORYS (503aa), Protein Betaine aldehyde dehydrogenase 2. Dehydrogenase that can use N-acetyl-c-aminobutyraldehyde (NAGABald), gamma-guanidinobutyraldehyde (GGBald), betaine aldehyde (Bet-ald), gamma-aminobutyraldehyde (GAB-ald), acetaldehyde, 4-aminobutylaldehyde (AB-ald), 3-aminopropionaldehyde (AP-ald), 4-N-

trimethylaminobutyraldehyde (TMAB-ald) and 3-N-trimethylaminopropionaldehyde (TMAP-ald) as substrates. It catalyzes the oxidation of GAB-ald more efficiently than Bet-ald which mediates the conversion of GAB-ald into gamma-aminobutyric acid (GABA) and prevents the formation of 2-acetyl-1-pyrroline (2AP) which gives fragrant rice its aromatic properties. The *fgr* (396 bp) gene fragment from *Chakhao Poireiton* was found to be present on the locus LOC_Os08g32870.1 (http://www.tigr.org). Aromatic character of rice is considered as a special trait with huge economic importance that determines the premium price in global trade. The availability of molecular maps and genome sequences led to the identification of a major gene for fragrance (*badh2*) on chromosome 8 and an 8-bp deletion in the exon 7 of this gene resulted in truncation of betaine aldehyde dehydrogenease enzyme whose loss-of-function lead to the accumulation of a major aromatic compound, 2-acetyl 1-pyrroline (2AP) in fragrant rice (Sakthivel et al. 2009). There are several studies showing exception to this mutation. In the present study, the isolated *fgr* gene fragment from *Chakhao Poireiton*, the deletion has not been detected (Fig. 18). Thus, the present result also showed that there are exceptions to this mutation. Shi et al. (2008) also showed an exception to this mutation, they discovered a new null *badh2* alleles which differed from the known functional *badh2* allele (having an 8-bp deletion and three SNPs in exon 7) and this new allele has a 7-bp deletion (5'-CGGGCGC-3') in exon 2 and then, thereafter, the new null *badh2* allele is designated as *badh2-E2* and the known null *badh2* allele as *badh2-E7*. Shi et al. 2008 reported that the 7-bp deletion in exon 2 similar to the 8-bp deletion in the *badh2-E7* allele causes a reading frame shift, thereby rendering the *badh2-E2* allele nonfunctional. In their study, 24 fragrant rice varieties tested, 12 have the *badh2-E2* alleles, a 7-bp deletion in exon 2 and the others have the *badh2-E7* alleles, an 8-bp deletion in exon

7 was observed at the *badh2* allele. Srivong et al. (2006) reported the Thai aromatic rice cultivars, KDML105 and RD6 showed deletion of 8-bp and 3 SNPs and the mutation at exon 7 of aromatic rice led to the production of truncated BADH2 and loss of its activity. The *fgr* gene encodes *badh2* (betaine aldehyde deydrogenase homologue 2) and reported an eight base-pairs (8-bp) deletion in exon 7 as the cause of fragrance in many of the fragrant rice. As the loss-of-function of the enzyme leading to development of fragrance corresponded well with the recessive nature of the trait, they suggested that *badh2* was indeed *fgr* (Bradbury et al. 2005). Sakthivel et al. (2009) reported that the 8-bp deletion and 3 single nucleotide polymorphisms (SNPs) in exon 7 of *badh2* have led to the introduction of premature stop codon to produce a truncated protein resulting in the abrogation of the function of the enzyme *BADH2* which accumulate substrate 2AP in fragrant varieties, while the functional *Badh2* gene codes for a 503 amino acid mature protein which consumes the substrate in non-fragrant varieties. Lorieux et al. (1996); Bradbury et al. (2005) suggested that single recessive gene, *fgr* gene encodes *badh2* (betaine aldehyde deydrogenase homologue 2) and reported an eight base-pairs (8-bp) deletion in exon 7 as the cause of fragrance in many of the fragrant rice including Basmati and Jasmine rice. In addition to 8-bp deletion in exon 7, several variations including a 7-bp insertion in exon 8 (Amarawathi et al. 2008); a 7-bp deletion in exon 2 (Shi et al. 2008); absence of MITE (miniature interspersed transposable element) in promoter (Bourgis et al. 2008); two new SNPs in the central section of intron 8 (Sun et al. 2008); a TT deletion in intron 2 and a repeated (AT)n insert in intron 4 (Chen et al. 2008) of badh2 were reported in various fragrant varieties.

