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High-Protein Rice for Nutritional Security : Genesis and Impacts

K. Chattopadhyay, S.G. Sharma, T. B. Bagchi,
S. Sarkar, A. Das, M. Chakraborti, N. Basak,
S. Ray, B.C. Marndi, S.K. Pradhan, A.K. Mukherjee,
L.K. Bose, A. Ghosh, A. Poonam and O. N. Singh

ICAR-National Rice Research Institute
Cuttack - 753 006, Odisha, India

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त्रिलोचन महापात्र, पीएच.डी.
एफ एन ए, एफ एन ए एस सी, एफ एन ए ए एस
सचिव एवं महानिदेशक
TRILOCHAN MOHAPATRA, Ph.D
FNA, FNASc, FNAAS
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GOVERNMENT OF INDIA
DEPARTMENT OF AGRICULTURAL RESEARCH & EDUCATION
AND
INDIAN COUNCIL OF AGRICULTURAL RESEARCH
Ministry of agriculture and farmers welfare
Krishi Bhavan, New Delhi 110001
Tel. : 23382629, 23386711 Fax : 91-11-23384773
E-mail : dg.icar@nic.in

Foreword

Rice is a staple food for the majority of poor population of India which suffers the most misery of malnutrition and related deficiency diseases. Among the problems of nutritional disorder, protein energy malnutrition (PEM) is quite severe in the country. Although both children and adults are affected by this disorder, the children the brain and its functioning. Therefore, development of biofortified rice enriched with protein and other micronutrients is of great significance. Biofortification is the most economically viable and ecologically sustainable option to address this serious problem of malnutrition.

The development of high protein rice variety CR Dhan 310 by ICAR-National Rice Research Institute, Cuttack, Odisha and its release by the Central Variety Release Committee (CVRC) in 2016 has been a momentous step in this direction to combat protein energy malnutrition. CR Dhan 10 is the first biofortified high protein (10.2%) rice variety developed in the background of popolar rice variety Naveen. This is a high yielding (4.5 tha^{-1}) variety released for cultivation in Odisha, Uttar Pradesh and Madhya Pradesh. Subsequently, another nutrient rich variety Mukul (CR Dhan 311) with high protein (10.1%) and moderately high Zn (20 ppm) content has been released by State Variety Release Committee (SVRC) of Odisha in 2016. These high protein varieties had significantly higher glutelin content than Naveen. The high protein rice varieties can easily by distiguished from the low protein recurrent parent like Naveen with the help of the Xanthoproteic test as the former gives more intense yellow or orange color that the latter.

The variety once promoted and grains made available to the people would serve as an important and ready supplement for the malnourished rice-eating population of the country. However, policy and administrative support is very crucial for its promotion and utilization. Further the use of nutri-grains of this verity into Government programmes such as mid-day meal scheme and public distribution systems would enhance it reach and benefit the majority of the needy population of India and other countries.

I am delighted for this development and congratulate the members of the team involved in the variety development and promotion. I hope this informative research bulletin on High-Protein Rice for Nutritional Security : Genesis and Impacts will help in understanding the process involved and activities undertaken during development of these new varieties and the potential impacts it could it could have on the society. This publication would definitely serve as a reference material for researchers, policy makers and civil society on high-protein rice.

My compliments to the ICAR-National Rice Research Institute, Cuttack, Odisha for supporting and facilitating visionary research leading to the release of these varieties.

Dated the 7th May, 2018
New Delhi

(T. Mohapatra)

Preface

Protein malnutrition is predominant in Asia where rice is the staple food for more than 70% of population. Although rice is deficient in protein (6-7%), due to higher digestibility and better nutritive value of glutelins, major fraction of seed protein of rice is nutritionally superior to other cereals. Higher nitrogen assimilation ability of a crop and variety mainly determines their grain protein content (GPC). Many QTLs are associated with higher GPC without affecting the plant morphology and grain yield. Through bulk-pedigree and backcross breeding, development of high yielding varieties of rice with high GPC was initiated at ICAR-National Rice Research Institute (NRRI), Cuttack, Odisha in late 2000s. Using a high GPC donor (ARC10075) several introgression lines in high yielding varieties such as Swarna and Naveen were developed and tested in multi-locations. Most of them had significantly higher level of lysine, threonine, leucine, isoleucine, valine, phenylalanine, alanine, proline, glutamic acid, arginine and total amino acid compared to recurrent high yielding parents. Among them a high yielding (4.5 t ha^{-1}) variety in Naveen background, CR Dhan 310 has been released as the first biofortified high protein (10.2%) rice variety by Central Variety Release Committee (CVRC) for cultivation in Odisha, Uttar Pradesh and Madhya Pradesh. Subsequently, another nutrient rich variety Mukul (CR Dhan 311) with high protein (10.1%) and moderately high Zn (20 ppm) content has been released by State Variety Release Committee (SVRC), Odisha in 2016. These high protein varieties have significantly higher glutelin content than Naveen. It has been validated that the high protein rice varieties can be easily distinguished from the low protein recurrent parent, like Naveen with the help of the *Xanthoproteic test* as the former gives more intense yellow or orange color than the latter. Wide spread cultivation of high yielding rice varieties with high nutritional values can significantly contribute towards the better nourishment of millions of undernourished people in India and the world.

(H. PATHAK)
Director, NRRI

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AUTHORS

Contents

Introduction

Milled rice grains generally contain 6-7% protein, which is the lowest among the cereals including wheat (12-14%) and maize (8-9%). However, rice protein is considered to be the best among the cereal proteins due its better balance of essential amino acids and higher digestibility. Protein digestibility corrected amino acid score (PDCAAS), which indicates the presence of essential amino acids and overall protein quality, is higher for rice (0.55) compared to that of the other popular cereal wheat (0.40). Rice is the staple food for about 50% of global population and 70% of the Indians. Therefore, the impact of increasing the protein content in rice would be enormous, particularly in the scenario where more than a third of world's children are affected by protein-energy malnutrition (PEM), 80% of whom belong to Asia, 15% to Africa and 5% to Latin America. In India, 80% children under five-year age are under-nourished, for whom the recommended intake of protein is 13-19 g/day/child. As the recommended calorie intake is 1000-1400 cal/ day/ child (200-300 g of rice) out of which 150-450 calories are to be supplied by protein, even 1% increase in grain protein content would add significant amount of protein to the diet.

Next to starch, protein is the second major chemical constituent of cereal grains such as rice and also that of legumes and oilseeds. Nitrogen (N), the major structural element of proteins is obtained by the rice plant from soil in the form of nitrate or ammonium. During the grain filling stage, N stored in leaves is remobilized to the developing seed. Remobilizing efficiency of N is highly dependent on the genotype. Therefore, manipulating N remobilization may be a good strategy to improve the grain protein content (GPC) without affecting the grain yield. The diversity observed for GPC in rice genotypes widens the scope for the quantitative improvement of high yielding rice varieties for this trait. Rice grain contains large amount of storage proteins. They are classified into glutelins, albumins, globulins, and prolamins, based on their solubility properties. Glutelin is the principal protein of rice grain, which is concentrated in the milled fraction, constituting at least 80% of the total protein in the starchy endosperm. The nutritional value of glutelins is superior to that of prolamins due to the greater digestibility (by humans) of the former and the higher lysine content. Therefore, the emphasis of rice breeding is not only on the quantitative but also on the qualitative improvement of rice proteins. This research bulletin aims to elaborate on (i) the physiological, biochemical and molecular basis of high GPC; (ii) diversity of rice germplasm collection at ICAR-National Rice Research Institute (NRRI), Cuttack; (iii) estimation procedure followed for medium and high throughput screening of GPC, fractionation of seed protein, SDS-PAGE for visualization of peptide and amino acid profiling of selected lines; (iv) improvement of rice varieties for higher GPC through bulk-pedigree and backcross breeding methods and the varieties so developed and released and (v) a rapid method to detect high grain protein rice varieties.

Physiological, biochemical and molecular basis of high grain protein content in rice

Utilization of N by plants involves several steps such as uptake, assimilation, translocation and remobilization including recycling and remobilization in the ageing plant. As rice is largely grown under waterlogged condition the nitrogen is taken

up mainly in the form of ammonium ions (NH_4^+). Moreover, the metabolism of NH_4^+ requires lesser energy compared to that of nitrate (NO_3^-) (Bloom *et al.*, 1992). The uptake of NO_3^- is repressed by NH_4^+ (Kronzucker *et al.*, 1999). Ammonium transporters present across the membranes of root hairs are responsible for the uptake of NH_4^+ from soil and its translocation to the plant system. Different types of transporters are involved during uptake, some of which have high affinity to NH_4^+. After the NH_4^+ is taken up by the transporter into the epidermal cytosol, it combines with glutamate in a reaction catalyzed by glutamine synthetase (isoform GS1; EC 6.3.1.2) to form one molecule of glutamine which carries two atoms of N. Though GS1 is homogeneously present in cytosol of all cell types in the roots of rice plant, this reaction is predominant in epidermal or exodermal cytosol. Later, this glutamine combines with 2-oxoglutarate in the presence of GOGAT (Glutamine: Oxoglutarate Amino Transferase) to form two molecules of glutamate, one of which serves as substrate for GS, while the other is available for transport, storage and further metabolism. The reaction is catalyzed by NADH-GOGAT (EC 1.4.1.14) in cytosol and Fd-GOGAT (EC 1.4.7.1) in plastids. The 2-oxo glutarate used in the reaction is provided from the mitochondrial NAD^+-isocitrate dehydrogenase reaction. This completes the GS/GOGAT pathway for assimilation of N in rice. The NADH-GOGAT is strongly induced by the glutamine produced by the reaction catalyzed by GS. The Fd-GOGAT is irresponsive to NH_4^+ concentration and is present in younger cells. As the cells mature, they get concentrated to central cylinder and secondary leaf primordia. The NH_4^+ assimilation in rice roots is highly influenced by the age of the tissue, cell type and availability of exogenous N. The GS2 assimilates any NH_4^+ produced through photorespiration in leaf and is immediately converted to glutamate through GS/ GOGAT cycle in leaf as per the following equation.

Glutamate + NH_4^+ → Glutamine +H_2O + ADP +Pi (catalyzed by GS)

Glutamine + 2- oxoglutarate → 2 Glutamate + NAD^+ (catalyzed by NADH/ Fd-GOGAGT)

Nitrogen is further transported to the shoot tissues through xylem vessels either in the form of glutamine or asparagine, which are produced from glutamate by GS /GOGAT cycle in the presence of amino transferases. After uptake, the NH_4^+ takes almost an hour to translocate to other parts of the plant. Younger leaves are stronger N sink than the older ones. After reaching the shoot parts, N that was transported in the form of glutamine or asparagines is either further metabolised or stored as storage / structural proteins or used to synthesize enzymes (Fig. 1).

