

## BREEDING FOR LOW PHYTIC ACID MUTANTS IN RICE (*Oryza sativa* L.)

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### ABSTRACT

Phytic acid, myo-inositol 1, 2, 3, 4, 5, 6 – hexakisphosphate (IP 6) is the major storage compound of phosphorous (P) in plants. Rice grain contains anti-nutritional factors, which reduce the bioavailability of iron and zinc. For a long time, phytate has been known to lower the absorption of these and other minerals in humans and non-ruminant animals. Induced mutations in the most important cereals have been utilized to reduce the phytate content of resting grains. All efforts to develop mutants with low phytic acid content that were recognized in four rice genotypes from different wild genotypes as OMCS2000, OM1490. Their rice grains were induced by gamma radiation (20 kr) then screened for high levels of free phosphate in order to identify low phytic acid (LPA). The LPA was analyzed in mutants, wild types, 101 improved varieties and 600 landraces. LPA mutants derived from OM1490, OMCS2000 in M3, M4 and M5 showed uniform agronomic traits. Some released genotypes were 47, 64, 144, 158 and 274. Besides that, three genotypes OM4498, OM2517, OM5731 and six landraces as Nang Quot Do, Ca Ro, Lua Lun, Nep Ao Vang, Nep Mau Luon, Nep Hat To were LPA phenotypes with 0.465  $\mu\text{g P}$ . Especially, Lua Vang obtained 0.930  $\mu\text{g P}$  and Lua Hoang (*Oryza rufipogon*) offered 1.395  $\mu\text{g P}$

Our findings may help rice breeders develop low phytic acid genotypes with improved nutritional value to overcome anemia syndrome and meet the demand of biofortification

**Keywords:** Low phytic acid (LPA), mutant, phytate

### INTRODUCTION

Phytic acid, myo-inositol 1, 2, 3, 4, 5, 6 – hexakisphosphate (IP 6) is the major storage compound of phosphorous (P) in plants. It exhibited mostly in cereal grains and pollens. The low phytic acid trait addresses an urgent goal for genetic improvement of rice because of anemia syndrome in rice eating countries. In cereal, non-mutant seed, phytic acid typically represents about 75% and inorganic acid P about 5% of seed total P (Lott *et al.* 2000). In the *lpa* mutants isolated in last few years in maize (*Zea mays* L.), barley (*Hordeum vulgare* L.), and rice (*Oryza sativa* L.), such ratio are altered: mutant seeds have normal levels of total P, but greatly reduced levels of phytic acid P (Rayboy *et al.* 2000).

During germination, phytates are broken down by the action of phytase, releasing their P, minerals and myo-inositol, which become available to the growing seedling. Phytic acid dose in cereal grains highly expresses. It is considered as a compound which can strongly absorb metal irons, then it can reduce the absorption potential of minerals by human and animal such as Fe, Zn, Ca, Mg. Some recessive genes, which control to reduce phytic acid, were created through chemical mutation. Mutant gene *lpa* was isolated in maize and barley genomes. Mutant gene *lpa-1* reduces phytic acid based on molecular balance mechanism. It located in chromosomes 1S, and 2H in maize and barley genomes, respectively. Mutant gene *lpa-2* controls low phytic acid with low inositol phosphates, and increased free phosphates.

**OBJECTIVE**

To create and detect rice mutants and landraces, which contain low phytic acid in grains.

**MATERIALS AND METHODS**

Two rice mutant populations were created from high yielding varieties OM1490, OMCS2000 (Table 1).

Seeds from M1, M2, M3, M4, M5 and M6 were separately collected to analyze phytic acid content in grains.

All of 600 accessions of landraces and 101 improved varieties were also analyzed.

Two varieties were used as checks of *lpa-1*, *lpa-2* from China.

**Table 1.** Parental materials for mutagenic treatment

Designation	Type	Feature	Treatment
OM1490 (M2-M6)	indica	High yield, wide adaptability, short duration (90-95 days)	Gamma ray, 20 Gy
OMCS2000 (M2-M6)	indica	High yield, short duration (less 90 days), good grain quality	Gamma ray, 20 Gy

***Assay for high phosphate(HIP) levels in the kernel of rice***

Simple screening procedure for detecting putative low phytate rice mutants was adopted from a study on rice low-phytate mutants by Raboy *et al.* (2000). Eight seeds were sampled from each M3 and individually ground in a mortar with a steel pestle. The resulting flours were then extracted with 1ml of 0.4 M HCl at 4°C overnight. Samples were mixed briefly and 100µl were removed and supplemented with 900 µl of Chen' reagent in microtiter plates. In the case of high phosphate content, a dark-blue coloured phosphomolybdate complex formed in 1-2 h.

