RESULTS

1. Concentration of inorganic P in variety of wild-type seed

The seeds of KDML105, IR68144, Azucena, Nipponbare, and Jao hom Nin rice cultivars were analyzed for inorganic phosphorus. In practice, the visual result of these rice showed to be equal to or less than 0.15 μg P of colorimetric standard (Figure 4).

2. Qualitative screening of low phytic acid

To estimate 8-12 seeds from each 1,274 non-lethal M_1 mutagenized plants were screened low phytic acid by the qualitative method which free phosphate was detected as phosphomolybdenum blue. Initial screening based on color intensity observation of M1 population showed high inorganic phosphorous (HIP) in the most of M2 seeds. Only 6 plants (M_1 331, M_1 672, M_1 971, M_1 783, M_1 1161, M_1 1234) showed HIP in M_2 seed generation. This results showed that M_1 plants were heterozygous groups (Table 2). M_2 seeds were selected and planted in M_2 plant generation to investigate HIP characteristics. The survival rate of low phytic acid mutants were lower than Jao Hom Nin (wild-type) (Table 3, Figure 5).

Subsequent seed developing, individual M_3 seeds of each M_2 plant were used to analyzed for HIP phenotype. The HIP phenotype of M_3 331-4 and groups of M_3 783 (M_3 783-1 and M_3 783-5) seeds remained HIP value and segregated at 3:1 ratio frequencies that fit the expected 1:2:1 genotypic ratio for a recessive allele (Figure 6, 7). M_2 672, M_2 971, $M_2$1161, and M_2 1234 were negative effect for HIP.

M3 plants of heterozygous low phytic acid groups were generated and analyzed HIP in M4 seeds. Segregation of HIP phenotype seeds of M 331 and M 783 in the M1

through M3 plants generation showed in Table4 and Table 5. The segregation of HIP phenotype seeds in each generation indicate that M 331 and M 783 was not obtained as homozygote recessive of low phytic acid plant.

To investigation of seed characteristic of low phytic acid groups observed that the seeds of M783 heterozygous have defective embryo in 3:1 ratio for a recessive allele which these seeds were effective for HIP (Figure8). The seeds of M_3 331 heterozygous low phytic acid were cut in two portion. The half grains without embryo were used for HIP screening and the rest half was planted. The germination results showed that embryo portion of HIP seed did not grow as usual (Figure 9). The wrinkle embryo of M3 783 mature seeds could not sprout.

3. Seed Viability

Seed viability was an indication of the capability of seeds to germinate and producing a normal plant. Tetrazolium (TZ) test was used to examine for viable seeds by respiratory enzyme. Respiratory enzyme reduced the colorless of tetrazolium salt solution into a chemical compound called formazan. Formazan stained living cell with a red color while dead cells remain colorless. In this result, the seed of wild-type IR 68144, KDML 105, Nippon bare, Jao Hom Nin were tested for seed viability, which found that the virtual embryo appeared intensely stained areas of red color (Figure 10).

The control of experiment, activity of respiratory enzyme was destroyed. Then, the seeds which were blocked enzyme activity, could not stain while normal seed remained having enzyme activity (Figure 11).

Testing seed viability of mutagenic low phytic acid groups (M_3 783 and M_3 331), one spike were isolated according to HIP value. The result demonstrated that HIP

phenotype seed of wrinkle embryo (M_3 783) was lethal while normal seed was viable seeds (Figure 12).

For groups of M_3 331 plant, seeds were screened HIP and tested seed viability. This experiment found both high and low inorganic P seeds remained viable but HIP phenotype seed showed weakly stain when compared to seed of wild-type and low inorganic P seeds (LIP) (Figure 13). Comparing % viability of seed between wild-type and low phytic acid mutant showed in Table 6

4. <u>High-performance thin layer chromatography (HP-TLC) analysis</u>

4.1 The visually estimation of standard concentration

The smallest visible quantities of D-myo-inositol-1-monophosphate ($InsP_1$), D-myo-inositol-1,4,5 trisphosphate($InsP_3$), D-myo-inositol-3,4,5,6-tetrakisphosphate ($InsP_4$), D-myo-inositol-1,3,4,5,6-pentakisphosphate ($InsP_5$), phytic acid ($InsP_6$) and phosphate (Pi) of each standard were 48, 5.95, 10, 2.15, 2.1 and 2.5 nmol respectively. Mobility of standard $InsP_1$ to $InsP_6$ could be separated by HP-TLC. This result found that $InsP_6$ migrated slower than $InsP_5$, $InsP_4$ and $InsP_3$ which increase in a steady mobility while $InsP_1$ and Pi migrated rapidly (Figure 14).

4.2 Degradation of phytic acid

HP-TLC method was used to isolate low phytic acid which this method was confirmed by phytic acid degradation. Phytic acid was degraded by phytase enzyme. Products of phytase activity were isolated with HP-TLC. This result indicated that phytic acid could be degraded after incubation for 30 min. After an incubation of 60 min, only trace amounts of inositol phosphate products could be detected and decreased

phytic acid content while inorganic P was high level, reflecting a high level of enzymatic activity (Figure 15). Thus, decreasing of phytic acid would be correlated with high inorganic phosphorus.

4.3 Determination phytic acid content of wild-type by HPTLC

The result of the KDML 105, IR68144, Azucena, Nippon bare and Jao Hom Nin wild-type were analyzed phytic acid , other inositol phosphate and Pi content by HP-TLC method. This method found that each wide-type gave similar phytic acid content while Pi content not differed and no appeared of other inositol phosphate from visually observation (Figure 16).

