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Full Length Article

Agronomic, Transcriptomic and Metabolomic Expression Analysis of Aroma Gene (*badh2)* under Different Temperature Regimes in Rice

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Abstract

Aroma of rice is controlled by the *badh2* gene and its expression is highly influenced by the environmental factors, genotypic condition and cultivation practice. The effects of environmental factors such as salinity, water, aging, heat, cold and shading on 2AP concentration or *badh2* gene expression or agronomic traits were investigated individually but the effects of the optimum temperature (20°C to 30°C) were not studied extensively. Therefore, this study investigated the consequences of three different temperatures (ambient or 28.29 ± 0.91°C, 25°C and 20°C) on agronomic performance, *badh2* gene expression, 2AP concentration and phenotypic aroma score of five aromatic and a non-aromatic (control) rice genotype. The agronomic performance such as the flowering days (121.60), days to maturity (151.20), plant height (119.80 cm), panicle length (34.80 cm), fertile grain per panicle (151.40), 1000 grain weight (32.67 g) and grain yield per plant (76.73 g) were higher at 25°C temperature compared to the ambient and 20°C temperature. The maximum down-regulation of *badh2* gene (-12.20 ± 0.01 fold), the highest concentration of 2AP (0.14 ± 0.02 ppm) and the excellent phenotypic aroma score (score 4) was also observed at 25°C temperature. Hence, the *badh2* gene expression level and 2AP concentration were influenced by the temperature, which also regulated the phenotypic expression of aroma and agronomic performance of aromatic rice. This information will enlighten the consequences of optimum temperature and the possible reasons of variation in aroma quality of rice, which could open the possibility of high quality aromatic rice production around the globe. © 2017 Friends Science Publishers

Keywords: Temperature; Aroma gene; *badh2*; 2AP; Rice

Introduction

Aromatic rice is one of the most valuable agricultural crops across the world and often considered as the premium group of rice in the rice producing countries. The characteristics that have made it valuable crop socio-economically are the attractive grain quality, pleasant aromatic flavor, and high nutrient contents. It has been earmarked for containing essential amino acid, particularly lysine, leucine, phenylalanine, and methionine (Sekhar and Reddy, 1982; Cruz and Khush, 2000). Recently it has been reported that aromatic rice contains lower amount of arsenic compared to plain rice (Haris, 2013), thereby reducing the risk of arsenic intake in the arsenic polluted area. They also suggested that switching to aromatic rice will not only reduce arsenic intake but also will help to increase as much as 69% intake of beneficial zinc and selenium elements.

Aroma of rice is controlled by a major gene known as *fgr* or *badh2* gene present on chromosome 8 and expressed popcorn-like aroma only at the homozygous recessive condition (Kim *et al.*, 2003; Bradbury *et al.*, 2005). Chen *et al.* (2008) mentioned that homozygous recessive allele of *badh2* gene also induced the formation of 2AP and rice becomes aromatic. Thus, gene expression analysis of *badh2* gene using reverse transcription quantitative PCR (RTqPCR) with relative quantification method could explore the genetic condition of aromatic rice as well as the influences of temperature on aroma gene by analyzing biochemical and physiological changes of aromatic rice due to the changes of *badh2* gene expression level (Livak and Schmittgen, 2001; Kim *et al.*, 2003). In addition, the quantitative analysis of 2AP can correlate aroma gene expression and phenotypic aroma of rice.

However, aroma quality of aromatic rice depends on the genetic condition, environmental factors, their interaction (G×E) effects, and cultivation procedure. Shamim (2013) and Singh *et al.* (2000) reported that high humidity (70 to 80%) within a temperature range of 25°C to 35°C during vegetative growth stage, bright and clear sunny day with a temperature range of 25°C to 32°C at the initial flowering stage and comparatively, cooler night temperature (20°C to 25°C) with moderate humidity and gentle wind

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velocity at flowing and ripening stage are essential for proper grain and aroma development of aromatic rice (Chen *et al.*, 2008). Rohilla *et al.* (2000) also stated that grain quality of aromatic rice is highly influenced by the temperature but both the critically low (below 20°C) and high temperature (above 30°C) are destructive and adversely affects grain quality. However, within the range of optimum temperature (20 to 30°C) (Yoshida, 1981), the growth rate increase linearly until 25°C and after 26°C rice grain yield decrease progressively (Baker and Allen, 1993; Baker, 2004). In a study, Wang *et al.* (2014) stated that gene expression also affected by temperature and more than 50% genes (50.4% of all genes) expressed at 25°C while slightly fewer genes expressed at 30°C but they did not studied aroma gene expression in aromatic rice.