During the grain filling stage, N that was stored in the leaves is remobilized to the developing seed, which poses a strong sink for the photosynthate and nutrients. However, a negative correlation exists between grain protein concentrations and yield (Beninati and Busch, 1992). Remobilizing efficiency is highly genotype dependent (Kichey *et al.*, 2007). Therefore, manipulating N remobilisation may be a good strategy to improve the nutritional quality of grain without affecting the yield. However, N remobilization is not triggered by this stage and is a continuous process that is triggered by each emerging organ that provides a potential sink for N *viz.*, N fluxes from older leaves to younger leaves during vegetative phase. Remobilization is always associated with higher protease activity and leaf senescence.

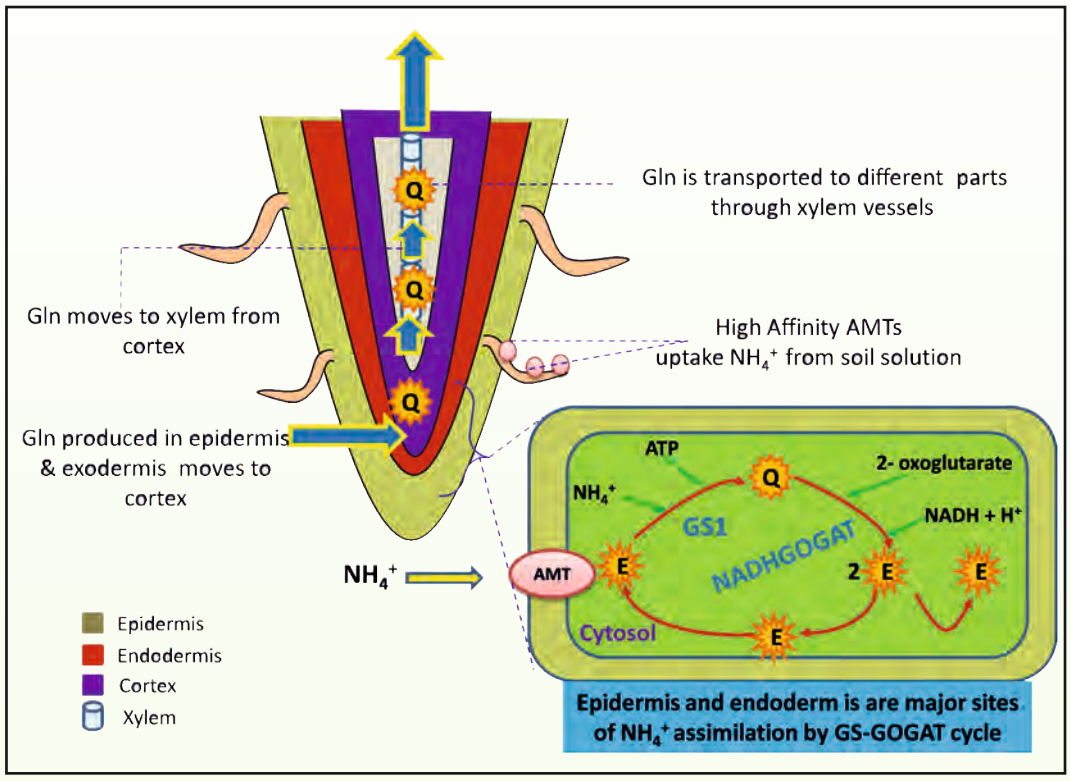

Fig. 1. Assimilation of N in rice root
Note: Q, Glutamine; E, Glutamate; AMT, Ammonium transporter; GS, Glutamine synthetase; GOGAT, Glutamine:oxoglutarate aminotransferase

The remobilization efficiency of N mainly depends on sink strength of seed, efficiency of transfer processes located in the source leaves, stems and reproductive structures, phloem pathway efficiency and growth environment of the plant. The N remobilization preferentially occurs in the form of glutamine through phloem sap and accelerates with leaf aging. The concentration of free amino acids in the phloem sap is in the range of 50-200 mM (Tilsner *et al.*, 2005) during grain filling. Apoplasmic phloem loading depends on amino acid transporters in the sieve elements / companion cell complexes (Tilsner *et al.,* 2005). It is an energy dependent process. Membrane bound amino acid transporters transport amino acids into sieve element / companion cell complexes of minor veins. Amino acid permeases (AAP), being non-specific to amino acids, are involved in the phloem loading process (Fischer *et al.,* 2002). The AAP are localised on plasma and internal membranes of main veins of mature leaves. Specific amino acid transporters are also reported (Hirner *et al.,* 2006; Van der Graaff *et al.,* 2006), which may be involved in the phloem loading process. Phloem loading, therefore, is the key step in the process of N remobilization.

Amino acid and peptide transporters expressed in the seed are involved in endosperm/ cotyledon loading of N compounds during embryogenesis (Rentsch *et al.,* 2007; Tsay *et al.,* 2007). Filling of N in seed mostly depends on efficiency of phloem loading by amino acid

transporters in source organs and sink strength of developing seed.

Amino acids to be loaded in phloem for N-remobilization are produced by the degradation of proteins synthesized before the onset of reproductive phase (Patrick and Offler, 2001). Rubisco being the major soluble protein, provides the major source of N for remobilization along with other photosynthesis related proteins. Proteases required for the proteolysis are present in cytosol and cell sap. Over-production of reactive oxygen species in chloroplasts (major), peroxisomes or mitochondria (Zimmermann and Zentgraf, 2005) during ageing triggers the process of proteolysis. Several other proteolytic mechanisms operating in different cell compartments may be involved in N-remobilization. Bassham *et al.* (2006) reported the role of autophagic recycling for N-mobilization during grain filling.

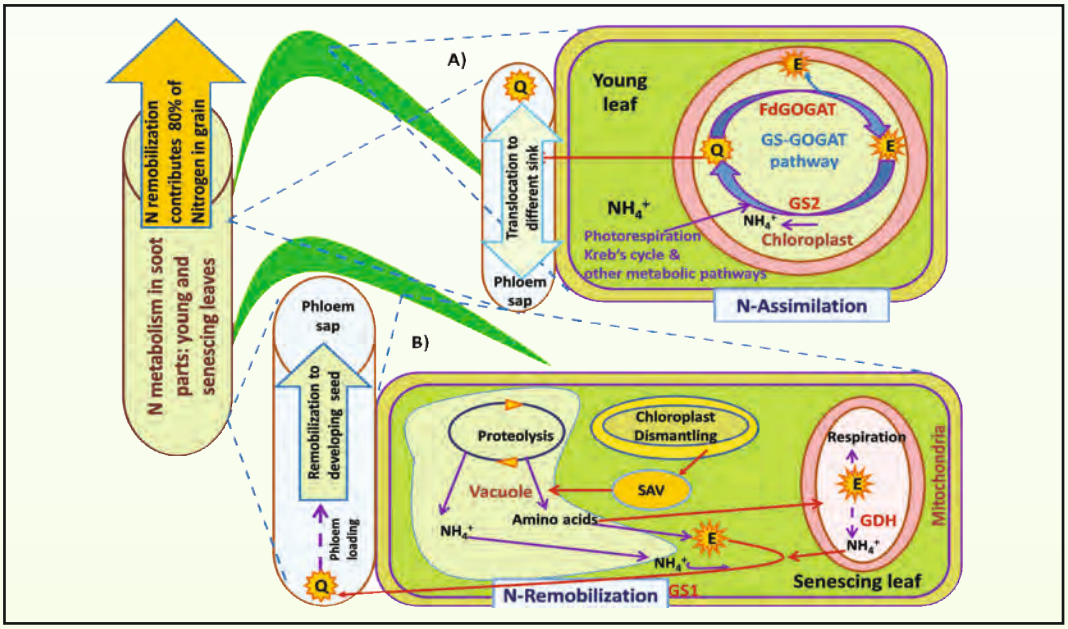

Fig 2. Mechanism of N- assimilation and transport in young leaves (A); Represents mechanism of degradation, N-remobilization and transport to developing seed from senescing leaves (B). SAV stands for Senescence-associated vacuole, Q represents Glutamine, E represents Glutamate, and GS represents Glutamine synthetase, GOGAT for Glutamine: oxoglutarate aminotransferase, GDH for Glutamate dehydrogenase. (Adapted from Masclaux-Daubresse *et al.*, 2010)

Several studies have emphasized upon the role of GS1 in N management, growth rate, yield and grain filling (Hirel *et al.,* 2001; Obara *et al.,* 2001; Tabuchi *et al.,* 2005; Martin *et al.,* 2006). However, the mobilization process still remains complex due to involvement of several isoforms of enzymes, unknown functions of proteins and multi-dimensional activity of enzymes. In rice, three GS1-encoding genes (*OsGS1;1*, *OsGS1;2*, *OsGS1;3*) have been identified (Ishiyama *et al.,* 2004) that have important roles in determining the growth rate and grain filling (Fig. 2). The *OsGS1;1* gene product located in companion cells and parenchyma cells of leaf tissues, is responsible for the generation of glutamine for remobilization via the phloem. Both germination and senescence require

huge amount of protein remobilization with improvement of higher activity of amiotransferases. Role of transaminases has also been documented during senescence which leads to greater accumulation of amino acids like tyrosine, leucine, isoleucine and the non-protein amino acid, γ-aminobutyric acid (GABA) with aging thereby reducing the concentration of glutamate, which is abundant in young leaves (Diaz *et al.*, 2006). Transaminases are reversible pyridoxal-5-phosphate containing enzymes that catalyze the transfer of an amino group to 2-oxo acids. The reversibility of their catalytic activity probably accounts for the relative stability of glutamate concentrations in plant tissues (Forde and Lea, 2007).

In rice, many QTLs along with associated markers have been identified that can be transferred to enhance the GPC of high yielding varieties (Aluko *et al.*, 2004; Yang *et al.*, 2015; Yoshida *et al.*, 2002; Zheng *et al.*, 2011; Qin *et al.*, 2009; Zhong *et al.*, 2011; Peng *et al.*, 2014b). But due to low heritability and significant influence of crop nutrient management practices, improvement of rice varieties for this quantitative trait through simple breeding scheme is a real challenge. In spite of that, it was demonstrated that both protein content and yield might be improved simultaneously up to a certain extent. It was reported that the higher expression of *OsAAP6*, a putative amino acid transporter, was correlated with higher GPC (Peng *et al.*, 2014a). Interestingly, *OsAAP6* situated at the long arm of chromosome 1, controlled GPC with no effect on plant morphology and grain yield, suggesting that GPC could be improved without reduction in grain yield. The ICAR-NRRI, Cuttack has also identified a QTL for GPC (q*GPC1.1*) on chromosome 1 through SNP genotyping using a 40894 SNP chip which has been found stable over the environments and associated with the locus, *Os01g0111900* on chromosome 1, encoding a glutelin family protein.

