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NUTRITIONAL QUALITY UPGRADING OF INDIGENOUSLY DEVELOPED RICE MUTANT THROUGH EXPLORATION OF PHYTIC ACID AND AMYLOSE REGULATORY GENES

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Keywords:

Rice mutant, Nutritional quality, Phytic acid, Amylose, Microarray

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ABSTRACT: Phytic acid and amylose in rice food play an essential role in human health. The best-practiced approach is necessary for nutritional quality improvement of rice grain. The mutational breeding approach and chemical treatment are powerful methods to enhance rice quality by managing the concentration of phytic acid and amylose contents. The analysis of glycemic index and amylose contents is also significant to reduce the risk of diseases. The present research investigates nutrient quality such as phytic acid and amylose content in the developed rice mutant (PBEF20). The developed mutant shows a 50% reduction in phytic acid with a 37.73% increment in amylose contents compared to the control i.e., untreated sample. The Inositol phosphate kinase (IPK-1) and ABC-2 type transporter domain-containing protein differentially downregulated in the early flowering mutant. IPK-1 gene is responsible for converting inositol 5-phosphate (IP5) to inositol 6-phosphate (IP6). The ABC-2 type genes are vacuolar transporters to transport the phosphate that assists in synthesizing phytic acid. The identified genes, including beta-amylase and chloroplast precursor, regulate various functions in the starch sucrose metabolic reference pathway, and their upregulation controls the expression of downstream genes involved in amylose synthesis. The expressions of the genes responsible for phytic acid, starch synthesis, and nutrient reservoir activity during the rice maturity stage are examined using microarray technology to track gene regulation, while the grain filling pattern of the amylose mutant is analyzed using scanning electron microscopy (SEM). Results indicate significant variation in carbohydrate granules size and starch compactness.

INTRODUCTION: Rice is the great source of calories in human diet after wheat which compensates more than 20 % of total calories obsessive globally [1, 2]. The era of growing population requires more attention on development of healthy food grain. A number of nations are facing challenges in the production of rice at minimum cost with better quality in a declining environment [3].

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Apart from quantity of grain, the nutrient quality is also important for better human health [4]. Rice has some anti-nutrients such as phytic acid (PA) that chelating nutrients that decrease its availability in the non-rumients animals and humans [5]. In developing countries, where 40-45% of growing children are anemic.

The more than half of this anemia results due to nutrient deficiency in food [5]. Rice is an excellent source of energy comprising of 77.5% carbohydrate, as starch. Starch consists in two forms amylose and amylopectin that made up of repeated units of glucose linked together in very large numbers. During the digestion process, these links are broken down that released glucose absorbed in to the body and excess glucose convert

into glycogen with the help of insulin [6]. The amylopectin is a branch structure which is easy to digestion whereas amylose is a straight chain molecule and harder for the digestive system to break up into glucose. It indicates that rice varieties with a greater proportion of starch in the form of amylose content have a lower glycemic index [7].

The glycemic index makes the people compelled to re-think for rice consumption. The IP6 is a phytic acid. The PA, myo-inositol-hexakisphosphate (InsP6) is a storage form of phosphorous in plants. The IPK-1 is responsible for conversion of inositol 5-phosphate (IP5) to inositol 6-phosphate (IP6). The ABC-2 transporters are essential for transportation of glutathione conjugates and different physiological responses [6]. Many researchers involved worldwide for the investigation of nutrition of rice based on different nutrients. A brief journey of analysis on these constituents is summarized by Shahid *et al* [7]. Literature shows that rice with low amylase content has the higher risk of diabetes mellitus [1, 7]. The study of Rohman *et al* [6] focused the biochemical estimation of nutrient quality in term of phytic acid, amylose content, Fe and Zn. Microarray analysis used to analyze expressions of genes which are responsible for the low phytic acid, starch synthesis and nutrient reservoir activity examined during the maturity stage. Li *et al* exercised to find the high-amylose starch through ultrasound extraction (Radix Puerariae) technology with high-intensity low-frequency settings. The results show that increased amylose content also influence the gelling properties [8]. The study of Kumar et al also concludes the significance of nutrients for human nutrition. The authors used transgenic breeding approach for the investigation of Fe, Zn and provitamin A elements [9]. The present research study focused to investigate and improve the nutrient quality of indigenously developed rice mutant. The observations and analysis have been performed through different methods such as experiments, Microarray technique and scanning electron microscope (SEM).