4.4 Confirmation of mutant phenotypes by HP-TLC

Eight to twelve seeds of each spike of M_3 331 and M_3 783 were used to analyze by HPTLC for investigated really decreasing of phytic acid. The success for isolated low phytic acid with HP-TLC method found difference between mutagenic seeds of M_3 331 and M_3 783 were divided as two characteristic low phytic acid phenotype. Some seeds of M_3 331 seeds (seed no. 4, 5, 6 and 9) were consisted of reducing phytic acid and very high inorganic P in recessive allele which the pattern differed from wild-type (Figure 17). For remnant seeds were similar to wild-type

In groups of M_3 783 seeds found some seed (seed no. 5 and 6) consisted of very high levels of free phosphate, low levels of phytic acid and trace amounts of other inositol phosphate compounds which the pattern differed from both wild-type and remnant seeds (Figure 18).

5. **Quantitative analyses of seed**

HPLC analysis was used to analyze quantity of phytic acid ($InsP_6$) and other inositol phosphates ($InsP_4$, $InsP_5$) in mature rice seed. Seeds of each heterozygous low phytic acid mutants were pooled and crushed for analysis. To quantitative analyses of M_3 331-4 heterozygous seeds were 0.94 mg/100g of $InsP_4$, 17.14 mg/100g of $InsP_5$ and 734.06 mg/100g of $InsP_6$ while homozygous nonmutant of M_3 331-1 (LIP) gave results as 0.97 mg/100g of $InsP_4$, 23.13 mg/100g of $InsP_5$ and 768.03 mg/100g of $InsP_6$. This result demonstrated that $InsP_4$, $InsP_5$ and $InsP_6$ content of M_3 331-4 heterozygous mutant seeds were lower than homozygous non mutant as a little (Table 7).

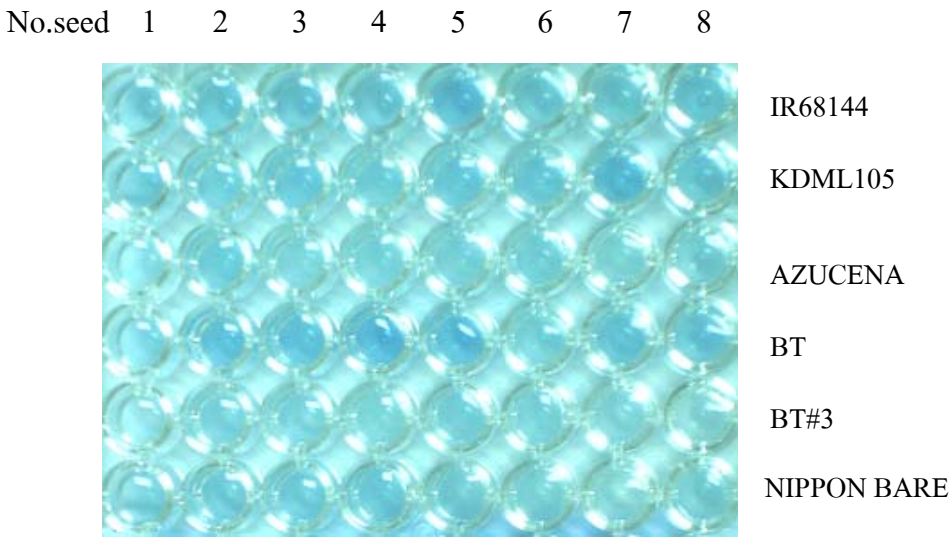
In the same way, seeds of M_3 783-2 and M_3 783-5 were analyzed to phytic acid ($InsP_6$) and other inositol phosphates ($InsP_4$, $InsP_5$) content. To quantitative analyses of M_3 783-5 heterozygous seeds were 18.95 mg/100g of $InsP_4$, 48.91 mg/100g of $InsP_5$ and 474.14 mg/100g of $InsP_6$ while homozygous nonmutant of M_3 783-2 (LIP) gave results as 2.32 mg/100g of $InsP_4$, 47.41 mg/100g of $InsP_5$ and 628.5 mg/100g of $InsP_6$. This result found that inositol tetraphosphate ($InsP_4$) content of M_3 783-5 heterozygous seed were higher than homozygous nonmutant of M_3 783-2 while phytic acid was decreased (Table 7).

Owing to, the defective embryo of low phytic acid groups (M_4 783) could be visually observed, M_4 783 heterozygous seeds were divided seed quality between normal embryo and wrinkle embryo for HPLC analysis. Quantity of $InsP_4$, $InsP_5$ and $InsP_6$ content of the wrinkle seeds were 38.77, 75.98, 530.23 mg/100g respectively while the normal seeds were 6.99, 40.56, 748.99 mg/100g respectively. This result found that inositol tetraphosphate ($InsP_4$) and inositol pentaphosphate ($InsP_5$) content of the wrinkle seeds higher than the normal seeds, on the contrary, phytic acid was decreased (Table 8)

The M_4 331 seeds of low phytic acid heterozygous were cut in two portion and the half grains without embryo were used for HIP screening. The remnant seeds were divided between high inorganic P (HIP) and low inorganic P (LIP) groups. Each groups were pooled , crushed and quantitative analyze with HPLC. The result showed that phytic acid content of the portion of HIP seeds were 677.62 mg/100g while the portion of LIP seeds were 991.11 mg/100g.

Seeds of M_4 331 (HIP) , M_4 331 (LIP) , M_4 783 (wrinkle embryo) M_4 783 (normal embryo) and BT were analyzed for total P which gave result as 362.17, 398.69, 315.57, 315.80 and 320.46 mg/100g respectively.

A.