Generally, the high-quality aroma producing rice variety demonstrated lower agronomic performance and yield (Berner and Hoff, 1986). The grain yield of rice also depends on several agronomic traits such as the number of fertile tillers, days to flowering and maturity, grain filling period, plant height, panicle length, the number of fertile grain per panicle, 1000 grain weight and grain yield per plant (Halil and Necmi, 2005). For this reason, the production of excellent aroma producing rice with better agronomic performance is a difficult task for rice breeder and requires integrated approaches.

Previous researchers studied the effects of cold (Ghadirnezhad and Fallah, 2014), high-temperature (Islam, 2011; Shrivastava *et al.*, 2012; Rani and Maragatham, 2013; Aghamolki *et al.*, 2014), salinity (Fitzgerald *et al.*, 2008; Gay *et al.*, 2010; Wijerathna *et al.*, 2014), aging (Pisithkul *et al.*, 2010) and shading (Mo *et al.*, 2015) on 2AP concentration of aromatic rice. Some researchers tried to investigate the genetic and molecular basis of aroma (Sakthivel *et al.*, 2009), genetic and environment interaction for yield performance (Wirnas *et al.*, 2015). All these studies were limited to the agronomic performance or 2AP concentration or *badh2* gene expression analysis in aromatic rice but none was considered the integrative approach or observed the effects of temperatures belongs to the optimum temperature ranges. Therefore, this study investigated the consequences of three different temperatures (ambient or 28.29 ± 0.91°C, 25°C and 20°C) on morpho-agronomic performance, *badh2* gene expression, 2AP concentration and phenotypic aroma score of five aromatic and a non-aromatic (control) rice genotype.

Materials and Methods

Plant Materials

In the present research, five aromatic and a non-aromatic genotype were used to assess the agronomic performance, expression level of the *badh2* gene, quantification of 2AP and phenotypic aroma expression. The genotypes were collected from the Malaysian Agricultural Research Development Institute (MARDI) and the International Rice Research Institute (IRRI) as listed in Table 1.

Experimental Design and Growing Condition

The small plastic pots were filled with 500 g black soil and sown with rice seeds. After 3 weeks, the seedlings were transplanted into medium sized pails (30 × 90 × 30 cm) filled with loam soil. The recommended fertilizer dose of Razak *et al.* (2012) and Chatterjee and Maiti (1981) were followed as 15:15:15:2 of 100 g NPKS and the pails were placed inside the glass house of the Institute of Biological Sciences, University of Malaya, Malaysia, from April 2014 to August 2014 at a Latitude 3°8' N and a Longitude 101°40' E with an elevation of 104.0 m from the sea level. The rice plants were distributed into three temperature conditions (ambient or 28.29 ± 0.91°C, 25°C and 20°C) and the environmental weather data (Fig. 1) was also collected from the Meteorological Department of Malaysia (Meteorologi, 2014). The Completely Randomized Design (CRD) was used as the experimental layout with 3 replications of each treatment and recommended intercultural operations were followed for the normal growth of rice plant. The samples were collected at physiological maturity stage when rice grain formed brown color and kept at -80°C for investigation.

Growth Chambers

Three growth chambers (ambient, 25°C and 20°C) were made under transparent plastic tin shade room, orientated to east–west direction and were surrounded by the net. The growth chambers were 4.57×1.83×2.13 m in length, width and height, respectively while each chamber was 1.83 m away from another chamber to avoid mutual shading and to obtain sufficient sunlight. The structure of growth chamber consisted of thick and transparent plastic with steel structure. Each growth chamber was equipped with two air conditioners (Wall Mounted 1.5HP, 12,000 BTU/h, A5WM 15N/A5LC, Acson Malaysia Sales & Service Sdn. Bhd., Malaysia) except the ambient chamber. Two inlet and two outlet fans were placed at an upper portion of the chamber to adjust relative humidity. Four tube lights also placed at the top level for light supply and four doors were kept for cultural practice. The chambers remained closed and the air conditioner was turned on alternatively every 4 h to maintain 25°C and 20°C temperature while the ambient chamber did not contain air conditioner.