The results are found with a significant improvement in response parameters. The complete paper is organized as follow; the first section of the study provides the introduction of research area with conclusions of former research. The second section describes the methodology used to achieve the aim of study. The findings of research are explained in section [4] and finally the last section of study concludes the results with future recommendations.

MATERIAL AND METHODS: The seeds of Basmati rice are collected from an authorized company and grown with chemical treatment in green house conditions. The quality of chemical treatment and untreated samples tested for phytic acid and amylose content. The results of treated samples are analyzed and further compared with control i.e., untreated samples. A complete methodology flow is graphically provided in **Fig. 1**. It started with the review of former research to identify the need of study and suitable techniques for the data analysis.

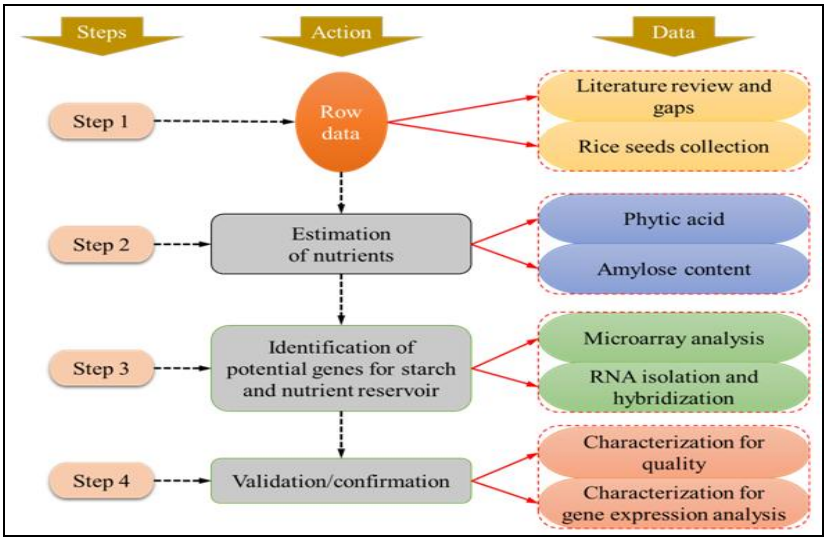

FIG. 1: METHODOLOGY FLOW CHART

The microarray technique is one of the powerful tool for the gene expression analysis of rice grain. The SEM is used for comparative study of carbohydrate granules size and shape in rice. It provides the altering in grain filling pattern to discuss the observations. The sample for microarray analysis collected from premature spikelet's of mutant at maturity stage.

Assessment of Phytic Acid: The wade reagent test is one of the accurate method to find the Phytic acid concentration in the rice sample [10]. The early flowering mutant and control seeds are subjected to wade reagent test for the assessment of Phytic acid. The absorbance was taken in ELISA plate with ELISA reader. The standard was prepared using 0.1 gm sodium phytate in 10 ml HCl (of molarity 0.65). The absorbance as recorded at different phytate concentrations. The synthesis pathway of phytic acid is shown in **Fig. 2**. Phytic acid, myo-inositol-hexakisphosphate ($InsP_6$) is a storage form of phosphorous in plants. The synthesis of Ins (1,4,5) P_3 from phosphatidylinositol1-4,5 bis-phosphate *via* Phospholipase C (PLC) using action of two kinases inositol 1,4,5-triphosphate kinase (IPK-2) and inositol 1,3,4,5,6-pentabisphosphate 2-kinase (IPK-1). IPK-1 gene product is responsible for conversion of inositol 5-phosphate (IP_5) to inositol 6-phosphate (IP_6). IP_6 is phytic acid.