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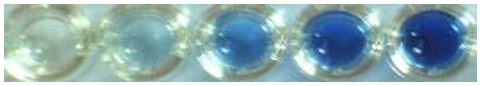


Figure 4 Estimate concentration of High Inorganic P (HIP)

A. Single seed of wild-type were tested HIP

B. Standard P concentration : (i) 0.0 µg P; (ii) 0.15 µg P, (iii) 0.46 µg P (IV) 0.93 µg P, (V) 1.39 µg P

Table 2 Relative of soluble inorganic P level for screened M_2 low phytic acid mutagenized seed ; by random sample estimate 8 seed of each plants

Plant designation	No. M_2 seed	Seed weight (mg)	Level P (μg) content
M_1 331	1	18	0.15
	2	16	0.15
	3	17	0.15
	4	14	0.15
	5	18	0.93
	6	17	0.15
	7	17	0.15
	8	17	1.93
M_1 672	1	18	0.15
	2	17	0.46
	3	17	0.46-0.93
	4	16	0.15
	5	17	0.93
	6	17	0.15
	7	16	0.15
	8	14	0.15

Table 2 (continued)

Plant designation	No. M_2 seed	Seed weight (mg)	Level P (μg) content
M_1 783	1	17	0.15
	2	16	0.15
	3	18	0.15
	4	16	1.39
	5	18	0.15
	6	18	0.15
	7	17	0.15
	8	14	1.39
M_1 971	1	14	0.15
	2	17	0.15
	3	15	0.15
	4	18	0.15
	5	16	0.15
	6	15	0.15-0.46
	7	16	0.15
	8	15	0.46
M_1 1161	1	19	0.46
	2	19	0.15
	3	16	0.15

Table 2 (continued)

Plant designation	No. M_2 seed	Seed weight (mg)	Level P (μg) content
	4	19	0.15
	5	19	0.15
	6	18	0.15
	7	17	0.15
	8	16	0.46-0.93
M_1 1234	1	18	0.15
	2	20	0.15
	3	19	0.15
	4	19	0.15
	5	17	1.93
	6	19	0.15
	7	17	0.15
	8	15	0.46-0.93

Note ; inorganic P > 0.46 μg P assumed that the plants would be low phytic acid,
Visually scored for content of level P by compared with P standard dilution.

Table 3 Survival rate of M2 population of low phytic acid plants

Plant designation	seeds planted	% survival
M2 331	8	50
M2 672	8	75
M2 783	12	41.7
M2 971	8	25
M2 1161	8	37.5
M2 1234	12	58.3
Jao Hom Nin (wild-type)	12	92

