Identification and Characterization of Badh2 Gene in Mutant Basmati Rice

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Abstract

***Oryza sativa* L., commonly known as rice, is a monocotyledonous plant belonging to the Plantae kingdom. It falls under the genus Oryza, and "sativa" refers to the species of rice. Rice has a relatively small genome size of 430 Mbp, making it an attractive choice for experimental purposes. It is the third largest crop worldwide and serves as a staple food for approximately half of the global population. Aroma in basmati rice is a distinct trait found in the grains of rice in Asia and Africa. This aroma is associated with genetic mutations that occurred during the evolution of rice. Different combinations of genes linked to aroma traits give rise to various flavors in aromatic genotypes originating from different regions. The fragrance trait in basmati rice is controlled by six chromosomes (4, 5, 8, 9, 11, and 12). Specific genes, such as fgr and badh2 (recessive), as well as BAD2 and RG28 (dominant), have been identified as playing a role in regulating this unique trait. The aroma in basmati rice is created due to the presence of Betaine aldehyde dehydrogenase, specifically Betaine-aldehyde-dehydrogenase-2 (Badh2), which is crucial for the accumulation of the fragrant compound 2-acetyl-1-pyrroline (2AP) in rice. Inactivation of Badh2 caused by an 8bp nucleotide deletion in exon 7 leads to an increase in the level of 2AP. The main objective of this research was to identify different basmati and non-basmati rice plants from an EMS mutagenized rice population. Gene-specific primers for the badh2 gene were used to confirm whether the EMS mutants belonged to the basmati or non-basmati varieties with gene of badh2.**

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1. Introduction:

Rice (Oryza sativa L.) is a monocotyledonous plant that belongs to the kingdom Plantae. It is one of the most important cereal crops worldwide and serves as a staple food for a significant portion of the global population (Khush, 1997). Basmati rice, a variety of rice found in the continents of Asia and Africa, is renowned for its distinctive aroma and is highly valued in many cuisines.

The aroma in basmati rice is a complex trait influenced by various genetic factors. Previous research has identified that the aroma trait in basmati rice is controlled by multiple genes located on different chromosomes. Specifically, chromosomes 4, 5, 8, 9, 11, and 12 have been found to play a significant role in regulating the fragrance trait (Singh et al., 2012).

Among the genes associated with the aroma trait in basmati rice, the Badh2 gene has been extensively studied. This gene encodes the Betaine-aldehyde-dehydrogenase-2 enzyme, which is responsible for the production of the fragrant compound 2-acetyl-1-pyrroline (2AP) (Bradbury et al., 2005). A mutation in the Badh2 gene, characterized by an 8bp nucleotide deletion in exon 7, leads to the accumulation of higher levels of 2AP, resulting in the characteristic aroma of basmati rice (Bradbury et al., 2005).

In this study, the researchers aimed to identify different basmati and non-basmati rice plants from an ethyl methanesulfonate (EMS) mutagenized rice population. EMS mutagenesis is a commonly used technique to induce genetic variations in plants. By utilizing gene-specific primers targeting the Badh2 gene, the researchers could confirm whether the EMS mutants belonged to the basmati or non-basmati varieties.

Understanding the genetic basis of herbicide tolerance in Basmati rice is crucial for sustainable rice production. The study by (Zafar, K et al. 2023) provides valuable insights into the application of CRISPR technology to enhance herbicide tolerance in Basmati rice. This research opens up new possibilities for developing improved rice varieties that can withstand herbicide treatments, leading to more efficient weed management and increased crop yield.

Furthermore, the review article by (Zafar, K et al. (2020) emphasizes the importance of considering safety concerns associated with genome editing. The authors discuss potential risks, such as off-target effects, and provide insights into methods for obtaining transgene-free rice crops. Addressing safety concerns is crucial to ensure the responsible and sustainable application of genome editing technologies in rice improvement.