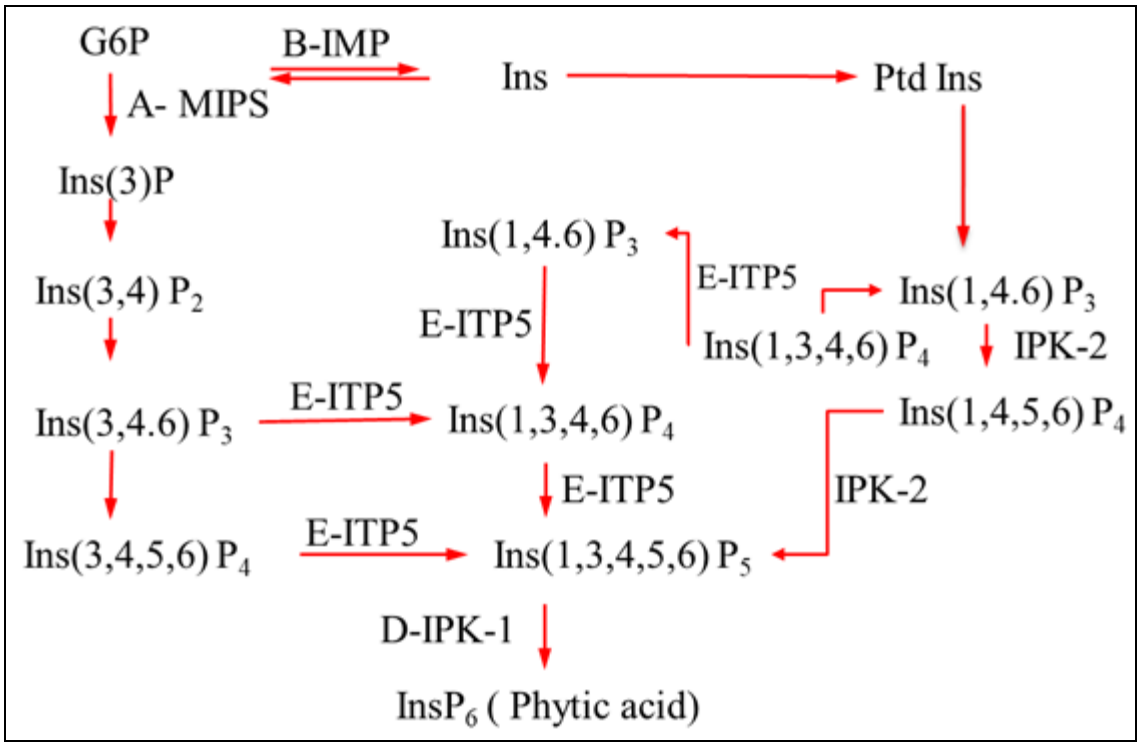

FIG. 2: PHYTIC ACID SYNTHESIS PATHWAY

The graph obtained using standard phytic acid concentration resulted with a negative slope as shown in **Fig. 3**. The plot shows an inversely proportional relation between phytic acid concentration and absorbance.

The increase in phytic acid concentration decreases the absorbance value. It chelates Fe present in Wade reagent makes it unavailable for colour development. Decreasing the absorbance value calculated by using Eq. 1.

$$Y = -0.003X+0.432$$

Where, Y= Absorbance value, X= Phytic acid conc.

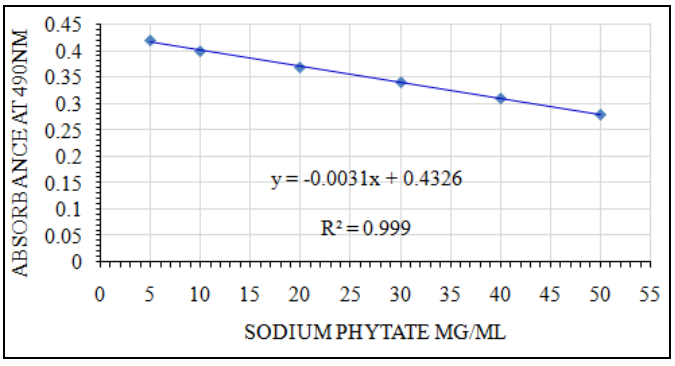

FIG. 3: STANDARD GRAPH OBTAINED KNOWN CONCENTRATION OF SODIUM PHYTATE SOLUTION

Absorbance value was used to find the phytic acid concentration from samples by using wade reagent protocol. A 10 mg of rice flour added to 200µl HCl of 0.65 M and kept for 12hour incubation at room temperature in shaker. Then centrifuge at 3000 rpm for 20 minutes. The 30µl extracted and mixed with 200µl diluted wades reagent and again reserved to incubation at 25°C for 15-20 minutes. The spectroscopic readings are recorded at 490 nm through ELISA reader. Result was observed on the basis of intensity of colour develop in the well as related to phytic acid concentration. The sample contain high phytic acid shows less colour intensity because it chelates Fe in Wade's reagent. **Fig. 4** shows the samples of control and mutant with color variation due to the concentration of phytic acid.

