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DETECTION OF GENE BADH2 IN CHARGE OF AROMATICS IN IRAQI RICE VARIETIES

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Abstract

Two field experiments were carried out during two agricultural seasons 2016 and 2017 in the rice research station in Mashkhab (province of Najaf Al-Ashraf) related to the Agricultural Research office. The agricultural season of 2016 included the cultivation of three varieties of rice, which they were Anber -33, jasmine, and Mashkhab -2, for the purpose of selection basis on aromatic for plants characterized by high aromatic of the two aromatic varieties (Anber 33 and Jasmine), and the detection of the badh2 gene in charge of aromatics in the three varieties using Polymerase Chain Reaction (PCR) technology and analysis of nucleotide sequences. While in the agricultural season 2017, and for the purpose of evaluating the performance of genotypes (selected and origin) by gene expression of badh-2 gene, and to know the role of chemical fertilization NPK by gene expression of badh-2 gene in Anbar 33 using Real time-quantitative polymerase chain reaction (RT-qPCR) technique. Randomized Complete Block Design (RCBD was used with three replicates, included 8 treatments was (Mashkhab 2 under the fertilization level NPK0, Jasmine origin under the fertilization level NPK0, Anber 33 origin under the fertilization level NPK0, Jasmine selected based on aromatic under the fertilization level NPK0, Anber 33 selected based on aromatic under the fertilization level NPK0, Anber 33 origin under fertilization level NPK1(70, 23, 25 kg N, P_2O_5, K_2O h^{-1}), Anber 33 origin under fertilization level NPK2(140, 46, 50 kg N, P_2O_5 and K_2O h^{-1}), Anber 33 origin under fertilization level NPK3(280, 92, 100 kg N, P_2O_5 and K_2O h^{-1}). The results of PCR reaction showed that there are bands with a molecular weight of 752bp in the three varieties representing the badh-2 gene, the nitrogenous base sequence identical revealed that the badh2 gene in two aromatic varieties Anber 33 and Jasmine was truncated and incomplete, while it was completely in the non - aromatic variety. The results of RT-qPCR showed that the gene expression of badh2 gene decreased in the two aromatic varieties compared to the non - aromatic variety, in addition, the chemical fertilization (NPK2 and NPK3) caused a significant increase in the gene expression of badh2 in Anber 33. Therefore can conclude that the presence of allele badh2 dominant biosynthesis of 2acetyl-pyrrolne (2AP) which is the main aromatic compound causing aromatic for rice, whereas the truncated recessive allelees of the badh2 stimulate the formation of 2AP, and the expression of the badh-2 gene increases with increasing levels of chemical fertilization therefore decreases the aromatic degree.

Key words: Gene Badh2, Aromatic, Iraqi Rice

Introduction

Aromatic is one of the most important characteristics of the food product because of its preference by consumers, aromatic and non-aromatic rice contains more than 100 volatile compounds, which includes alcohols, aldehydes, ketones, acids, esters, alveolate, pyridines, and other compounds (Grimm *et al.*, 2001). Whereas, Acetyl - 1Pyrrolin (2AP) is the most compound that contributes to gives smell to aromatic varieties compared to its low concentrations in non-aromatic varieties, and It is characterized by a lower odor threshold than other volatile compounds (Hashemi *et al.*, 2013). The fgr gene plays an important role in 2AP biosynthesis (Sakthivel *et al.*, 2009), where the only inhibitor of 2AP formation is the complete enzyme badh2 which coded by a completed transcript of the badh2 gene (Juwattanasomrau *et al.*, 2011). The badh2 gene is located on the long arm of the chromosome 8 (Jewel *et al.*, 2011) which coding the BADH2 enzyme (Amarawathi *et al.*, 2008). (Vanavichit *et al.*, 2010) observed that, this position in non-aromatic varieties contain 15 exons, consisting of coding sequences translated into 503 amino acid sequences, and thus the protein synthesis or betaine aldehyde dehydrogenase enzyme. While in aromatic varieties, this region, and within the seventh exon there is an omitting of a number of

