# THESIS APPROVAL

## GRADUATE SCHOOL, KASETSART UNIVERSITY

Doctor of Philosophy (Plant Breeding)
**DEGREE**

Plant Breeding
**FIELD**

Interdisciplinary Graduate Program
**PROGRAM**

**TITLE:** Mapping of Quantitative Trait Loci Conferring Phosphorus Compound Contents in Seed and Seedling of Mungbean (*Vigna radiata* (L.) Wilczek)

**NAME:** Miss Utumporn Sompong

**THIS THESIS HAS BEEN ACCEPTED BY**

**THESIS ADVISOR**
( Professor Peerasak Srinives, Ph.D. )

**THESIS CO-ADVISOR**
( Associate Professor Sontichai Chanprame, Ph.D. )

**THESIS CO-ADVISOR**
( Mr. Sutkhet Nakasathien, Ph.D. )

**GRADUATE COMMITTEE CHAIRMAN**
( Associate Professor Voravit Siripholvat, D.Agr. )

**APPROVED BY THE GRADUATE SCHOOL ON** _____

**DEAN**
( Associate Professor Gunjana Theeragool, D.Agr. )

THESIS

# MAPPING OF QUANTITATIVE TRAIT LOCI CONFERRING PHOSPHORUS COMPOUND CONTENTS IN SEED AND SEEDLING OF MUNGBEAN (*Vigna radiata* (L.) Wilczek)

UTUMPORN SOMPONG

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Plant Breeding)
Graduate School, Kasetsart University
2011

Utumporn Sompong 2011: Mapping of Quantitative Trait Loci Conferring Phosphorus Compound Contents in Seed and Seedling of Mungbean (*Vigna radiata* (L.) Wilczek). Doctor of Philosophy (Plant Breeding), Major Field: Plant Breeding, Interdisciplinary Graduate Program. Thesis Advisor: Professor Peerasak Srinives, Ph.D. 90 pages.

Phytic acid (PA) is the principal storage form of phosphorus in seed of cereals and legumes. It is a powerful inhibitor against the absorption of proteins and certain mineral nutrients. Mungbean is an important Asian legume which has only little research on PA. The objectives of this study were to (1) identify mungbean germplasm with low and high seed PA, (2) estimate narrow-sense heritability ($h_n^2$) of phosphorus (P) compound contents in seed, (3) investigate changes of P compound contents during germination, and (4) identify QTLs for the contents in seed and seedling. P compound contents were determined by colorimetric methods. Eleven accessions each of cultivated and wild mungbean were assessed for PA content. V1137BG, V1725BG, AusTRCF321936, and Sukhothai had highest PA content of about 16 $mg.g^{-1}$, while AusTRCF321925 had the lowest content of 8.68 $mg.g^{-1}$. Two high PA accessions, V1725BG and Sukhothai were crossed with two low PA accessions, AusTRCF321925 and AusTRCF322012 resulting in four population for estimating $h_n^2$ of the P contents. The $h_n^2$ estimates of phytic acid P (PAP) content in seeds of the four populations ranged from 0.12 to 0.88, those of inorganic P (IP) content ranged from 0.30 to 0.61, and those of total P (TP) content varied from 0.06 to 0.69. All accessions showed a dramatic increase in IP content following seedling ages at 12, 24, 48 and 72 hr, however they showed decreased PAP content and stable TP content. QTL analysis was conducted using $F_{2:3}$ population of 170 lines derived from a cross between V1725BG and AusTRCF321925. A linkage map consisting of 101 SSR markers on 13 linkage groups was developed**.** Four QTLs were identified conferring the content of IP in seed, three for PAP in seedling, two for PAP in seed and TP in seedling, one for TP in seed and IP in seedling, and ten QTLs were identified for three agronomic traits.

_____ _____ ___ / ___ / ___

Student's signature Thesis Advisor's signature

# ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and deep appreciation to Prof. Dr. Peerasak Srinives, my thesis advisor, for teaching, guidance and encouragement which enable me to carry out my research successfully. I would gratefully like to thank Assoc. Prof. Dr. Sontichai Chanprame and Dr. Sutkhet Nakasathien, my committee members, Assoc. Prof. Dr. Julapark Chunwongse, the defense examination chairman, and Dr. Theerayut Toojinda, a BIOTEC expert, for valuable comments and suggestions.

