CHAPTER III

MATERIALS AND METHODS

3.1 Material

The black scented rice, *Oryza sativa* L. cv. *Chakhao Poireiton* and *Chakhao Amubi* were kindly provided by the Department of Plant Breeding and Genetics, Central Agricultural University, Imphal, India and were used in the study.

Fig.5: A) Black scented rice (*Chakhao*) in the field B) White rice and black scented rice

Fig 6: Black scented rice cultivars *Chakhao Poireiton* and *Chakhao Amubi*

3.2 Methods

3.2.1 Sample preparation for bioactive compounds analysis

Chakhao Poireiton and *Chakhao Amubi* were dehulled and ground using a grinder. Acidified Methanol Extraction was used for the extraction of the sample. 5g of sample powder was extracted with 300 ml of acidified methanol (1N HCl, 85:15, v/v) (Kim et al. 2008) using Soxhlet (apparatus) for 16 hours at 60ºC (Gholivand and Piryaei, 2014; Huang et al.2009). The change in the temperature between 25-60ºC during extraction of anthocyanin and storage of the anthocyanin extracts at 4ºC does not seem to have a significant effect on the absorbance readings, instead there is an 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 extracted extracts were evaporated/concentrated using a rotatory evaporator to dryness and reconstituted in acidified methanol (5ml).

3.2.2 HPLC analysis (Extraction and Purification)

The solvents: methanol, acetonitrile and phosphoric acid were collected for HPLC grade (Sigma, USA). The standard cyanidin 3- O glucoside was purchased from Sigma, USA. Cyanidin-3-glucoside or delphinidin-3-glucoside are the most common anthocyanins in grains. The reconstituted extract was filtered through 45 micron syringe filter prior to HPLC analysis. The standards were dissolved in acidified MeOH (1N HCl, 85:15, v/v) to obtain the concentrations of 1mg/ml. The HPLC method for anthocyanin measurement was made based on methods reported earlier with some modifications (Mazza et al. 2004; Naczk & Shahidi, 2006; Jing et al. 2007; Jia et al. 2008; Wang et al. 2014). Chromatographic analysis was performed on the Thermo Spectrasystem HPLC System (Thermo Scientific, USA) equipped with an (autosampler/injector end) UV-VIS 3000 (AS 3500 detector) HPLC P-4000 pump,

SN 4000 controller and chromleon 4.2 software. The separation of anthocyanins was done by C18 column (250X4.6mm). Elution was performed using mobile phase A (96% buffer solution: Buffer solution is 20 mM sodium dihydrogen phosphate adjusted to pH 2.5 by the addition of phosphoric acid) and mobile phase B (4% acetonitrile). The gradient condition was as follows:- 0-10 min, 15%B, 10-20 min, 25% B, 20-30 min 35% and 30-40 min 45%. The flow rate was 1 ml/min and elution of compounds of interest was monitored at wavelength 517nm.

3.2.3 Determination of the total phenol content

Total phenol contents were measured using a modified Folin-Ciocalteu method (Waterhouse, 2001; Jing et al. 2007). Briefly, a series of tubes was prepared with 15 ml of water and 1 ml of Folin–Ciocalteu reagent. Then, 1 ml of samples, Folin–Ciocalteu reagent dilutions (standards) and water blank was added into tubes, mixed well and left to stand at room temperature for 10 min. The 20% Na_2CO_3 solution (3 ml) was added to each test tube and mixed well before they were put in a dry-bath incubator at 40 °C for 20 min. After incubation, tubes were immediately cooled down in an ice bath. The absorbance of samples and standards were measured at 755 nm using a Cecil UV–visible spectrophotometer. Total phenol contents were calculated as Gallic acid equivalents based on a Gallic acid standard curve.