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nitrogenous bases, which causes the early termination of gene, as well as inhibition of gene expression and the production of truncated non-functional enzyme BADH2. Therefore, it will not be able to oxidize 4-aminobutanl, causing 1-pyroline accumulation and thus increase the 2AP biosynthesis. More than 18 allelees of badh2 were recorded in the chromosome 8 that associated with aromatic rice (Kovach *et al.,* 2009; He *et al.,* 2017; Priyadarshini *et al.,* 2018), where (Shi *et al.,* 2008) pointed out that the badh2-E2 allele involves omitting of 7bp linked to show the aromatic trait. In addition to the allele badh2-E7, the badh2-E7 is the main allele which showed more correlated with the aromatic trait (Bradbury *et al.,* 2005), and it includes the omitting of 8 nitrogenous bases and 3 SNPs in the aromatic varieties (Karami *et al.,* 2017). Therefore, this study aimed to investigate the variations in the badh2 gene for some Iraqi rice varieties, as well as study the effect of selection and chemical fertilization (NPK) on gene expression of badh -2.

Materials and Methods

Two field experiments were carried out during two agricultural seasons 2016 and 2017 in the rice research station in Mashkhab (province of Najaf Al-Ashraf) related to the Agricultural Research Office, in order to detect the gene badh2 in charge of aromatic in rice and to know the effect of the selection and chemical fertilization on gene expression of badh2.

Agricultural season 2016

Three varieties, Anber-33, Jasmine and Mashkhab2, were planted with the aim of selecting on the basis of aromatic under intensity of 10% for plants characterized by high aromatic, which were evaluated according to (Sood and Sadic, 1978) method, for the aromatic varieties (Anber 33 and Jasmine), to represent the seeds of the first selection cycle. All soil and crop service operations were carried out in accordance with agricultural recommendations. Moreover, in order to detect the gene badh2, which is in charge of aromatic in the three varieties, Polymerase Chain Reaction PCR technique was used to amplify the aromatic gene and analyze the sequence of nucleotide of the three varieties by following these steps:

Extraction of Deoxyribose Nucleic Acid (DNA) from plant

DNA was extracted from the plant's leaves for three varieties of rice two aromatic varieties, Anber 33 and Jasmine with a non-aromatic variety, Mashkhab2, in the flowering stage. The clean leaves and free of pathogens were taken, then marked, washed, sterilized and stored in sterile plastic bags using Quick-DNA ™ Plant / Seed Miniprep Kit, manufactured by zymo research USA company, then the instruction manual was used to extract the DNA (kit No D6020), while the purity of the extracted DNA is assessed by the spectrophotometer. Furthermore, the absorption of the DNA sample was calculated at the wavelength of 260 nm ($O.D_{260}$), and the absorption of the same sample then calculated at the wavelength of 280 nm ($O.D_{280}$), where the ratio between the wave reading (260 nm) to (280 nm) helps to evaluate the purity of DNA, and it ranging from 1.8 to 2.0 for pure DNA (Sambrook *et al.,* 1989), as the following equation:

$$purity\ of\ RNA = \frac{O.D_{260}}{O.D_{280}} \geq 2$$

Polymerase Chain Reaction (PCR) technique to amplify the aromatic gene

PCR test was conducted using Maxime PCR PreMix kit (i-Taq) (Kat. No 25025) supplied by Korean company iNtRoN to the extracted DNA for the three rice varieties (Mashkhab2, Jasmine and Anber 33). The polymerase chain reaction was perform with a total volume of 25 ìl contained with the components listed in Table 1, and the volume was completed with Nuclease-free water to 25 µl.

