

DISCUSSION

Rice is a commonly consumed staple food crop of Thailand's people. The development of rice breeding, which increased a high nutritional value, will be promoted to health's consumer. However, several reports suggest that phytic acid (InsP_6) is the single most important factor hindering the uptake of a range of minerals and stored in rice grain (Juliano, 1972; O' Dell, 1972; Kennedy and Schelstraete, 1975 and Yoshida *et al.*, 1999).

Phytic acid is considered a major inhibitor of mineral intake because phytic acid structure is a polyanion at physiological pH and an effective chelator of nutritionally important mineral cation such as calcium, zinc, and iron. Once consumed in human foods or animal feeds, phytic acid binds to these minerals in the intestinal tract to form mixed salts that are largely excreted (Raboy *et al.*, 2001). Therefore, the content of phytic acid in the rice grain may have important nutritional implication for the bioavailability of the minerals present in the rice. Thus, reduction of the phytic acid content in the rice grain is likely to increase the bioavailability of nutrient absorption.

The reduction of phytic acid content can be degraded by phytase enzyme which is a phosphatase that catalyses the stepwise hydrolysis of phytic acid to orthophosphate, a series of lower phosphate esters of myo-inositol and eventually myo-inositol. Phytase activity will be appeared in plant and microorganism while mono-gastric animals have no or limited phytase activity in their digestive tract (Brinch-Pederson *et al.*, 2002).

In plants, several reports showed that phytic acid is degraded during seed germination by phytase. Most plant phytase belong to either an acidic group with a pH optimum around pH 4 or to an alkaline group with a pH optimum around pH 8.0 and temperature optimum approximate 45-60 °C (Yoshida *et al.*, 1975 ; Hayakawa *et al.*,

1989; Laboure et al, 1993; Greiner et al, 2001; Brinch-Pedersen et al, 2002). In study of phytase gene in several plants found the presence of mRNA accumulated during germination but phytase transcripts could not be detected in dry seeds. Then, phytase would be have the role in seed germination (Maugenest et al, 1997; Maugenest *et al.*, 1999; Hegeman and Ggrabau, 2001). The naturally, phytic acid occur in rice seed, although the phytase is found existing in rice but it will hydrolyze phytic acid when seeds is soaked until germination in water. In addition to, phytase will hydrolyze phytic acid present at lower pH levels and Tm optimum estimate at 45 °C.

Since phytase has activity at low pH and Tm optimum approximate 45 °C. Phytic acid can not digested at this condition in mono-gastric animal. Under meal condition, rice cooking may be destroyed phytase activity. Then, phytic acid can not degraded by phytase. These cause may be lost minerals in form of macromolecule of phytic acid excretion. Then, decreasing of phytic acid in dry seed will be help to solve micronutrient deficiency. Using the mutational breeding approach is taken to reduce phytic acid content which this procedure would be impairing phytic acid biosynthesis. Several reports revealed isolating of low phytic acid mutant in cereal grains such as maize, barley, wheat, soybean and rice (Guttieri *et al.*, 2003; Larson *et al.*, 2000; Raboy *et al.*, 2000; Shi *et al.*, 2003; Wilcox *et a.l.*, 2000). The reports suggested that low phytic acid (lpa) mutants are divided as two phenotypic distinct types. In lpa1-like mutants, seed phytic acid were reduced which are matched by corresponding increases in inorganic P. For lpa2-like mutants, seed phytic acid were reduced which are matched by increases in both inorganic P and in inositol (Ins) phosphates containing five or fewer P esters (compared with phytic acid's six P esters).

HIP phenotype

The selecting of low phytic acid mutant seeds used determining of high in inorganic P which was primary screening. The rationale behind this screening was that a high free phosphate level is indicative of a low phytic acid mutant since phosphate, as one of the structural components of phytic acid, is likely to accumulate when phytic acid synthesis is compromised or disrupted as a result of a mutation. This method was expected to provide a fast and simple procedure to screening of low phytic acid grains from a large number of mutagenic population.