3.2.4 Determination of the total monomeric anthocyanin

The total monomeric anthocyanin content was measured according to the methods described earlier with some modifications (Guisti & Wrolstad, 2001; Jing et al. 2007; Hosseinian et al. 2008). Two dilutions of the samples were prepared, one for pH 1.0 using 0.025M potassium chloride buffer and the other for pH 4.5 using 0.4 M sodium acetate buffer. The samples were diluted 10 times to a final volume of 2 ml. The absorbance of each sample was measured at 520 nm against distilled water as

blank. The concentration (mg/L) of each sample was calculated according to the following formula and expressed as cyanidin-3- glucoside (Cy-3-glc) equivalents:

$$\frac{A \text{ X MW X DF X } 10^3}{€ \text{ X } 1}$$

Where A is the absorbance = $(A_{\lambda vis\text{-}max})_{pH\ 1.0}$ - $(A_{\lambda vis\text{-}max})_{pH\ 4.5}$, MW is the molecular weight=449.2g/mol for Cy-3-glc, DF is the dilution factor (0.2ml sample is diluted to 2ml, DF = 10), and € is the extinction coefficient (L x cm^{-1} x mol^{-1})=26,900 for Cy-3-glc, where L (pathlenght in cm) =1.

3.2.5 Determination of the DPPH radical scavenging activity

The determination of the quenching of free radical activity was done using 2,2-diphenyl-1-picryl hydrazyl (DPPH) described by Devi et al. (2015) with some modifications. DPPH is a stable free radical due to its spare electron delocalization over the whole molecule (Szabo et al. 2007). One ml of 0.1 mM solution of DPPH free radical in methanol was mixed with 1 mL of the extract (50, 100, 150 μg/mL) and after mixing the solutions were incubated for 30 min and then absorbance was measured at 517 nm. Similarly 1 ml of methanolic solution of ascorbic acid (50, 100, 150 μg/mL) were mixed with 1 ml of DPPH methanolic solution and absorbent were recorded at the same wavelength. The radical scavenging activity was calculated using the following formula:

DPPH free radical scavenging activity (%) = [(A0 − A1/ A0) × 100]

A0 = the absorbance of the control

A1 = the absorbance in the presence of anthocyanin extracts or standards.

3.2.6 Sample Preparation for GC-MS analysis and GC-MS analysis

The main requirements for the black scented rice extract preparation are water and volatile oil assembly (Clavenger Apparatus). 500 mg of the powder sample was transferred to 1 liter flask. Water was added to fill the flask half full and put the flask

on heat mantle. Antiforming agent was added and the trap was filled with water and heated to boil. The trap was set in such a way that the condensate will not drop directly on the surface of the liquid in the trap but run down the sides. Distilled, untill two consecutive readings taken at 1 hour intervals shows no change in oil content. The volume of the volatile oil collected was 2 ml. The samples thus prepared were used to check the volatile compounds profile of *Chakhao Poireiton* and *Chakhao Amubi* using Gas Chromatography-Mass Spectrophotometer (Shimadzu GCMS Qp 2010 Ultra).

3.3 Statistical analysis

Samples were analysed in triplicate & one way analysis of variances performed using SAS 9.1 significant differences were detected at $P < 0.05$.

3.4 Molecular characterization of the pericarp color and scent genes

3.4.1 DNA extraction

The black scented rice cultivar, *Oryza sativa* cv. *Chakhao Poireiton* was collected for molecular characterization of the genes responsible for pericarp color and scent. The samples were germinated on the petriplates and were grown in the pots; the young tender leaves were collected for genomic DNA extraction. A modified CTAB method was used for isolation of genomic DNA from rice leaf samples. There are only few modifications from the CTAB method by Doyle & Doyle (1987; 1990) methodology.

Purity of DNA was checked using Thermo NanoDrop. The DNA extracted was analyzed on 1% agarose gel and was visualized by staining with ethidium bromide and transillumination under short-wave UV light of BioRad gel doc system. Electrophoresis was performed at constant power of 100 Watt for 3.5 h including a 1 h pre-run to warm the gel to 50-60 °C.