Chen's Reagent: 1 volume 6N H<sub>2</sub>SO<sub>4</sub>; 1 volume 2.5% ammonium molybdate; 1 volume 10% ascorbic acid ; 2 volumes H<sub>2</sub>O

We usually prepare just enough Chen's Reagent for a given day's use. Allow the assays to develop for 2 hr at ambient (room) temperature, shorter development times like 1 hr will also work. Note: in the original method the colorimetric assays were heated. We find it works well without the heating step.

**Phosphorus Standards for Microtiter Plate PI assay**

No	µl 1mM K <sub>2</sub> HPO <sub>4</sub>	µl 0.4M HCl	µl H <sub>2</sub> O	ng P
1	0	10	90	0
2	5	10	85	155
3	15	10	75	465
4	30	10	60	930
5	45	10	45	1395

A wild type, non-mutant, normal seed typically gives a result less than or equal to the second standard, 155ng P. A seed homozygous for low phytic acid (LPA) has enough inorganic P that the result is usually off scale darker than the highest standard given above. Therefore, when following the inheritance of *lpa*,

the results can be read from the micro-titer plate visually. Normal seeds (either homozygous wild type or heterozygous produce an intermediate or light blue color, seeds homozygous) for *lpa* produce a dark blue colour, and the difference is very clear cut. To obtain qualitative data, the assays must be read in a spectrophotometer at wave length of 820nm. If seeds homozygous for *lpa* are tested using the above method, the results will be off-scale. To get accurate quantitative results, these seeds may have to be extracted in a larger volume per unit weight (in 15 or 20µl rather than 10µl).

Another alternative method is useful if the objective is to do a large number of tests in the shortest time possible. Individual seeds can be crushed, then extracted and assayed in one test tube. For example, one can crush a seed, extract it in 0.5ml of dilute acid, and add 0.5ml of Chen's reagent. Within one or two hours, the difference between normal, non-mutant seeds and LPA seeds is usually apparent.

### ***Rapid isolation of rice DNA***

DNA isolation for PCR analysis, which does not require liquid nitrogen, needs only small amount of tissue sample and protocols, was done (Lang 2002). DNA suitable for PCR analysis was prepared using a miniscale procedure in a labeled 1.5 ml centrifuge tube in ice. The young leaf was ground using a polished glass rod in a well of a Spot Test plate (Thomas Scientific) after adding 400 µl of extraction buffer (50 mM tris-HCl pH 8.0, 25 mM EDTA, 300mM NaCl and 1% SDS). Grinding was done until the buffer turned green which is an indication of cell breakage and release of chloroplasts and cell contents. Another 400µl of the extraction buffer was added and mixed into the well by pipetting. 400µl of the lysate was transferred to the original tube of the leaf sample. The aqueous supernatant was transferred to a new 1.5 ml tube and DNA precipitated using absolute ethanol. DNA was air-dried and resuspended in 50µl of TE buffer (10mM Tris- HCl pH 8.0, 1mM EDTA pH 8.0). An aliquot of 1µl is sufficient for PCR analysis. DNA quality and quantity were spectrophotometrically determined.

### ***PCR amplification***

The genomic DNA from both of the F<sub>2</sub> plants and the parents were subjected to PCR amplification using the synthesized primers. The PCR buffer consist of: 10mM Tris pH 8.4, 50mM KCl, 1.8mM MgCl<sub>2</sub> 0.01mg/ml gelatin. 5 unit *Taq* of *Taq polymerase* in a volume of 25µl. Template DNA were initially denatured at 94°C for 5 min, followed by 30 cycles of PCR amplification under the following parameters, 1 min denaturation at 94°C, 1 min primer annealing at 55°C and 2 min primer extension at 72°C. Final 5 min incubation at 72°C was allowed for completion of primer extension on a 480-thermalcycler. The amplified products were electrophoretically resolved on 1.2 % agarose gel using 1X TAE buffer.

## **RESULTS AND DISCUSSION**

### ***Identification of seed high inorganic P (HIP) donors***

A total of 101 improved varieties was evaluated for seed high inorganic P based on color intensity.

The results showed that three improved genotypes as OM4498, OM5731, DS2000 had low level of phytate (0.465 µg P), then golden rice of 0.930 µg P, Lua Hoang (*Oryza rufipogon*) of 1.395 µg P (Figure 1).

For landraces, 3,200 seeds of 600 accessions of local rice varieties uniform in agronomic traits were screened for HIP (Table 2). Nang Quot Do, Ca Ro, Lua Lun, Nep Ao Vang, Nep Mau Luon, Nep Hat To exhibited low phytic acid level of 0.465 µg P.