In this study, Jao Hom Nin rice seeds were treated with fast neutron and planted in M1 generation. The M2 seeds were screened for high in inorganic P which this method was modified from Larson's method but add of 3% H₂O₂ for fading of anthocyanin color. Initial screening was identifying high in inorganic P (HIP) seed, based on color intensity observation from M2 seeds of 1,274 M1 plants. The results found that M331 and M783 gave high inorganic phenotypic segregating in M2, M3, M4 seed generation while M672, M971, M1161 and M1234 were regarded as false positive because no found HIP phenotypic in M3 seed of all these plants. This rather high proportion of false positives may be due to the extraction method used in the primary screening where crushed grains were soaked overnight in 0.4 M HCl in order to extraction of the most soluble phosphate. Although the samples were kept at 4 °C during overnight incubation, it seems likely that prolonged exposure to HCl supported hydrolysis of a wide variety of phosphorylated cellular compounds, resulting in artificially increased levels of free phosphate in the extracts. The other reasons, these plants which, low phytic acid may be lethal between growing or not sprout, owing to, the observation of survival rate in Table 3 found that the low phytic acid mutants have low survival rate by comparing with wild-type.

An investigation of high in inorganic P in low phytic acid mutant seeds found that seeds of M₂, M₃, M₄ generation of both M331 and M783 were segregated as low inorganic P : high inorganic P in 3 : 1 ratio while wild type was not appearance for high inorganic P characteristic. These results expected that putative low phytic acid were recessive heredity because of the studying of genetic testing by backcrossing between putative low phytic acid of barley (*Hordeum vulgare* L.) mutant and wild type showed that all putative low phytic acid mutations were recessively inherited (Rasmussen and Hatzack, 1999).

Characterization of HIP phenotype

Low phytic acid mutants were examined for viable seed by tetrazolium (TZ) test. The tetrazolium test is a biochemical test, which differentiates live from dead seeds based on the activity of the respiration enzymes in seeds. Upon seed hydration, the activity of dehydrogenase enzyme increase resulting in the release of hydrogen ion, which reduces the colorless tetrazolium salt solution (2,3,5-triphenyl tetrazolium chloride) into a chemical compound called formazan. Formazan stains living cells (respiring) with a red color while dead cells (not respiring) remain colorless (Elias and Garay, n.d.) In this study found that all low phytic acid of M₃ 331 (HIP seeds) showed a weak staining in embryo while low inorganic P seeds were strong staining of formazan in embryo (Figure 13) and seed germination of M₃ 331 found that seeds could be sprouted but not growing (Figure 9). Then, HIP phenotype of M₃ 331 remained occurring respiration but not strong staining which correlation with seed germination. For, HIP phenotype of low phytic acid mutant M₃ 783 were embryo completely unstained altogether (Figure 12) and could not sprouted. Owing to, phytic acid (InsP₆) was first known as the storage form of phosphorous in seeds. During germination, phytic acid was broken down by endogenous phytase enzyme, releasing their P, inositol and mineral contents for use by the growing seedling. Then, this compound could serve

