CHAPTER IV

RESULT

RESULT

4.1 Phytochemical analysis using DPPH assay, Follin colcalteau method, pH differential method and HPLC analysis

Organic solvents such as methanol and acetone are usually used for extraction of anthocyanins, the acidified MeOH showed the highest extraction efficiency (70-100%) (Naczk & Shahidi, 2006; Mazza et al. 2004). In the present study, anthocyanins were extracted from black scented rice using acidified methanol by the Soxhlet Apparatus. The anthocyanin was obtained from 300 ml methanol for 16 hrs at the boiling point. The total anthocyanin content in *Oryza sativa* cv. *Chakhao Poireiton* was found to be 740 mg/kg and *Oryza sativa* cv. *Chakhao Amubi* was 692 mg cyanidin 3-glucoside/kg of powdered rice (Table 5: Fig. 9). And the total phenol content were 577 and 500 mg/100g of the powdered sample as gallic acid equivalents in *Chakhao Poireiton* and *Chakhao Amubi*, respectively (Table 5: Fig. 10). In the DPPH free radical scavenging assay, anthocyanin extract of *Chakhao Poireiton* exhibited 42.91% scavenging activity at 50 µg/ml, 55.20% scavenging activity at100 µg/ml, and 70.28% scavenging activity at 150 µg/ml, respectively (Table 6: Fig. 11) and that of the *Chakhao Amubi* exhibited 39.35% scavenging activity at 50 µg/ml, 53.86% scavenging activity at100 µg/ml, and 69.73% scavenging activity at 150 µg/ml, respectively (Table 6: Fig. 11). The standard ascorbic acid showed 46.06%, 89.03% and 93.73% scavenging activity at 50 µg/ml, 100 µg/ml and 150 µg/ml, respectively (Table 6: Fig. 11). Also, extracts were analysed with HPLC method using gradient system. Anthocyanins were identified according to the HPLC retention time by comparison with authentic standards and published data (Jing et al. 2007; Hosseinian et al. 2008; Jia et al. 2008; Lee et al. 2009). Fig. 7 and Fig. 8 show the HPLC chromatogram of *Chakhao Poireiton* and *Chakhao Amubi*, respectively. The

total anthocyanins reported, includes both identified and non-identified HPLC peaks (Table 4). Due to lack of corresponding anthocyanin standards and published data corresponding to the peak, some of the peaks remain unlabelled. By comparing with the previously reported data (Jing et al. 2007; Hosseinian et al. 2008; Jia et al. 2008; Lee et al. 2009), four main anthocyanis, i.e. delphinidin 3-galactoside (Dp-3-gal), delphinidin 3-arabinoside (Dp 3-ara), cyanidin 3-galactoside (Cy-3-gal) and cyanidin 3-glucoside (Cy-3-glc) were identified in *Chakhao Poireiton* and *Chakhao Amubi* (Table 4). Three main anthocyanins, delphinidin 3-galactoside (Dp-3-gal), delphinidin 3-arabinoside (Dp-3-ara) and cyanidin 3-galactoside (Cy-3-gal) were identified in *Chakhao Amubi* (Table 4). In both the samples, Dp-3-gal was found to be the most predominant anthocyanin.

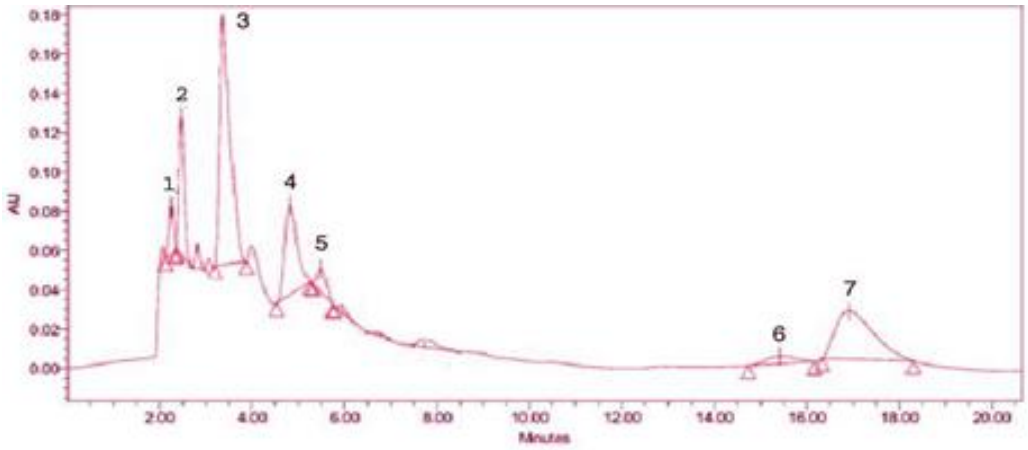

Fig. 7: HPLC chromatogram (517 nm) for anthocyanin distribution in *Chakhao Poireiton*

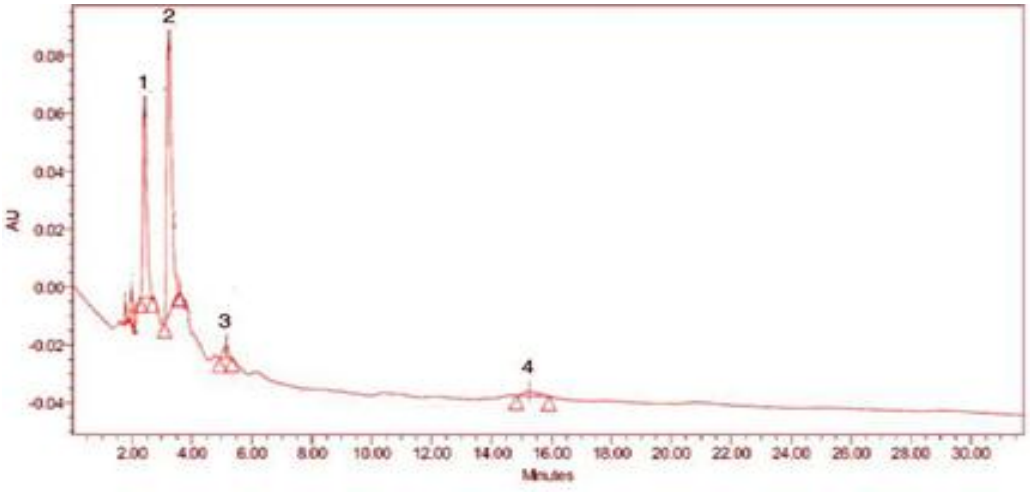

Fig. 8: HPLC chromatogram (517 nm) for anthocyanin distribution in *Chakhao Amubi*