Data Collection for Agronomic Traits

The agronomic data for days to maturity, grain filling periods, flowering days (as soon as 50% of the flowers appeared), plant height (measured from ground level to the top of the node in cm), panicle length (cm), the number of fertile tillers, fertile grain per panicle, 1000 grain weight (g) and grain yield per plant (g) were collected. The SAS 9.3

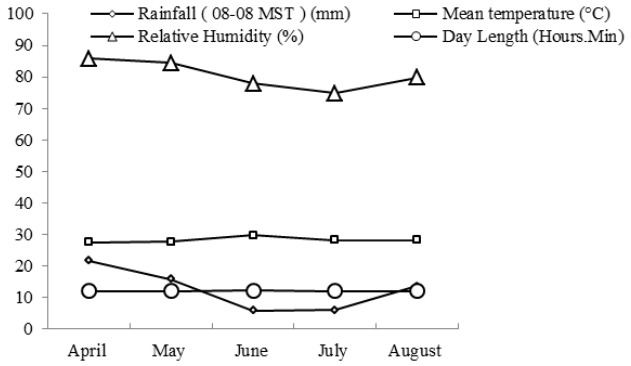

Fig. 1: Meteorological information of the experimental site (Average ambient temperature was recorded 28.29 ± 0.91°C during the study period) Source: Meteorological Department of Malaysia (Meteorologi, 2014)

Statistical Software (SAS Institute, Cary, NC) was used for the Duncan Multiple Range Test (DMRT) and the Minitab software (Minitab 16 Statistical Software, Sydney, Australia) was used for Analysis of Variance (ANOVA) measurements of agronomic traits.

RNA Extraction from Rice Grain

Rice grains contain a high level of polysaccharide so a modified CTAB method was followed for total RNA extraction from grains using Transzol™ plant RNA extraction kit (Transzol™ plant, Beijing Transgen Biotech Co. Ltd, China) following manufacturer's instruction. The quality and concentration of extracted total RNA were determined spectrophotometrically (Nano Drop 2000, Thermo Scientific, USA) at 260, 280, 260/280 and 260/230 nm. The reliability and purity of extracted RNA sample were also observed by 1% agarose gel electrophoresis system.

Primer

For this investigation, primers were designed by using Primer quest tools (Integrated DNA Technology Inc., USA, http://sg.idtdna.com/Primerquest/Home/Index) and prepared by My TACG Bioscience (Malaysia) as in Table 2. The complete nucleotide sequences of *Badh2* gene and *Actin* gene were obtained from the NCBI (National Center for Biotechnology Information, USA) with [Gene Bank Acc. No. EU770321 and X16280, respectively].

Real-Time Quantitative PCR

Real-time quantitative PCR amplification was conducted using RTqPCR (CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad, USA) on a total of 20 μl reaction volume containing SYBR green mix (GoTaq 1-step RTqPCR reaction mix, Promega Corporation, USA), primer (forward and reverse), nuclease free water and RNA template (100 ng). Negative control (without reverse transcriptase), no template control (without RNA) and positive control (without primer) were included with each reaction set, assayed triplicate for 3 biological replicates. The thermal cycling condition was 48°C for 15 min (reverse transcription), 95°C for 10 min (reverse transcription inactivation) followed by 40 cycles of 95°C for 10 sec (denaturation), 60°C/52°C (*Actin*/*badh2* respectively) for 30 sec (annealing), 72°C for 30 sec (extension). After amplification, the samples were kept at 95°C for 10 sec and 65°C for 5 sec then raised gradually by 0.5°C every 5 sec to obtain a melting curve.

Gene Expression Analysis

The amplification results from CFX Manager Software (Included with CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad, USA) were exported to Excel file and the quantification of gene expression was conducted according to the relative quantification methods of Livak and Schmittgen (2001). The C_T values of *Actin* gene was used as internal control (endogenous reference) and C_T values of non-aromatic rice genotype (MR 219) were used as calibrator (control). The Minitab software (Minitab 16 Statistical Software, Sydney, Australia) was used for the estimation of significance differences by ANOVA and descriptive statistics of the gene expression.