Chen et al. (2006) reported that a single recessive gene (*fgr*) on chromosome 8 is responsible for the production of 2AP, initially they placed the *fgr* locus between

RM8264 and RM3459 with the physical distance of 800 kb. Later, they restrictedly mapped the *fgr* locus to an interval of 69 kb flanked by the left marker L02 and the right marker L06. They also reported that the sequence analysis of the *fgr* region revealed three candidate genes encoding respective eukaryotic-type carbonic anhydrase, 3-methylcrotonyl-CoA carboxylase beta chain, and betaine aldehyde dehydrogenase which is found similar with the present result that the *fgr* gene fragment from *Chakhao Poireiton* showed 99% identical sequence with Badh2 (betaine aldehyde dehydrogenase) gene and methylcrotonyl-CoA carboxylase beta chain gene. Chen et al. (2008) further localized *fgr* to an interval between L04 and L06 and the sequence alignment between the *Fgr*-containing nonfragrant rice varieties indica cv 93-11 and japonica cv Nipponbare revealed considerable variation within this L04/L06 interval, which had a physical distance of 62 kb in 93-11. But in Nipponbare, a 30-kb DNA segment rich in transposition related genes was inserted inside an interval of L02/L03. Both 93-11 and Nipponbare had three putative genes *Cah*, *Mccc2*, and *Badh2*, encoding putative eukaryotic-type carbonic anhydrase,3-methylcrotonyl-CoA carboxylase b-chain and betaine aldehyde dehydrogenase, respectively in the L04/L06 interval were considered to be candidates for the *Fgr* gene in these non fragrant varieties. After screening the indica Nanjing11 and japonica Suyunuo BAC libraries, they obtained two and four positive BAC clones in the *Fgr* (Nanjing11; nonfragrant) and *fgr* (Suyunuo; fragrant) regions, respectively. They subcloned three *Fgr* candidates and three *fgr* candidates from these BAC clones (corresponding to *Cah*, *Mccc2*, and *Badh2* in each case) into the expression vector pCAMBIA1302 (pCAM). The inserts of these six *Fgr/ fgr* constructs were sequenced and compared with their counterparts in the indica cv 93-11 (http://rise.genomics.org.cn) and the japonica cv Nipponbare

(http://www.gramene.org). Sequence alterations specific in the fragrant variety Suyunuo, Chinese aromatic rice were observed only in the *badh2* gene, including an 8-bp deletion (5'-GATTATGG-3') and three single nucleotide polymorphisms in exon 7. They further sequenced another 23 fragrant rice varieties at their *badh2* loci. Sakthivel et al. (2009) reported that availability of high-density molecular marker maps and genome sequences in rice has allowed mapping, fine mapping and positional cloning of gene for fragrance, they also reported the studies of various workers which put up many systematic attempts to map fragrance trait at molecular level (Table 1). The single recessive gene (*fgr)* have been mapped on chromosome 8 (Ahn et al. 1992; Wanchana et al. 2005; Chen et al. 2006, 2008; Sun et al. 2008). Out of the twelve chromosomes, at least six (4, 5, 8, 9, 11 and 12) were implicated to harbor the gene which further complicated the genetic basis of fragrance in rice (Sun et al. 2008). Various workers have attempted to map fragrance trait at molecular level, Ahn et al. (1992) mapped the single recessive gene (*fgr*) on chromosome 8 based on its close linkage of a RFLP clone RG28. Many studies by varoius workers on fragrance (Wanchana et al. 2005; Chen et al. 2006; 2008; Sun et al. 2008) have supported the results of Ahn et al. (1992), despite of the contradictory genetics.

The present study on the *Chakhao Poireiton* is an exception to the 8-bp deletion reported as the genetic cause for aroma, here, the results showed that there is no deletion. Similarly, exceptions were observed on five indigenous aromatic rice genotypes viz., Tarunbhog, Ganjeikalli, Bishnubhog, Bansphool A and Adamchini did not carry this deletion (Sakthivel et al. 2006). Exceptions have also been reported by others in some fragrant varieties (Kuo et al. 2005; Navarro et al. 2007; Shi et al. 2008; Fitzgerald et al. 2008). Thus, the present study is also an indicative of the existence of allelic/genic diversity for fragrance in aromatic rice gene pool.