Table 4: Chromatographic characteristics of anthocyanins from *Chakhao Poireiton* and *Chakhao Amubi*

Peak no.	Retention Time (min)	Area (%)(517nm)	Compound
Chakhao Poireiton			
1	2.2-2.4	2.9	unidentified
2	2.4-2.7	10.4	unidentified
3	3.0-3.9	37.5	Delphinidin3-galactoside
4	4.6-5.2	16.1	unidentified
5	5.2-5.7	3.1	Delphinidin 3-arabinoside
6	14.6-16.2	3.4	Cyanidin 3-galactoside
7	16.4-18.4	26.7	Cyanidin 3-glucoside
Chakhao Amubi			
1	2.2-2.5	32.0	unidentified
2	3.0-3.9	62.1	Delphinidin 3-galactoside
3	5.2-5.7	3.0	Delphinidin 3-arabinoside
4	14.6-16.2	2.9	Cyanidin 3-galactoside

Table 5: Total anthocyanin and phenolic content in *Chakhao Poireiton* and *Chakhao Amubi*

	Chakhao Poireiton	*Chakhao Amubi*
Total Anthocyanin Content (mg/kg)	740	692
Total Phenolic Content(mg/100g)	577	500

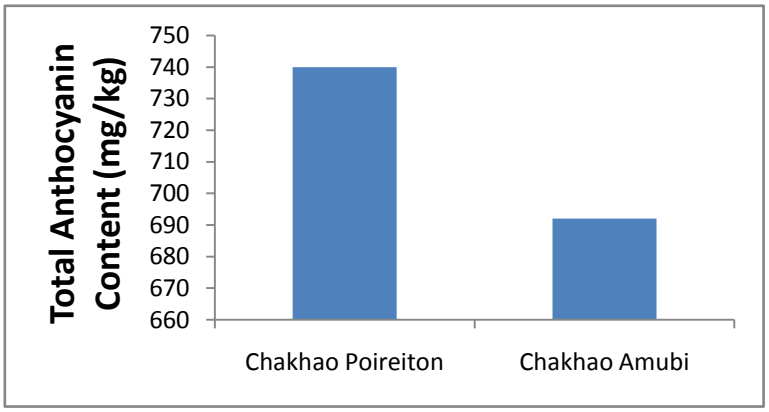

Fig. 9: Bar diagram of the total anthocyanin content in *Chakhao Poireiton* and *Chakhao Amubi*

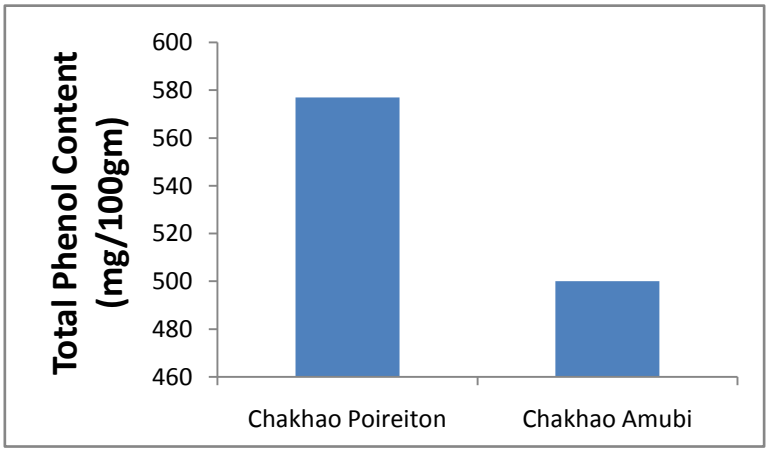

Fig. 10: Bar diagram of total phenol content in *Chakhao Poireiton* and *Chakhao Amubi*

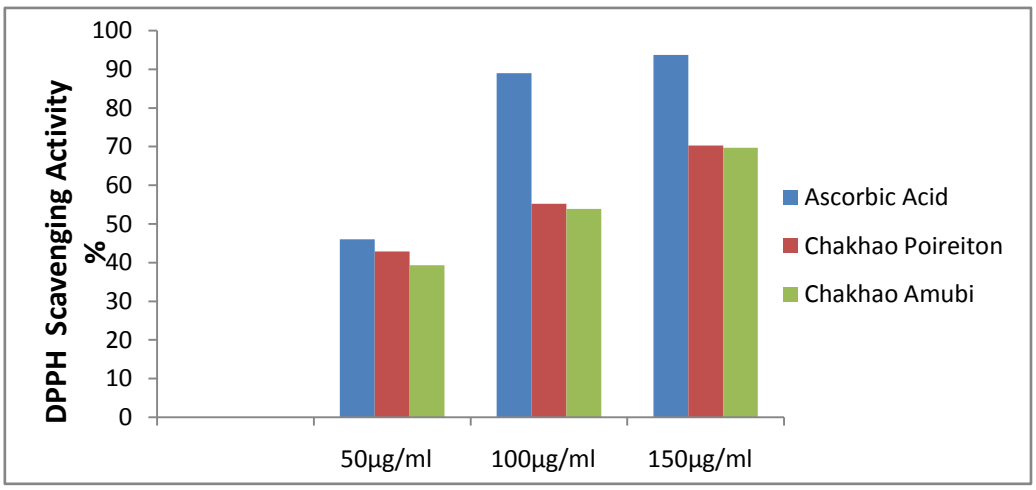

Fig. 11: Bar diagram of the DPPH scavenging activity % of the methanol extracts of *Chakhao Poireiton* and *Chakhao Amubi*

Table 6: DPPH scavenging activity % of the methanol extracts of *Chakhao Poireiton* and *Chakhao Amubi*

DPPH Scavenging Acitivity %	Ascorbic Acid	*Chakhao Poireiton*	*Chakhao Amubi*
50µg/ml	46.06	42.91	39.35
100µg/ml	89.03	55.20	53.86
150µg/ml	93.73	70.28	69.73

4.2 GC-MS analysis of the volatile oil compositions of *Chakhao Poireiton* and *Chakhao Amubi*

The volatile oil profiles of the black scented rice cv. *Chakhao Poireiton* (Fig. 12) and cv. *Chakhao Amubi* (Fig. 13) were studied and reported in the study. Twenty-six volatile compounds were identified by gas chromatography-mass spectrometry from *Chakhao Poireiton* (Table 7) and from *Chakhao Amubi* (Table 8) eleven volatile compounds were identified. Out of the twenty-six compounds from *Chakhao Poireiton*, N- hexadecanoic acid were the most abundant contributing 22.92% followed by 9,12- Octadecadienoic acid (11.66%) (Table 7). And out of the eleven volatile compounds from *Chakhao Amubi*, 17- Pentatriscontene contributed 40.60% followed by Octadecenal, 20.00% (Table 8).