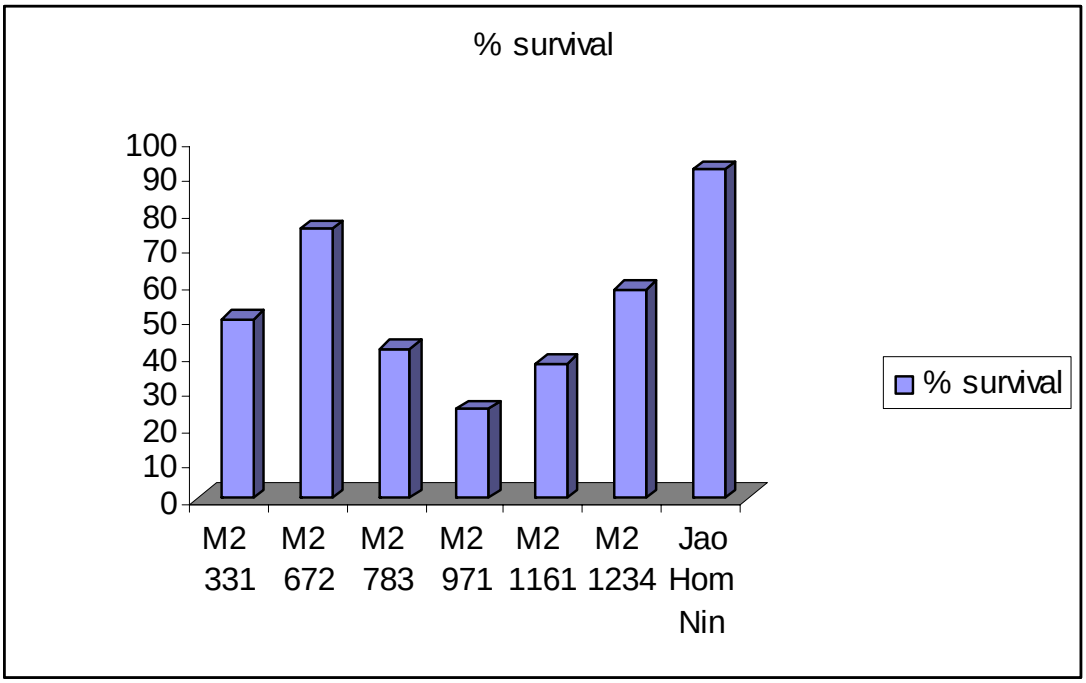


Figure 5 Showed a chart of % survival

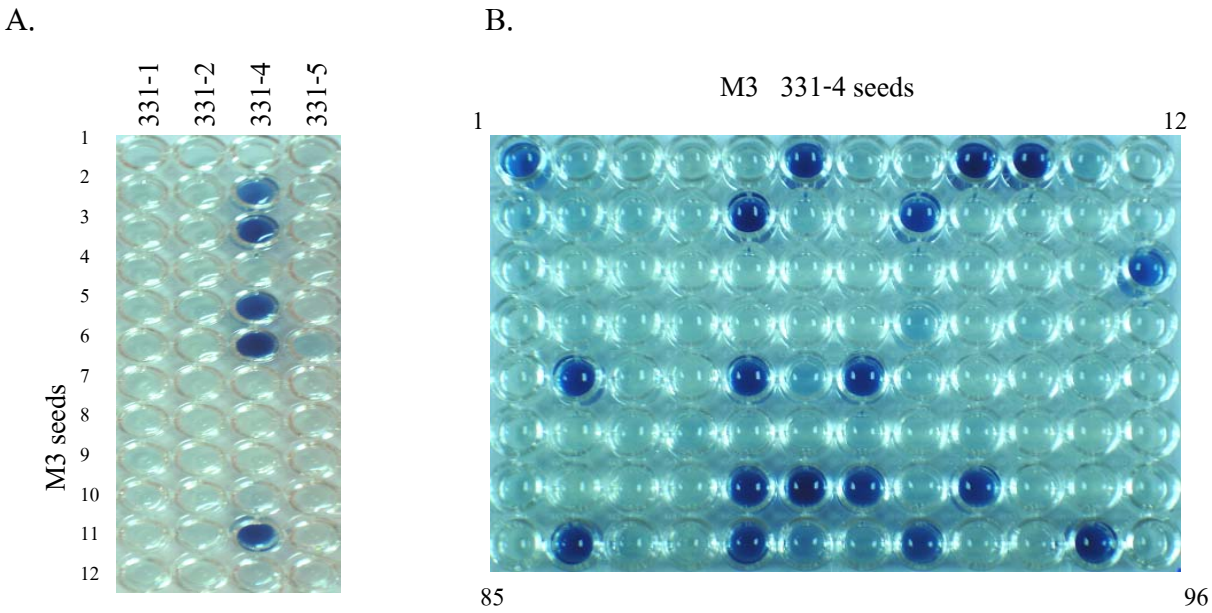


Figure 6 Segregation of M_2 331 mutagenized low phytic acid phenotype in the M3 seed generation. Estimated 10-12 seeds of each plant were analyzed high inorganic P. Individually seeds were crushed and assayed for inorganic P.

A. A single M3 seed from M2 plant were analyzed for HIP phenotype

B. One spike of M_3 331-4 seed were analyzed HIP of seed, indicated that segregation ratio as 3:1 (LIP:HIP)

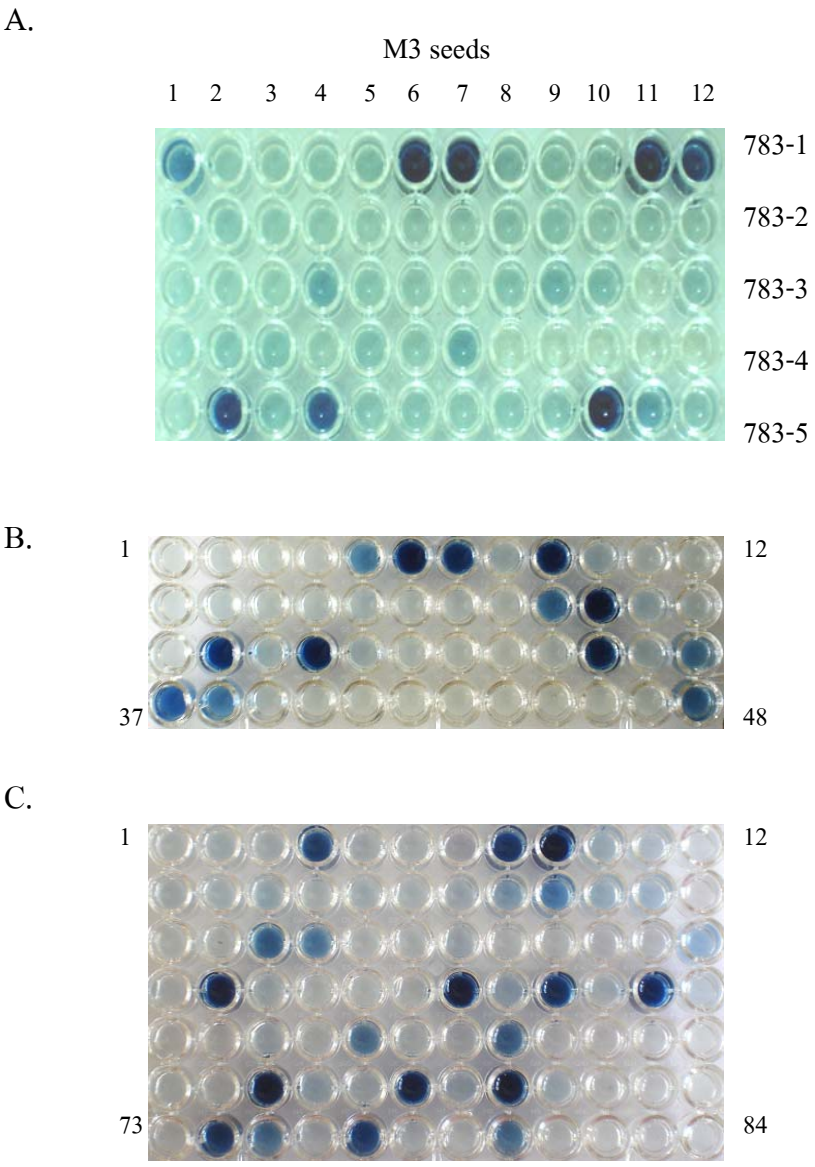


Figure 7 Segregation of M_2 783 mutagenized low phytic acid phenotype in the M3 seed generation. Estimated 10-12 seeds of each plant were analyzed high inorganic P. Individually seeds were crushed and assayed for inorganic P.