The reaction mixture was prepared in a sterile tube (a tube for each genotype and a Negative DNA tube), where its components were mixed using a micro pipette and placed in a centrifuge to preserve the final volume of the reaction mixture, then placed in the thermal polymerization device PCR, and the program that described in Table 2 was implemented for the purpose of amplifying the badh2 gene. Finally, the tubes were lifted after the end of time and placed in the refrigerator until electrophoresis

Electrophoresis of PCR product using Agarose gel

Electrophoresis was carried out to determine DNA fragments after the polymerase reaction process sequence of the resulting samples and the DNA Ladder marker. A 1 g of Agarose was mixed and dissolve it in 100 ml of electrophoresis buffer (0.5x TBE), then placed in the beaker of (500 ml) and the volume completed with distilled water. It was heated till boiling and left to decrease at the temperature of (40-50 ^{0}C), then a 2 µl of Red Save dye was added to it. In the meantime, DNA samples (3µl) was prepared by mix it with a (5µl) loading buffer, and the template was prepared then the comb placed in one of its ends to create holes inside the Agarose gel layer and then dissolved Agarose poured into the template and left to harden at room temperature. After completing the hardening of the Agarose gel layer, the comb was

Table 1: PCR reaction mixture components concentrations.

Seq.	Components	Concentration
1	Taq PCR PreMix	5µl
2	Forward primer	1ML
3	Reverse primer	1ML
4	DNA	1-1.5µl
5	Distill water	16.5 µl
Final volume	25µl	

Table 2: Program of PCR conditions.

Seq.	Stage	Temperature (^{0}C)	Time	No. of cycle
1	Initial Denaturation	95R"C	3 min.	1
2	Denaturation -2	95R"C	45sec	
3	Annealing	64R"C	1 min	
4	Extension-1	72R"C	1 min	
5	Extension -2	72R"C	7 min.	1

removed carefully without distortion or crushing of the holes, the template returned in its place at the Electrophoresis device and the buffer solution (1X TBE) was added to electrophoresis basin until the Agarose layer has been submerged in about 1mm height. Moreover, 5 µm of the amplified DNA by PCR was added from each sample to each hole of the Agarose gel layer, and 5 µl was added from the 100bp DNA Ladder produced from the Kapa /USA (Cat. KK No6302) company, to the hole on the left side of the added samples to help determine the volumes of the amplified DNA and then the electrodes was connected into the power and operate the power supply at 120 mA for 1 hour and half. After completing the samples electrophoresis, the Agarose gel layer containing the amplified DNA products and dyed with the Red Save dye was tested under ultraviolet rays and took pictures to it.

Analysis of nucleotide sequences of DNA for rice varieties

For the purpose of knowing the sequence of nucleotide of amplified DNA products by PCR of the three rice varieties (Mashkhab 2, Jasmine and Anber 33), the DNA products were sent to the Korean company (Macrogen) with the forward Primer which is used in the process of DNA amplification. The nucleotide sequences for amplified DNA product was then entered into the available database at the National Center of Biotechnological Information (NCBI) using the Finch Tv program, as well as, the sequence of nitrogenous bases is also matched by the Bio Edit program.

Primer

Primer (NC008401) which is specific for detection the badh2 gene, and its sequence are shown in Table 3.

Agricultural season 2017

For the purpose of evaluating the performance of genotypes (selected and origin) by gene expression of badh-2 gene, and to know the role of chemical fertilization NPK with gene expression of badh-2 gene in var Anber 33 using Real Time-quantitative polymerase chain reaction (RT-qPCR) technique. The Randomized Complete Block Design RCBD was used with three replicates, including 8 treatments as follows:

1- Mashkhab 2 under the fertilization level NPK0.

2- Jasmine origin under the fertilization level NPK0.

3- Anber 33 origin under the fertilization level NPK0.

4- Jasmine selected based on aromatic under the fertilization level NPK0.

5- Anber 33 selected based on aromatic under the fertilization level NPK0.

6- Anber 33 origin under the fertilization level NPK1 (70, 23, 25 kg N, P_2O_5, K_2O h^{-1}).

7- Anber 33 origin under the fertilization level NPK2 (140, 46, 50 kg N, P_2O_5 and K_2O h^{-1}).