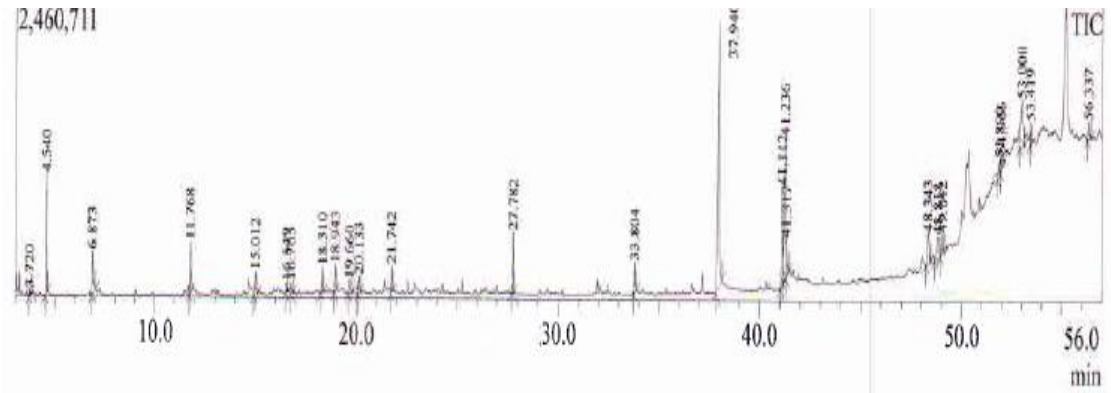

Fig. 12: GC-MS chromatogram of *Chakhao Poireiton*

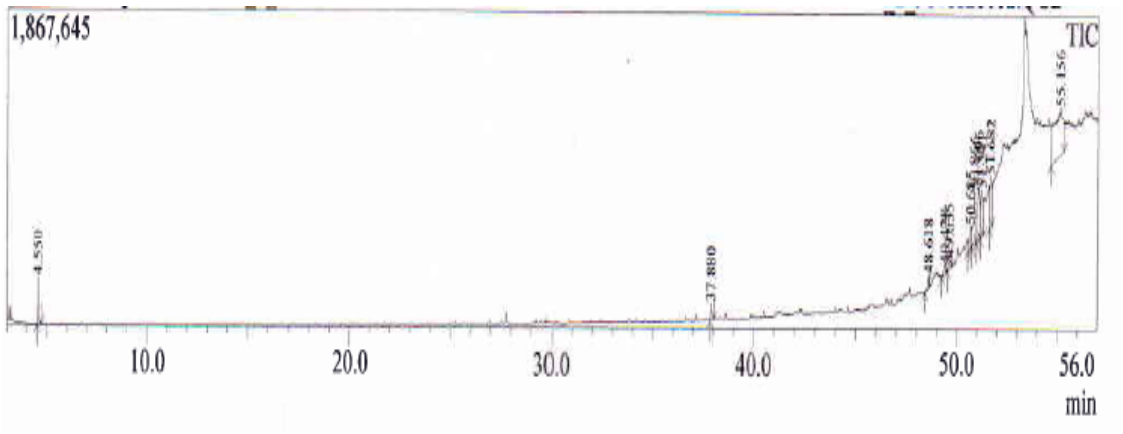

Fig. 13: GC-MS chromatogram of *Chakhao Amubi*

Table 7: Volatile oils profiling using GC-MS of *Chakhao Poireiton*

Peak	R.Time	Name	Area	Area%
1.	3.720	Butanenitrile. 3-methvl	104009	0.25
2.	4.540	Benzene methyl	2295838	5.61
3.	6.873	2-Furancarboxaldehyde	1393622	3.41
4.	11.768	2-Furancarboxaldehyde 5- methyl	1603643	3.92
5.	15.012	Benzene butyl	588613	1.44
6.	16.549	Undecane	289797	0.71
7.	16.765	Benzofuran. 2-methvl- 3	l 8639	0.78
8.	18.310	Benzene, pentyl	31571	1.30
9.	18.943	Furan 2-(2-furanylmethyl)-5-methyl	619648	1.51
10.	19.660	Dodecane	649180	1.59
11.	20.133	Benzofuran 4, 7-dimethyl	491294	1.20
12.	21.742	Furan, 2,2'-methylenebis[5-methyl]	649017	1.59
13.	27.782	Pentadecane	1188621	2.90
14.	3 3.804	Tetradecanoic acid	781382	1.91
15.	37.940	n-Hexadecanoic acid	9379913	22.92
16.	41.112	9, 12-Octadecad ienoic acid (Z.Z)	33 l 7895	8.11
17.	41.236	Octadec-9-enoic acid	477 1521	11.66
18.	41.317	Oleic acid	l 336655	3.27
19.	48.343	g-Hexadecenoic acid Octadecyl ester. (Z)	1588616	3.88
20.	48.818	9-Hexadecenoic acid, eicosyl ester, (Z)	1535517	3.75
21.	49.042	I 7-Pentatriacontene z	1059294	2.59
22.	51.890	9-Octadecenoic acrd (Z)-, 9-octadecenyl ester, (Z)	684629	1.67
23.	51.966	9-Hexadecenoic acid. 9-octadecenyl ester. (Z.Z)	647022	1.58
24.	53.000	4-Beta-H-Pregna	3591642	8.78
25.	53.419	Stigmast-5-En.3-oL. Oleat	687915	1.68
26.	56.337	Stigmasts 5-En-3-oL. (3.Beta.24S)	8 18755	2.00
			40924254	100.00

Table 8: Volatile oils profiling of *Chakhao Amubi*

Peak	R. Time	Name	Area	Area%
1.	4.550	Toluene	748852	3.38
2.	37.880	l-(+)-Ascorbic acid 2,6-dihexadecanoate	4900 l 1	2.21
3.	48.618	Cyclononasiloxane octadecamethvl	346471	1.56
4.	49.428	9-Octadecenoic acid, 1,2,3 -propanetriyl ester, (E,E,E)	427185	1.93
5.	49.635	Z.Z-3 -13 -O ctadecedien- I -o I	434892	1.96
6.	50 685	9-Octadecenoic acid (Z) - tetradecvl ester	950550	4.29
7.	50.866	l3-Octadecenal, (Z)	2667664	12.03
8.	51.096	9-Hexadecenoic acid. eicosvl ester- (Z)	2655953	11.98
9.	51.241	Tetracosamethvl-cvclododecasiloxane	2277003	10.27
10.	51.682	Z-9-Pentadecenol	2169969	9.79
11.	55.156	17-Pentatriacontene	9001555	40.60
			22170105	100.00

