

MATERIALS AND METHODS

Materials

1. Mature rice seed, variety KDML105, IR68144, Azucena, Nipponbare, Jao Hom Nin (wild-type), and M2 mutagenic of Jao Hom Nin. All rice will be supplied by Rice Gene Discovery and DNA Technology, Kasetsart University, Kamphaensaen Campus, Nakhon Pathom, Thailand.

2. Reagent

- hydrochloric acid
- diethyl ether
- hydrogen peroxide
- perchloric acid
- sulfuric acid
- trichloroacetic acid
- 1-propanol
- 25% ammonia solution
- activated charcoal
- ascorbic acid
- ammonium heptamolybdate tetrahydrate
- 2,3,5-triphenyl tetrazolium chloride
- etc.

3. Equipments for analysis

- Shaker
- Centrifuge
- TLC autospotter
- HP-TLC plate

- 96-well microtitre plates
- microtube
- etc.

Methods

1. Plant Culture

About 2,000 seeds of Jao Hom Nin (M1 seeds) were treated with Fast Neutron and grown to produce the following generation of M1 population. The cultivation of plants were arranged for 10 plates. The plants of each plate were put along 12 rows and each row has 8 plants. Then, each plates will be have all 96 plants. M2 Seeds from individual plants will be collected and analysis phenotyping for screening putative low phytic acid.

2. Mutant Isolation

2.1 Screening for High Inorganic P (HIP) seed phenotype

M2 mutagenic of Jao Hom Nin seed from a total of 1,274 M1 plants were individually tested for HIP phenotype (Larson et al., 2000).

2.1.1 Extraction of soluble inorganic P

Single seeds were weighed, crushed and extracted overnight in 10 µl of 0.4 M HCl per mg of seed at 4 °C.

2.1.2 Colorimetric method

The extracts were allowed to settle for 30 min and aliquots of each single seed extract were assayed for inorganic P by modification of the method of Larson *et al.* (2000), which designed for use in 96-well microtitre plates. Sampling a 20 µl aliquot of each single seed extract was placed in a 96-well microtitre plates, and added 79 µl distilled water, 1 µl of 3% H₂O₂ in each pit. The mixture was set at room temperature until red color of Jao Hom nin extract was faded. Inorganic P soluble was detected by using a 100 µl of colorimetric reagent that mixture of 1 volume 3M H₂SO₄, 1 volume 2.5% (w/v) ammonium molybdate, 1 volume 10% (w/v) ascorbic acid and 2 volume distilled water and incubated at room temperature for approximately 1 hr. Soluble P will be appeared blue color, so that individual seed extracts could be visually scored for the presence or absence of HIP by compared with P standard dilution. Any seed extracts testing higher than 0.46 µg P were deemed HIP which mean as low phytic acid. If the HIP value in a standard set corresponds to laboratory data, the HIP phenotype will be performed in mutant seed. Groups of seed which effective high inorganic P will be selected and planted in next generation. Compared inorganic P soluble content between variety seeds of wild-type rice and M2 Jao Hom Nin mutagenic seeds.

2.2 Preparation of P standards

One milimolar (1mM) of K₂HPO₄ was diluted as five P standards dilution to achieve (i) 0.0 µg P, (ii) 0.15 µg P, (iii) 0.46 µg P, (iv) 0.93 µg P and (v) 1.39 µg P.

3. Analysis low phytic acid mutant by high-performance thin layer chromatography (HPTLC)

3.1 Extraction of inositol phosphate and phosphate

M3 grains were analyzed for decreasing of phytic acid and increasing of other inositol phosphate and phosphate content for screening low phytic acid groups. Single rice grains were crushed with a pair of pliers, transferred to micro-centrifuge tubes and homogenized in 10 x (v/w) of cold 10% (w/v) TCA, 5 mM NaF and 5 mM EDTA using a small pestle. Homogenated were vortexed for 2 hr. and centrifuged at 5000 g for 5 min. TCA was removed from the supernatants by a three-fold extraction with two volumes of water-saturated diethyl ether. Contaminated nucleotides as ATP, CTP were removed by adding 1 µl of activated charcoal suspension and vortexing for 15 min at 4 °C. After centrifugation at 5000 g for 5 min, removed supernatant and repeated three times. Supernatants were stored at -20 °C. (Rasmussen and Hatzack, 1998; Hatzack and Rasmussen, 1999).

3.2 Detection of phosphorylated compounds by HP-TLC

A 10 µl volume of supernatant was applied to HP-TLC cellulose precoated glass plates (without fluorescent indicator). HP-TLC plates were developed in mobile phase consisting of 1-propanol-25% ammonia solution-water (5:4:1). Plates were developed at room temperature until the solvent front moved near the upper plate border. HP-TLC plates were air dried and sprayed with a molybdate reagent containing 8 mM ammonium heptamolybdate tetrahydrate, 0.1 M HCl and 0.5 M HClO₄. Plates were subsequently incubated at 85 °C for 6 min and exposed to UV-light (254 nm) at a distance of 10 cm for another 6 min. Faint blue spots were immediately visible after UV exposure and maximal color intensity of spots was reached after 2 hr. To avoid background formation and fading of spots processed plates were kept out of bright light. (Rasmussen and Hatzack, 1998; Hatzack and Rasmussen, 1999).

3.3 Phytate degradation

Jao Hom Nin grains were ground using a mortar and pestle. Twenty of milligram portions of flour were suspended in 200 μ l of 50 mM sodium acetate, pH 4.6. Each sample was shaken and incubated at 37 °C all time. Samples were kept in each aperiod of time at 30 min and 60 min and stopped phytase activity by snap-freezing in liquid nitrogen. Degradation products from phytase activity were isolated by adding 200 μ l of 10% (w/v) TCA, 5 mM NaF and 5 mM EDTA to each sample. Homogenated were vortexed, followed by diethyl ether extraction and treated with activated charcoal. Isolated degradation product were detected by HP-TLC and sprayed with a molybdate reagent as described above.

3.4 Detection sensitivity of inositol phosphate standards

The visual detection limit of the molybdate staining method was estimated by employing a series dilution of several inositol phosphate standards concentration, chromatographed on HP-TLC plates. For example of inositol phosphate standards were used as 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)

4. **Tetrazolium testing**

The wild-type and mutagenic rice grain was detected viability of embryo. Grains were soaked overnight and discarded one half. Placed the one half of seed in a petri dish containing 0.1% tetrazolium and incubated for 1 hr at room temperature. The embryo will be stained red color, if embryo remained viability.

Controlled experiment by blocked activity of respiratory enzymes, the embryo will be discolored.

$$\% \text{ of viability} = \frac{\% \text{ stained seed} \times 100}{\text{total seed}}$$

5. Quantitative analysis

The amount of phytic acid and inositol phosphate content from grains of Jao Hom Nin (wild-type) and Jao Hom Nin mutagenic rice will be determined by HPLC. Analyze quantitative of phytic acid and inositol phosphate content received coassistance from Food Chemistry Division, Institute of Nutrition, Mahidol university at Salaya, Nakhon Pathom, Thailand.