8- Anber 33 origin under the fertilization level NPK3 (280, 92, 100 kg N, P_2O_5 and K_2O h^{-1}).

Conducted a number of contrasts comparisons between the treatment previous. The focus has been on the local Anber33 variety, which is the Iraq's most common aromatic rice, and adapted to the Iraqi environment since long times, as the area under cultivation were approximately 90% of the total area cultivated with rice.

RNA extraction

When the plants reach the flowering stage, 10 samples (flag leaf) were taken for each treatment of the experimental treatments, clean leaves and free of pathogens were taken, then marked, washed, sterilized, and stored in sterile plastic bags using ZR Plant RNA MiniPrep™ kit, manufactured by ZymoUSA companyaccording to the manual instruction to extract the RNA (Kat. No R2024). The purity of the RNA was evaluated by a spectrophotometer device, where the

Table 3: Sequences of nucleotide for specific primer.

Primer	Sequence	Tm (R"C)	GC (%)	Reaction volume	Reference
Forward	5'- TTTTTCCACCAAGTTCCAGTG- 3'	52.2	45	752base pair	Karamia *et al.*, (2016)
Reverse	5'- TGAGAATCATGTTCGGGATG- 3'	53.5	42.9		

absorption of the RNA sample was calculated at the wavelength of 260 nm (O.D260) and then the absorption of the same sample at the wavelength of 280 nm (O.D280) were also calculated. The ratio of wave reading (260 nm) to (280 nm) helps to evaluate the purity of the RNA. This ratio ranges between or greater than or equal to 2 for pure RNA (Sambrook *et al.*, 1982) according to the following equation:

$$purity\ of\ RNA = \frac{O.D_{260}}{O.D_{280}} \geq 2$$

Relative gene expression for badh2 gene

Real Time-quantitative polymerase chain reaction (RT-qPCR) test for the study treatments were conducted using the GoTaq® Probe RT-qPCR Master Mix (Cat. No A6120) kit equipped from Promega Company. The required volume of all components of the (RT-qPCR) was calculated according to Table 4. The components were mixed with a vortex device at a speed of 3000 cycles / min for 10 seconds, then placed in RT-qPCR and the program was executed as listed in Table 5.

At the end of reaction, the data were analyzed according to the Livak and Schmittgen (2001) method to estimate the relative gene expression of badh2 gene through the following equations:

$\Delta_{ct} = Ct_{badhz} - Ct_{GAPDH}$

$\Delta\Delta_{ct.control} = \Delta_{ct.test} - \Delta_{ct.control}$

Table 4: Components of the Real Time-quantitative polymerase chain reaction (RT-qPCR).

Seq.	Components	Concentration (µl)
1	GoTaq® Probe RT-qPCR Master Mix	10 µL
2	Forward primer of reference gene	1.8 µL
3	Reverse primer of reference gene	1.8 µL
4	Probe of the reference gene	0.4 µL
5	Forward primer of target gene	0.4 µL
6	Reverse primer of target gene	0.4 µL
7	Probe of the target gene	0.4 µL
8	Reverse transcriptase	0.4 µL
9	Nuclease-free water	Up to 20 µL
10	RNA Sample Volume	2µL
Final volume		20 µL

Table 5: RT-qPCR Conditions Program.

Step	Temperature ^{0}C	Time	Number of cycle
Reverse transcription	42 °C	10 min	Hold
Enzyme activation	95 °C	3 min	Hold
Denaturation	95.0 °C	15 sec	40
Annealing/Extension	58.0 °C -	15 sec	

Relative gene expression = $2^{-\Delta\Delta Ct}$

Since:

Ct_{badhz} : Is the cycle threshold for the target gene (badh2)

Ct_{GAPDH} : Is the cycle threshold for the reference gene (GAPDH).

$\Delta_{ct.test}$: Is the difference between cycle threshold for the gene badh2 and the reference gene for the tested samples.

$\Delta\Delta_{ct.control}$: Is the difference between the cycle threshold for the badh2 gene and the reference gene for comparison samples.