4.3 Quantification and the quality checking of the genomic DNA

The absorbance ratio A260/A280 was 1.95 and A260/A230 was 1.73. The DNA quantification absorbance was measured at 260 nm giving a concentration of 115.7 ng/µl. 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 (Fig. 14). There are only few modifications from the CTAB method by Doyle & Doyle (1987; 1990) methodology. Here, polyvinyl pyrrolidine (PVP) has been added in the powder form which is one main modification (1% w/v). Almost all the steps remained same with some few modifications in the incubation period, centrifugation and the volume of the reagents taken. The absorption ratio (A260/A280) of extracted DNA samples ranged in between 1.8-1.9 showing that the DNA was free from protein and polyphenols. DNA yield is important in molecular studies. The quality and quantity of the DNA extracted from the black scented rice cv. *Chakhao Poireiton* in the study was pure and concentration was good enough which could be stored for further used in molecular studies like polymerase chain reaction amplification, restriction digestion, etc.

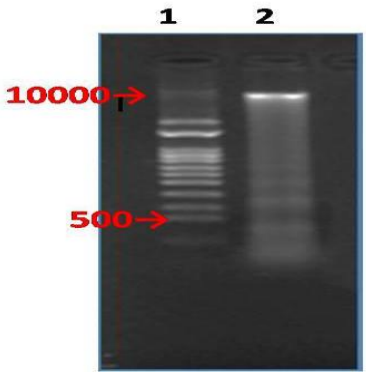

Fig. 14: Genomic DNA run on agarose gel. Lane-1 1 KB ladder Lane-2 Genomic DNA

4.4 Electrophoresis of the PCR products

The gel photographs of the PCR products using the specific primers for *Pb* and *fgr* gene are shown by the fig. 15 and 16, respectively.

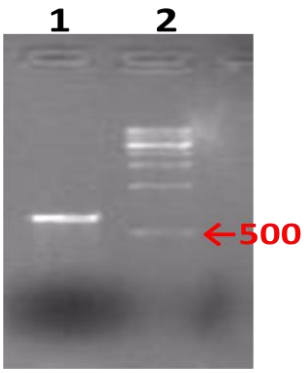

Fig. 15: Agarose gel of the PCR product with the *Pb* primer. Lane-1 *Pb* PCR product Lane-2 1 KB ladder

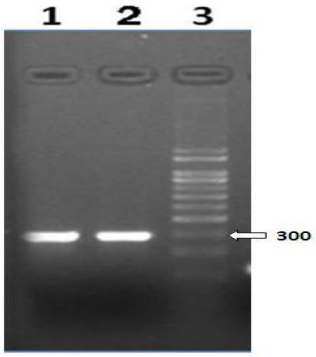

Fig. 16: Agarose gel of the PCR product with *fgr* primer. Lane 1 & 2: *fgr* PCR Lane 3: 100 bp laddder

4.5 Sequence analysis of the isolated *Pb* and *fgr* gene fragments using bioinformatics software and tools

The sequences of the *Pb* (1126 bp) and *fgr* (396 bp) gene fragments were submitted to Genebank and the sequences are as follow with their genebank accession numbers.

>PB (Genebank accession no. KP830117)

TCACAGGGTAATACTAATTACTACTTCATCCGTTTCACAATGTAAGTCATTCTAGTATTTTACATATTCATATTGACGGTAATGA
ATCTACATTCAAATTGATGTTAATAAATCTAGATTCATTACCATCAATATGAATATGGGAAATGCTAGAATGACTTACATTATG
AAACGGAGGGAGTGTAATTTTTTTAGAACCAAATTTGTCAAGGTAATTGGAGTAGTACTAGCTATTTAGTGCTTCCAAAATG
GAATTTCAAGATGATTTGAATTCACGAGATGTCGCGTGCTGCAGGTGGACAAAGCATCCATTCTCGCAGAAACGATAGCCTA
CCTCAAAGAGCTGGAGAAAAGAGTGGAAGAGCTGGAATCCAGCAGCCAACCATCGCCATGTCCATTGGAAACAAGAAGCA
GGCGAAAGTGCCGTGAGATCACTGGGAAGAAGGTTTCTGCAGGAGCGAAGAGAAAGGCGCCGGCGCCGGAGGTGGCCAG
CGACGCGACACCGACGGGGAGCGGCGCCATTGTGTGAGCAACGTGAACGTCACCATCATGGACAACAAGGAGGTTCTCCTC
GAGCTGCAATGCCAGTGGAAGGAATTGCTGATGACGAGAGTGTTCGACGCGATCAAGGGAGTCTCCCTGGATCCTCTCGGT
GCAGGCATCAACATCGGATGGTCTCCTTGGACTGAAGATACAAGCCAAGGTCGTCATCTCAGCGGCTAAGTAGAGCTCGCA
GCAGAAATGCAGCATCGTCTATCTATCTATCAATCTCTGTATCTATACGTCTGTTTGGAGTTCTGATATTGGCCATATTATTGTT
GCATGGATGCAGTTTGCCTCATCTGCTGCCGTCGAACCTGGGATGATTACAGAAGCTCTCCGGAAAGCTATAGCAAGCTAGC
TAGCTAGAAGGTTGAGATCAGTATCGTCTTGGTCATTTGCTCTCATCTTGTTTAGGAGGTACCGGGAAATAAATCTTGTCGCT
CTGATCAGTGCCTCCTTACTAGTCTCGTGATCTGTTGTAAGTAATAGTTTGACATGCATATATATCATACAACAACGTTTGATC
TTGCATGGTAGCATATATATGTACACCACATTTCTTATATCTTACTCCTTAAA

>FGR (Genebank accession no. KP830118)