Solvent Extraction of 2AP Compound

Rice grains were hulled by hand and kept at -20°C to isolate 2AP volatile chemical component using solvent extraction method. One gram rice grains were grind into mortar and pastels using liquid nitrogen then transferred in a 125 mL conical flask containing 40 mL of 20 ppm 2, 4, 6-trimethylpyridine (TMP, Sigma-Aldrich Chemical Co., Germany) which was used as an internal standard (Tanchotikul and Hsieh, 1991; Mahatheeranont *et al.*, 2001). The concentration of TMP was prepared as 20.00 ppm by dissolving absolute TMP in a precisely measured volume of 0.1 M HCl. The mixture (40 mL TMP and 1 g rice) was stirred for 30 min and filtered into 50 ml centrifuge tubes. A total of 3 mL of 1.0 M NaOH was added to 25 mL filtrate to make the solution slightly basic then centrifuged at 6000 rpm for 10 min. The supernatant liquor was transferred to a 250 mL pear-shaped separatory funnel then 50 mL of dichloromethane was immediately added as an organic solvent. The extraction was conducted twice, resulting in 100 mL of dichloromethane solution. After drying over anhydrous sodium sulfate, the extract was concentrated to 1 mL using a rotary evaporator under reduced pressure (300 hPa) and a temperature of 26°C. The concentrated extract was transferred to a vial and 1 µL was taken for qualitative analysis by GC-MS and quantitative analysis by GC-FID.

Table 1: Description of studied six rice genotypes

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

Table 2: List of primers with expected RTqPCR product sizes

Primer name	Accession No.	Primer sequence	Sizes (bp)
Actin	X16280	Forward: 5C'- CTTCATAGGAATGGAAGCTGCGGGTA-3C' Reverse: 5C'-CGACCACCTTGATCTTCATGCTGCTA-3C'	196
badh2	EU770319	Forward: 5C'- TAGGTTGCATTTACTGGGAGTT-3C' Reverse: 5C'- ACAAACCTTAACCATAGGAGCA-3C'	75

Gas Chromatography-Mass Spectrometry

The samples were analyzed on a GC/MS system (GCMSQP2010W, Shimadzu, Japan) where Helium gas (purity 99.99%) at a pressure of 80 Kpa was used as the GC carrier gas. The injector and the GC/MS interface temperatures were set at 250°C and 220°C, respectively. The temperature of a DB5 capillary column (30 m × 0.25 mm id, film thickness 0.25 μm, J & W scientific, Folsom, CA) was programmed starting at 30°C after split less injection of samples. The initial temperature of 30°C held for 1 min, it was ramped to 185°C at 5°C/min. After hold for 2 min at 185°C, it increased to 220°C at a rate of 7°C/min and held there for 20 min. The effluent from the capillary column went directly into the mass spectrometer, operated in the electron impact (EI) mode with an ionization voltage of 70 eV and the ion source temperature was 220°C. The extracted and concentrated 2AP volatile compound from rice grains were identified by comparing its mass spectra with mass spectral data of standard compound in the Wiley 10 + NIST 14 combined library. The 2AP further confirmed by the GC retention time with standard 2AP compounds. The similar protocol was used for quantitative analysis of 2AP using GC-FID (GC-FID QP2010W, Shimadzu, Japan) except the synthetic and purified 30 ppm of 2AP standard solution which was made by using precisely measured volume of 0.1 M HCl and was collected from BOC Science (BOC Science, Ramsey Road Shirley, NY 11967, USA).

Sensory Aroma Evaluation

The aroma score of the studied genotypes was estimated according to the methods of Golam *et al.* (2010). At room temperature, forty grains of each genotype were soaked in a covered glass petri dish containing 10 ml 1.7% KOH solution for an hour. The sample was scored on 1–4 scale where 1, 2, 3 and 4 corresponding to an absence of aroma, mild aroma, moderate aroma, and strong aroma, respectively. All the samples were sniffed by three well-trained panelists.