Results and Discussion

Amplification of the badh-2 gene using PCR technique

After the PCR reaction conditions were prepared, the products of rice varieties (Mashkab 2, Jasmine and Anber 33) were electrophoresis over to the Agarose gel in the existence of 100bp DNA Ladder. The results of Figure 1 showed the presence of a band with a molecular weight of 752bp in the three varieties, representing the gene badh-2 in charge of the appearance of the aromatic trait. (Karami *et al.*, 2016) confirmed through their study of 5 aromatic varieties and 6 non-aromatic rice varieties that the gene badh2 with a molecular weight of 752bp was in charge of aromatic trait in rice varieties. This was consistent with the recommendation of (Bradbury *et al.*, 2005) to design a specific Primer for the exon 7 to amplify

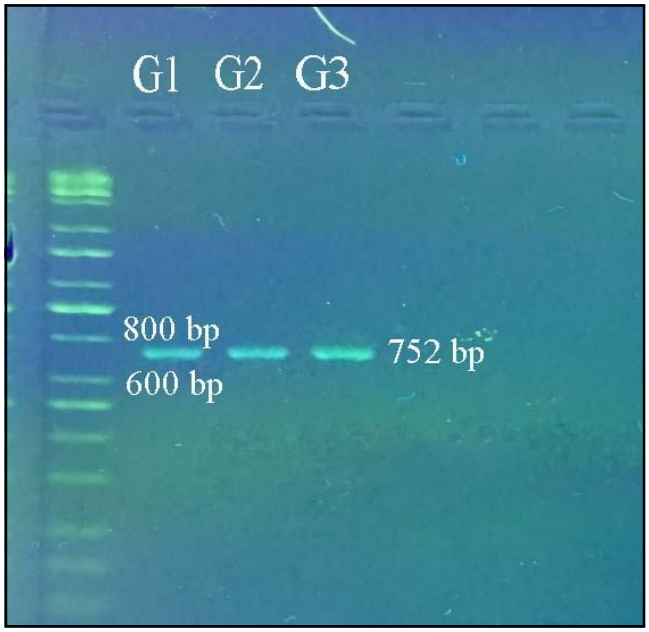

Fig. 1: PCR reaction products electrophoresis of the NC008401 primer for the three genotypes of rice: Anber 33 (G1), Jasmine (G2) and Mashkhab 2 (G3).