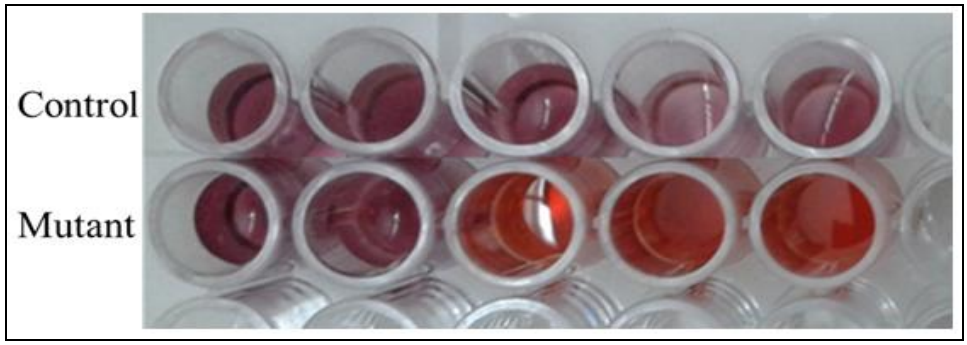

FIG. 4: SCREENING OF LOW PHYTIC ACID MUTANT RICE PLANTS BY WADE'S REAGENT

Assessment of Amylose Contents: Amylose content estimation performed in two steps. In the first step isolation of starch and second amylose content determination. The protocol for starch isolation is well defined in former research studies [11]. The similar procedure is adopted in this study for starch isolation. A 1gm of rice flour was mixed with 3 ml of 0.5% NaOH solution. The mixture was constant stirred for 4 hours and left to stand for 24 hour at 10°C.A solid phase taken and washed several times with distilled water. The isolated starch of rice was dried in oven at 40°C for 48 hours. Then it grinds by mortar pestle and passes through a 0.55 mm mesh. This isolated starch was used for amylose determination. To determine the amylose, a 0.5 gram of isolated starch was mixed 10 ml of DMSO and warm up in boiling water bath for 1 hour. One ml of this aliquot solution was mixed with 1ml of iodine solution (1mg I_2 and 10 KI for 1 ml of this solution). Dilute the solution and make final volume to 25 ml after 30 min after the incubation, absorbance of solution recorded at 600 nm wavelength. Various concentration of starch were prepared and amylose content was estimated in them by taking absorbance at 600nm. Using this optical density, standard graph was prepared, which will further be used to estimate amylose content in mutants. The standard graph obtained for known concentration of I_2 and KI solution is shown in **Fig. 5.** The graph showing the increasing value of absorbance.

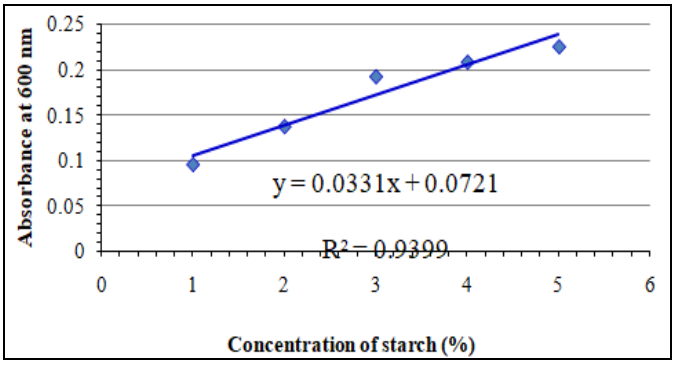

FIG. 5: STANDARD CURVE FOR AMYLOSE

Microarray Analysis: Microarray analysis is performed for identification of gene related to starch and nutrient reservoir. The analysis performed by using chip rice genome array that is performed at Agilent's Pvt. Ltd. The gene spring GX software issued to normalize the microarray data at 75th percentile shift and the values of expression evaluated for the array transversally to all spots (n = 0-100 and $n_{(median)}$ =75). Thereafter the obtained results segregated from expression values and normalize to control sample. The significant genes synchronized up and down representing a fold (log_2).