I would sincerely like to thank Dr. Victor Raboy, my collaborator from the Laboratory of Seed Chemistry and Genetics, Agricultural Research Service - United State Department of Agriculture (ARS-USDA), Aberdeen, Idaho, USA for his advice, and providing facilities for chemical analysis and proof-reading of my research manuscript. I am deeply indebted to Dr. Prakit Somta, Senior Lecturer at the Department of Agronomy, for his guidance in genotypic and QTL analysis and proof-reading of the manuscript.

This research was funded by the Royal Golden Jubilee (RGJ) Scholarship of the Thailand Research Fund (TRF), and the Project on Biotechnology for Varietal Development of Thai Mungbean of the National Science and Technology Development Agency (NSTDA).

Finally, I wish to express my deepest gratitude to my mother and sister for their understanding, supporting and encouragement, which enable me to accomplish my study.

Utumporn Sompong

April 2011

# TABLE OF CONTENTS

**Page**

# LIST OF TABLES

# LIST OF TABLES (Continued)

# LIST OF FIGURES

# LIST OF ABBREVIATIONS

ABA = abscisic acid
ABC = ATP - binding cassette
AFLP = amplified fragment length polymorphism
ANOVA = analysis of variance
ARS-USDA = Agricultural Research Service- United State Department of Agriculture
ATP = adrenosine triphosphate
AVRDC = Asian Vegetable Research and Development Center
ax = axial
bp = base pairs
C = concentration
Ca = calcium
CIM = composite interval mapping
cM = centiMorgan
CRD = completely randomized design
CTAB = cetyl trimethyl ammonium bromide
*df* = degree of freedom
DFL = days to 50% flowering
DMRT = Duncan's multiple range test
DMT = days to 50% maturity
DNA = deoxyribonucleic acid
dNTP = deoxyribonucleotide triphosphate
EDTA = ethylene diaminetetraacetic acid
ems = expected mean square
EtBr = ethydium bromide
eq = equatorial
F = filial generation
Fe = iron
g = gram
GluB = glutilin B protein

## LIST OF ABBREVIATIONS (Continued)

| | |
|---|---|
| *Gm* | = *Glycine max* |
| GRAS | = generally recognized as safe |
| HL | = Haug and Lantzsch |
| $h_n^2$ | = narrow-sense heritability |
| IAA | = indole-3-acetic acid |
| Ins | = inositol |
| IP | = inorganic phosphorus |
| K | = potassium |
| kb | = kilo base pairs |
| LG | = linkage group |
| LOD | = logarithm of odd |
| lpa | = low phytic acid |
| M | = mutant |
| mg | = milligram |
| Mg | = magnesium |
| MIK | = *myo*-inositol kinase |
| MIPS | = D-*myo*-inositol 3-phosphate synthase |
| ml | = milliliter |
| MRP | = multidrug resistance - associated protein |
| MS | = mean square |
| $MS_B$ | = between $F_2$ family mean square |
| $MS_w$ | = within $F_2$ family mean square |
| MYMV | = mungbean yellow mosaic virus |
| µg | = microgram |
| µl | = microliter |
| n | = number of $F_2$ family |
| N | = nitrogen |
| Na | = sodium |
| $N_C$ | = number of classes |
| ng | = nanogram |

# LIST OF ABBREVIATIONS (Continued)

$N_S$ = number of $F_3$ plants
NS = non-significance
NSTDA = Natural Science and Technology and Development Agency
*p* = probability
P = phosphorus
PA, Pha, Phy = phytic acid
PAP = phytic acid phosphorus
PCR = polymerase chain reaction
$P_i$ = phosphate group
PP = polyphosphate
Ptd = phosphatidyl
PVE = phenotypic variance explain
PVP = polyvinylpyrrolidone
QTL = quantitative trait loci
r = number of $F_3$ plants per $F_2$ family
*r* = Pearson's correlation coefficience
RAPD = random amplification of polymorphic DNA
RFLP = restriction fragment length polymorphism
RGJ = Royal Golden Jubilee
RIL = recombinant inbred line
RNAi = ribonucleic acid interference
SD = standard deviation
*SD* = seed
SD100WT = 100-seed weight
SE = standard error
*SL* = seedling
SOV = source of variation
SSR = simple sequence repeat, microsatellite
Sum = summary
$\sigma^2_B$ = between $F_2$ family variance