Results

Effect of Temperature on Agronomic Traits

The agronomic traits of the studied genotypes such as days to flowering and maturity, the number of fertile tillers, fertile grains per panicle, grain filling period, plant height, 1000 grain weight and grain yield per plant showed that (Table 3) all rice genotypes exhibited differential grouping under the same temperature for different traits as well as for the same trait at the different temperature. Moreover, the highest and the lowest values for a trait were observed in different genotypes under different temperature (Table 3). The lowest values for the number of fertile tiller per hill in Rato Basmati, grain filling periods (8.20 d) in MRQ 50 and days to maturity (117.00 d) in Ranbir Basmati genotype were observed at ambient temperature. While, at 25°C temperature, the maximum value was observed for the number of fertile tiller (43.00), fertile grain per panicle (151.40), 1000 grain weight (32.67 g) and grain yield per plant (76.73 g) in E 13 genotype. Similarly, at 25°C, the highest value was for the flowering time (121.60 d), days to maturity (151.20 d), plant height (119.80 cm) and panicle length (34.80 cm) in Rato Basmati genotype. Conversely, the lowest value for flowering time (79.20 d), panicle length (16.60 cm) and 1000 grain weight (15.19 g) in E 13 genotype, for plant height (60.00 cm) in MRQ 50, while for fertile grain per panicle (33.20) and grain yield per plant (24.97 g) in Rato Basmati genotype were observed at 20°C temperature. The prolong grain filling periods (26.60 d) was observed in E 7 genotype at 20°C temperature. Thus, temperature influenced the agronomic performance of rice genotypes, which were distributed into different groups.

Effects of Temperature on Transcriptomic Expression of *badh2* Gene

The relative expression of the *badh2* gene transcriptome in aromatic rice genotypes was compared to non-aromatic rice genotype (control, MR 219 genotype) normalized by the *Actin* gene (internal control) demonstrated different levels of

Table 3: Differences of agronomic performance of rice genotypes at different temperature

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

Means with the same letter are not significantly different at 5% level ($p = 0.05$) by Duncan's Multiple Range Test (DMRT). Here, NFTH = Number of fertile tiller per hill, FD = Flowering days, GFP = Grain filling periods, DM = Days to maturity, PH = Plant height (cm), PL = Panicle length (cm), GP = Grain per panicle, FGP = Fertile grain per panicle, TGW = Thousand Grain weight (g), GYP = Grain yield per plant (g)

Table 4: The *badh2* gene expression (fold), 2AP concentration (mg kg^{-1}) and aroma (score) in rice genotypes under different temperature

Genotype	Ambient			25°C			20°C		
	badh2 (mean ± SD)	2AP (mean ± SD)	Aroma (score)	*badh2* (mean ± SD)	2AP (mean ± SD)	Aroma (score)	*badh2* (mean ± SD)	2AP (mean ± SD)	Aroma (score)
MRQ 50	-2.66 ± 0.03	0.09 ± 0.01	3	-7.32 ± 0.01	0.08 ± 0.02	4	-1.91 ± 0.03	ND	2
Ranbir Basmati	-1.15 ± 0.05	0.01 ± 0.01	3	-10.08 ± 0.01	0.10 ± 0.01	4	-1.10 ± 0.23	0.04 ± 0.02	2
Rato Basmati	-2.49 ± 0.04	ND	3	-12.20 ± 0.01	0.13 ± 0.02	4	1.15 ± 0.22	ND	2
E 7	-1.57 ± 0.08	ND	3	-10.78 ± 0.03	0.09 ± 0.03	4	-1.05 ± 0.15	ND	2
E 13	-1.10 ± 0.05	ND	3	-7.29 ± 0.02	0.14 ± 0.02	4	-1.21 ± 0.28	ND	2
MR 219	1.01 ± 0.18	ND	1	1.00 ± 0.07	ND	1	1.02 ± 0.22	ND	1

ND = not detected, - values = down-regulated, aroma score 1 = no aroma, 2 = mild aroma, 3 = Moderate aroma, 4 = strong aroma

expression (Table 4), while the control has unique fold measurement (almost 1.00 fold). A medium down-regulation of the *badh2* gene (-2.66 ± 0.03 fold) was observed in MRQ 50 genotype in ambient condition. The maximum down-regulation of the *badh2* gene (-12.20 ± 0.01 fold) was observed in E 7 genotype at 25°C temperature. The minimum expression was observed in MRQ 50 genotype (-1.91 ± 0.03 fold) at 20° temperature.

All aromatic genotypes demonstrated higher down-regulation of the *badh2* allele at 25°C temperature compared to 20°C and ambient condition. Therefore, the molecular analysis of *badh2* gene expression revealed that the down-regulation of the recessive *badh2* allele was responsible for aroma status of the studied genotypes and the expression of the *badh2* gene was regulated by the environmental temperature.

Effect of Temperature on Metabolomic Concentration (2AP Concentration)

During quantitative analysis of 2AP metabolite, genotype Ranbir Basmati produced 2AP in all three conditions and MRQ 50 genotype produce 2AP at ambient and 25°C temperature while all other aromatic genotypes produced 2AP at 25°C only (Table 4). The non-aromatic genotype MR 219 did not produce 2AP in any of the three conditions. The quantitative analysis represented that the 25°C temperature was suitable for the production of the highest amount of 2AP in all aromatic genotypes.