**Table 2.** Heterozygote and homozygote HIP of local rice varieties

Designation	Number of seeds	Score				
		1	2	3	4	5
Waxy	368	325	37	6	0	0
Aroma	360	349	11	0	0	0
Landrace	2472	2350	110	12	0	0

### Screening for rice seed low phytate mutants (M2)

Gamma ray is an useful physical measure to create mutant rices.

In late 2003, mutated populations were screened for low phytic acid at CLRRI using 2,000 samples of M2 of OMCS 2000 and 800 samples of M2 lines of OM1490. They were treated by irradiation with 20Gy gamma ray. Four M2 lines with low phytic acid (level 4) and 36 M2 lines with relatively low phytic acid (level 3) were selected as compared to the relatively high phytic acid (level 2) of OM 1490 and OMCS 2000.

In the case of high phosphate content, a dark-blue coloured phosphomolybdate complex formed in 2 hours. Putative *lpa* mutant grains were scored for phytate and orthophosphate levels using TLC analysis as described by Rasmussen and Hatzack (1998).



**Figure 1.** Assay for free phosphate (HIP phenotype) in some varieties. Single seeds from a given panicle were crushed, extracted and assayed for free P based on colorimetric molybdenum staining assay. To allow for direct comparison, 100mg of flour were extracted in 10 vol. of HCl 0.4 N and an equal aliquot vol. was tested. The standards contained 0.0  $\mu\text{g P}$ ; (ii) 0.155  $\mu\text{g P}$ ; (iii) 0.465  $\mu\text{g P}$ ; (iv) 0.930  $\mu\text{g P}$  and (v) 1.395  $\mu\text{g P}$ .

**For selection of LP in mutant rice OM 1490 and OMCS 2000 (M3)**

Phosphorus, phytic acid and phytate are found in significant amounts in crop seeds. Most of the P in seed is stored in the form of phytic acid (*Myo*-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate), and phytic acid P is nutritionally unavailable to human and livestock.

Dwarf mutant of OM1490 and OMCS2000 were induced by 20-gamma ray treatment. It is very difficult to identify LP in the genotypes due to non-uniform exhibition of dark blue color among individuals.

For mutant rices in M3: we used 257 individuals of OM1490 mutants and 367 of OMCS2000 mutants. Only five lines showed homozygous HIP genotype with almost seeds represented equal or higher than the standard P, No. 3 (0.46 $\mu$ g P). These lines encoded 47, 64, 144, 158 and 274.

These plants phenotypically exhibited LPA and they were continued to grow in the experimental field up to M5 and M6 for evaluation.

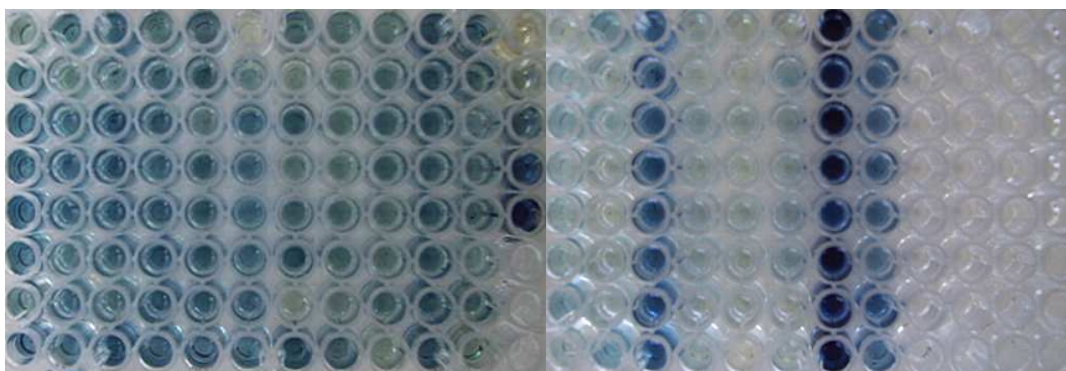
In M5, LPA screening was conducted among OM1490 and OMCS 2000 mutants (Table 3)

On the other hand, when observing HIP test at the scale 1, 2, 3 and 4; OM1490 mutant showed that many seeds offered high phytic acid and made up 64.4% higher than OMCS2000 mutant (53.4%) at scale 1. On the contrary, OMCS2000 mutants gained lower phytic acid seeds than OM1490 mutants at scale 2. However, OM1490 mutants gained LPA (5.2%) at scale 3, lower than IR64 mutants.