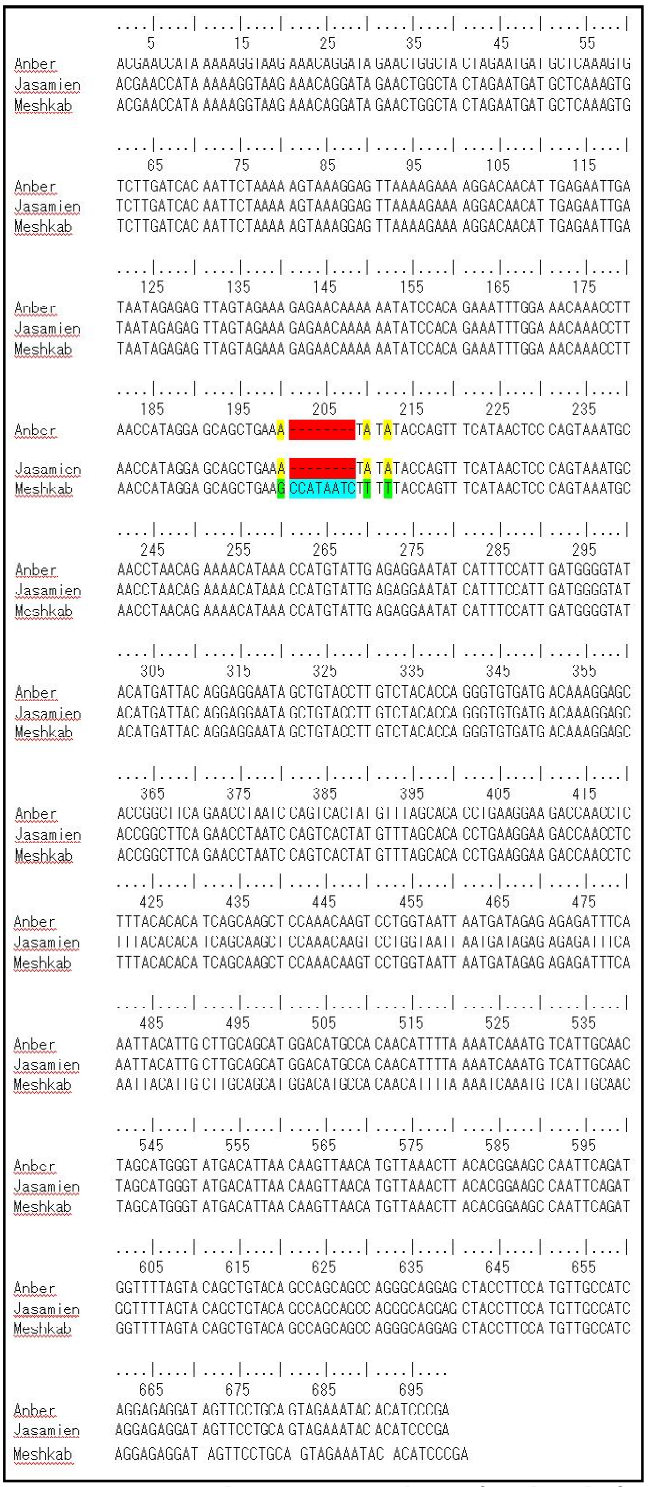

Fig. 2: Comparing the sequence analysis of nucleotide for rice varieties (Mashkhab 2, Anber 33 and Jasmine).

the badh2 gene, the results observed that the appearance of a band with a molecular weight of 752-bp representing this gene, the emergence of this gene in Mashkhab 2, which required analysis for nucleotide sequences.

Analysis of nucleotide sequences of badh2 gene in rice varieties

The results of the analysis of nucleotide sequences of PCR-amplified product of three rice genotypes (Anber 33, Jasmine and Mashkhab 22) using the BLAST program proved that its belong to the badh2 gene that in charge of aromatic trait. By comparing the sequence of nucleotide of the badh2 gene of Anber 33, jasmine and Mashkhab 2 with the sequences of nucleotide of other rice genotypes that previously recorded in the NCBI database, that the two varieties, Anber 33 and jasmine, gave a highest similarity amounted 100% with the aromatic genotype SyYuNuo cultivated in China and registered under the reference number EU770320.1. The non-aromatic variety Mashkhab, gave the highest similarity of 100% with the non-aromatic (Nipponbare) genotype grown under the reference number AP014964.1. The results of comparing sequence of nucleotide for badh2 gene in Fig. 2 showed that the similarity between Anber 33 and Jasmine was 100%, and between Anber 33 and Mashkhab 2 amounted 98.71%, and between Jasmine and Mashkhab 2 reached 98.71%. Where the two varieties of aromatic (Anber 33, and Jasmine) were different from the non-aromatic variety (Mashkhab) with the presence of a single nitrogen base substitution mutation on site 195 in aromatic varieties (A) replaced by (G) for a non-aromatic varieties, as well as the omitting of 8 nucleotide (CCATAATC) at site 196 - 203 in aromatic varieties compared to its presence of non-aromatic varieties. The results also showed the presence of a single nucleotide replacement mutation at two sites 205 and 207 for the aromatic varieties (A) were replaced by (T) in non-aromatic varieties.