3.4.2 PCR analysis and gel electrophoresis

The PCR reaction was carried out using PCR master mix (Sigma, USA) in 25 µl reaction volume containing 1X PCR buffer incorporates 1x PCR buffer, 200 µM dNTPs, 0.4 µM of each forward and reverse primer (Table 3), 1.2 mM $MgCl_2$, 1 unit Taq DNA polymerase and 10 ng template DNA (PCR machine, Applied Biosystem). Profile used was as follows: an initial hot start and denaturing step at 94°C for 5min followed by 35 cycles at 94°C for 30 sec, appropriate annealing temperature 56°C for 30 sec and primer elongation at 72°C for 1 min. Final extension step at 72°C for 10 min was performed. The PCR product was analyzed on 1.8% agarose gel and was visualized by staining with ethidium bromide and transillumination under short-wave UV light of BioRad gel doc system.

Table 3: Gene specific primers (Synthesized by GCC Biotech, India)

Gene	forward primer 5'-3'	reverse primer 5'-3'	Aplicon size	Reference
Pb	GGGAGAAGCTCAACGAGATG	GGGTGGCAGATTCATCACTT	700/ 1000	Wang and Shu, 2007
fgr	GCAAGTGACGGAGTACGCCT	GCTAACTTCCGCTCACGCAA	348/391	Chen et al. 2008

3.4.3 Cloning of PCR Products

The desired PCR product was visualized and purified by eluting from the gel (Sigma Elution kit). The purified PCR product (insert) was ligated into pGEM-T Easy plasmid (Promega, USA) using T4 DNA ligase (Promega Kit). The ligation mixtures were transferred into *Escherichia coli*. DNA were transformed and allowed the colonies to grow overnight and need to determine if they contain the insert of interest. Screening them could be done by colony PCR or the more traditional plasmid miniprep followed by restriction digestion. Though colony PCR is the most rapid initial screening method, here, plasmid miniprep has been used. This is because a

plasmid miniprep will provide a lot of material for further analysis although it takes an extra day to grow up the culture. A plasmid miniprep followed by restriction digestion is the classic method for screening colonies. Well-isolated colonies are picked from a plate and transferred to culture medium containing the appropriate antibiotic for selection with proper sterile technique. There are many different culture media formulations which are commonly used for minipreps. During the study, LB media supplemented with ampicillin was used for miniprep cultures to insure that the bacteria do not outgrow the ability of the antibiotic to select for the plasmid. If a rich medium like Terrific Broth is used, the bacteria can grow to very high cell densities and deplete the antibiotic. Once the antibiotic is depleted, the selection pressure to keep the plasmid is removed and the plasmid may be lost. The colony was inoculated into 2 ml of culture medium. A high-copy plasmid was used. The culture was incubated overnight (12–16 hours) with shaking (~250rpm). Once the DNA is purified, a portion of the plasmid is screened by restriction digestion

3.4.4 Sequencing of the genes

The overnight culture was taken and centrifuged to remove the culture media, the cells were resuspended and lysed. The clear lysate was taken to elute the plasmid DNA. The direction of ligation and proper orientation of cloning was determined by restriction enzyme digestion EcoRI and NcoI. Digestions were performed with 10 units of the indicated enzymes for 1 hour at 37°C. The digested samples were resolved on a 1% agarose gel and stained with ethidium bromide. Uncut plasmid was also run on the same gel for comparison.

The clones were further confirmed by sequencing reaction using primers designed from the vector backbone, they wereT7 and SP6 primers and gene-specific primers (Table 3). In this reaction, 1.25 units Taq DNA polymerase, 20 pmol primers

and 200 mM dN*TPS* were added to 15 ng of plasmid in a buffer containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris -HCl-pH 9 and 0.1% Triton® X-100. The PCR cycle condition consists of three segments. Firstly, there was a pre-denaturation for 4 min at 94°C. The second variable segment was of 40 cycles each one was 1 min at 94°C, 1 min at 55°C and 2 min at 72°C; the last segment was an extension for 7 min at 72°C. Sequencing was done by the Sequencer (Applied Biosystem).