Estimation of GPC in rice

Micro-Kjeldahl method

Total nitrogen content was determined by the method of FAO (1970). Exactly 200 mg of rice grain was digested with concentrated H_2SO_4 followed by distillation using 5 ml of 4% boric acid and 10 ml of 40% NaOH solution and titration against standard 0.01N HCl solution. Amount of nitrogen in the sample was calculated using the following formula:

$$\%N = \frac{(\text{ml HCl in sample}) - (\text{ml HCl in blank}) \times \text{normality of acid} \times 14.01 \times 100}{\text{Weight of sample (mg)}}$$

Percentage of GPC = %N × 5.95.

Single grain protein content was measured as the average of the 10 grains expressed in mg/grain.

Calibration of near infrared spectroscopy (NIRS) and estimation of apparent GPC

Near-infrared (NIR) spectroscopy has proved effective in predicting GPC and is a reliable tool for genetical analysis and high-throughput selection (Shao *et al.*, 2011). This is based on the absorption of molecular overtone and combination vibrations of hydrogenous groups X-H (X =C, N, O) in the near-infrared region (750-2500 nm) of the electromagnetic spectrum.

The NIRS was calibrated with the help of three software related to NIR spectroscopy (model: FOSS- NIRSDS 2500, FOSS Analytical, Sweden). The software ISI Nova scan, Mosaic solo and WinISI III Project Manager v 1.50e (Windows Infra Soft International, USA) were used for scanning, configuration and calibration of samples respectively. A small cup (size: inner diameter 66 mm and height 25 mm) was used for scanning of 160 samples with full spectrum (400-2500 nm) by taking about 15 g of sample for analysis. The reflectance spectra (log1/R) from 400-2500 nm were recorded at 2 nm intervals. After incorporating the laboratory value in spectra

file, the regression equation was developed and simultaneously various trial and error methods of mathematics (eg.-"1,4,4,1", "1,2,3,1" etc.) under modified partial least square (mPLS) were also developed to find out the best regression equation for prediction of different parameters. The first digit of these mathematics indicates the order of the derivative (0 represents no derivative, 1 is first derivative of log 1/R and so on), the second digit is the gap in data points over which the derivative was calculated, the third and fourth digits refer to the number of data points used in the first and second smoothing, respectively (Fig. 3). After making different equations with different mathematics, another set of known samples (24) was scanned for prediction to get external validation and pair t-test was performed to obtain any significant variation between laboratory value and predicted value (Fig. 4). A subsequent external validation of the initial calibration model using samples independent from the calibration set led to further NIRS performance values for each constituent. 1,4,4,1 and 1,4,3,1 both are good calibration equations based on Lowest SEC (standard error of calibration) and SECV (standard error of cross validation) and highest RSQ (coefficient of determination), 1-VR (1 minus variance ratio) (Bagchi *et al.,* 2015).

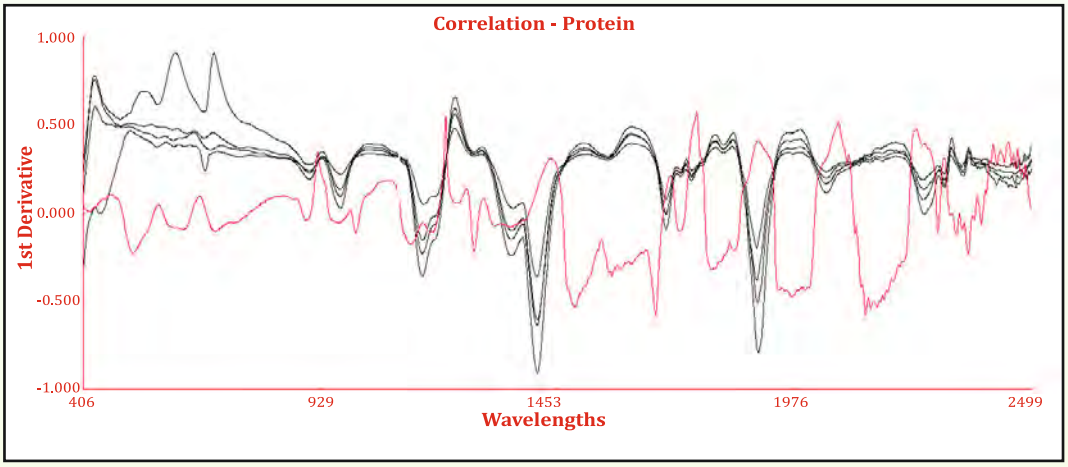

Fig. 3. Regression coefficient along with correlation plot of modified partial least squares model (MPLS) calibration equations under first derivative for grain protein content

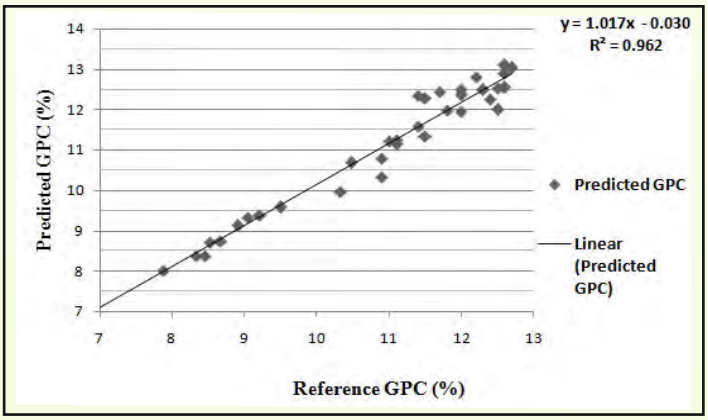

Fig. 4. Reference versus NIR predicted values plots for protein content of grain.

Extraction, fractionation, quantification and SDS-PAGE analysis of storage proteins

Extraction of rice grain proteins was done as per Ju *et al.* (2001) and Krishnan and Okita (1986). Rice flour (6-7 g) was defatted with n-hexane. The defatted flour was extracted by stirring with 20 mL of distilled water at room temperature for 2 hrs and centrifuged at 3,000×g for 30 min to get the albumin fraction. The residue was repeatedly re-extracted with various solvents in a stepwise manner to get globulin, prolamin and glutelin fractions. The protein fractions were freeze-dried and stored at -70°C. The protein content of each fraction was measured according to Lowry *et al.* (1951). The partially purified glutelins and prolamins were fractionated on preparative SDS-PAGE gels carried out according to the standard protocol (Sambrook and Russel, 2001) with 5% stacking and 12% resolving polyacrylamide gel. The BioRad Mini-PROTEAN 3 Electrophoresis Cell (BioRad, USA) was used for the purpose. Peptide pattern in the gel was visualized through staining with coomassie brilliant blue and photograph was taken using a gel documentation system.

Amino acid profiling

Polished rice samples were hydrolyzed with 6N HCl at 110°C under anaerobic condition for 24h. The hydrolyzed samples were neutralized with 6N NaOH and were derivatized using a kit (AccQ-Fluor Reagent, WAT052880, Waters). The derivatized samples were injected in high performance liquid chromatography (HPLC) (1525, Waters) equipped with a C18 RP column and a fluorescence detector (2475, Waters). The amino acids were identified and quantified by comparing with the retention times and peak areas of standards (WAT088122,Waters) (Pal *et al.*, 2016).

Diversity of rice germplasms for GPC

The International Rice Research Institute (IRRI) Philimines evaluated 13089 *indica* accessions, wherein protein content ranged from 4.3% to 18.2% (dry season) and 3.5% to 15.9% (wet season). This indicated wide genetic variability of this trait and also the feasibility of developing high-protein rice cultivars. At NRRI, Cuttack, about 2000 germplasm of *Indica* rice were found to exhibit wide range of variation for the GPC. While ARC 5973 contained lowest GPC (4.01%), PB -312 contained the highest amount (14%). A list of high and low GPC germaplsm in brown rice is given below (Table 1).

Table 1. Rice germplasm with their GPC in brown rice

Sl.No.	Varieties with high GPC	GPC (%)	Varieties with low GPC	GPC (%)
1	Kalinga-III	12.80	Naveen	8.60
2	Heera	10.50	Basmati	7.60
3	Bindli	13.20	Nuadhusara	7.90
4	PB140	12.80	Annada	7.50
5	Mamihunger	13.60	Chakhao	6.90
6	ARC10075	11.70	Nalbora	6.40
7	ARC10063	12.00	Assambiroi	6.60
8	PB170	12.60	Aghonibora	6.80
9	PB- 84	13.05	Swarna	7.60
10	PB-312	14.10	IR64	7.00
11	PB-318	11.02	Geetanjali	7.80
12	CR Dhan 310	11.50	Pooja	6.90
13	Manipuri	10.50	Lalat	6.50
14	Tara	10.20	ARC 5973	4.01
15	Kalabiroin	10.20	ARC 6023	4.89

Utilization of germplasm with high GPC for development of breeding population

Two high-protein (11-13%) rice germplasm, ARC 10063 and ARC 10075, identified from the stock of the Assam Rice Collections of the NRRI Rice Gene Bank, were evaluated thoroughly for the seed protein fractions. Three glutelin bands (near 21 kD, 29 kD and 43 kD) were highly expressed in the high-protein cultivars. They showed higher activity of nitrate reductase (NR) and glutamic dehydrogenase (GDH) at seedling stage (one-week-old) and maximum tillering stage (three week-old). These high protein landraces were used in the breeding programme to improve high yielding varieties for GPC.

Breeding for high protein rice

Several efforts were made over the past few decades to improve the protein content of rice, which involved mainly conventional breeding and induced mutagenesis approaches (Mahmoud *et al.*, 2008; Khush and Juliano, 1984). However, these efforts were largely unsuccessful, as indicated by the lack of modern high-protein rice cultivars (Vasal, 2002). Initial efforts made by IRRI to develop high protein elite lines employed pedigree and long cycle recurrent selection. But the high protein lines so developed were not accepted in long run probably either due to deviation in grain type and cooking qualities from the adapted parent, IR 8, or due to low stability of their protein yields. The ICAR-NRRI has developed some high yielding breeding lines with substantial improvement of grain protein content over the high yielding varieties (Photo 1) through bulk-pedigree and back cross breeding methods (Chattopadhyay *et al.*, 2013).

Photo 1. Field view of evaluation of breeding lines for high GPC at NRRI, Cuttack in 2013

High protein rice developed through bulk-pedigree method

In our breeding program, efforts were made to improve GPC through modified bulk-pedigree method of selection. Thus, 1780 F_8 recombinant lines were derived in the year 2013 from five different cross combinations involving two high-GPC landraces, namely ARC10075 and ARC10063; three high-yielding parents, namely Swarna, Naveen and IR64; and one parent, i.e. Sharbati, known for superior grain quality with high micro nutrient content (Fig. 5). NIR spectroscopy was used to facilitate high-throughput selection for GPC. Significant selection differential response to selection and non-significant difference between the predicted and observed response to selection for GPC and protein yield indicated the effectiveness of this selection process. This resulted in lines with high GPC, protein yield and desirable level of amylose content. Further, based on high mean and stability for GPC and protein yield over the environments in wet season 2013, 2014 and dry season 2014, twelve elite lines were identified (Chattopadhyay *et al.*, 2018). Higher accumulation of glutelin fraction (Fig. 6) without any significant change in prolamin/glutelin ratio in the grain suggested safe guarding of the nutritional value of rice grain protein of most of these identified lines (Photo 2).