The presence of Nucleotides omitting in the aromatic allele makes BADH2 free of many C-terminal SKL carboxylic acids endings, which believes that C-terminal SKL to be important in linked the substrate with enzyme badh2 (Chen *et al.*, 2008), where the C-terminal SKL acts as peroxisomal targeting signals of the first type (PTS1) and thus directs and recognizing the enzyme to organelle peroxisome and thus performs its work. In the case of aromatic allele badh2-E7, the product cannot recognize its position in the organelle as a result of its non-conformity with the substrate, which causes formation of heterodimers and it is therefore digested and converted to Acety-Co-A by Peroximse to contain the B-oxidization system and thus the allele becomes Non-functional.

Relative gene expression for badh2 gene badh2 gene was amplified using RT-qPCR technology to study the relative gene expression for badh2 gene (aromatic trait) of two variety jasmine and Anber 33 compared to non-aromatic Mashkhab 2. As well as, study the effect of selection on expression of this trait in the two varieties

Table 6: Cycle threshold and relative gene expression for badh2 gene in rice genotypes and relative gene expression in *var.*

Treatment			Chemical fertilization (NPK)	CT of gene badh2	Relative gene expression	contrasts comparisons	Mean
	Mashkhab	G2	NPK0	19.13	-	G7	29.30
						G8+G5+G6	25.70
						P.Value	0.002
	Jasmine Origin	G3	NPKO	23.97	0.1505	G6	24.77
						G8	27.67
						P.Value	0.029
	Anber33 origin	G1	NPK0	27.57	0.0282	G2	19.13
						G1+G3	25.77
						P.Value	0.001
	Jasmine selected based on aromatic	G4	NPKO	24.60	0.1237	G1	27.57
						G3	23.97
						P.Value	0.009
	Anber 33 selected based on aromatic	G7	NPK0	29.30	0.0150	G1	27.57
						G7	29.30
						P.Value	0.167
	Anber 33 selected based on aromatic	G8	NPK1	27.67	0.0230	G3	23.97
						G4	24.60
						P.Value	0.602
	Anber 33 selected based on aromatic	G5	NPK2	24.67	0.0584		
	Anber 33 selected based on aromatic	G6	NPK3	24.77	0.0413		

and study the gene expression of badh2 gene in Anber 33 under different levels of chemical fertilization (NPK). Table 6 and Fig. 3 showed a significant increase in the cycle threshold amount (CT) of jasmine and Anber 33, which gave two averages amounted to 23.97 and 27.57 cycles compared to Mashkhab 2, which gave an average of 19.13 cycles. This is an initial indicator of the decrease in the relative gene expression of these two varieties, as confirmed by the calculation of the relative gene expression, where the results observed that jasmine and Anber 33 had decreased their relative gene expression by 0.1501 and 0.0282 times, respectively, compared to Mashkhab 2, and by this result can conclude that the amount of aromatic in Anber 33 is higher than in the jasmine. The decrease in relative gene expression is attributed to the fact that the badh2 gene in Jasmine and Anber 33 was truncated and incomplete as shown in (Fig. 2) causing gene expression to be discontinued. This is consistent with (Wang, Li 2016), (Hinge, *et al.*, 2016), findings, indicated that aromatic varieties are carriers the