RNA Isolation and Hybridization: A 100 mg of rice spikelet's of 5 mm length stored at -20°C in the RNA later the total RNA isolation carried out by using Trizol reagent method. RNA concentration, integrity and purity was checked by using nanodrop spectrophotometer. The integrity of the extracted

RNA analyzed with the help of Bioanalyzer. The 500ng of total RNA was rescind transcribed using oligod T primer tagged to T7 promoter sequence. Then obtained cDNA was transformed in to double stranded cDNA and converted into cRNA by *in-vitro* transcription stage using T7 RNA polymerase enzyme. The Cy3 dye was added into the newly synthesized strands during synthesis. The samples that pass the quality control for précised action subjected for hybridization. A 1650 ng of labelled cRNA hybridized on the array with the help of gene expression hybridization insuring the chambers at 65º C for 16 hours.

RESULTS AND DISCUSSION: The chemical mutagenesis subjected to introduce an indigenous rice mutant. After the iterative exercise of treatments, the early flowering mutant has been developed with enhanced quality parameters and grain filling pattern speed. It can be observed from **Fig. 6(A)** and **(B)** that the control has larger awn. Comparatively, the developed early flowering mutant has minimum awn as shown in **Fig. 6(B).** The rice grain of both control and mutant are shown in **Fig. 7**.

It shows clear physical improvement in rice. **Fig. 7(A)** shows the grain of mutant which is larger grain length than the control sample as shown in **Fig. 7(B).** The analysis of rice nutrient quality parameters resulted the different concentrations of amylase content and phytic acid. **Table 1** shows the biochemical analysis of nutrient quality parameters.

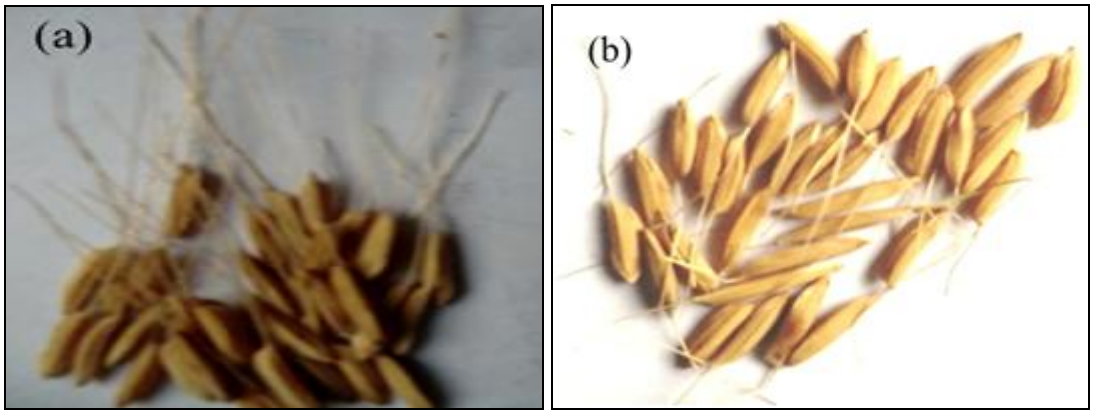

FIG. 6: SEEDS (A) CONTROL (B) PBEFL20 MUTANT

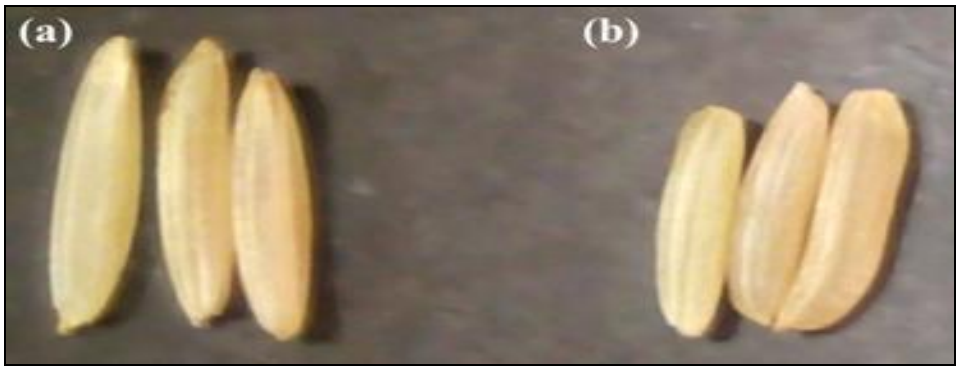

FIG. 7: RICE GRAIN (A) MUTANT (B) CONTROL

TABLE 1: ASSESSMENT OF NUTRIENT QUALITY PARAMETERS

S. no.	Parameters	Control	Early flowering mutant
1	Phytic acid %	0.39 ± 0.01	0.15 ± 0.01
2	Amylose content %	25.39± 0.81	34.97± 1.63