The results of the present study also help the complexities in understanding fragrance. Though it is reported that loss of single recessive gene function results in fragrance in most of the fragrant varieties, the emerging exceptions suggest that the 8-bp deletion in *badh2* is not the exclusive explanation for fragrance in all fragrant rice suggesting that certain varieties are still fragrant even without this mutation. Now it is clear that it is not always the case that the 8-bp deletion in the *badh2* gene could be considered the only factor responsible for the accumulation of 2AP leading to fragrance, as there are certain fragrant rice which are devoid of this deletion and there are no accumulation of 2-AP where they are still fragrant. The aroma profiling study of Rao et al. (2006) found that the five varieties which are exceptional to 8-bp deletion possessed low levels of 2AP than those carrying the deletion. Despite the low levels of 2AP, these genotypes are fragrant indicating that the flavor compounds other than 2AP may be responsible for the fragrance and hence, can be classified as non-2AP associated fragrance. Similarly, the aroma profiling of the *Chakhao Poireiton* using GC-MS also does not show the presence of 2-AP and the molecular sequence study of the *fgr* gene region also does not show the deletion. Thus, *Chakhao Poireiton* is a great exception from the other fragrant rice where the 8-bp deletion in exon 7 of *badh2* have led to the introduction of premature stop codon to produce a truncated protein which result in abrogation of the function of the enzyme BADH2 which consequently accumulate substrate 2AP in fragrant rice. Here, in *Chakhao Poireiton* there is a complex mixture of volatile compounds imparting the completely different aroma and the sequence of the *fgr* region which is completely different from the previous reports cause the aroma which suggests a more diverse set of rice fragrance. The exceptions might be the genotypes did not carry the mutation and the absent of 2-AP indicate that neither 2AP nor 8-bp deletion on *badh2* could be the cause of

fragrance indicating the involvement of different gene(s)/loci or mutation. There is also possibility that the presence of modifiers and the genetic background of the variety may play a role in producing different or modified aroma from the typical popcorn-like aroma produced by 2AP. Similarly, Jezussek et al. (2002) reported many new aroma compounds and suggested that 2AP was not an important constituent of fragrance in a fragrant variety called 'Indica' which differed in over all fragrance from the rest of fragrant varieties including Basmati 370. Bradbury et al. (2005) explained that the different flavors in some genotypes may be due to the modifying influence of other secondary metabolic consequences of 8-bp mutation in different genetic backgrounds. However, this explanation cannot be applied to all or at least to the black scented rice, as they do not have the deletion at all and are still fragrant and as well there is no accumulation of 2-AP. Available evidences on the existence of exceptions to *badh2* mutation, different aroma and flavor, compounds other than 2AP for fragrance have all contributed to the complexity on the molecular understanding of fragrance in rice. The variations in the rice fragrance may be due to several factors, as rice fragrance is easier to be influenced by many elements, such as genetic background, environmental condition, storage time, and etc. As for example the 2AP concentration was higher in brown rice ripened at low temperature than that ripened at high temperature and the strength of rice fragrance would gradually fade away as the storage time lasted (Itani et al. 2004). The Thailand fragrant rice variety Khao Dawk Mali 105 when grown at Tung Kula Rong Hai region suffering from drought has the strongest aroma and the best quality (Yoshihashi et al. 2002). All these factors could render rice fragrance varying wildly among individuals carrying the same *fgr* gene, producing many plausible individuals.

The study showed the exceptions from the mutation reported earlier, this indicates the involvement of other genetic loci in controlling fragrance trait. The present results emphasize the need to characterize the fragrance and its underlying factors in a wide range of genetic resources available for this trait and summarize the new insights gained on the genetic and molecular understanding of fragrance in rice.