# LIST OF ABBREVIATIONS (Continued)

| | |
|---|---|
| $\sigma^2_w$ | = within $F_2$ family variance |
| TBE | = Tris-boric acid-EDTA |
| TCA | = trichloroacetic acid |
| TEMED | = tetramethyl ethylenediamine |
| TP | = total phosphorus |
| TRF | = Thailand Research Fund |
| Tris | = 2-amino-2-hydroxymethyl-propane-1,3-diol |
| Univ. | = University |
| USA | = United State of America |
| $V_A$ | = additive genetic variance |
| $V_D$ | = dominant genetic variance |
| $V_e$ | = extracted volume |
| $V_E$ | = environmental variance |
| $V_{EC}$ | = common environmental variance |
| $V_s$ | = sample volume |
| W | = sample weight |
| Zn | = zinc |

# MAPPING OF QUANTITATIVE TRAIT LOCI CONFERRING PHOSPHORUS COMPOUND CONTENTS IN SEED AND SEEDLING OF MUNGBEAN (*Vigna radiata* (L.) Wilczek)

## INTRODUCTION

Many children and children-bearing women in rural communities in developing countries are facing with malnutrition caused by mineral and protein deficiencies and the present of anti-absorption substances in foods. Phytic acid is a powerful inhibitor preventing absorption of protein and certain mineral nutrients in human and non-ruminant livestock. They must excrete a large fraction of phytate salts and thus can contribute to water pollution. Phytic acid is found in high amount in grain cereals and legumes. However, there is not much research done in mungbean, one of the most important legumes in Asia.

To analyze phytic acid content is a tedious job. One approach is to use molecular marker to identify its level through gene mapping and marker-assisted selection. Linking traits to markers using genetic information of a population can identify the gene location. The number and locations of loci controlling the trait can be discovered from the genetic map. DNA markers can add significantly more value than just improving the speed, costs or quality of existing breeding programs. The limitation is not the available technology, but rather the challenge facing the breeder to find creative approaches for developing new products. This research is aiming to map quantitative trait loci (QTL) conditioning phosphorus (P) compound contents using molecular marker technology. The information will be useful for breeding of optimum content of phytic acid in mungbean seed and seedling in the future.

# OBJECTIVES

1. To identify high and low seed phytic acid germplasm

2. To estimate narrow-sense heritability of seed phosphorus compound contents

3. To assess changes in phosphorus (P) compound contents during germination

4. To analyze quantitative gene loci (QTL) controlling phosphorus compound contents in seed and seedling and agronomic traits of mungbean

# LITERATURE REVIEW

## Phytic acid

Phosphorus (P) is an essential macronutrient for all living organisms. It serves various basic biological functions as a structural element in nucleic acid and phospholipid, in energy metabolism, in the activation of metabolic intermediates, as a component in signal transduction cascades, and in the regulation of enzymes (Wang *et al*., 2004b). P is one of the three macronutrients that plants must obtain from soil. It is a major component of compounds whose functions relating to growth stimulation, root development, flowering, and ripening.

P cycle in natural and agricultural ecosystems (Figure 1) begins after inorganic P is absorbed by plant roots from the soil fluid and translocated by the xylem and the phloem to all parts of the plant. Only a limited amount of the soil inorganic P is available to the plant because the mobility of inorganic P is low. Yet it has high affinity for organic and inorganic compounds, and soil particles. In natural ecosystems, seeds germinate or decompose like vegetative parts of the plant do, returning inorganic P back to the soil by chemical, microbial and plant enzymatic actions on organic P compounds. Up to 80% of P supplied via fertilizers becomes fixed in the soil (Pederson *et al*., 2002). Consequently, to ensure crop productivity, a surplus of inorganic P fertilizer is often applied. Many years of intense applications with industrially produced inorganic P and manure P has resulted in strong P accumulation in the soil. Increased soil P content increases the inorganic P loss to the aquatic environment.