The phytic acid in control sample is 0.39± 0.01%. Whereas, the phystic acid in developed early flowering mutant is 0.15 ± 0.01 which is 37.7% lower than control. These results are also represented graphically in **Fig. 8**. The amylose content measured in control and early flowering mutant are 25.39± 0.81% and 34.97± 1.63% respectively. It is found that the amylose content in early flowering mutant is improved by 36.88%. Both the nutrients are resulted with suitable

improvement and safe for human health. The concentration of amylose content is shown in **Fig. 9**. Which is showing the similar interpretations. The results obtained in this paper are reliable and comparable with the findings of former researchers [12].

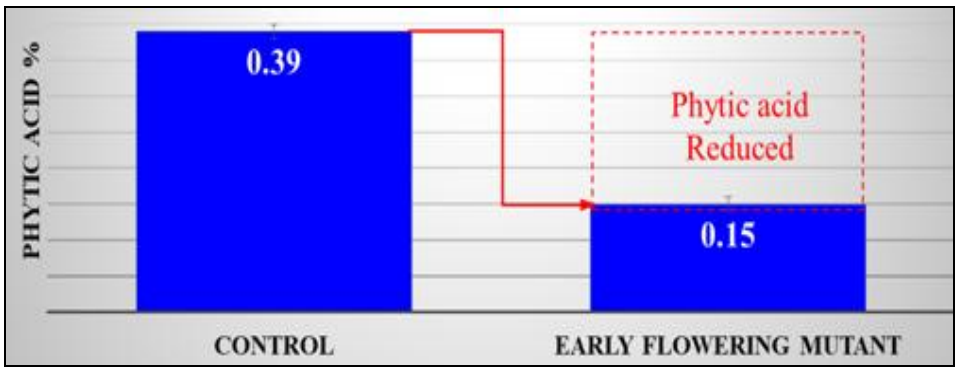

FIG. 8: PHYTIC ACID ESTIMATION

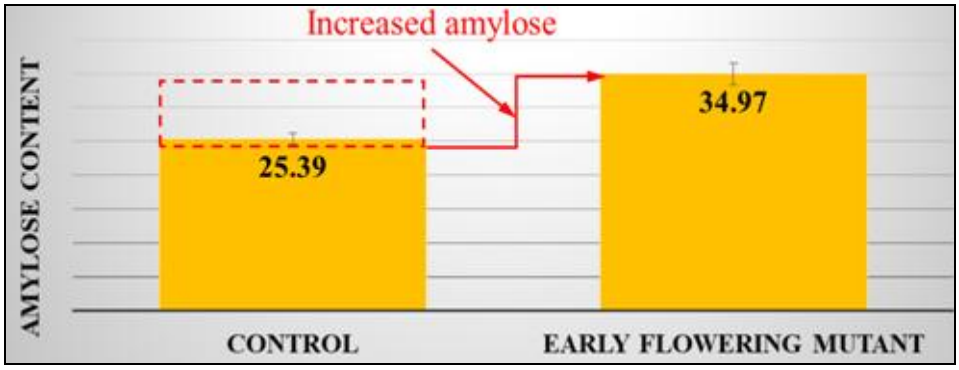

FIG. 9: AMYLOSE CONTENT ESTIMATION

Characterization of Mutant Grain for Carbohydrate Quality by SEM: Comparative study of carbohydrate granules size and shape in rice is done by SEM analysis. The starch granules are smaller in case of control compared to PBEF20 mutant. It indicates the change in grain filling pattern during maturity of seeds. **Fig. 10(A)** shows the grain filling pattern for control sample. The SEM graph shows that the granules are dense with smaller size due to the slow grain filling. On the other hand, **Fig. 10(B)** shows the grain filling pattern for PBEF20 mutant. It shows the larger size of granules due to the faster grain filling speed. The effect of treatment can be clearly observed from the SEM analysis. The results are improved significantly.