TGCAAGTGACGGAGTACGCCTCCGATGAGCCGTGGGGATGGTACAAATCCCCTTCCAAGCTGTAATGTAATATGCGCATACG
CATGACACGCCCATCTGTTCTGATCGTTTGGAATAAGAGGCCATAGTATGACGGGATGGACTTTGTGCATATCGATGTTTGAT
TTGCCATGTGCTGAGCCTGGAAATAAAAAAGGTTATAGTTGGAGACTTGTGAGAGTTGTACGACTTGTACTGATGGTTACGC
GACAATTTGTGCATGGATAGTTTGTGTGCTTGTACCGGGGTTCTCTCCGTATCGGCTTGTGGTGTTTCAACCTTAAGACCCCT
GCAGTGCAGGAGCCTGCAGGTCATCCTGTTCTGCAGCGAAGGTTTTGCGTGAGGGTAAGTTAGCAA

Sequence data was aligned to Blastn and established the significant aligned sequences. BLAST search for the 1122 bp of *Pb* gene fragment reported 99% identical sequence with partial *Oryza sativa* Japonica Group putative anthocyanin regulatory *Lc* protein (*Ra*) gene (Genbank ID: gb| EU095986.1) *Oryza sativa* Indica Group putative anthocyanin regulatory *Lc* protein (*Ra*) gene, gb|EU095985.1|Oryza sativa Plw-OSB1 mRNA for R-type basic helix-loop-helix protein, dbj|AB021079.1| *Oryza sativa* transcriptional activator (*Ra*) mRNA gb|U39860.1|OSU39860.

BLAST search for the 396 bp of *fgr* reported 99% identical sequence with partial *Oryza sativa* voucher MSB_807 *Badh2* gene (Genbank ID: gb|JQ308433.1|) *Oryza sativa* Japonica Group putative methylcrotonyl-CoA carboxylase beta chain (05K17.1) gene gb|EU155083.1|.

Fig. 17 and 18 showed the multiple sequence analysis of the *Pb* and *fgr* gene fragments, repectively performed by using Clustal W.

```
PEIAI             TCGACGCGATCAAGGGAGTCTC CCTGGATGTCCTCTCGGTGCAGGCATCAACATCGGATG
9311              TCGACGCGATCAAGGGAGTCTC CCTGGATGTCCTCTCGGTGCAGGCATCAACATCGGATG
NIPPONBARE        TCGACGCGATCAAGGGAGTCTC CCTGGATGTCCTCTCGGTGCAGGCATCAACATCGGATG
Chuanheinuo       TCGACGCGATCAAGGGAGTCTC CCTGGAT--CCTCTCGGTGCAGGCATCAACATCGGATG
Yunanheixiannuo   TCGACGCGATCAAGGGAGTCTC CCTGGAT--CCTCTCGGTGCAGGCATCAACATCGGATG
Chakhaopoireiton  TCGACGCGATCAAGGGAGTCTC CCTGGAT--CCTCTCGGTGCAGGCATCAACATCGGATG
                  ********************* *******  ****************************
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Fig. 17: Multiple sequence analysis of the *Pb* gene. The purple rice lines Yunanheixiannuo, Chuanheinuo and the *Chakhao Poireiton* contained 2 bp (GT) deletion at the end of exon 7 compared with white rice lines Pei'ai 64S, Nipponbare and 9311.

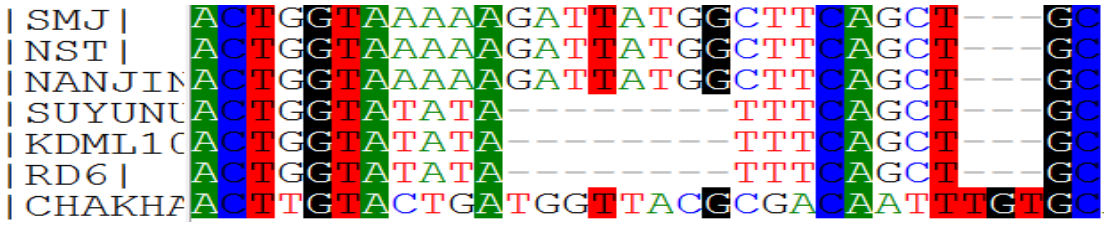

Fig. 18: Multiple sequence analysis of *fgr* gene of aromatic and non-aromatic rice. Comparison is focused on *Badh2* gene within exon 7 of aromatic rice of KDML105, RD6, SuYuNuo and *Chakhao Poireiton* and non-aromatic rice of SMJ, NST. Nipponbare and Nanjing. The aromatic variety shows 8-bp deletion and 3 single nucleotide polymorphisms (SNPs). But *Chakhao Poireiton fgr* gene fragment sequence is completely different from the other fragrant rice.

4.6 Mapping of the *Pb* and *fgr* gene fragments on the chromosome

The BLAT Alignment Tool (Kent, 2002) was used which quickly finds alignments to DNA sequences using Ensembl BLAT database. BLAT (BLAST like alignment tool) (www.gramene.org) mapped out the *Pb* and *fgr* gene sequence isolated from black scented rice cultivar (*Chakhao poireiton*). 1122 bp *Pb* gene fragment and 396 bp *fgr* gene fragment were mapped on chromosome 4 and chromosome 8, respectively (Fig. 19 & 22).

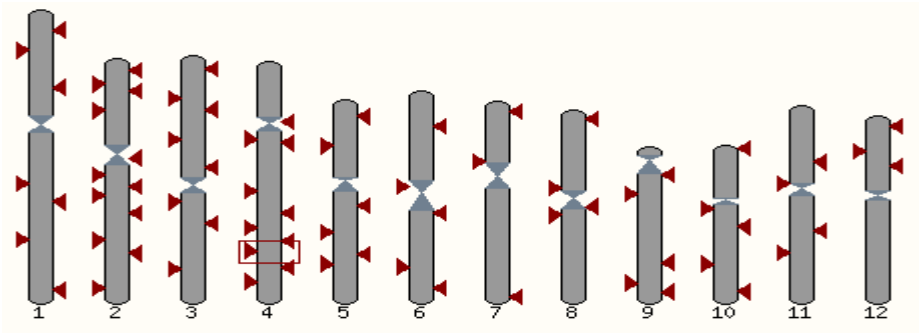

Fig. 19: Alignment Locations vs. Karyotype of the *Pb* gene fragment

Table 9: Alignment Summary of the *Pb* gene fragment on the chromosome

Links	Query		Chromosome				Stats			
	Start	End	Name	Start	End	Ori	Score	E-val	%ID	Length
[A] [S] [G] [C]	1	1119	Chr:4	27949448	27950569	+	1099	0.	99.47	1123
[A] [S] [G] [C]	499	908	Chr:11	8583849	8584259	+	336	0.	95.19	416
[A] [S] [G] [C]	258	407	Chr:11	8584340	8584489	+	102	0.	92.00	150
[A] [S] [G] [C]	22	182	Chr:6	28985018	28985177	+	86	2.4e-36	88.27	162

BLAST Genomic Sequence

BLAST/BLAT type	BLASTN
Query location	Query_1 1122 to 1 (-)
Database location	4 26781084 to 26782210 (+)
Genomic location	4 26781084 to 26782210 (+)
Alignment score	98.0
E-value	1.5E-243
Alignment length	1127
Percentage identity	98.0

Exons All exons