A. One spike of M_3 783-1 seed were analyzed HIP of seed, indicated that segregation ratio as 3:1 (LIP:HIP)

B. One spike of M_3 783-5 seed were analyzed HIP of seed, indicated that segregation ratio as 3:1 (LIP:HIP)

Table 4 Relative segregation of high or low inorganic P seeds of M 331 from M1 through M3 plant generation

Generation	Plant designation (M1-M3)	Segregation of seed (M2-M4)	Range of level inorganic P
M1	M1 331	low : high	0.15-1.39
M2	M2 331-1	all low	0.15-0.46
	M2 331-2	all low	0.15-0.46
	M2 331-4	low :high	0.15-1.39
	M2 331-5	all low	0.15-0.46
M3	M3 331-4-1	all low	0.15-0.46
	-4-2	low : high	0.15-1.39
	-4-3	all low	0.15-0.46
	-4-4	all low	0.15-0.46
	-4-5	low : high	0.15-1.39
	-4-6	all low	0.15-0.46
	-4-7	all low	0.15-0.46
	-4-9	all low	0.15-0.46
	-4--10	low : high	0.15-1.39
	-4-11	all low	0.15-0.46
	-4-12	all low	0.15-0.46
	-4-13	low : high	0.15-1.39
	-4-14	low : high	0.15-1.39

Table 5 Relative segregation of high or low inorganic P seeds of M 783 from M1 through M3 plant generation

Generation	Plant designation (M1-M3)	Segregation of seed (M2-M4)	Range of level inorganic P
M1	M1 783	low : high	0.15-1.39
M 2	M2 783-1	low : high	0.15-1.39
	M2 783-2	all low	0.15-0.46
	M2 783-3	all low	0.15-0.46
	M2 783-4	all low	0.15-0.46
	M2 783-5	low : high	0.15-1.39
M3	M3 783-1-1	low : high	0.15-1.39
	-1-2	all low	0.15-0.46
	-1-4	all low	0.15-0.46
	-1-5	low : high	0.15-1.39
	-1-6	all low	0.15-0.46
	-1-7	low : high	0.15-1.39
	-1-8	low : high	0.15-1.39
	-1-9	low : high	0.15-1.39
	-1-10	low : high	0.15-1.39
	-1-11	all low	0.15-0.46
	-1-12	low : high	0.15-1.39

Table 5 (continued)

Generation	Plant designation	Rate Segregated of seed	Range of level inorganic P
M3	M3 783-5-1	all low	0.15-0.46
	-5-2	all low	0.15-0.46
	-5-3	all low	0.15-0.46
	-5-4	low : high	0.15-1.39
	-5-5	low : high	0.15-1.39
	-5-7	low : high	0.15-1.39
	-5-8	all low	0.15-0.46
	-5-9	all low	0.15-0.46
	-5-10	low :high	0.15-1.39

A.

B.

Figure 8 Showed characteristic of M783 low phytic acid seeds

A. Homo - and heterozygous dominant

B. homozygous recessive of M783

A.

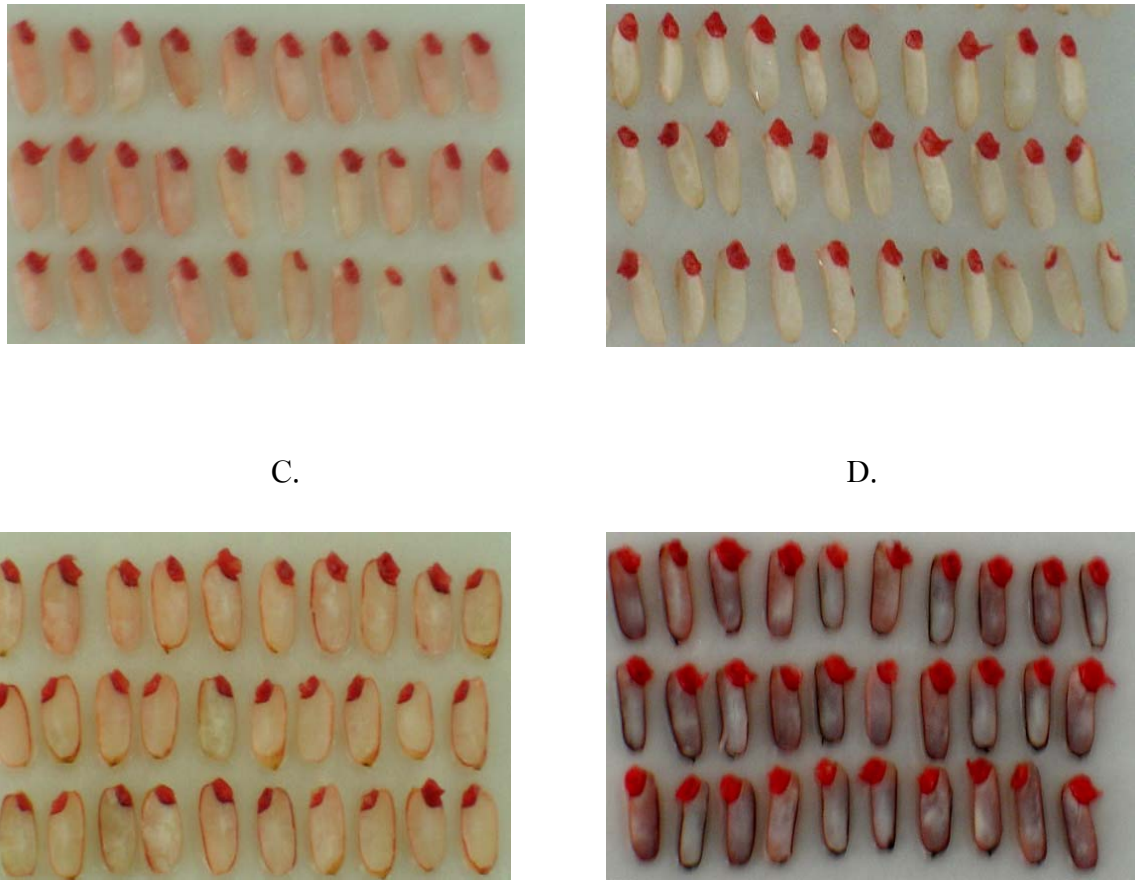
B.