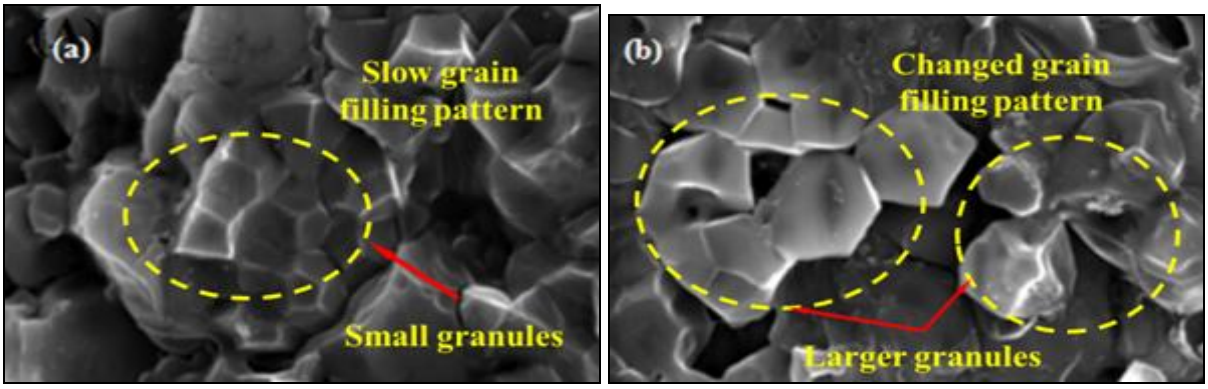

FIG. 10: CHARACTERIZATION OF MUTANT GRAIN CARBOHYDRATE BY SEM IMAGE (A: CONTROL) (B: PBEF 20)

Characterization for Gene Expression Analysis: The characterization of PBEF20 mutant and control for gene expression analysis has been performed. Microarray technology is an important strategy for genome wide expression pattern and functional analysis of basmati rice. It is obtained that some

genes related to phytic acid like Inositol phosphate kinase (IP_6) and ABC-2 type transporter domain containing protein differentially down regulated in early flowering mutant with fold change -1.97 and-3.62 respectively. These genes responsible for phytic acid synthesis. Mutation in ABC-2 type transporter domain containing protein causes reduction of phytic acid synthesis [12]. Some other genes for starch and nutrient reservoir differentially up regulated such as beta-amylase (Os07t0543100) with fold change 4.62 involved in processes such as carbohydrate metabolic, starch catabolic, beta-amylase activity *etc*.