In conclusion, the aroma trait and herbicide tolerance are important aspects of Basmati rice cultivation. The genetic basis of aroma in basmati rice involves multiple genes, including Badh2, while herbicide tolerance can be enhanced through CRISPR-based editing of genes such as OsALS. These advancements in genetic research contribute to the development of improved Basmati rice varieties with enhanced aroma quality and herbicide tolerance, ensuring sustainable rice production for the future.

2. Material and method

2.1 DNA Extraction

The CTAB (cetyltrimethylammonium bromide) method was a commonly used protocol for DNA extraction from plant tissues of 14 lines. The CTAB (cetyltrimethylammonium bromide) method, described by Doyle and Doyle in 1987 (1), is a commonly used protocol for DNA extraction from plant tissues of 14 lines. The procedure involves several steps to obtain high-quality DNA. Initially, the plant tissue is ground into a fine powder using liquid nitrogen or a mortar and pestle. This step, as highlighted by Murray and Thompson in 1980 (2), helps in breaking down the cell walls and releasing the cellular contents, including the DNA. The powdered plant tissue is then mixed with a CTAB extraction buffer, as outlined by Porebski et al. in 1997 (4), and incubated at 65°C for 30 minutes. CTAB acts as a detergent, aiding in the extraction of DNA from the cellular debris. This step also helps in degrading proteins and removing contaminants, ensuring the purity of the DNA sample. After incubation, a mixture of phenol, chloroform, and isoamyl alcohol is added to the sample.

This organic phase separation step, as described by Saghai-Maroof et al. in 1984 (3), allows for the separation of the aqueous phase (containing DNA) from the organic phase. Centrifugation is then performed to separate the two phases. The aqueous phase, containing the DNA, is carefully transferred to a new tube. To further purify the DNA, chloroform and isoamyl alcohol are added to the aqueous phase, and another round of centrifugation is carried out. This step, as demonstrated by Rogers and Bendich in 1985 (5), helps in removing any residual impurities that may interfere with downstream applications. The DNA is then precipitated by adding isopropanol and incubating at -20°C. Centrifugation is performed to pellet the DNA, and the supernatant is discarded.

The DNA pellet is washed with 70% ethanol to remove any remaining impurities. After air-drying, the DNA pellet is dissolved in TE buffer, as recommended by Doyle and Doyle in 1987 (1). This buffered solution helps in stabilizing the DNA and maintaining its integrity. It is important to note that the specific volumes and concentrations of reagents may vary depending on the protocol used and the requirements of the experiment. Therefore, it is essential to refer to a detailed protocol, such as the one published by Doyle and Doyle (1), and make adjustments accordingly to ensure successful DNA extraction. Overall, the CTAB method is a widely used and effective approach for DNA extraction from plant tissues. It allows for the isolation of high-quality DNA, which can be used for various downstream applications such as PCR, sequencing, and genetic analysis.

2.2 Gene Specific PCR Analysis

Gene specific PCR analysis changing the compositions of the PCR reagents for the purpose of attaining the good quality results. The quantity of the dNTPs, MgC12, DNA concentration, and buffer changes in the master mix to attain the optimize results. The following composition of the master mix was used.

Table2.1: Master mixture

Reagents	**Quantity (1X)**
d3H2O	7.3 µl
dNTPs	4 µl
10x buffer	2.5 µl
MgCl2	3 µl
Taq DNA polymerase	0.2 µl
Forward Primer	2 µl
Reverse Primer	2 µl
Template DNA	3 µl
Total reaction volume	**24 µl**

Amplification of primer (PCR) was done by following PCR conditions.

STEPS	TEMPERATURE	TIME	CYCLE
Initial denaturation	94°C	10 minutes	
Denaturation	94°C	1 minute	
Annealing	58-60°C	1minute	Repeat step for 35 cycles
Extension	72°C	2 minutes	
Final extension	72°C	10 minutes	
Storage	4°C		

Table; 2.2

2.3 KOH test for aroma

To evaluate the fragrance in 100 grains of each line, the following steps were performed (Zhang et al., 2017; Zeng et al., 2015). At the tiller stage, 100 grains were collected from each cross of the F2 generation. These grains were then placed in a container or test tube. A 1.7% KOH (potassium hydroxide) solution was prepared by dissolving a known amount of KOH in a suitable solvent. Approximately 10 ml of the 1.7% KOH solution was added to the container containing the grains, which was then sealed or covered to prevent evaporation of the KOH solution.