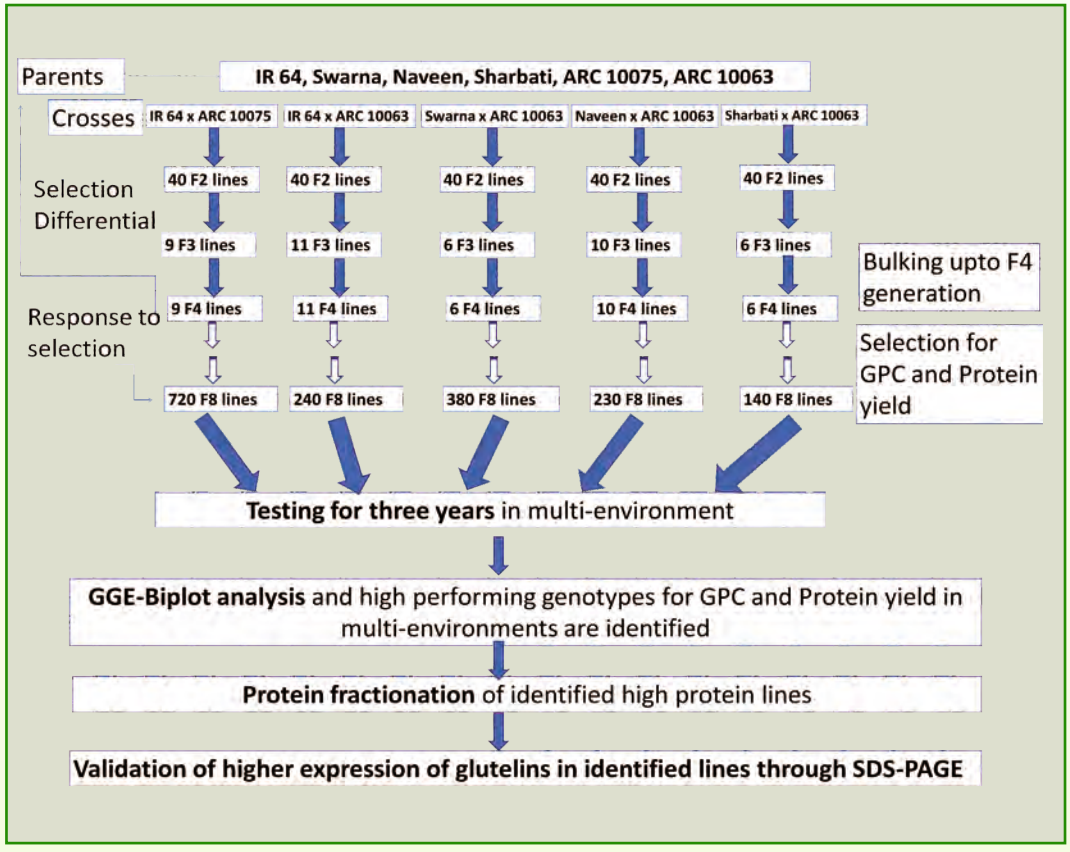

Fig 5. Schematic presentation of bulk-pedigree breeding procedure and qualitative and quantitative improvement of GPC in rice at NRRI, Cuttack.

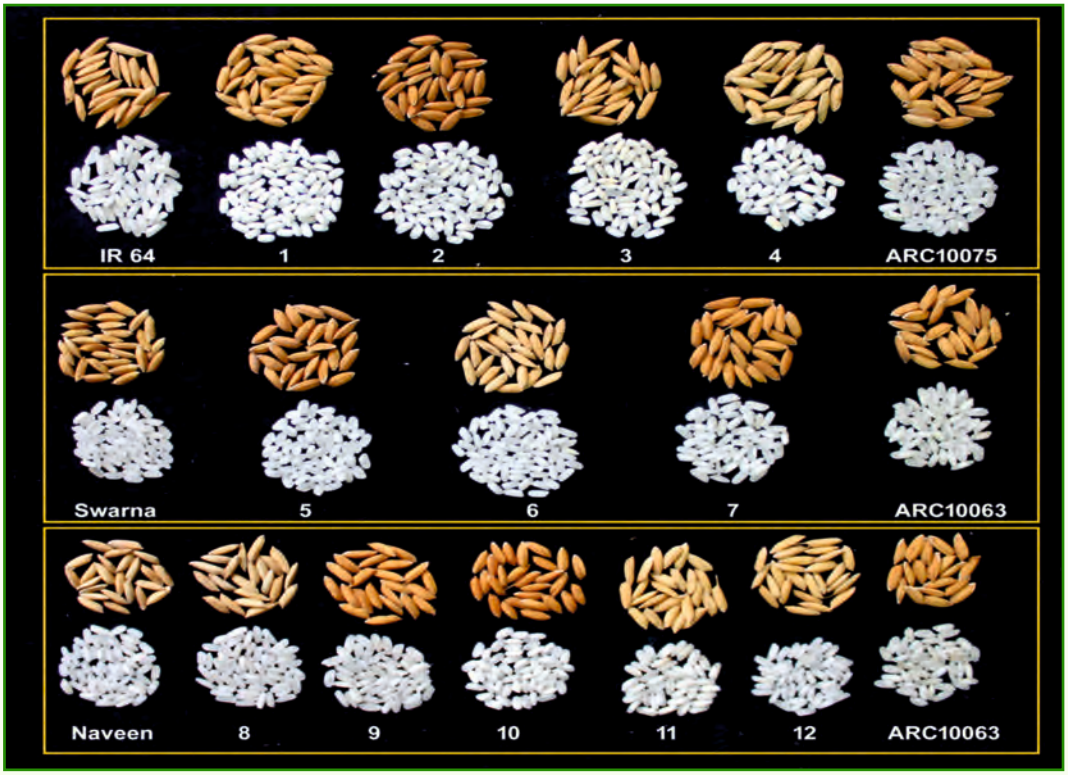

Photo 2. Grain and polished rice of high GPC breeding lines

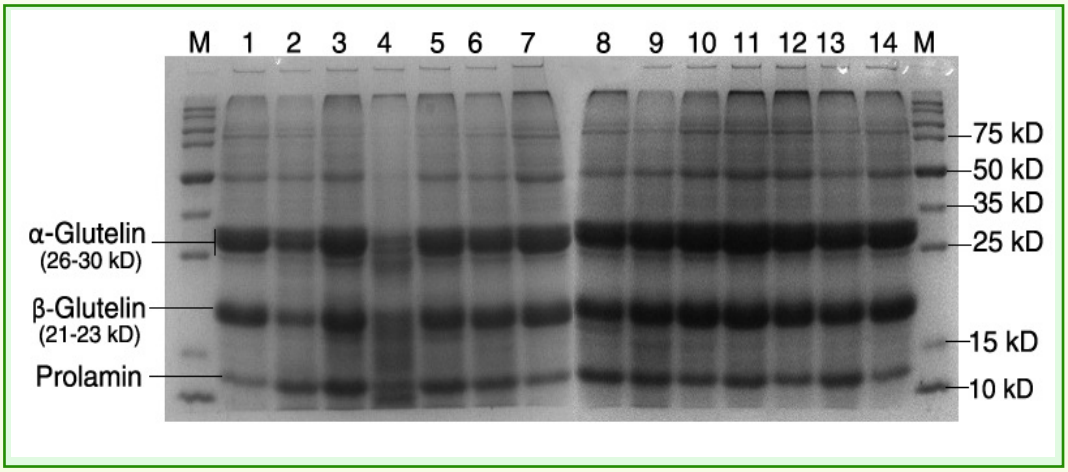

Fig. 6. The SDS-PAGE profiles of partially purified glutelin fraction showing α (~29 kD) and β-glutelin (~21 kD) sub-unit and a prolamin band (~13-14kD) in rice genotypes, viz., lane1: ARC1063, lane 2: Swarna, lane3: CPL-D- F_8-824, lane 4: CPL-A- F_8-1045, lane 5: CPL-D- F_8-891, lane 6: CPL-D- F_8-905, lane 7: CPL-D- F_8-884, lane 8: CPL-D- F_8-887, lane 9: CPL-C- F_8-972, lane 10: CPL-C- F_8-966, lane 11: CPL-C- F_8-588, lane 12: CPL-A- F_8-1049, lane 13: CPL-A- F_8-1091, lane14: CPL-A- F_8-1031 and lane M: ladder of 10-225 kD molecular weight. Glutelin samples were loaded at 15 µg on each lane and proteins were detected with standard coomassie brilliant blue stain after electrophoresis.

High protein rice development through backcross method of breeding

Backcross method of selection is not only a reliable method to validate the additive effect of a quantitative trait locus (QTL) or a candidate gene, but also proved useful for developing elite introgession lines. This method is quite effective in generating transgressive segregants for the desired trait in the background of the high yielding recurrent parent. Backcross also helps to reduce the effect of undesirable traits. Although land races are, in general, agronomically inferior to crop plants, transferring favourable alleles from land races to crop plants with positive effects on quality-related traits has proved feasible in several crops including rice (Yang *et al.*, 2004; Mahmoud *et al.,* 2008; Xie *et al.,* 2014). We used one such land race (ARC10075) as high GPC donor (Chattopadhyay *et al.,* 2011) in our breeding programme. We exercised two/three repeated backcrossing with recurrent parents, Swarna and Naveen for developing two backcross populations (Fig 7). The BC_3F_1 plants were selfed and population was carried by single seed descent (SSD) method, which was reported efficient not only for speeding up and economizing the breeding process, but also for producing wide range of phenotypic variation with high level of transgressive segregation. The present breeding programme coupling backcrossing with SSD, delivered a large number of transgressive lines for GPC. We found more than 20% introgression lines for GPC with substantial phenotypic similarities (plant architecture, maturity duration, etc.) with recurrent parents, Naveen and Swarna.