```
>chromosome:ASM465v1:4:26780784:26782510:-1
26782510 TTTAGACGGTAGCTCCATTGATGGATCTTGCAGGCCGTCGCCGTCGAGTTTTGTGGCGTG 26782451
26782450 GAAGAGGACGGCGGACTCGGACGAGGTGCAGGCCGTGCCGCTCATCAGCGGAGAGCCGCC 26782391
26782390 ACAGAAGTTGCTGAAGAAAGCTGTCGCCGGAGCCGGTGCCTGGATGAACAATGGTGACAG 26782331
26782330 CAGCGCGGCGGCGATGACGACTCAAGGAAGCAGCATCAAGAACCATGTCATGTCAGAGAG 26782271
26782270 AAGGCGCCGGGAGAAGCTCAACGAGATGTTCCTGATTCTCAAATCAGTTGTCCCGTCCAT 26782211
26782210 TCACAAGGTAATACTAATTACTACTTCATCCGTTTCACAATGTAAGTTATTCTAGCATTT 26782151
26782150 TCCATATTTATATTAACGGTAATGAATCTACATTTAAATTGATGTTAATGAATCTAGATT 26782091
26782090 CATTACCATCAATATGAATATGAGAAATGCTAGAATGACTTACATTATGAAACGGAGGGA 26782031
26782030 GTAGTAATTTTTTTAGAACCAAATTTGTCAAGGTAATTGGAGTAGTACTAGCTATTTAGT 26781971
26781970 GCTTCCAAAATGGAATTTCAAGATGATTTGAATTCACGAGATGTCGCGTGCTGCAGGTGG 26781911
26781910 ACAAAGCATCCATTCTCGCAGAAACGATAGCCTACCTCAAAGAGCTGGAGAAAAGAGTGG 26781851
26781850 AAGAGCTGGAATCCAGCAGCCAACCATCGCCATGTCCATTGGAAACAAGAAGCAGGCGAA 26781791
26781790 AGTGCCGTGAGATCACTGGGAAGAAGGTTTCTGCAGGAGCGAAGAGAAAGGCGCCGGCGC 26781731
26781730 CGGAGGTGGCCAGCGACGACGACACCGACGGGGAGCGGCGCCATTGTGTGAGCAACGTGA 26781671
26781670 ACGTCACCATCATGGACAACAAGGAGGTTCTCCTCGAGCTGCAATGCCAGTGGAAGGAAT 26781611
26781610 TGCTGATGACGAGAGTGTTCGACGCGATCAAGGGAGTCTCCCTGGATGTCCTCTCGGTGC 26781551
26781550 AGGCATCAACATCGGATGGTCTCCTTGGACTGAAGATACAAGCCAAGGTCGTCATCTCAG 26781491
26781490 CGGCTAAGTAGAGCTCGCAGCAGAAATGCAGCATCGTCTATCTATCTATCAATCTCTGTA 26781431
26781430 TCTATACGTCTGTTTGGAGTTCTGATATTGGCCATATTATTGTTGCATGGATGCAGTTTG 26781371
26781370 CCTCATCTGCTGCCGTCGAACCTGGGATGATTACAGAAGCTCTCCGGAAAGCTATAGCAA 26781311
26781310 GCTAGCTAGCTAGAAGGTTGAGATCAGTATCGTCTTGGTCATTTGCTCTCATCTTGTTTA 26781251
26781250 GGAGGTACCGGGAAATAAATCTTGTCGCTCTGATCAGTGCCTCCTTACTAGTCTCGTGAT 26781191
26781190 CTGTTGTAAGTAATAGTTAGACATGCATATATATCATACAACAACGTTTGATCTTGCATG 26781131
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26781070 TGGTAAGTGATGAATCTGCCACCCCATCACCAACTCCCTAAAATTCTGACATGTGAAACG 26781011
26781010 TCGACCACTGTCTATCCTCTCTCTAAACAAAACATACAGTAAAAAAAAATAGAAGTCACA 26780951
26780950 GATAGACTCTGTAGCTCTCAACCATAATCATCCTTACATGCTACTCCTCTAATAAATGGA 26780891
26780890 GACTTTATGTGTAGTGGTGAAAACTTGCAACCAAAAATCTCTCTAGTTAATTCAGAAATT 26780831
26780830 AATAATGCAAATTTGATTTCCTCTTTGCAATTTGTTTTTTTAATTCC              26780784
```

Fig. 20: Alignment Locations of the *Pb* gene fragment vs. Genomic Sequence of *Oryza sativa Indica* ASM465v1

Name	Transcript ID	Length	Protein	Biotype	Flags
RA	BGIOSGA014565-TA	1662 bp	553 aa (view)	protein coding	

Protein summary

Protein domains for BGIOSGA014565-PA.1

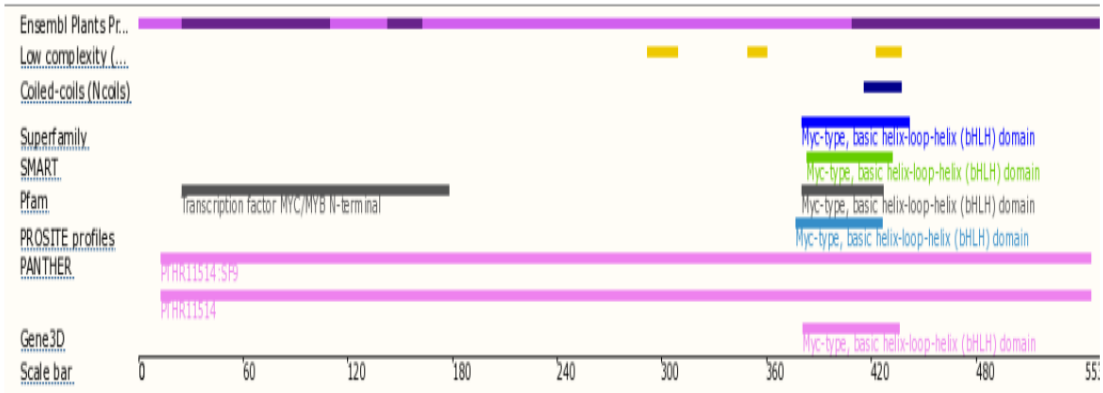

Fig. 21: The Protein domain of the overlapping gene of the *Pb* gene fragment

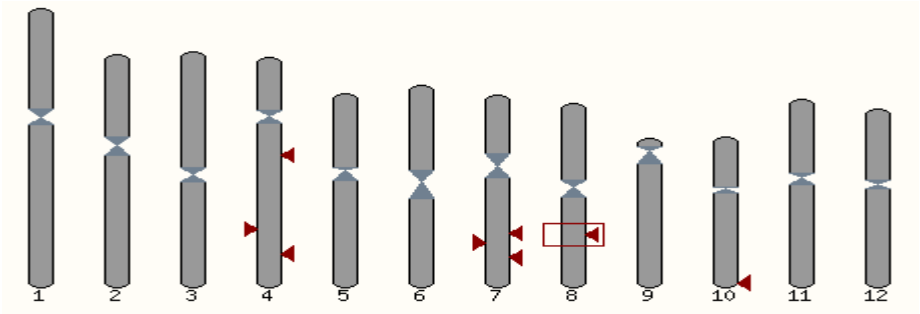

Fig. 22: Alignment Locations vs. Karyotype of the *fgr* gene fragment

Table. 10: Alignment Summary of the *fgr* gene fragment on the chromosome

Links	Query		Chromosome				Stats			
	Start	*End*	*Name*	*Start*	*End*	*Ori*	*Score*	*E-val*	*%ID*	*Length*
[A] [S] [G] [C]	*2*	*394*	*Chr:8*	*20385765*	*20386156*	*+*	*382*	*1.4e-219*	*99.24*	*394*
[A] [S] [G] [C]	*195*	*214*	*Chr:4*	*30332300*	*30332319*	*+*	*20*	*0.35*	*100.00*	*20*
[A] [S] [G] [C]	*14*	*32*	*Chr:10*	*22562950*	*22562968*	*+*	*19*	*1.1*	*100.00*	*19*
[A] [S] [G] [C]	*283*	*304*	*Chr:7*	*25136706*	*25136727*	*+*	*18*	*3.3*	*95.45*	*22*

BLAST Genomic Sequence

BLAST/BLAT type	BLASTN
Query location	Query_1 2 to 286 (+)
Database location	8 21706850 to 21707134 (+)
Genomic location	8 21706850 to 21707134 (+)
Alignment score	100.0
E-value	1.1E-57
Alignment length	285
Percentage identity	100.0

Exons All exons