Phytic acid (Figure 2b), known as *myo*-inositol-1, 2, 3, 4, 5, 6-hexa*kis*phosphate, is the principal storage form of P in seeds of cereals and legumes. It is a simple ring-carbohydrate, *myo*-inositol (Figure 2a), with attached six phosphate groups (Figure 2c). *Myo*-inositol is the cyclic alcohol derivative of glucose utilized by several pathways in plant cell including indole-3-acetic acid (IAA) metabolism, cell wall polysaccharide and cyclitol synthesis, and phosphatidylinositol (PtdIns)/Ins

phosphate pathway (Raboy, 2003). The phosphate groups are responsible for characteristic properties of phytic acid, allowing it to form very stable complexes with multivalent cations, i.e. $Ca^{2+}$, $Fe^{3+}$, $Mg^{2+}$, $Zn^{+}$ and protein. Barrientos and Murthy (1996) reported that phytic acid adopts the sterically stable one phosphate at $2^{nd}$ carbon in the axial position and five phosphates in the equatorial position (1ax/5eq) in a pH range of 0.5 - 9.0, and over pH 9.5 sterically hindered five phosphates in the axial and one phosphate in equatorial position (5ax/1eq) (Figure 3).

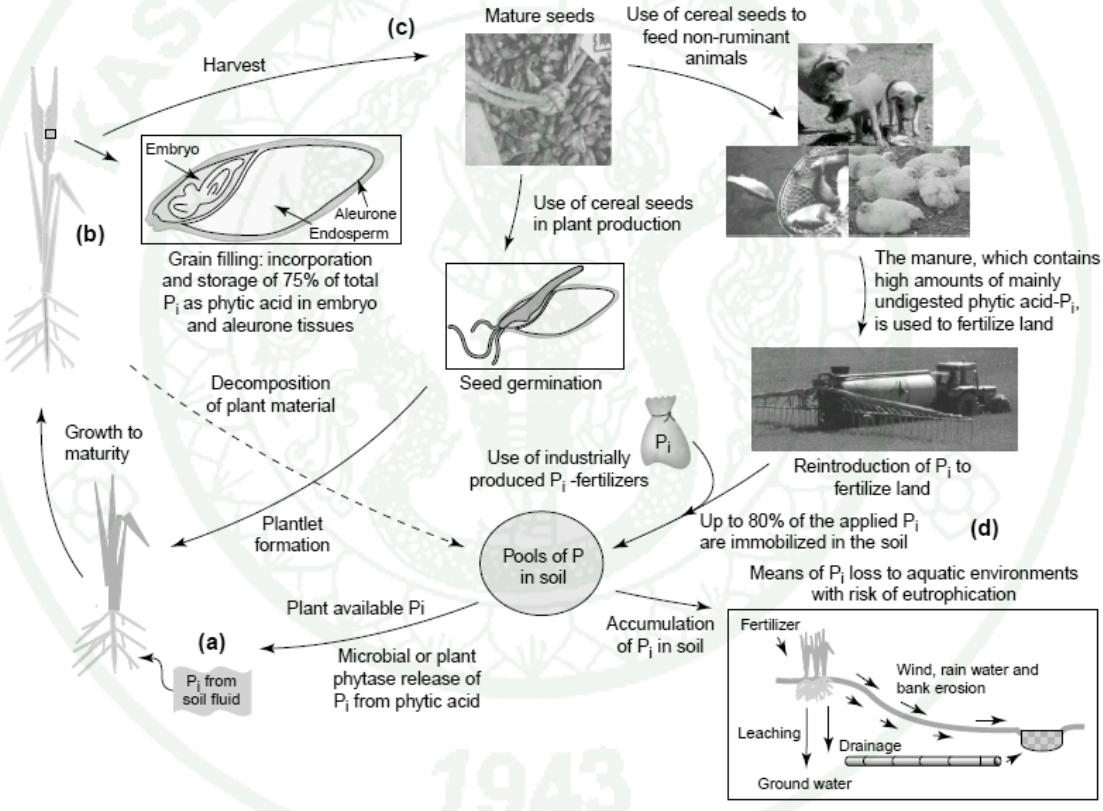
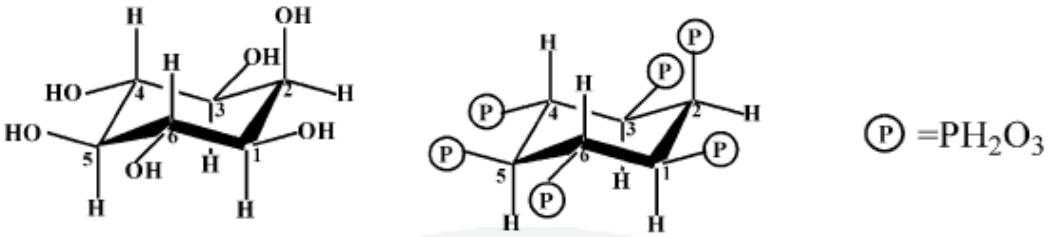