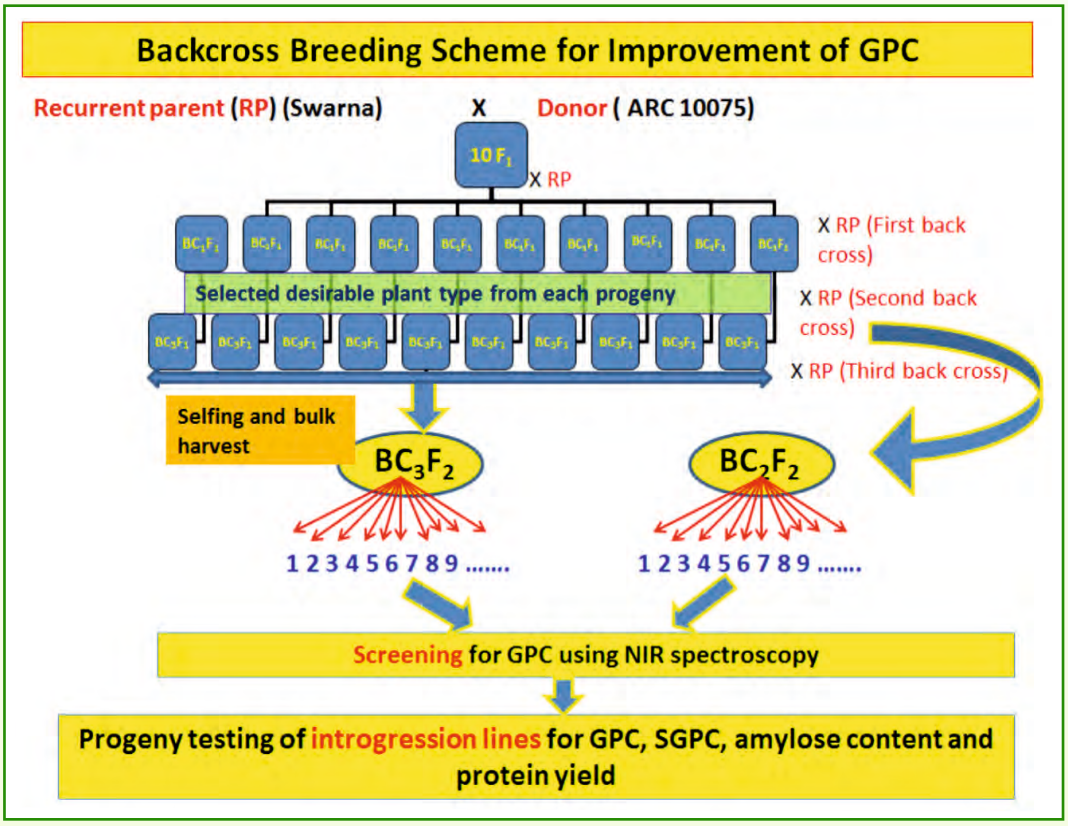

Fig. 7. Breeding scheme for improvement of GPC of high yielding rice through backcross breeding.

Normal distribution was noted in both the populations for GPC with a range of 7.13%-13.6% in Population-1 (ARC10075/Naveen) and 6.09%-14.47% in Population-2 (ARC10075 /Swarna) (Fig. 8). BC_3F_3 lines were evaluated for GPC and lines with high GPC (>10%) in the high yielding backgrounds, 'Swarna' (Photo 3) and 'Naveen' were detected through high throughput screening using calibrated NIR spectroscopy. Progenies were then screened for higher GPC, grain yield, protein yield and desirable amylose content (20-25%). This breeding procedure was found effective in generating transgressive segregants for protein yield.

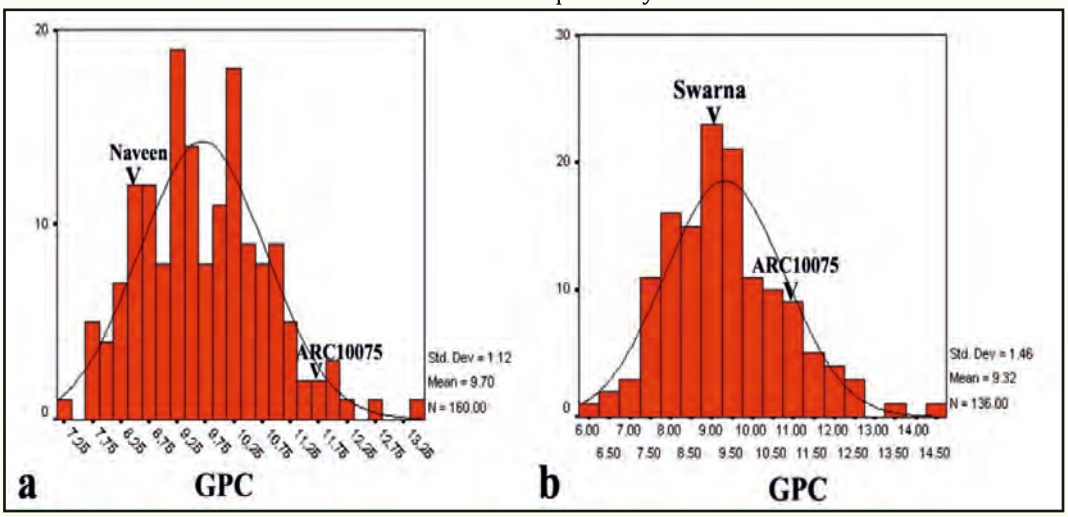

Fig. 8. Distribution of grain protein content (GPC) in rice in (a) backcross Population -1 (ARC10075/Naveen) and in (b) Population-2 (ARC10075/Swarna)

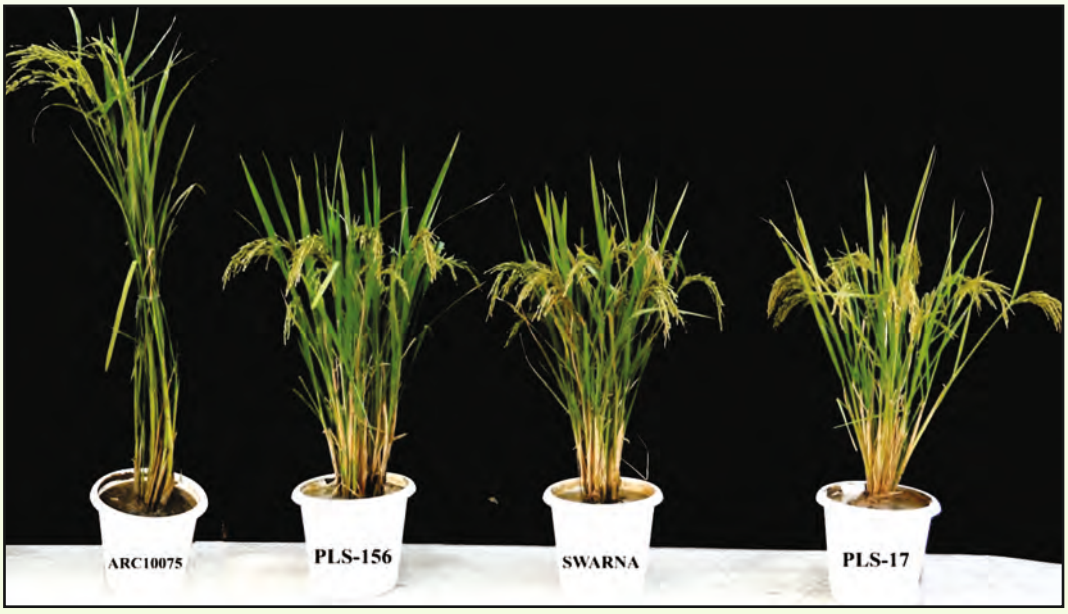

Photo 3. High protein lines in Swarna background (PLS-156, PLS-17) and their parents (Swarna, ARC 10075)

Evaluation of high protein lines in Naveen and Swarna background

Ten high yielding selected introgression lines ($BC_3F_{2:4}$) from the Population-1 and Population-2 were grown in the same experimental plot of the NRRI, Cuttack in *rabi* 2013 and *kharif* 2014. Nitrogen, phosphorus, and potassium were supplied at 80 kg, 60 kg, and 40 kg per hectare, respectively in *kharif* and 120 kg, 60 kg and 60 kg per hectare, respectively in *rabi*. Phosphorus (as single super phosphate) was applied as a basal dose, and nitrogen (as urea) and potassium (as muriate of potash) were applied in two equal doses at 30 days after transplanting and at 50% flowering. The GPC was estimated in polished rice by the standard micro-Kjeldahl method (Yoshida *et al.,* 1976). Single grain protein content (SGPC) was also estimated (from the average of 10 grains). The latter was observed to be a more stable parameter than GPC with higher percent of heritability than the latter. All lines had significantly higher GPC, SGPC and protein yield than their corresponding high yielding parents (Table 2). They also had acceptable grain type (Photo 4, 5) and other quality traits (Table 3).

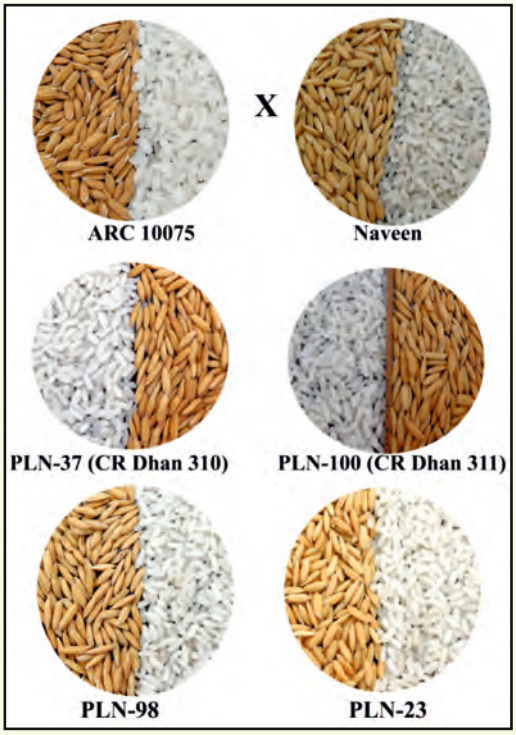

Photo 4. Grain and polished rice of parents, Naveen and ARC 10075 and the derived high protein lines in Naveen background.