```
>chromosome:ASM465v1:8:21706550:21707434:1
21706550 GACTTTATATTTGACCCCTTTTTTTTGCAAAAGAAAGGGAAATACTTAACGAAAATTTCC 21706609
21706610 TACTGCAGGAGATCGATGCCGGAATTATCTGGGTGAACTGCTCGCAACCCTGCTTCTGCC 21706669
21706670 AAGCTCCATGGGGCGGGAACAAGCGCAGCGGCTTTGGACGCGAGCTCGGAGAAGGGTGGG 21706729
21706730 TAGCACACAACAATCTCACTTTAAAACACCATTTCGATCGTCTGATGATCTCGACCTGAC 21706789
21706790 ATCATGCCTTTGGTATTTTCATTCACTTTTCAGGGGCATTGACAACTACCTAAGCGTCAA 21706849
21706850 GCAAGTGACGGAGTACGCCTCCGATGAGCCGTGGGGATGGTACAAATCCCCTTCCAAGCT 21706909
21706910 GTAATGTAATATGCGCATACGCATGACACGCCCATCTGTTCTGATCGTTTGGAATAAGAG 21706969
21706970 GCCATAGTATGACGGGATGGACTTTGTGCATATCGATGTTTGATTTGCCATGTGCTGAGC 21707029
21707030 CTGGAAATAAAAAAGGTTATAGTTGGAGACTTGTGAGAGTTGTACGACTTGTACTGATGG 21707089
21707090 TTACGCGACAATTTGTGCATGGATAGTTTGTGTGCTTGTACCGGGTGCAGTGCAGGAGCC 21707149
21707150 TGCAGGTCATCTGTTCTGCAGCGAAGGTTTTGCGTGAGCGGAAGTTAGCTGATGACCGGT 21707209
21707210 TCGGTTTAGCTAAAACCGGATGATGGATGATGGATTATTTATCAAGAGGATTACGTGCCA 21707269
21707270 CGCTTCTAAAAATATAACTTAAATTTTTCTCATCACCAAACGACTTCTTCAAAAACTTTT 21707329
21707330 TCCATCGGCATTGGTTAGCTTTATATTATATGCTATTTTATTTATTTTATAGGGAGATAT 21707389
21707390 TATATTCAATTAAACCTGAACTGATAGATTATCCATCATCCTTCA               21707434
```

Fig. 23: Alignment Locations of the *fgr* gene fragment vs. Genomic Sequence of *Oryza sativa Indica* ASM465v1

Name	Transcript ID	Length	Protein	Biotype	RefSeq	Flags
BADH2	BGIOSGA028697-TA	1512 bp	503 aa (view)	protein coding	NM_001068368	

Summary

UniprotKB	This gene has proteins that correspond to the following Uniprot identifiers: B3VMC0
Gene type	Protein coding
Prediction Method	Gene annotation by BGI through a process of automatic and manual curation

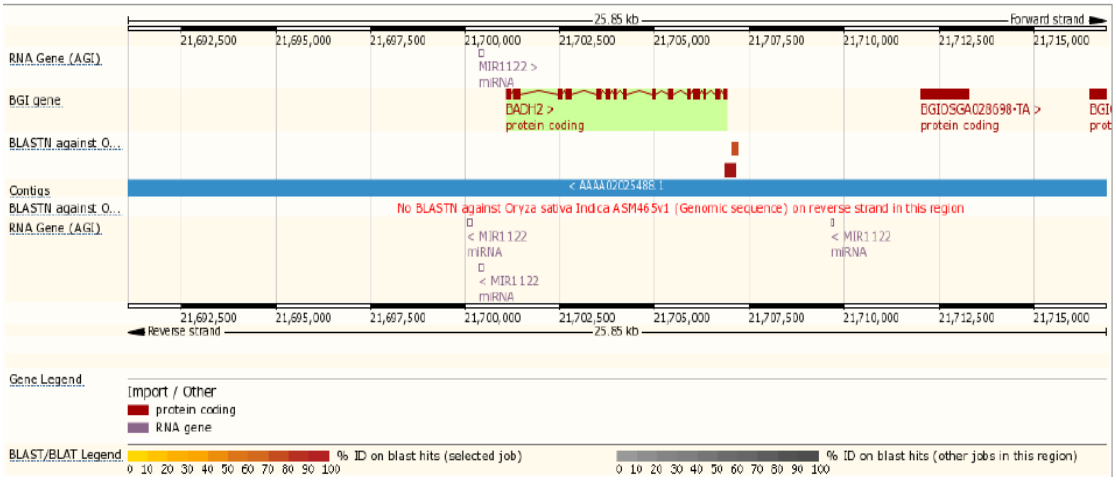

Fig. 24: Protein of the overlapping gene of the *fgr* gene fragment