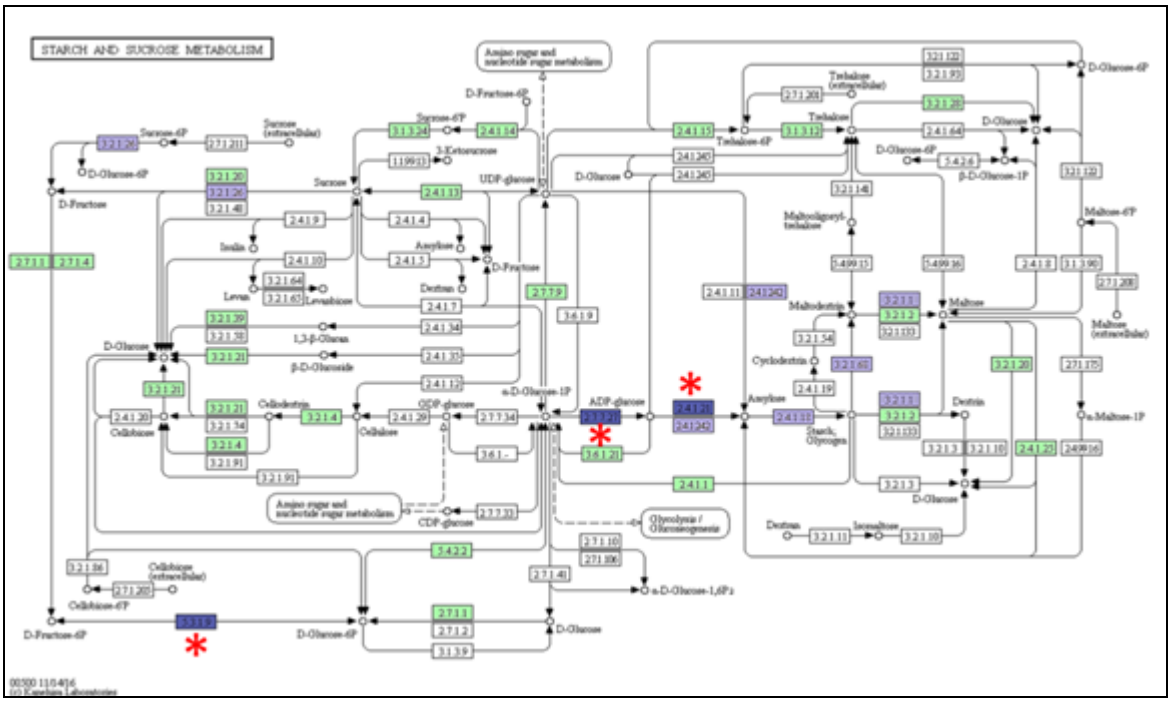

FIG. 11: THE UP AND DOWN REGULATED GENE OF STARCH SYNTHESIS AND METABOLIC GENES, INVOLVE IN METABOLIC NETWORK PATHWAY

Similarly, sedoheptulose -1, 7-bisphosphatase (Os04t0234600) with fold change 2.50, present on Chromosome number 7 play a vital role for chloroplast precursor carbohydrate brake down, carbohydrate anabolic process, defense response to bacterium, starch biosynthetic process, sucrose biosynthetic process, thylakoid, phosphoric ester hydrolase activity, phosphatidylinositol phosphatase activity and sedoheptulose-bisphosphatase activity. Glutelin subunit mRNA, (Os01t0762500) with fold change 3.69 present on chromosome number1 nutrient reservoir activity. Starch synthase isoform zstsii (Os02t0744700) with fold change 2.41 present for starch biosynthetic process, amyloplast, chloroplast, and transferring glycosyl groups. Sedoheptulose-1,7-bisphosphatase (Os04t0234600) with fold change 2.37 found for Carbohydrate metabolic process, starch biosynthetic process, phosphatidylinositol phosphatase activity. The nutrient reservoir genes differentially up and down regulated are amidase family protein, (Os04t0184100) with fold change 5.99, present on chromosome number 4 carbon-nitrogen ligase activity, with glutamine as amido-N-donor and nutrient reservoir activity. Glutelin type-A III precursor (Os03t042730The up and down regulated genes in metabolic network pathway shown by different colors which indicates that the genes with reference id 2.7.7.27, 2.4.1.21 and 5.3.1.9 follow the up regulation whereas the genes with reference id 3.2.1.26, 2.4.1.242, 2.4.2.18 and 3.2.1.1 turned to down regulation. These genes performed different multiple functions in the pathway to starch synthesis and metabolism as shown in **Fig. 11.**

The results of study show the significant improvement in nutrient quality parameters of basmati rice. The developed mutant provides the improved amylose with reduced phytic acid. The findings will benefit to the farmers, society and researchers of similar domain.

CONCLUSION: The results of the chemical analysis indicate that early flowering mutant has approx 50% reduction of phytic acid with 37.73% increment in amylose contents. The reduction in phytic acid increases the availability of micronutrients such as Fe and Zn in the diet.

The sufficiency of these micronutrients prevents the human from diseases like anemia. The rice with high amylose causes glucose's persistent release, which is beneficial for diabetes mellitus patients. It also helps prevent retinopathy, neuropathy, nephropathy, and other complications due to the quick release of glucose.

The microscopic analysis helped to understand the improvement in parameters through the change in starch pattern. Gene expression analysis was performed using microarray to identify the genes responsible for phytic acid synthesis, starch synthesis and nutrient reservoir. The up and down-regulation of the genes is analysed during the maturity stage. The Inositol phosphate kinase (IP6) and ABC-2 type transporter domain-containing protein differentially down regulated in early flowering mutant, indicating the lowering of phytic acid in the rice. Some other genes such as beta-amylase and chloroplast precursor were upregulated, controlling the expression of other downstream genes in the metabolic pathway to amylose synthesis. Overall, the study results are positive for human health and further research.

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