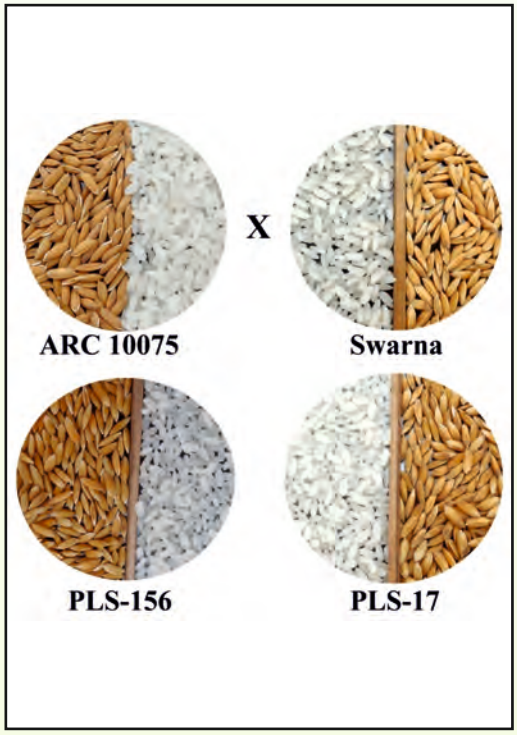

Photo 5. Grain and polished rice of parents, Swarna and ARC 10075 and the derived high protein lines in Swarna background

Table 2. Evaluation of selected high yielding introgression lines in Naveen and Swarna background for GPC, single grain protein content (SGPC), grain yield per sq meter and protein yield per sq meter

Trait (season wise)	Parameters	High GPC donor ARC10075	Check -1	Introgression lines in Naveen background							Check -2	Introgression lines in Swarna background		
			Naveen	PLN-100	PLN-116	PLN-23	PLN-32	PLN-37	PLN-98	PLN-99	Swarna	PLS-124	PLS-156	PLS-17
Rabi 2013	GPC (%)	10.80	7.74	10.40	11.17	12.10	11.90	10.51	10.15	9.86	8.42	10.65	10.79	11.76
	SGPC (mg)	2.40	1.56	1.98	1.68	2.15	1.85	1.93	1.99	1.94	1.43	1.62	1.60	1.56
Kharif 2014	GPC (%)	10.88	8.13	9.47	10.43	9.72	9.47	9.79	10.45	10.25	8.20	10.48	9.69	10.10
	SGPC (mg)	2.58	1.60	2.10	2.14	2.16	2.00	2.07	2.08	2.14	1.50	2.22	1.70	1.82
Mean	GPC (%)	10.84	7.93	9.93	10.80	10.91	10.68	10.15	10.30	10.06	8.31	10.57	10.24	10.93
	SGPC (mg)	2.49	1.58	2.04	1.91	2.16	1.93	2.00	2.04	2.04	1.47	1.92	1.65	1.70
% Improvement of protein content over check	GPC	28.61		21.17	28.19	25.87	28.66	24.45	25.25	24.27		20.35	17.81	22.99
	SGPC	35.91		24.63	21.40	27.48	23.83	25.02	23.58	25.71		23.70	11.21	13.82
Grain yield (kg/ha) *Kharif* 2014	Yield (kg/ha)	2450	4060	3890	3870	3900	4310	4246	3990	3880	5320	5300	5175	5260
% Improvement of grain yield over check				-4.19	-4.68	-3.94	6.16	4.58	-1.72	-4.43		-0.38	-2.73	-0.75
Protein yield (kg/ha) *Kharif* 2014		266.56	330.26	368.34	403.62	379.24	408.11	415.77	416.82	397.74	436.24	555.47	501.46	531.15
% Improvement protein yield over checks				11.53	22.21	14.83	23.57	25.89	26.21	20.43		27.33	14.95	21.76

Table 3. Evaluation of introgression lines for high GPC for grain quality parameters

Quality parameter	PLN-100	Check-1 (Naveen)	Check-2 (ARC10075)	PLN-116	PLN-23	PLN-32	PLN-37	PLN-98	PLN-99	Swarna	PLS-17	PLS-124	PLS-156
Kernel length (mm)	6.17	5.53	6.44	5.33	6.38	6.22	5.83	6.28	6.74	5.27	5.33	5.37	5.43
Kernel breadth (mm)	2.05	2.39	2.24	2.05	2.22	2.18	2.2	2.04	2.08	1.56	1.44	1.59	1.8
Kernel length and breadth ratio	3.01	2.31	2.87	2.6	2.87	2.85	2.65	3.08	3.24	3.38	3.7	3.38	3.02
Grain type	Long slender	short bold	medium slender	short bold	medium slender	medium slender	short bold	long slender	long slender	short slender	short slender	short slender	short slender
Amylose content (%)	23.61	22.51	24.35	22.73	23.06	23.87	22.89	23.48	21.55	23.2	22.4	22.8	

Except two, all high protein lines had significantly higher glutelin content than Swarna and Naveen. Glutelin contains essential amino acids such as lysine and is the major constituent of protein body II, which is more digestible than protein body I, which contains mostly prolamins. Therefore, higher accumulation of glutelins ensures better protein quality in most of these lines. The ratio of prolamin to glutelin fractions ranged from 0.02 to 0.037 (Table 4). All high protein lines had similar or slightly lower values of prolamin/glutelin ratio than the high yielding varieties, Swarna and Naveen, which ensures that these introgression lines retained good cooking quality. Finally, amino acid composition is also a key factor in determining the nutritional quality of cereal grains. Most of the selected high protein lines in Population-1 had significantly higher level of lysine (Lys), threonine (Thr), leucine (Leu), isoleucine (Ile), valine (Val), phenylalanine (Phe), alanine (Ala), proline (Pro), glutamic acid (Glu), arginine (Arg) and total amino acids as compared to the recurrent high yielding parent. Elevated level of some of the essential amino acids improved the quality of the storage protein in these derived lines. This qualitative improvement was earlier largely restricted to the maize crop through enrichment of grain Lys (Ufaz and Galili, 2008).

Table 4. Fraction of soluble protein (percent in 100g polished rice sample) in introgression lines for GPC in Naveen and Swarna background and their parents

Sl. No.	Genotype	Albumin	Globulin	Prolamin	Glutelin	Prolamin/ glutalin ratio	Total
1	ARC-10075	0.434	1.415	0.443	12.864	0.034	15.156
2	Naveen	1.406	1.02	0.244	9.297	0.026	11.967
3	PLN-37	0.44	1.263	0.352	13.519	0.026	15.575
4	PLN-100	0.565	1.292	0.356	12.49	0.029	14.703
5	PLN-32	0.988	1.283	0.24	11.058	0.022	13.57
6	PLN-99	0.798	1.28	0.296	10.584	0.028	12.959
7	PLN-23	1.48	1.575	0.333	8.889	0.037	12.276
8	PLN-116	0.283	0.9	0.242	11	0.022	12.425
9	PLN-98	0.823	1.507	0.365	11.61	0.031	14.306
10	Swarna	0.629	0.85	0.351	10.1	0.035	11.93
11	PLS-156	0.845	1.231	0.324	10.2	0.032	12.6
12	PLS-17	0.764	0.925	0.283	10.5	0.027	12.471
13	PLS-124	0.449	1.078	0.218	7.769	0.028	9.514
Mean		0.785	1.238	0.322	11.141	0.029	13.486
CD (5% level)		0.06	0.11	0.08	0.35	-	0.41

High protein rice varieties

Release of CR Dhan 310 and CR Dhan 311 (Mukul)

Among seven high protein lines in Naveen background, tested under Biofortification trials, PLN-37 had average protein content of 10.2% in polished grain which was 21.6% and 12.44% higher than quality checks, Kalanamak and Chittimuthyalu, respectively in the trial conducted at ten locations. Performance with respect to grain yield was also good in all zones with an average of 4483kg/ha. It outperformed the yield-check; Samba Mahsuri by registering yield superiority of 6.81%. It has long panicle with medium slender grains. This high yielding variety (CR 2829-PLN-37) with high GPC has been released by the Central Variety Release Committee in 2016 for Odisha, Uttar Pradesh and Madhya Pradesh as CR Dhan 310 (Gazette notification, Govt. of India, S.O. 1438(E), No. 927, dated 19/04/2016). Another line PLN-100 (CR 2829-PLN-100) has been released by the State Variety Release Committee of Odisha (Govt. of Odisha, No 2M (05) 126pt/2016, 40863 dated 25/11/2016)in the name of Mukul (CR Dhan 311) for high protein content (10.1%) and moderately high Zn content (20 ppm) with grain yield of 4330 kg/ha (Photo 6).

Photo 6. Field view of CR Dhan 310 and CR Dhan 311 (Mukul)

Characteristics of high protein rice varieties

The high protein rice varieties are medium early (122-125 days) with semi-dwarf (108-115 cm), compact plant type and good initial growth and tillering ability. They are found to be better or at par with the checks for their response to important biotic stresses. The protein yield per unit area of CR Dhan 310 and CR Dhan 311 were 457 kg/ha and 438 kg/ha, respectively as compared to that of Naveen (367 kg/ha) and the high GPC donor ARC10075 (305 kg/ha). In the agronomic trial held at NRRI farm during *kharif* 2014, these two varieties also performed at par with their high yielding parent, Naveen. They had high head rice recovery (>60%) and acceptable grain and cooking quality as shown by intermediate amylose content (22-24%). They have been well accepted by the farmers due to their resemblance for grain and plant type with the recurrent parent Naveen, a well adopted popular rice variety of the eastern India (Photo 7).

Photo 7. Field view showing agro-morphological and phenotypic similarity of Naveen and CR Dhan 310 (CR 2829-PLN-37)

Genetic architecture and associated QTLs

Genotypic similarity of these varieties with Naveen was also established by employing more than 400 SSR primers distributed over all the 12 chromosomes. Sixty eight primers including some markers linked with reported QTLs were found polymorphic between Naveen and ARC 10075. Finger printing pattern revealed that these two high protein rice varieties were around 95% genetically similar with Naveen (Fig.9). Both these introgressed lines carried a genomic region from ARC 10075 of the marker RM11 in chromosome 7 which was reported to be associated with QTL *pro 7* for GPC (Guo *et al.,* 2007). CR Dhan 311 (PLN-100) also contained a genomic region from ARC 10075 for the marker RM 167, which was reported to be associated with the QTL pro11(Aluko *et al.,* 2004) for GPC on chromosome 11. On the other hand, another variety CR Dhan 310 (PLN-37) contained a genomic region from high protein donor for the marker RM 232, which was associated with QTL *pro2* for GPC (Aluko *et al*., 2004; Yu *et al.,* 2009) on chromosome 2.

The marker trait association analysis was done for 200 germplasm, which detected multiple QTLs governing protein content in rice. The strongly associated markers with grain protein controlling QTLs namely, *qPC2.2, qPC4.2, qPC4.3, pro1, pro2, qPC3* and *qProt8* may be useful for pyramiding in high yielding varieties to develop protein rich rice.