**Figure 1** P cycle in natural and agricultural ecosystems

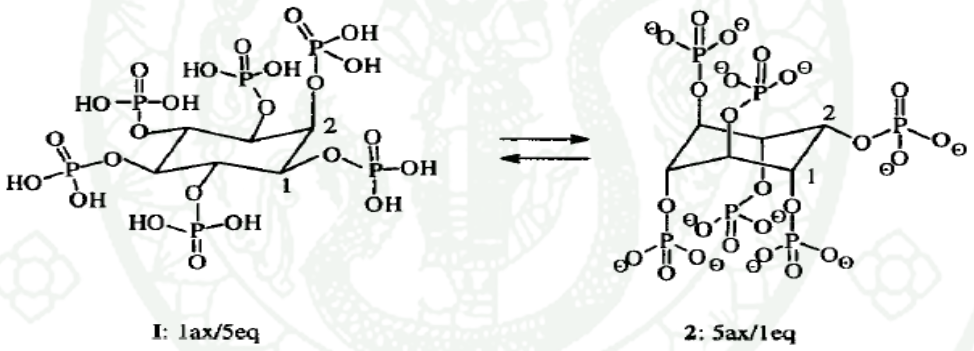
**Source**: Pederson *et al*. (2002)




a b c

**Figure 2** *Myo*-inositol (a), Phytic acid (b), and Phosphate group (c)

**Source**: Raboy (2003)




(pH 0.5-9.0) (pH >9.5)

**Figure 3** Conformations of phytic acid

**Source**: Barrientos and Murthy (1996)

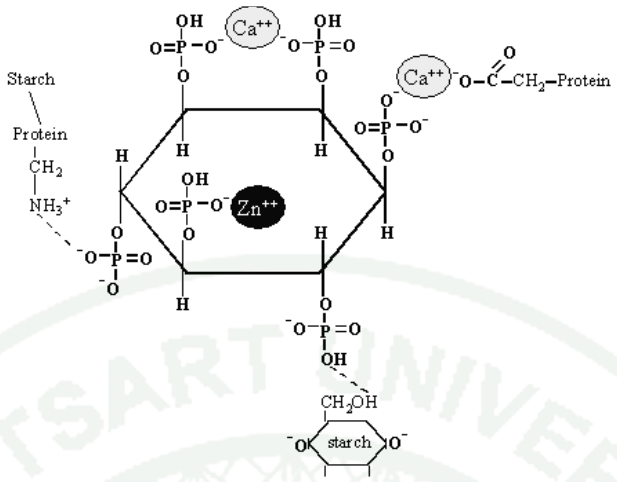



**Figure 4** Phytin or phytate molecule

**Source**: Sutton *et al.* (2004)

Phytic acid is found in plant tissues and organs, including seeds, pollens, roots and tubers (Dost and Tokul, 2006). Almost all phytic acid is presented as phytin or phytate (Figure 4), i.e. mixed salts (usually with $K^+$, $Ca^{2+}$, $Mg^{2+}$ and $Zn^+$) that are deposited as globoid crystals in single-membrane vesicles together with protein (Hatzack *et al*., 2001), especially in β-conglycinin (7S) fraction of storage proteins (Brooks and Morr, 1984).

Phytin is broken down following germination and provides *myo*-inositol, and mineral cations that are needed for seedling growth and development (Oatway *et al*., 2001). Phytic acid is found in high amount in grain cereals and legumes. In small grain cereals, 90% of the seed phytic acid is in aleurone and the remaining 10% in scutellum. In the contrary, in maize, 90% is found in scutellum and 10% in aleurone. In dicots, it is deposited in both endosperm and cotyledons (Pederson *et al*., 2002). In cereals, approximately 0.4 - 6 % by weight of the seed is phytic acid (Harland and Oberleas, 1987).