5.9 Gene expression study of the *Pb* and *fgr* genes of *Chakhao Poireiton*

RT-PCR analysis of the *Pb* gene using the gene specific primer showed that the mRNA of this gene is transcribed more than the Actin gene (Table 12) in the cv. *Chakhao Poireiton* which indicates its association with pigmentation metabolism. Kim et al. (2011) evaluated anthocyanin biosynthesis in rice cultivars taking one white cv. *Dongjin* and two black rice cv. *Heugjinju* and *Heugseol* using RT-PCR analysis on the 17 unknown and hypothetical genes. Among these 17 genes, nine showed an upregulated pattern similar to white rice. They observed that four unknown genes (Os07g0184633, Os03g0247300, Os11g0539600, and Os07g0486400) were highly induced during the early heading stage in the two black rice cultivars and the Os01g0780900 gene was highly induced during late heading stage. They also observed that the gene Os12g0425800 was highly expressed in the *Heugseol* cultivar with high anthocyanin contents, weakly induced in the *Heugjinju* cultivar with low anthocyanin and this gene was not expressed in the cv. *Dongjin* without anthocyanin, which implies that this gene play a role in controlling anthocyanin levels. Kim et al. (2011) also observed that the MYB and GT transcription factor families showed the highest expression levels with these groups accounting for approximately 37% of the predicted transcription factors and other putative groups of anthocyanin production included the NAC families, basic helix-loop-helix (bHLH) and peroxisome proliferator-activated receptor binding protein (PBP). They illustrated the functional

diversity of the transcription factor families and concluded these factors are highly activated during anthocyanin pigmentation. They reported MYB family members activate flavonoid pigmentation, are involved in specific steps of the anthocyanin pathway and have evolved specific functions with different biochemical properties and the GT factors interact with multiple sequences within the promoter of the *Tdc* gene and express a trans-acting factor possessing a GT motif. NAC factors play an important role in regulating the expression of flavonoid biosynthesis-related genes (Morishita et al. 2009) and the bHLH factors function in determining red seed color in anthocyanin biosynthesis (Gonzalez et al. 2008). Reddy et al. (1998) reported that PBP factors may be closely related to the flower-specific Myb305 factor and MYB-like protein. Many previous studies reported that the Myb and GT families, NAC, bHLH and PBP transcription factors in colored tissues are involved (Martin and Paz-Ares, 1997; Yujie et al. 2010; Reddy et al. 1998; Borevitz et al. 2000; Sheng et al. 2005).

It is also observed that the transcribed mRNA of the *fgr* gene in the *Chakhao Poireiton* is similar with that of the actin gene (Table 12). Similarly, Fitzgerald et al. (2007) analysed the *BAD* gene expression in *Oryza sativa* using qRT-PCR, taking three fragrant (Kyeema, Basmati 370, Khao Dawk Mali 105) and three non-fragrant (Amaroo, Nipponbare, Teqing) rice varieties. They assayed the relative expression levels of the *BAD1* and *BAD2* encoding genes using quantitative real time PCR (qRT-PCR) in leaf tissue and the expression data for the *BAD* genes was normalized to expression data for Actin, a widely used 'housekeeper' gene in expression studies. *BAD1* and *BAD2* are expressed in leaf tip tissue of all fragrant and nonfragrant rice varieties analysed. The present study also assayed the relative expression levels of the *fgr/BADH2* encoding gene using quantitative real time PCR (qRT-PCR) in leaf tissue

of *Chakhao Poireiton* using Actin as the housekeeping gene. Fitzgerald et al. (2007) observed the differences in expression levels within and between homologues, alleles and varieties. They found the *BAD1* expression was similar across all varieties, with the greatest difference occurring between Khao Dawk Mali 105 (0.27) and Amaroo and Teqing (0.14). On average, *BAD1* expression was slightly, higher in the fragrant varieties than the non-fragrant varieties. They observed more variation in *BAD2* expression: In all non-fragrant rice varieties they analysed (Amaroo, Nipponbare, Teqing) and in fragrant variety Khao Dawk Mali, *BAD2* expression was higher than *BAD1* expression. However, in fragrant varieties Basmati 370 and Kyeema, *BAD1* and *BAD2* were expressed at similar levels. The present study showed that the *fgr* gene in the *Chakhao Poireiton* might be related with the fragrance/scent. Furthermore, the full-length isolation of the genes could be done and usage of these valuable genes in the transgenic crop production would be applicable.