Effect of Temperature on Phenotypic Expression of Aroma

The phenotypic aroma expression evaluated by the organoleptic test of grain represented different aroma score of the same genotype under different temperature (Table 4).

All aromatic genotypes demonstrated strong aroma (score 4) at 25°C temperature, which was higher than other two conditions (ambient and 20°C) in case of all aromatic genotypes (Table 4) except the non-aromatic MR 219, scored 1 in all conditions. The organoleptic evaluation of aroma also represented that the 25°C temperature was a suitable temperature for the maximum phenotypic expression of aroma in aromatic rice genotypes.

Table 5: Analysis of variance for the *badh2* gene expression (fold), 2AP concentration (mg kg^{-1}) and aroma (score) of rice genotypes under different temperature

Source	df	*badh2*		2AP		Aroma	
		F-value	P-value	F-value	P-value	F-value	P-value
Genotype	5	2.21^{ns}	0.13	0.90^{ns}	0.51	12.00^{**}	0.00
Temperature	2	15.59^{**}	0.00	8.90^{**}	0.00	25.00^{**}	0.00

Statistical significance $^{**}p \leq 0.01$ at 1% level, df = degrees of freedom, F = F value, p = probability value, ns = non-significant

Table 6: Quantitative analysis results for 2AP of previous researchers

Researchers	Experimental observation	2AP concentration (mg kg^{-1})
Wongpornchai *et al.* (2003)	KDML 105 brown rice seeds	3.000
Mahatheeranont *et al.* (2001)	Fresh brown rice	0.340
	Brown rice stored for 12 months	0.120
	Brown rice from local market (no brand)	0.320
	Milled rice (Surintip brand)	0.250
	Milled rice (Matusorn brand)	0.120
	Milled rice (changchuroungkhao brand)	0.050
Bergman *et al.* (2000)	Basmati easy cook (Tilde) Milled	0.019
	Jasmin (Fantastic foods) Brown	0.550
	Amber aromatic (Lundberg) Brown	0.345
Laksanalamai and Ilangantileke (1993)	Fresh aromatic	0.10 (Peak area ratio)
	Aged aromatic	0.05 (Peak area ratio)
	Non-aromatic	0.00 (Peak area ratio)
Buttery *et al.* (1988)	Cooked rice	0.0006

Differences of the Effects of Temperature on *badh2* Gene Expression, 2AP Concentration and Aroma Score

The significant levels of the effect of temperature on the expression of aroma gene, 2AP concentration and aroma score were analyzed by using ANOVA test (Table 5), where highly significant differences ($p \leq 0.01$) at 1% level were observed for different temperatures. Conversely, the genotypes did not show differences for *badh2* gene expression and 2AP concentration.

The Table 5 signifies that the expression level of *badh2* allele and 2AP concentration depends on the growing temperature and their level of expression did not differ significantly among aromatic genotypes within the same temperature.

Discussion

Experimental evidences show that aroma of rice is controlled by a major gene named as *badh2* known to be highly influenced by environmental components especially the temperature, which also might influence aroma quality, 2AP concentration and agronomic performance (Hashemi *et al.*, 2013). Hence, several researchers studied the effects of high and low temperature stress on 2AP concentration and agronomic performance of aromatic rice (Islam, 2011; Xu *et al.*, 2012), while some researchers tried to identify the range of the optimum temperature for rice production (Yoshida, 1981; Singh *et al.*, 2000). Until now, no comprehensive study has been conducted to observe the effects of optimum temperature on aroma gene expression, 2AP concentration as well as agronomic performance of aromatic rice, which might focus on the variation in aroma quality and aromatic rice production in rice growing countries all over the world. Therefore, this experiment was conducted to investigate the consequences of three different temperature i.e., ambient temperature of the rice growing area (28.29 ± 0.91°C), upper limit temperature for increasing growth rate of rice (25°C) and lower limit temperature for unaffected grain quality (20°C) that belongs to the range of the optimum temperature for aromatic rice production based on the information of Singh *et al.* (2000), Yoshida (1981), Baker and Allen (1993) and Baker (2004). The effects of these temperatures were assessed to evaluate superior aroma expression of aromatic rice in terms of the phenotypic, transcriptomic, metabolomic and agronomic performance analysis.