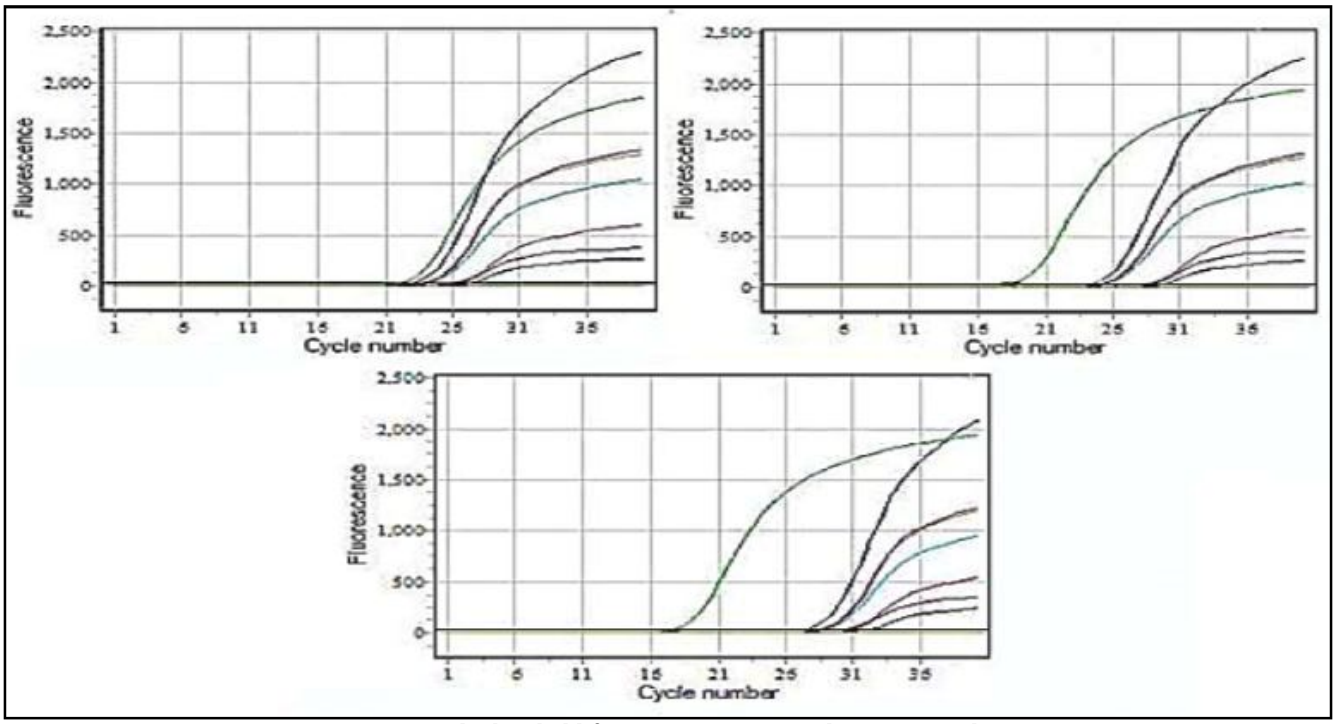

Fig. 3: Cycle threshold for Rice genotypes under NPK Levels.

uncompleted transcript badh2 causing inhibition of relative gene expression and thus promoting the accumulation of 2AP and increased the aromatic.

The results of Table 6 showed that the genotype selected based on aromatic for Jasmine and Anber 33 did not differ significantly from the original two varieties at the cycle threshold (CT), this means that they do not differ by the relative gene expression of the badh2 gene. Perhaps the selection was done in a sensory method. The results of Table 6 also showed that the chemical fertilization treatments NPK2 and NPK3 caused a significant decrease in the cycle threshold (CT), which gave an average of 24.67 and 24.77 cycles respectively, compared with non-addition treatment NPK0 that gave CT reached 29.3 cycles which did not differ significantly with NPK1 that gave an average of 27.67 cycles, which means that the relative gene expression at NPK2 and NPK3 is higher than in NPK0 and NPK1. This is confirmed by the relative gene expression calculations, which decreased by 0.0584 and 0.0413 times for NPK2 and NPK3 treatments, while at NPK0 and NPK1 treatments were 0.015 and 0.0230 times respectively. From that can conclude that high chemical fertilization (NPK2 and NPK3) caused a decrease in the aromatic level, and the increase in relative gene expression of high fertilization (NPK2, NPK3) compared to the treatments (NPK0, NPK1) may be due to the effect of high fertilization levels with transcription factors, which plays an important role in increasing or inhibiting gene expression (Wang *et al.*, 2016), (Dmitriev *et al.*, 2016) indicated that the role of chemical fertilization NPK by increasing gene expression by its effect on gene transcription factors in plants, including: histone acetyltransferase activity, iron ion binding, transcription cofactor activity, oxidoreductase activity, protein binding, ion channel activity. Thus, resulted in increased the gene expression of badh2 that caused the aromatic in rice.

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