3.4.5 Sequence analysis

The best way to identify an unknown sequence is to see if that sequence already exists in a public database. The BLAST nucleotide (www.ncbi.nlm.nih.gov) algorithm finds similar sequences by generating an indexed table both for the query and the database. BLAST search was performed for the sequences *Pb* and *fgr* gene fragments. Multiple sequence analysis of the *Pb* and *fgr* fragments were done using Clustal W (www.ebi.ac.uk).

3.4.6 Mapping of the Genes

Ensemble genome browser (http://asia.ensembl.org/) helps to find out the chromosomal location of a gene depending upon their position and nucleotide base description. The BLAT Alignment Tool developed by Kent, 2002 was used, the BLAST-like Alignment Tool, quickly finds alignments to DNA sequences using Ensembl BLAT database. The present study used the BLAST Tool on Grammene (www.grammene.org) which uses BLAT algorithm as a default to map out the chromosomal location of the *fgr* and *Pb* genes. The locus of the *Pb* and *fgr* gene fragments were identified against the database Genes in MSU Release 7- Genomic Sequence on TIGR: Rice Genome Annotation Project (http://www.tigr.org).

3.5 Gene expression profiling study

3.5.1 Total RNA extraction

Total RNA from rice leaf samples was extracted using Trizol method. Total RNA extraction from leaf samples was checked for the quality and quantity by Thermo NanoDrop machine.

3.5.2 cDNA synthesis from total RNA samples

Genomic clones represent a random sample of all of the DNA sequences in an organism and with very rare exceptions, are the same regardless of the cell type used to prepare them. By contrast, cDNA clones contain only those regions of the genome that have been transcribed into mRNA. Because the cells of different tissues produce distinct sets of mRNA molecules, a distinct cDNA library is obtained for each type of cell used to prepare the library. cDNA Syntheis kit was used for the reverse transcription reaction and the reaction was carried out according to the recommended protocol. 2 µg of total RNA was used as the template and the gene specific primers (Table 3) were used as primers for reverse transcription in 20 µl reaction volume. The RNA was annealed with the primer at 65°C for 5 min using Thermal Cycler (Applied Biosystem). This was incubated at room temp for 2 min in a reaction mix containing 1X RT reverse trancription buffer, DTT, dNTP and RNase inhibitor. The reverse transcription was carried out at 42°C for 90 min using M-MLV reverse transcriptase enzyme. The reaction was stopped by incubation at 70°C for 15 min. The reverse transcription product was used as template for PCR reactions in which the mRNAs of genes of interest were amplified using specific primers (Table 3).

3.5.3 Standardization of PCR amplification with single set of primer

The PCR reaction was carried out using PCR master mix in 25 µl reaction volume containing 1X PCR buffer incorporates 1x PCR buffer, 200 µM dNTPs, 0.4 µM of each of forward and revesrse primer, 1.2 mM MgCl2, 1 unit Taq DNA polymerase and 10 ng template DNA. Profile was used as follow: an initial hot start and denaturing step at 94°C for 5 min followed by 35 cycles at 94°C for 30 sec, appropriate annealing temperature 58°C for 30 sec, and primer elongation at 72°C for 1 min. Final extension step at 72°C for 10 min was performed. The PCR product was analyzed on 1.8% agarose gel and was visualized by staining with ethidium bromide and transillumination under short-wave UV light of BioRad gel doc system.

3.5.4 Gene expression study by qRT-PCR SYBR green based method

All the real-time PCR assays were performed on Step One™ Real-Time PCR System (Applied Biosystem). A typical 20 µl reaction mix consisted of 2X SyBR green dye, 1 pm of each primer, 1 µl of template and DNase and RNase free water. The PCR conditions were a two-step program: 95°C for 10 min and 40 cycles of 95°C for 15sec, 58°C for 30sec and 72°C for 30sec.