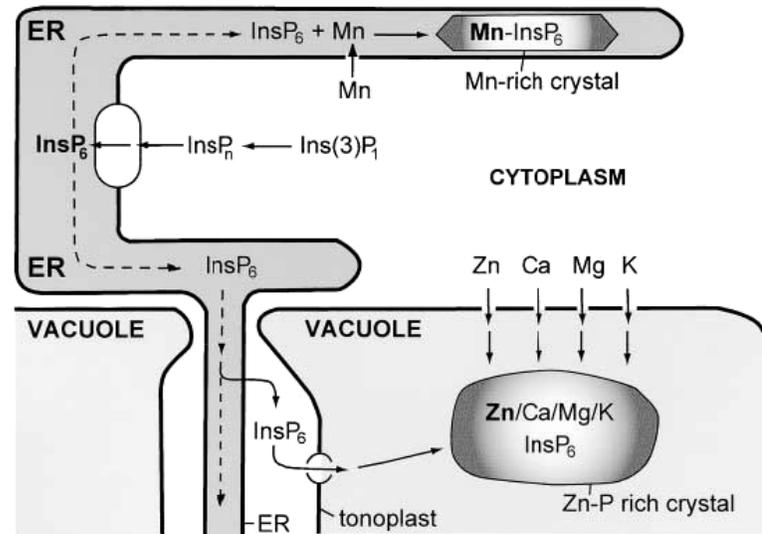
as phosphorus donors for regeneration of ATP from ADP (Raboy, 2001). These reason assumed that putative low phytic acid mutant could not growing as usual because of content of phytic acid was decreased in mutant.

Otegui *et al.* (2002) studied different minerals storing sites in developing seed of Arabidopsis. This report concluded model of phytic acid synthesis and its translocation to mineral storing sites in developing Arabidopsis seeds (Figure 19). This model showed that phytic acid accumulated in embryo and stored of mineral such as Ca, Mg, K. Thus, low phytic acid mutants have effect to mineral binding which these mineral were supplied as cofactor to plant growing. This reason supported that HIP phenotype seeds can not sprout or not germination

Tetrazolium testing in this research found strong staining of formazan in embryo of both wild type and non-mutant seeds (LIP seeds of M331 and M783) while mutant seeds (HIP seeds of M331 and M783) were weak staining or colorless. In this testing confirmed that respiration of rice in during germination would occur in embryo. So, germination seeds were probably relation with phytic acid content.

The report studied role of phytic acid in wheat grain found that in the embryo plus scutellum and aleurone fractions, there was a rapid build-up of phytic acid during grain development which suggested that the synthesis of phytic acid is associated with the onset of dormancy (Williams, 1970). In this reason hypothesized that the wrinkle embryo seed of M783 may be short dormancy because level of phytic acid less than normal seed. Thus, embryo may be germinated and died before during grain development until mature grain.

A.



B.

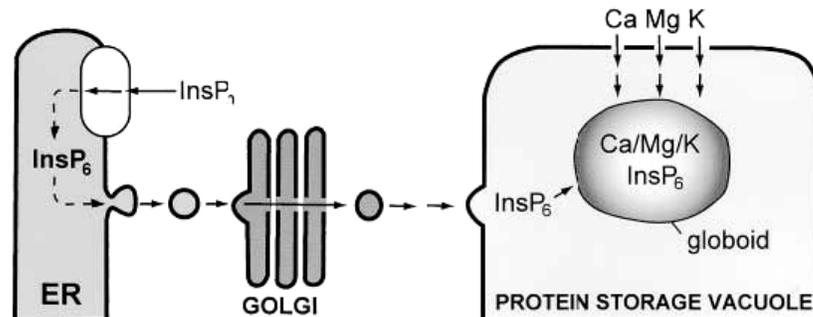


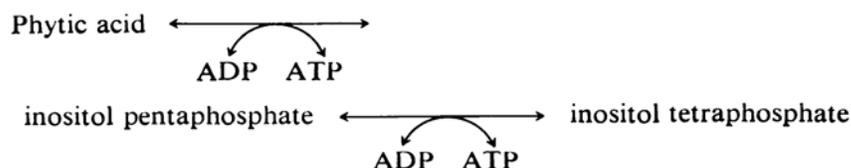
Figure 19 Model of phytic acid synthesis and its translocation to mineral-storing in developing Arabidopsis seeds.

A. In the chalazal endosperm, phytic forms crystals in two locations: Mn-rich crystals in the ER and Zn-rich crystals in the vacuoles.

B. In the embryo, the transport of phytic acid from the ER to PSV. Golgi to PSV transport of phytic acid occurs via multivesicular bodies.