Figure 9 Showed plant seedling of heterozygous M_3 331 seed for 5 days.

A. Germination of low inorganic P level (normal) of M_3 331 seed

B. Germination of high inorganic P level (mutant) of M_3 331 seed found that they could be sprouted but could not growing

A. B.



C. D.

Figure 10 Testing seed viability of wild-type by soaked seed for 1 day

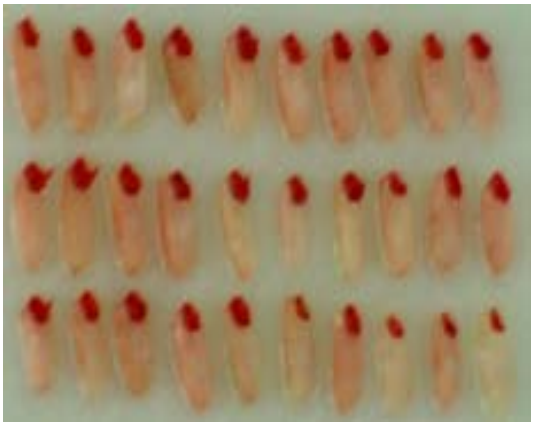
A. IR68144

B. KDML

C. Nippon bare

D. Jao Hom Nin (wild-type)

A.



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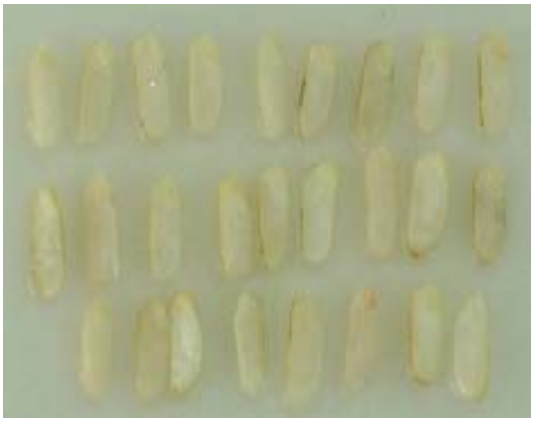
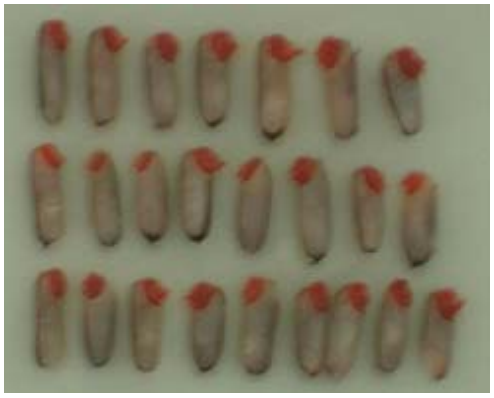


Figure 11 Controlled testing by blocked activity of dehydrogenase enzyme which this enzyme will be related to respiration

A. Sample of IR68144 seed, activity of dehydrogenase enzyme worked, embryo stilled viable

B. Sample of IR68144 were destroyed activity of dehydrogenase enzyme, embryo will be discolored; showed that no respiration

A.



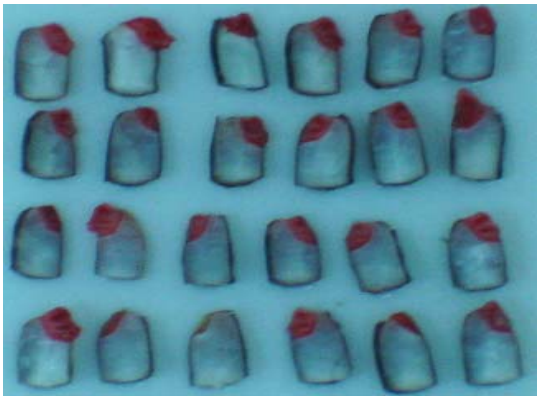
B.

Figure 12 Testing seed viability of heterozygous mutagenic low phytic acid M_3 783 by soaked seed for 1 day

A. low inorganic P seed (LIP seed)

B. high inorgsnic P seed (HIP seed)

A.



B.

Figure 13 Testing seed viability of heterozygous mutagenic low phytic acid M_3 331 by soaked seed for 1 day

A. low inorganic P seed (LIP seed)

B. high inorganic P seed (HIP seed)

Table 6 Comparing % viability of seed between wild-type and mutagenic low phytic acid

Plant designation	Total seed	Staining seed	% Viability
IR68144	50	50	100
KDML	50	47	94
BT	50	49	98
Nipoon bare	50	50	100
LIP of M_3 783-1 mutant	26	25	96
HIP of M_3 783-1 mutant	11	0	0
LIP of M_3 783-5 mutant	24	24	100
HIP of M_3 783-5 mutant	12	0	0
LIP of M_3 331-4 mutant	24	22	92
HIP of M_3 331-4 mutant	7	5	71