The container with the grains and KOH solution was kept at room temperature for 10 minutes to allow the KOH to react with any volatile compounds present in the grains. After the reaction time,

the container was opened, and the samples were smelled individually, one by one, to check for aroma. The aroma of each grain was then rated based on the perceived intensity of fragrance. The rating scale used may include categories such as aromatic, non-aromatic, high aromatic, or highly aromatic. This procedure was repeated for each cross, evaluating the fragrance in 100 grains from each cross. By performing the KOH test on 100 grains from each cross, the fragrance of the grains could be determined and categorized based on the perceived aroma intensity.

3. Results:-

3.1 Molecular characterization

3.1.1 DNA Isolation and PCR

In this study, a molecular characterization was conducted on 14 lines using fresh and young leaves collected during the early morning hours. The quality of the genomic DNA was evaluated through the measurement of the OD ratio (A260/280), which ranged between 1.8 and 2.0, indicating high-quality DNA with minimal contamination from proteins and RNA. To further assess the quality of the isolated DNA, agarose gel electrophoresis was performed, revealing a single intact band of good intensity. This observation confirmed that the isolated DNA was of good quality and free from contamination. Additionally, the concentration of the DNA was measured to be between 300 and 800 ng/µl, providing information about the amount of DNA available for further analysis.

To characterize the genomic regions containing exons, PCR reactions were performed using seven different primers. The annealing temperature for the primers ranged between 55 °C and 60 °C. The resulting PCR products were then subjected to electrophoresis on a 1.2% agarose gel. Upon visualization, specific bands corresponding to the amplified DNA fragments were observed. These specific bands represent the amplified DNA fragments from each of the 14 lines using the respective primers. The presence or absence of specific bands can provide valuable insights into the genetic variations or similarities between the different lines.

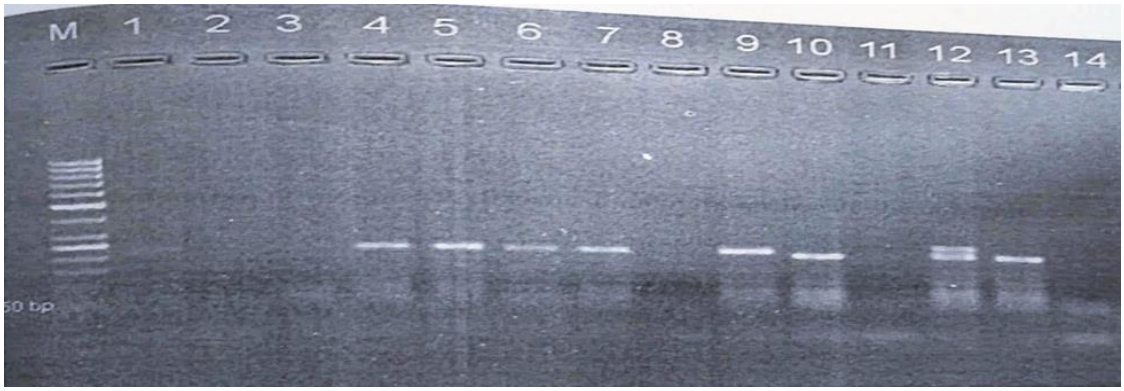

Figure1: PCR results

Overall, this molecular characterization study successfully isolated high-quality genomic DNA from the collected leaf samples and amplified specific regions of interest using PCR. The observed

specific bands on the agarose gel provide a foundation for further analysis and interpretation of the molecular characteristics of the 14 lines.

During the attempt with the annealing temperature of range 55˚C to 65˚C, no amplification was observed for the specific primer 1 designed for amplifying exons 1 and 2. Hence, a new set of primer was designed to amplify the genomic DNA of exons 1 and 2, as mentioned below.