Source: Otegui *et al.* (2002)

William (1970) suggested that the enzyme phytase, which can be purified from the mature seeds and is responsible for the degradation of the phytin during the first few days of germination, If this enzyme mediates ATP-linked reactions of the type then it might be expected that inorganic phosphate would change with the phytic acid via exchange reactions with the ATP



In William's reason, ATP may be not enough for respiration of low phytic acid seeds. This cause may be effect to low phytic acid that can not growing. In another reason may be relation to abnormal of phytase.

High-performance thin-layer chromatography (HPTLC) screening.

High-performance thin-layer chromatography (HPTLC) method was used for analysis of inositol mono- to hexakisphosphate and free phosphate on cellulose precoated glass plates (Hatzack and Rasmussen, 1999). This method was a reliable analyzed in order to investigate the phytic acid and free phosphate composition in the low phytic acid mutants and confirm the results of the primary screening. Our report applied the HPTLC system for analysis of grain extracts from wild type and groups of HIP grains of low phytic acid mutant.

The originated of HPTLC analysis, the variety of standard of inositol phosphate (InsP_1 , InsP_3 , InsP_4 , InsP_5 , InsP_6) and P_i were analyzed mobility on HP-TLC cellulose precoated glass plates. The result found that InsP_6 migrated slower than five or fewer inositol phosphate and P_i . The visual detection limit of the molybdate staining showed

in the level of nanomole (figure 14). Thus, the high detection sensitivity makes the HP-TLC system suitable for micro analysis. This research demonstrates that the HPTLC system can be applied to single grain analysis of Jao Hom Nin mutants.

The seeds of wild type (KDML105, IR68144, Azucena, Nippon bare, Jao Hom Nin (BT) and M3 grains of each putative M2 mutants (M_3 331 and M_3 783) were analyzed by HPTLC. Each grains of wild type gave result nearly of phytic acid content and free phosphate while grains analysis of each putative low phytic acid were divided as two different phenotypes. This result found that some grains of M_3 331 mutants was characterized by a large dark spot indicating a very high level of free phosphate while level of phytic acid lower than wild type and non mutant grains (Figure 17). For M_3 783 mutants found some grains compose of high level of free phosphate, a faint band indicating a small amount of phytic acid and a trace amounts of P-compound could be observed, which migrated faster than phytic acid but slower than free phosphate, suggesting that phosphoinositols with less than six phosphate groups. These compounds could not be detected in grain extracts from wild type, non mutant grain, nor in extract of M3 331 grains (Figure 18). Therefore these phosphoinositols were regarded as genuine metabolic intermediates rather than random breakdown products resulting from phytic acid hydrolysis during TCA extraction.

The heterozygous of M_4 331 and M_4 783 low phytic acid were isolated grain by dividing into two groups which consist of HIP groups and LIP groups. Each groups were analyzed amount of phytic acid (InsP_6) and other phosphoinositols (InsP_4 , InsP_5) by High performance liquid chromatography (HPLC) for a precise quantitative analysis of these compounds. This results found that quantitative analysis of low phytic acid grains by HPLC corresponded to HP-TLC analyze (Table 7, Table 8).

Hence, HPTLC system was suitable for mass screening of mutant rice grains because HPTLC analyses confirmed that putative low phytic acid mutants increased free phosphate content in grains which correlated with decreasing of phytic acid. In addition, simultaneous appearance of novel, bona fide inositol phosphates (M783) were found by HPTLC analysis. This research could isolated pattern of putative low phytic acid by HPTLC method which this method would be trending in detailed investigations of the enzyme involved in the biosynthesis and degradation of phytic acid during the plant life cycle. Screening of mutation rice by HPTLC would help us to select putative low phytic acid varieties and reduce number of large mutant population for identify gene concerning in biosynthesis pathway to phytic acid (Figure 20).