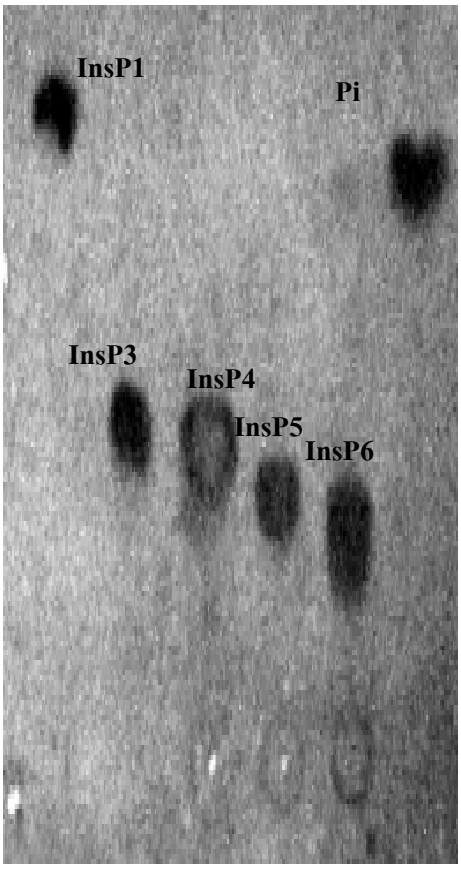


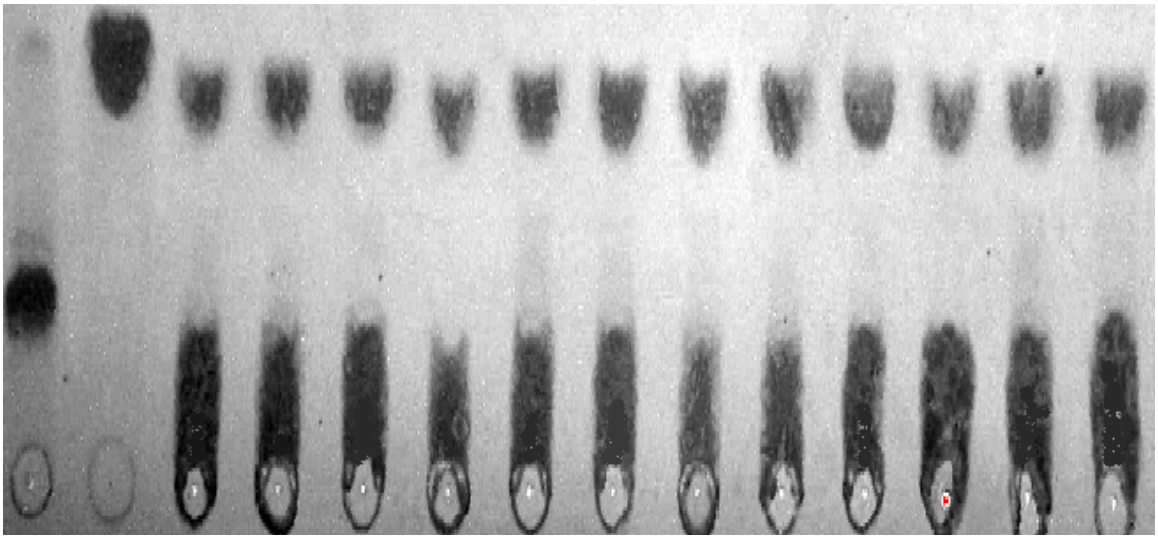
Figure 14 Standard of inositol phosphate ; D-myo-inositol-1-monophosphate (IP1), D-myo-inositol-1,4,5-trisphosphate(IP3), D-myo-inositol-3,4,5,6-tetrakisphosphate (IP4), D-myo-inositol-1,3,4,5,6-pentakisphosphate (IP5), phytic acid (IP6) and phosphate (Pi). The smallest visible quantities of each standard were 48, 5.95, 10, 2.15 2.1 and 2.5 nmol, respectively

A = BT normal

B = degraded by phytase , 30 min

C = degrade by phytase ; 60 min

Figure 15 Degradation phytic acid by phytase and developed product on HP-TLC plates



$InsP_6$ Pi KD KD IR IR AZ AZ BT BT BT#3 BT#3 NI NI

Figure 16 Analysis of phytic acid , other inositol phosphate and Pi content of wild-type by HP-TLC method