Gene expression analysis of *badh2* gene, gas chromatography-mass spectrometry assessment for quantitative analysis of 2AP combined with organoleptic analysis of phenotypic aroma represented that down-regulation of the recessive *badh2* allele is responsible for the significant elevation of 2AP concentration and phenotypic aroma expression in the studied aromatic rice genotypes. Though, Niu *et al.* (2008) observed that down-regulation of dominant *Osbadh2* transcripts in the transgenic plants resulted in higher 2AP concentration but the results of the present experiment were similar to the results of Chen *et al.* (2008) who stated that the presence of dominant *Badh2* allele encoding Betaine Aldehyde Dehydrogenase (BADH2) inhibited synthesis of 2AP, while its recessive alleles induced 2AP formation. The result of this study also revealed that the dominant *Badh2* was more abundant in non-aromatic rice compared to aromatic rice as similar the

information of Chen *et al.* (2008). Formerly, Bradbury *et al.* (2005) stated that aroma is a recessive trait and a loss of function of dominant *Badh2* gene is responsible for the accumulation of 2AP in aromatic rice. Perozich *et al.* (1999) studied the nature and character of *Badh2* allele and stated that it belongs to the aldehyde dehydrogenases (ALDH) superfamily which is also involved in the responses to environmental stresses and tolerance character of the plant (Sophos and Vasiliou, 2003). Although the biochemical synthetic pathway leading to aroma production in rice has not been recognized completely yet but the recessive condition of aroma allele (*badh2/badh2*) and the accumulation of aroma compounds due to the loss of function of dominant *Badh2* allele (Bradbury *et al.*, 2005; Chen *et al.*, 2008) were well explained by the present experiment (Table 4). Moreover, the down-regulation of recessive *badh2* gene expression, which reduced the mRNA transcripts and helped to accumulate the 2AP compound for the aromatic flavor was observed to be influenced by the temperature.

Previous researchers (Buttery *et al.*, 1988; Laksanalamai and Ilangantileke, 1993; Bergman *et al.*, 2000; Mahatheeranont *et al.*, 2001; Wongpornchai *et al.*, 2003) performed characterization, qualitative analysis and quantitative analysis of volatile compounds present in rice. The available information for quantitative estimation of 2AP concentration in different types of rice varieties is listed in Table 6 to compare the results of the present study.

In this investigation, the obtained concentrations of 2AP were ranged from 0.00 to 0.14 mg kg^{-1} depended on temperature condition and rice variety (Table 4), which were within the range and similar as the observation of previous researchers mentioned in Table 6. Similarly, the phenotypic aroma score of studied genotypes were also dependent on temperature and variety (Table 4).

The morphological and agronomic characters of all studied genotypes varied with temperature condition (Table 3). The ambient temperature highly influenced the number of fertile tillers per hill, grain filling period and days to maturity while 20°C temperature reduced days to flowering, plant height, panicle length, fertile grains per panicle, 1000 grain weight and finally the grain yield. On the other hand, 25°C facilitated better vegetative growth as well as higher grain yield which were according to observation of Golam *et al.* (2011) for aromatic rice varieties. Previously, Aghamolki *et al.* (2014) studied the influences of high temperature on different growth stages of rice and observed that heat stress reduced yield when it imposed during booting and flowering stage. They also mentioned that heat stress did not affect later growth stages (ripening stage) of rice. Zhang *et al.* (2013) stated that a mild increase of night-time temperature during reproductive growth stage reduced yield and performances of yield-related traits. Similar observation was also pointed out by Shrivastava *et al.* (2012) and Islam (2011) who mentioned that high temperature at booting and grain filling stage reduced growth rate and grain yield of aromatic rice.

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

Conclusion

The present investigation revealed the consequences of three different temperatures belong to the range of optimum temperature for normal growth of rice plant to evaluate the effects of optimum temperature on aroma quality of rice. The relative gene expression analysis of *badh2* gene, gas chromatography-mass spectrometry assessment for quantitative analysis of 2AP combined with organoleptic analysis of phenotypic aroma represented that the down-regulation of the recessive *badh2* allele was responsible for the significant elevation of 2AP concentration as well as the phenotypic aroma in the studied aromatic rice genotypes influenced by the temperature condition. This experiment also identified that the variations in the optimum temperature in the rice growing areas during aromatic rice growing seasons might be a possible reason for differences in aroma quality of the aromatic rice genotypes.

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