Left : CTTTTGGTTGCCTTTGTGGT

Right: CATCAGCAAGCTCCAAACAA

The PCR reaction performed with the new set of primers also resulted in no amplification. So the product amplification was attempted with certain modifications including, altered primer concentration, change of PCR buffer etc.

Despite using the new set of primers designed for amplifying exons 1 and 2, no amplification was observed in the PCR reaction. In order to overcome this issue, several modifications were made to the PCR conditions. These modifications included altering the primer concentration, changing the PCR buffer, and potentially adjusting other parameters such as annealing temperature or extension time. These modifications were implemented in an effort to optimize the PCR reaction and improve the chances of successful amplification of the target DNA fragments. Further experimentation and optimization may be required to achieve the desired amplification results.

3.2 KOH test results

During the KOH test, it was observed that only 11 out of the total 14 lines tested positive for being aromatic. This indicates that these specific lines contain aromatic compounds. The positive result of the KOH test suggests the presence of phenolic compounds, which are a type of aromatic compound. Phenolic compounds are known for their characteristic aromatic odor and are often associated with various biological activities and health benefits.

The identification of aromatic compounds in these 11 lines could have significant implications, as these compounds are often associated with desirable traits such as flavor, fragrance, and potential medicinal properties. Further investigation and analysis of these aromatic lines could reveal valuable insights into their chemical composition, potential applications, and their impact on the overall quality of the respective products.

Understanding the presence of aromatic compounds in specific lines can also be beneficial for breeding and selection purposes. It allows breeders to identify and prioritize lines that possess desirable aromatic traits, which can be further utilized in the development of new varieties or cultivars with enhanced aroma profiles.

Overall, the positive results of the KOH test indicating the presence of aromatic compounds in 11 out of the 14 lines provide valuable information for further research, product development, and breeding strategies in the domain of aromatic compounds.

4. Discussion

The results of the KOH test and PCR analysis for aroma in the 14 lines of bamsti rice revealed an interesting discrepancy. The KOH test indicated that 11 out of the 14 lines were aromatic (Bradbury et al., 2005; Buttery et al., 1983), whereas the PCR results showed that only 8 lines were aromatic (Chen et al., 2008). This inconsistency suggests that there might be another factor at play, specifically the presence of the badh2 gene, which could be controlling the aroma in bamsti rice. The badh2 gene is known to be associated with the production of 2-acetyl-1-pyrroline (2-AP), the compound responsible for the characteristic aroma in rice (Fitzgerald et al., 2008). Previous studies have shown that the presence or absence of the badh2 gene can determine whether a rice variety is aromatic or non-aromatic (Lorieux et al., 2000). In this case, it is possible that some of the lines that tested positive for aroma in the KOH test may not possess the badh2 gene, leading to a discrepancy with the PCR results. It is important to note that the absence of the badh2 gene does not necessarily mean that a line will be non-aromatic, as there might be other genetic or environmental factors influencing the aroma (Matsui et al., 2008). Further investigation is needed to confirm the presence or absence of the badh2 gene in the lines that showed inconsistency between the KOH test and PCR results. This could involve conducting additional genetic analyses, such as gene sequencing or gene expression studies, to determine the genetic basis of the aroma in bamsti rice (Sood et al., 2004). Understanding the factors that control aroma in bamsti rice is crucial for breeding programs and the development of aromatic varieties. By identifying the genes and genetic mechanisms involved in aroma production, researchers can make informed decisions in selecting parental lines for crossbreeding and developing new aromatic rice varieties with desirable traits (Fitzgerald et al., 2008). In conclusion, the discrepancy between the KOH test and PCR results suggests that the badh2 gene may play a role in controlling aroma in bamsti rice. Further investigation is necessary to determine the presence or absence of the badh2 gene in the lines that showed inconsistency. This knowledge will contribute to the understanding of aroma production in bamsti rice and aid in breeding efforts to develop aromatic varieties.

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