IP6 = Standard phytic acid Pi = Standard P

KD = KDML105

IR = IR68144

AZ = Azucena

BT = Jao Hom Nin

NI = Nippon Bare

A B

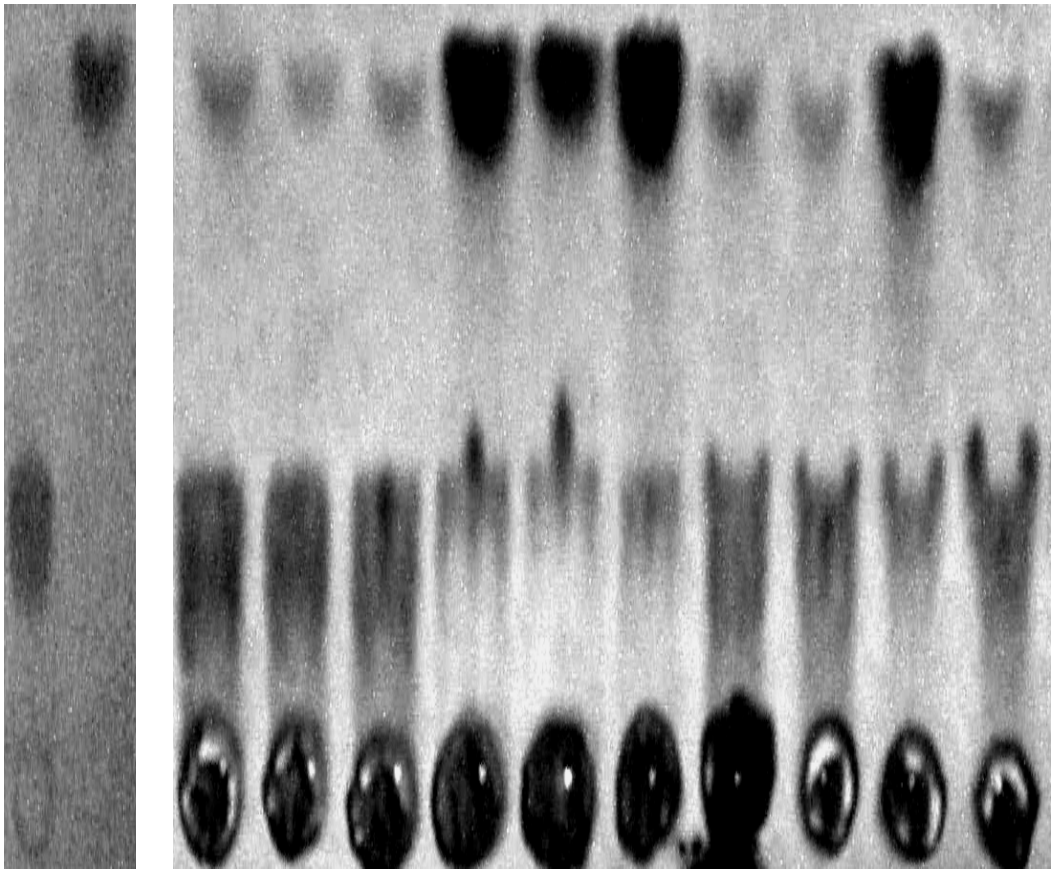


Figure 17 Screened of low phytic acid of M_3 331 by HP-TLC method ;

The result showed very high levels of free phosphate and decreased of phytic acid in homozygous recessive seed (4, 5, 6, 9) which remnant seeds were similar to wild-type

A. $InsP_6$ = standard phytic acid

Pi = Standard Pi

B. estimate 10 seeds of low phytic acid M3 331 were detected level of phytic acid, other inositol phosphate and Pi content.

A. B.

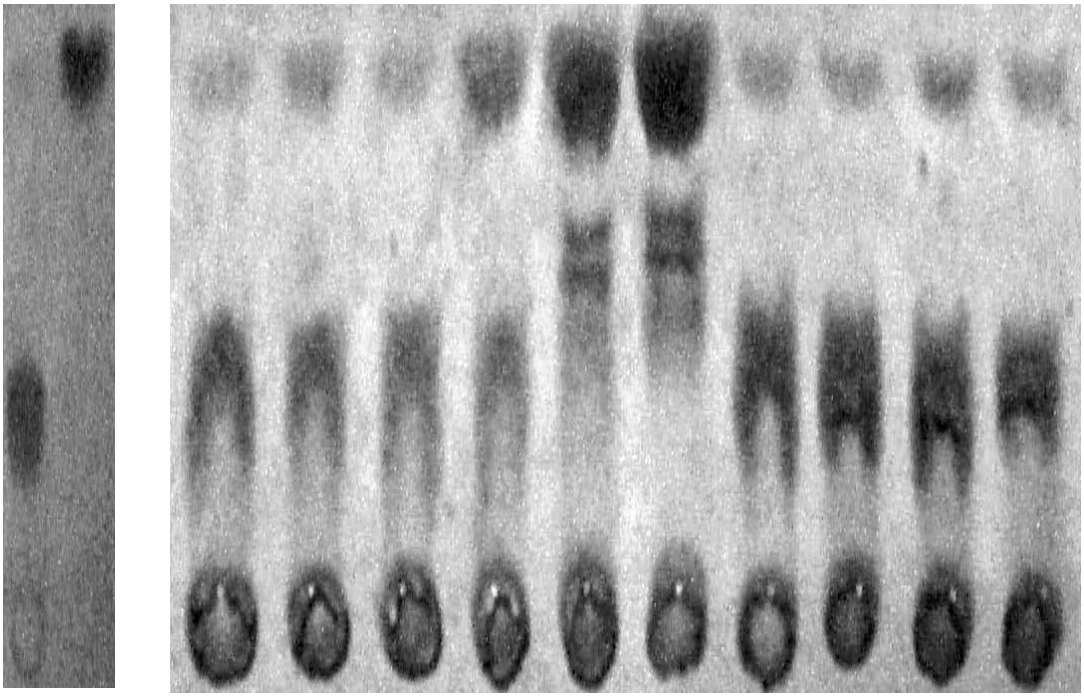


Figure 18 Screened of low phytic acid of M_3 783 by HP-TLC method ;

The result showed very high levels of free phosphate, decreased in phytic acid and trace amounts of other inositol phosphate ($InsP_4$, $InsP_5$) that not observe in wild type and non mutant seed

A. IP6 = standard phytic acid

Pi = Standard Pi

B. estimate 10 seeds of low phytic acid M3 783 were detected level of phytic acid, other inositol phosphate and Pi content.

Table 7 Quantitative analyzed of inositol tetraphosphate (IP4), inositol pentaphosphate (IP5) and inositol hexaphosphate (IP6) (mg/100g) by HPLC.

Name	IP4	IP5	IP6	Total IP5 + IP6
M_3 331-1	0.97	23.13	768.03	791.16
M_3 331-4	0.94	17.14	734.06	751.20
M_3 783-2	2.32	47.41	628.5	675.91
M_3 783-5	18.95	48.91	474.14	523.05

Note M_3 331-1 homozygous nonmutant

M_3 331-4 heterozygous low phytic acid

M_3 783-2 homozygous nonmutant

M_3 783-5 heterozygous low phytic acid

Table 8 To compared, inositol tetraphosphate (IP4), inositol pentaphosphate (IP5) and inositol hexaphosphate (IP6) content of M_4 783 (wrinkle embryo) and M_4 783 (normal embryo) by HPLC

M_4 783	IP4 (mg/100g)	IP5 (mg/100g)	IP6 (mg/100g)	IP5 + IP6 (mg/100g)
Wrinkle embryo	38.77	75.98	530.23	606.21
Normal embyo	6.99	40.56	748.99	789.55