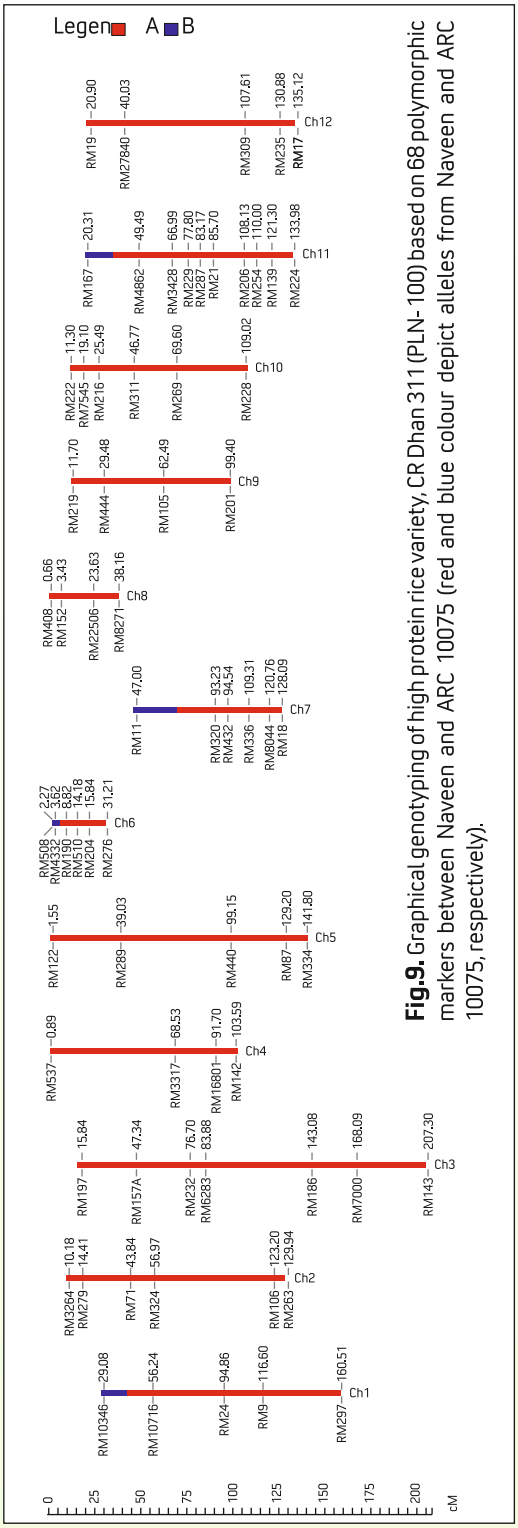

Fig.9. Graphical genotyping of high protein rice variety, CR Dhan 311 (PLN- 100) based on 68 polymorphic markers between Naveen and ARC 10075 (red and blue colour depict alleles from Naveen and ARC 10075, respectively).

New QTLs for GPC and SGPC

The high GPC donor ARC10075 was crossed with the high yielding popular variety Naveen which had on average 8% GPC (brown rice). A backcross derived population was developed by three consecutive backcrossing with recurrent parent Naveen, followed by single seed descent. Normal distribution was observed for GPC and single grain protein content of BC_3F_5 lines derived from this population. The population was genotyped using the high-resolution Affymetrix custom array 40894 SNP chip. The assay was designed on Affymetrix Axiom technology. Among 40894 markers in SNP chips, 5492 SNPs accounting 13.43% were found to be polymorphic between ARC10075 and Naveen. In *kharif* season 2014 (E1), one QTL (*qGPC1.1*) in 11cM position on chromosome 1 was identified for GPC with a LOD value of 3.13, which explained 12.18% phenotypic variance. In this environment, four other QTLs for SGPC were identified. One of the pleiotropic QTL (*qSGPC1.1*) shared the same position with *qGPC1.1* explaining 10.37% PVE with a LOD value of 2.9. The other three QTLs for SGPC *(qSGPC2.1, qSGPC7.1, qSGPC11.1*) with LOD values of 3.32, 3.31 and 2.88 explaining 6.7, 7.68 and 6.42 PVE, respectively. In *rabi* season 2014 (E2), more number of QTLs for both GPC and SGPC were detected. Out of the two QTLs for GPC, one (*qGPC1.1*) was common with the previous environment (E1) explaining 13.85% PVE with LOD value of 4.02. The new one (*qGPC 2.1*) explained 17.35% PVE with LOD value of 3.19. Eleven QTLs for SGPC were found in E2 on chromosomes 1, 2, 3, 7, 8, and 12. Two of them were common with previous environment (E1). They were *qSGPC2.1* and *qSGPC7.1* with LOD values of 3.53 and 3.33, respectively which explained 14.64% and 7.81% PVE. Finally a QTL for GPC (q*GPC1.1*) on chromosome 1 was found, which was stable over the environments andassociated with the locus, *Os01g0111900*, encoding a glutelin family protein. These two high

protein varieties (CR Dhan 310 and CR Dhan 311) also contained this QTL.

Suitable area /ecology for cultivation and fertilizer application

These two medium duration (120-125 days) high protein rice varieties, CR Dhan 310 and Mukul (CR Dhan 311) are suitable for cultivation in favorable rainfed upland /medium land and irrigated lands during *kharif* and *rabi* season. The projected grain yield and protein yield cannot be obtained from those lands affected by submergence and water stagnation.

Response of high protein rice to fertilizer application

Overall grain yield of CR Dhan 310 was *at par* with Naveen. Reducing NPK by 25% did not affect yield in CR Dhan 310 significantly, but grain yield in Naveen decreased by 7.5%. Increasing NPK by 50% increased yield by more than 5% in CR Dhan 310 and 7% in Naveen. The partial factor productivity (PFP) obtained by applying the higher dose of NPK was found to be 40.5 kg grain / kg N applied for CR Dhan 310 and 41.6 kg grain / kg N applied for Naveen.

Based on the above considerations, the recommended fertilizer dose (irrigated ecology) is 120:60:60 NPK kg/ha + 5 t/ha FYM. Zinc sulphate @ 25 kg/ha should be applied in zinc deficient soils as basal application. Leaf Colour Chart (LCC) based N application may be followed for increasing N use efficiency.

Demonstration of high protein rice in Odisha

High protein rice varieties were successfully demonstrated in mid-central table land and east & south-eastern coastal plains (non-saline) (Zone 3, 10).The average yield of Mukul (CR Dhan 311) was found to be 4645 kg/ha with protein yield of 469 kg/ha as compared to 4700 kg/ha and 382 kg/ha for Naveen at ten blocks (20 farmers fields) under Cuttack, Jagatsinghpur (Photo 8), Dhenkanal and Baramba districts in 2015-16. In 2017, more than 30 FLDs were taken in three districts. The average yield of CR Dhan 310 and Mukul were 5 t/ha. The farmer, Khetramohan Swain, vill. Mudupur, block-Jagatsinghpur, got award at state level in 2016-17 for his progressive contribution including adaptation of high protein rice 'Mukul'.

Photo 8 Cv. Mukul in Jagatsinghpur district in *kharif* 2016

Response of high protein rice to biotic stresses

CR Dhan 310 and CR Dhan 311 were found to be better or *at par* with the checks with regard to their response to important biotic stresses. CR Dhan 310 was found tolerant or moderately tolerant to leaf blast, brown spot, sheath rot, stem borer, gall midge biotype 1 and leaf folder, while CR Dhan 311 showed tolerance to leaf blast, glume discoloration, brown spot, RTD and bacterial leaf blight and moderate tolerance against brown plant hopper, gall midge and stem borer.

Special benefit of high protein rice

The rice Naveen was released more than 10 years back. Therefore, as per policy of the government, no subsidy is provided for its cultivation. The high protein rice varieties are phenotypically, genotypically and morphologically similar to Naveen. Moreover, yield of the released varieties are at par with that of Naveen. So, replacement of Naveen with high protein 'improved Naveen' (CR Dhan 310 and Mukul) will be financially beneficial for the farmers and nutritionally rewarding for the consumers.

A Rapid Method for detection of high protein rice over its adapted low protein parent

As protein content is not a phenotypic trait, it is desirable to suggest a simple and rapid method to differentiate between the parent Naveen (GPC-7.5%) and its high protein version CR Dhan 310 (GPC-10.3%) and Mukul (GPC- 10.1%). The *Xanthoproteic test* involves treating 0.2 g powdered rice grains taken in a test tube with 1 ml of concentrated nitric acid followed by heating the test tube on a low flame burner and addition of 40% alkali to neutralize the acid. The grains of the high protein rice varieties (CR Dhan 310 and CR Dhan 311) can be easily distinguished from the low protein types (Naveen) with this test as the former give more intense orange color

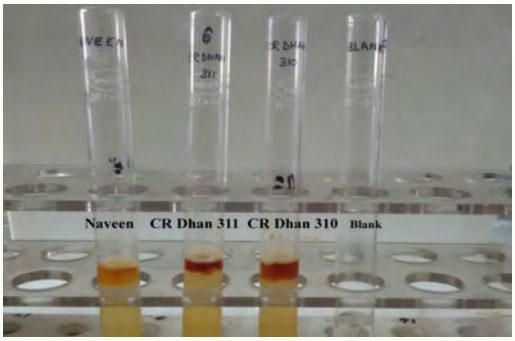

Fig. 10. Xanthoproteic test to detect high protein rice over the parent, Naveen

Conclusion

High yielding rice varieties with high nutritional value developed through breeding intervention have the potential to significantly contribute towards better nourishment of millions of underprivileged children in India, who depend mainly on rice for their nutrition. At present, no support price for farmers for high nutrient rich or any other specialty rice is available. Higher support price for growers and subsidy for mid-day meal rice are required to give benefits to both the poor rice-farmers and the underprivileged children in rural India. Moreover, as these varieties are not suitable for areas with water stagnation or deficit or those with low nutrient availability, the extension machinery has to be geared up to promote their cultivation in suitable lands and popularization among masses by creating awareness about the health benefits of these varieties. Institutional support and policy decisions would be needed to help the farmer get higher price for the nutrient dense rice grains and for their popularization.

References

Aluko G, Martinez C, Tohme J, Castano C, Bergman C and Oard HJ. 2004. QTL mapping of grain quality traits from the interspecific cross *Oryza sativa* × *O. glaberrima*. *Theoretical and Applied Genetics,* **109**, 630–639.

Bagchi TB, Sharma SG and Chattopadhyay K. 2015. Development of NIRS models to predict protein and amylose content of brown rice and proximate compositions rice bran. *Food Chemistry,* **191**, 21–27, http//dx.doi.org/10.1016/j.foodchem.2015.05.038.

Bassham DC, Laporte M, Marty F, Moriyasu Y, Ohsumi Y, Olsen LJ and Yoshimoto K. 2006. Autophagy in development and stress responses of plants. *Autophagy,* **2**(1), 2-11.

Blumenthal J M, Baltensperger D D, Cassman K G, Mason S C and Pavlista A D 2008. Importance and effect of nitrogen on crop quality and health. In *Nitrogen in the environment: sources, problems and management* (Eds J. L. Hatfield & R. F. Follett), Amsterdam: Academic Press/Elsevier, p. 23-78.

Chattopadhyay K, Das A and Das SP. 2011. Grain protein content and genetic diversity of rice in north eastern India. *Oryza,* **48**, 73-75.

Chattopadhyay K, Das A, Marndi BC, Bagchi TB, Sharma SG, Singh ON and Mohapatra T. 2013. Characterization of backcross derived lines of Naveen and Swarna having high grain protein content (GPC). *CRRI Newsletter*, **34**(4), 19.

Chattopadhyay K, Sharma SG, Bagchi TB, Molla KA, Sarkar S, Marndi BC, Sarkar A, Dash SK and Singh ON. 2018. Development of recombinant high yielding lines with improved protein content in rice (*Oryza sativa* L.). *The Journal of Agricultural Science, Cambridge,* 1–17. *https://doi.org/10.1017/*S0021859618000230.

Beninati NF, and Busch RH. 1992. Grain protein inheritance and nitrogen uptake and redistribution in a spring wheat cross. *Crop Science,* **32**(6), 1471-1475.