**Table 3.** Mutants showing LPA at five standard scales

M6	Number of lines	Score				
		1	2	3	4	5
OM1490	229	152	61	15	1	0
OMCS2000	232	124	94	12	2	0

233 262 78 100 286 223 250 232 80 C 1 C2 P 7 23 100 WT1WT2 250 WR C 1



**Figure 2.** Assay for free phosphate (HIP phenotype) in some varieties and M5 mutants from OMCS2000 and OM1490, P: standard; C1: XieQingZao, C2: Wild rice, OM1490 mutants : 7, 23, 78, 80, 100; OMCS2000 mutants :233, 262, 286, 223, 250, 232 ; WT1: OM1490 wild type; WT2: OMCS2000 wild type

The M6 seeds of 102 lines and 99 lines of OM1490 mutants and OMCS2000 mutants were screened for low phytic acid, respectively. Assay for free phosphate (HIP phenotype) was presented in table 4. Seeds with high inorganic P were identified based on color intensity.

**Table 4.** Mutants showing LPA at five standard scales

M6	Number of lines	Score				
		1	2	3	4	5
OM1490	102	711	113	0	0	0
OMCS2000	99	530	262	0	0	0

**PCR-based marker assisted selection (MAS)**

The advent of marker-assisted selection permits rapid identification of individuals that contain genes for low phytic acid. The presence or absence of the associated molecular marker indicates, at an early stage, the presence or absence of the desired target gene. Segregating populations were used to confirm co-segregation between SSR markers and the gene for LPA. Polymorphic marker RM207 detected LPA in OM1490 mutants (Figure 3). Lane 1 (IR 64) exhibited band of 200 bp and lane 2 showed *Lpa-1* at 220 bp. Lane 3-7 presented high phytic acid from wild type OM1490, lane 8-12 indicated LPA of landraces as Nep than, Nep hạt to, Nep ao vang, Nep thom and Nep mau luon.

**Figure 3.** Banding patterns of the SSR marker RM207 for LPA

Lane 1: IR64, Lane 2: *Lpa-1*, Lane 3-7: OM1490 mutants, Lane 9-12: Nep than, nep Hạt To, Nep Ao Vang, Nep Thom and Nep Mau luon.

**CONCLUSION**

Low phytic acid is one of important studies in rice to improve promising nutritional lines. Mutants from physical method can reduce phytic acid level in grain, it means that inorganic phosphorous content increases in seed. Low phytic acid was theoretically predicted by testing HIP (high inorganic phosphate) on mutants of OM1490 and OMCS2000 varieties. Phytic acid characters appear to differ types of lever P. HIP traits and associated characters is required for efficient use of these characters to increase. Most of mutant seeds showed low phytic acid at scale 2 among these varieties. At scale 3 of HIP testing, about 2.325  $\mu\text{g/ml}$  P, OM1490 (M5) gave 0.4% lower than OMCS 2000 (M5) at 0.9%. These lines will be useful in reducing grain phytic acid and improving the nutritional value of rice grain and/or milling by-products. Four low phytic acid cultivars such as Nep Than, Nep Hat To, Nep Ao Vang, Nep Thom and Nep Mau Luon are considered as good donors to transfer target gene of LPA into improved varieties in future. These characters will then need to be incorporated into high yield genotypes with others such as disease and insect resistance. These are also used for screening genotypes with HIP.

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**Chọn giống lúa có hàm lượng phytic acid thấp thông qua đột biến gen**

Phytic acid, *myo*-inositol 1, 2, 3, 4, 5, 6 – hexakisphosphate (IP 6) là hợp chất dự trữ quan trọng trong hạt mề cốc. Nó làm ngăn cản sự hấp thu sắt và kẽm và gây hội chứng anemia cho người dân dùng cơm là nguồn lương thực chủ yếu.

Viện Lúa ĐBSCL thực hiện phân tích đánh giá nguồn vật liệu từ ngân hàng gen, lúa hoang, lúa đột biến từ OM1490 và OMCS2000. Các dòng được ghi nhận có hàm lượng phytic acid thấp (LPA) là dòng 47, 64, 144, 158 và 274. Các vật liệu OM4498, OM2517, OM5731 và sáu mẫu giống lúa bản địa Nàng quýt đỏ, Cá rô, Lúa Lùn, Nếp Áo Vàng, Nếp Máu Lươn, Nếp Hạt To được ghi nhận là vật liệu có LPA (0,465 µg P). Đặc biệt, Lúa Vàng đạt giá trị 0,930 µg P và Lúa Hoang (*Oryza rufipogon*) 1,395 µg P.