The locus of the *Pb* and *fgr* gene fragments were LOC_Os08g32870 and LOC_Os04g47080, respectively against the database Genes in MSU Release 7-Genomic Sequence on TIGR: Rice Genome Annotation Project (http://www.tigr.org).

4.7 Quantification and quality checking of the RNA

A ratio of approx 1.8 to 2.0 (A260/A280 nm) means that the RNA is sufficiently pure and without polysaccharide contamination for use in most applications and is soluble. A lower ratio generally means polysaccharide contamination and/or insolubility. The concentration of the RNA was 173.5 ng/µl and ran on agarose gel (Fig. 25).

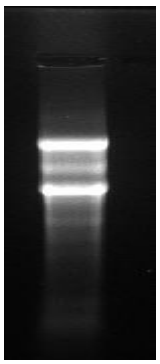

Fig. 25: RNA Gel

4.8 Gene expression profiling of the *Pb* and *fgr* genes

Total mRNA was extracted from leaf tissue and DNA copies (cDNA) of the mRNA molecules which were produced by the M-MLV reverse transcriptase enzyme. PCR amplification was carried out with the gene specific primers. The P (H1) value of the *Pb* gene was found to be 0.789 showing more than the normalized gene, 0.616 (Table 12) which implies that the mRNA of *Pb* gene is transcribed more than the actin. In the case of the *fgr* gene the P (H1) value was found similar with that of the actin, 0.616 (Table 12) which shows that the level mRNA transcribed of *fgr* gene and actin are same.

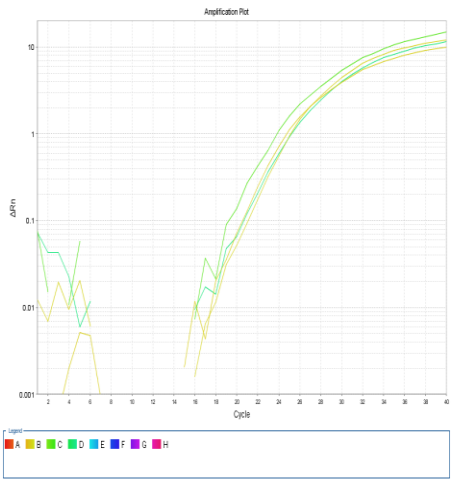

Fig. 26: Amplification plot of *Pb*

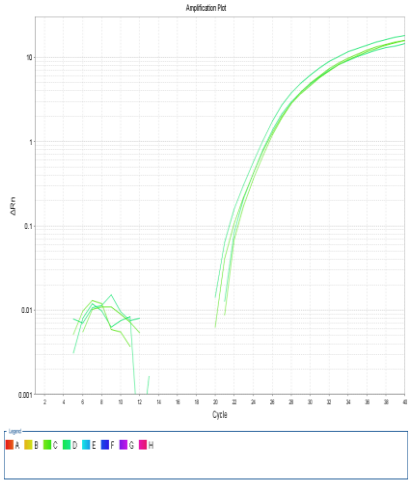

Fig. 27: Amplification plot of *fgr*

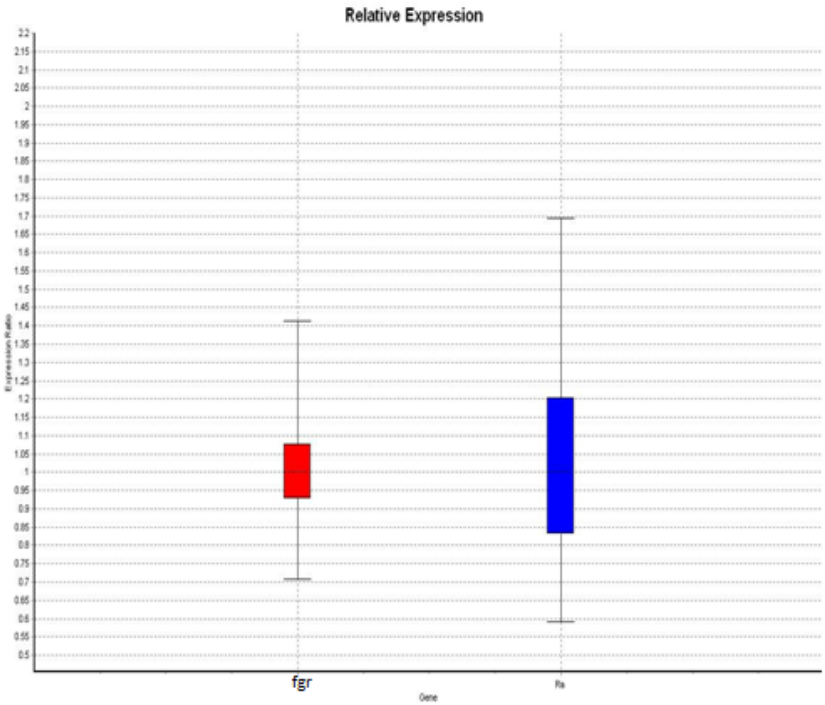

Fig. 28: Whisker-box plot

Table 11: Ct Value

	Actin (Reference)	*fgr*	*Pb*
Reaction-1	23.030	24.080	22.790
Reaction-2	23.500	24.440	22.770
Reaction-3	23.670	24.220	22.670

Table 12: Relative Expression Result (Fig. 28)

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)
Actin	REF	1.000	1.000	0.767 - 1.314	0.658 - 1.524	0.616
fgr	TRG	1.000	1.000	0.872 - 1.148	0.795 - 1.260	0.616
Pb	TRG	1.000	1.000	0.948 - 1.056	0.923 - 1.084	0.789