Bloom AJ, Sukrapanna SS and Warner RL. 1992. Root respiration associated with ammonium and nitrate absorption and assimilation by barley. *Plant Physiology,* **99**(4), 1294-1301.

Derycke V, Veraverbeke W S, Vandeputte G E, De Man W, Hoseney R C and Delcour J A 2005. Impact of proteins on pasting and cooking properties of nonparboiled and parboiled rice. *Cereal Chemistry,* **82**, 468–474.

Diaz C, Saliba-Colombani V, Loudet O, Belluomo P, Moreau L, Daniel-Vedele F and Masclaux-Daubresse C. 2006. Leaf yellowing and anthocyanin accumulation are two genetically independent strategies in response to nitrogen limitation in Arabidopsis thaliana. *Plant Cell Physiology,* **47**(1), 74-83.

FAO. 1970. Amino Acid Contents of Food and

Biological Data on Protein. FAO Nutritional studies No. 24. Food and Agriculture Organization of the United Nations FAO, Rome.

Fischer WN, Loo DD, Ludewig U, Boorer KJ, Tegeder M, Rentsch D and Frommer WB. 2002. Low and high affinity amino acid H^+ co-transporters for cellular import of neutral and charged amino acids. *The Plant Journal,* **29**(6), 717-731.

Forde BG and Lea PJ. 2007. Glutamate in plants: metabolism, regulation, and signalling. *Journal of experimental botany,* **58**(9), 2339-2358.

Guo Y, Mu P, Liu J, Lu Y and Li Z. 2007. QTL mapping and QxE interaction of grain cooking and nutrient qualities in rice under upland and lowland environments. *Journal of Genetics and Genomics,* **34**(5), 420-428.

Hirel B, Bertin P, Quilleré I, Bourdoncle W, Attagnant C, Dellay C and Gallais A. 2001. Towards a better understanding of the genetic and physiological basis for nitrogen use efficiency in maize. *Plant Physiology,* **125**(3), 1258-1270.

Hirner A, Ladwig F, Stransky H, Okumoto S, Keinath M, Harms A and Koch W. 2006. Arabidopsis LHT1 is a high-affinity transporter for cellular amino acid uptake in both root epidermis and leaf mesophyll. *The Plant Cell,* **18**(8), 1931-1946.

Ishiyama K, Inoue E, Watanabe-Takahashi A, Obara M, Yamaya T and Takahashi H. 2004. Kinetic properties and ammonium-dependent regulation of cytosolic isoenzymes of glutamine synthetase in Arabidopsis. *Journal of Biological Chemistry,* **279**(16), 16598-16605.

Ju Z, Hettiarachchy N and Rath N. 2001. Extraction, denaturation and hydrophobic properties of rice flour proteins. *Journal of Food Science,* **66**, 229-232.

Juliano BO. 1980. Rice: Recent progress in chemistry and nutrition. *In*: Inglett, G. E., Munck, L. (Eds.). Cereals for Food and Beverages, Recent Progress in Cereal Chemistry. Academic Press: New York.

Kichey T, Hirel B, Heumez E, Dubois F and Le Gouis J. 2007. In winter wheat (*Triticum aestivum* L.), post-anthesis nitrogen uptake and remobilisation to the grain correlates with agronomic traits and nitrogen physiological markers. *Field Crops Research,* **102**(1), 22-32.

Khush, GS, Juliano, BO. 1984. Status of rice varietal improvement for protein content at IRRI. *In* Nuclear Techniques for Cereal Grain Protein Improvement: proceedings of research coordination meeting, IAEA: Vienna, P. 199-202

Krishnan, HB and Okita TW. 1986. Structural relationships among rice glutelin polypeptides. *Plant Physiology,* **81**,748-753.

Kronzucker, HJ, Siddiqi MY, Glass ADM and Kirk GJD. 1999. Nitrate-Ammonium

Synergism in Rice. A Subcellular Flux Analysis. *Plant Physiology,* **119** (3), 1041–1045. (DOI: 10.1104/pp.119.3.1041).

Lowry OH, Rosebrough NJ, Lewis Farr A and Randall RJ. 1951. Protein measurement with the Folin Phenol reagent. *Journal of Biological Chemistry,* **193**, 265.

Martin A, Lee J, Kichey T, Gerentes D, Zivy M, Tatout C and Tercé-Laforgue T. 2006. Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production. *The Plant Cell*, **18**(11), 3252-3274.

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A. 2010. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Annals of botany,* **105**(7), 1141-1157.

Mahmoud AA, Sukumar S and Krishnan HB. 2008. Interspecific rice hybrid of *Oryzasativa* × *Oryzanivara* reveals a significant increase in seed protein content. *Journal of Agricultural Food Chemistry,* **52**, 476–482.

Obara M, Kajiura M, Fukuta Y, Yano M, Hayashi M, Yamaya T and Sato T. 2001. Mapping of QTLs associated with cytosolic glutamine synthetase and NADH glutamate synthase in rice (*Oryza sativa L.*). *Journal of Experimental Botany,* **52**(359), 1209-1217.

Pal P, Kaur P, Singh N, Kaur AP, Misra NN, Tiwari BK, Cullen PJ and Virdi AS. 2016. Effect of nonthermal plasma on physico-chemical, amino acid composition, pasting and protein characteristics of short and long grain rice flour. *Food Research International,* **81**, 50–57.

Patrick JW and Offler CE. 2001. Compartmentation of transport and transfer events in developing seeds. *Journal of Experimental Botany,* **52**(356), 551-564.

Peng B, Kong H, Li Y, Wang L, Zhong M, Sun L, Gao G, Zhang Q, Luo L, Wang G, Xie W, Chen J, Yao W, Peng Y, Lei L, Lian X, Xiao J, Xu C, Li X and He Y. 2014a. OsAAP6 functions as an important regulator of grain protein content and nutritional quality in rice. *Nature Communication,* **5**, 4847 (DOI: 10.1038/ncomms5847)

Peng B, Wang L, Fan C, Jiang G, Luo L, Li Y and He Y 2014b. Comparative mapping of chalkiness components in rice using five populations across two environments. *BMC Genetics,* **15**:49.

Qin Y, Kim SM, Sohn JK 2009. QTL analysis of protein content in double-haploid lines of rice. *Korean Journal of Crop Science*, **54**(2), 165–171.

Rentsch D, Schmidt S and Tegeder M. 2007. Transporters for uptake and allocation of organic nitrogen compounds in plants. *FEBS letters,* **581**(12), 2281-2289.

Sambrook J and Russell D. 2001. Molecular Cloning a laboratory manual. CSHL Press, New York, USA.

Shao Y, Cen Y, He Y and Liu F. 2011. Infrared spectroscopy and chemometrics for the starch and protein prediction in irradiated rice. *Food Chemistry,* **126**, 1856–1861. http//dx. doi:10.1016/ j.foodchem. 2010.11.166.

Sugimoto T, Tanaka K and Kasai Z. 1986. Improved extraction of rice prolamin. *Agricultural and Biological Chemistry,* **50**, 2409-2411.

Tabuchi M, Sugiyama K, Ishiyama K, Inoue E, Sato T, Takahashi H and Yamaya T. 2005.Severe reduction in growth rate and grain filling of rice mutants lacking OsGS1; 1, a cytosolic glutamine synthetase1; 1. *The Plant Journal,* **42**(5), 641-651.

Tilsner J, Kassner N, Struck C and Lohaus G. 2005. Amino acid contents and transport in oilseed rape (*Brassica napus* L.) under different nitrogen conditions. *Planta*, **221**(3), 328-338.

Tsay YF, Chiu CC, Tsai CB, Ho, CH, and Hsu PK. 2007. Nitrate transporters and peptide transporters. *FEBS letters,* **581**(12), 2290-2300.

Ufaz S and Galili G. 2008. Improving the content of essential amino acids in crop plants: goals and opportunities. *Plant Physiology,* **147**, 954–961.

Vasal SK. 2002. The role of high lysine cereals in animal and human nutrition in Asia. *In*: Protein Sources for the Animal Feed Industry, Food and Agriculture Organization: Rome, p. 97-108.

Van der Graaff E, Schwacke R, Schneider A, Desimone M, Flügge UI and Kunze R. 2006. Transcription analysis of Arabidopsis membrane transporters and hormone pathways during developmental and induced leaf senescence. *Plant Physiology,* **141**(2), 776-792.

Wang X, Pang Y, Zhang J, Wu Z, Chen K, Ali J, Ye G, Xu J and Li Z 2017. Genome-wide and gene-based association mapping for rice eating and cooking characteristics and protein content. *Scientific Reports,* **7**, 17203, doi:10.1038/s41598-017-17347-5.

Xie LH, Tang SQ, Chen N, Luo J, Jiao, GA, Shao GN, Wei XJ and Hu PS. 2014. Optimisation of near-infrared reflectance model in measuring protein and amylose content of rice flour. *Food Chemistry,* **142**, 92–100.

Yang LJ, Xu L and Li JY, 2004.Analysis of correlation between protein content, amylose content in the unpolished rice and 1000-grain weight in six different varieties' rice. *J Shanghai Normal University* (Natural Sciences) 10(supplement), 55-58.

Yang Y, Guo M, Li R, Shen L, Wang W, Liu M, Zhu Q, Hu Z, He Q, Xue Y et al., 2015. Identification of quantitative trait loci

responsible for rice grain protein content using chromosome segment substitution lines and fine mapping of *qPC-1* in rice (*Oryza sativa* L.). *Molecular Breeding,* **35**, 130, http//dx. doi: 10.1007/s11032-015-0328-z.

Yoshida S, Forno, DA, Cock JH and Gomez KA. 1976. Laboratory manual for physiological studies of rice, 3rd edn, IRRI, Manila, p. 1-83.

Yoshida S, Ikegami M, Kuze J, Sawada K, Hashimoto Z, Ishii T, Nakamura C and Kamijima O. 2002. QTL analysis for plant and grain characters of sake-brewing rice using a doubled haploid population. *Breeding Science,* **52**, 309–317.

Yu YH, Li G, Fan YY, Zhan KQ, Min J, Zhu ZW and Zhuang JY. 2009.Genetic relationship between grain yield and the contents of protein and fat in a recombinant inbred population of rice. *Journal of Cereal Science,* **50,** 121-125.

Zheng LN, Zhang WW, Chen ZG, Ma J, Chen WW, Zhao ZG, Zhai HQ and Wan J M. 2011. Dynamic QTL analysis of rice protein content and protein index using recombinant inbred lines. *Journal of Plant Biology,* **54**, 321–328.

Zhong M, Wang L, Yuan J, Luo L, Xu C and He YQ 2011. Identification of QTL affecting protein and amino acid contents in rice. *Rice Science*, **18**(3), 187–195.

Zimmermann P and Zentgraf U, 2005. The correlation between oxidative stress and leaf senescence during plant development. *Cell and Molecular Biology Letters,* **10**, 515–534.