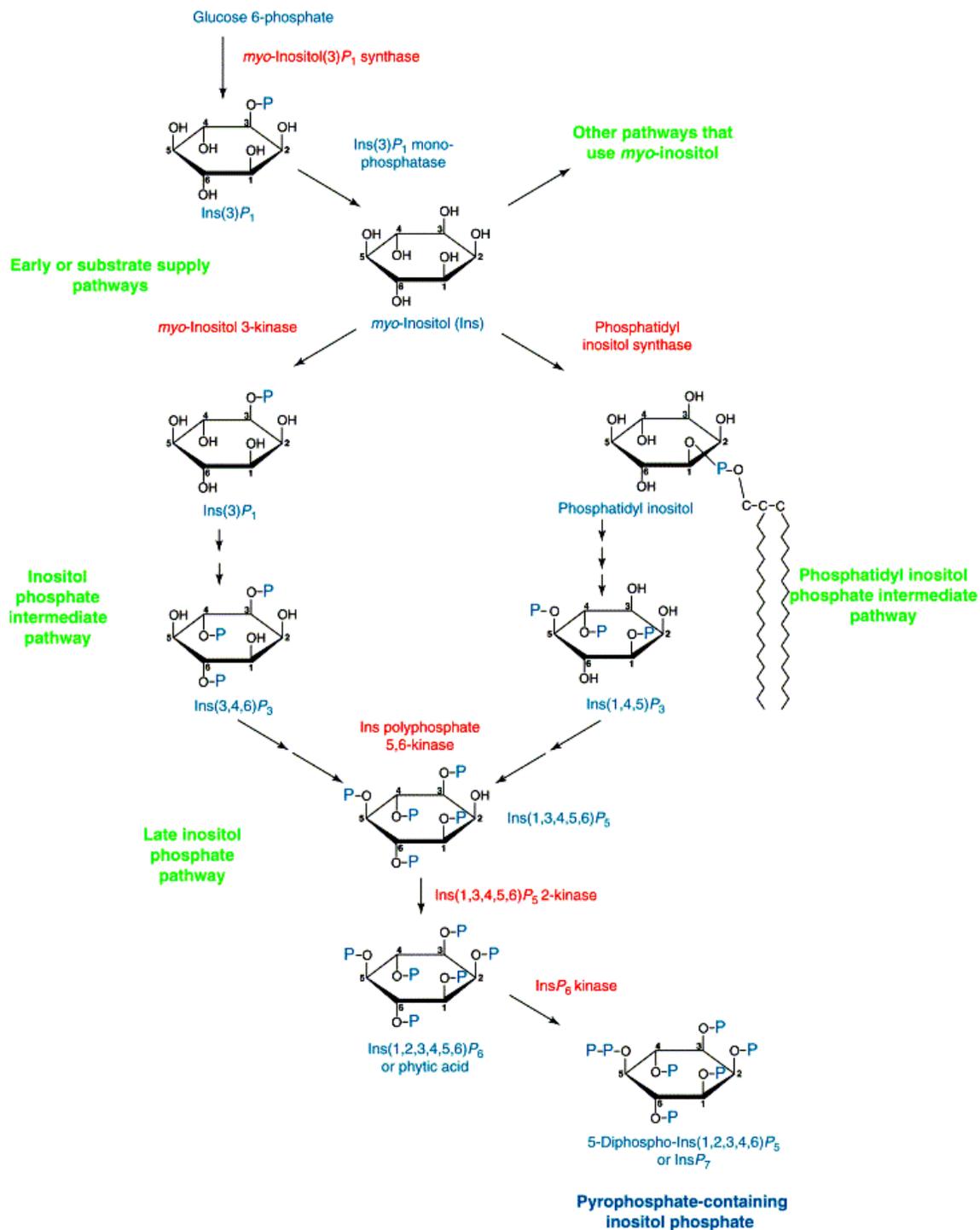


Figure 20 Showed phytic acid pathway

Source: Raboy (2001).