Phytic acid in plant is synthesized as depicted in Figure 5. Enzymes catalyzing critical steps in these pathways are illustrated. There are two pathways to synthesize

phytic acid, inositol (Ins) phosphate intermediate or lipid-independent pathway and phosphatidylinositol (PtdIns) phosphate intermediate or lipid-dependent pathway (Raboy, 2001 and Raboy, 2009). The early or substrate supply pathway proceeds from glucose 6-phosphate to Ins(1), Ins(3)$P_1$ and PtdIns. The late Ins polyphosphate (PP) pathways basically involve the conversion of Ins triphosphates to $InsP_6$ or phytic acid and the PP-Ins phosphates. Besides, inositol phosphates and *myo*-inositol, are the precursor of many other compounds, play a central role in several metabolic processes as well as signal transduction in the plant cell (Figure 6).

Phytic acid has a function in messenger ribonucleic acid (mRNA) export (York *et al*., 1999), signal transduction and regulation, energy transduction, adrenosine triphosphate (ATP) regeneration in rats (Safrany *et al*., 1999) and *in situ* test of wheat endosperm (Morton and Raison, 1963), a second messenger ligand (Sasakawa *et al*., 1995), DNA double-strand break repair (Hanakahi *et al*., 2000) and physiological response of guard cells to abscisic acid (ABA) (Lemtiri-Chlieh *et al*., 2000). While inositol polyphosphates play a role in controlling *PHO5* expression in the phosphate signal transduction (PHO) pathway (Auesukaree *et al*., 2005) and function in endocytosis and vesicular trafficking in yeast (Saiardi *et al*., 2002). Besides, Nelson *et al*. (1998) found that D-*myo*-inositol 3-phosphate synthase (MIPS), a first enzyme catalyzing in phytic acid synthesis pathway, is regulated in response to stress.

Phytic acid may play an important role as an anti-oxidant (Graf *et al*., 1987) by complexing iron and thereby reducing free radical generation and peroxidation of membranes (Coelho *et al*., 2002), an anti-carcinogen providing protection against colon cancer (Thompson and Zhang, 1991), an anti-inflammatory, selectin inhibitor, an energy store (Talamond *et al*., 2002) and hypocholesterolemic property (Urbano *et al*., 2000). In healthy adults, phytin produces good effects in the case of serious physical and mental fatigue and intensive sports when it is given as a prophylactic drug. Although phytic acid was not given the status of “generally recognized as safe” (GRAS) by the Food and Drug Administration of the USA in their 1995 list, it was used extensively as a food additive outside the USA (Dost and Tokul, 2006). Phytic

acid is routinely added to meats, fish meal pastes, canned seafoods, fruits, vegetables, cheese, noodle, soy sauce, juice, bread and alcoholic beverage to prevent product discoloration, increase nutritional quality and prolong shelf life. By 1997, sodium phytate was listed as a GRAS substance and has been used as a preservative for baked goods in the USA (Hix *et al*., 1997). Phytate has the potential to prevent dental calculi formation (Grases *et al*., 2009). Phytic acid can also be used in treating metals to prevent rust, as a replacement for cyanide or ammonium phosphate in etching solutions for offset printing, in toothpastes/mouthwashes, and in anti-freeze/cooling water or other closed systems for corrosion protection (Oatway *et al*., 2001).

On the other hand, phytic acid is a powerful inhibitor against the absorption of proteins and certain mineral nutrients. It is an effective chelator of positively charged divalent cations. The phytic acid-nutrient complexes are insoluble at neutral and alkaline pH of small intestine (Zhou and Erdman, 1995), and yet indigestible by humans and nonruminant livestock, such as swine, poultry and fish (Guttieri *et al*., 2004) because they do not produce adequate quantities of digestive enzyme phytase required for hydrolyzing phytate (Veum *et al*., 2002). This phenomenon can contribute to human mineral deficiencies. A subpopulation at greatest risk for mineral deficiencies caused in part by dietary phytic acid would be children and child-bearing women in rural communities in developing countries that depend on cereals and legumes as staple food (Raboy, 2002).

When phytic acid is consumed in feeds and foods, monogastric animals must excrete a large fraction of phytate salts. Yet, phytate in animal waste can contribute to water pollution, a significant problem in USA, Europe and elsewhere (Sharpley *et al*., 1994). The ultimate source of P in aquaculture effluent is feed of fish. In many freshwater systems, P is the first limiting nutrient against the growth of phytoplankton and algae, which reduce dissolved oxygen levels in aquatic ecosystems when they grow excessively (Miller *et al*., 1974).

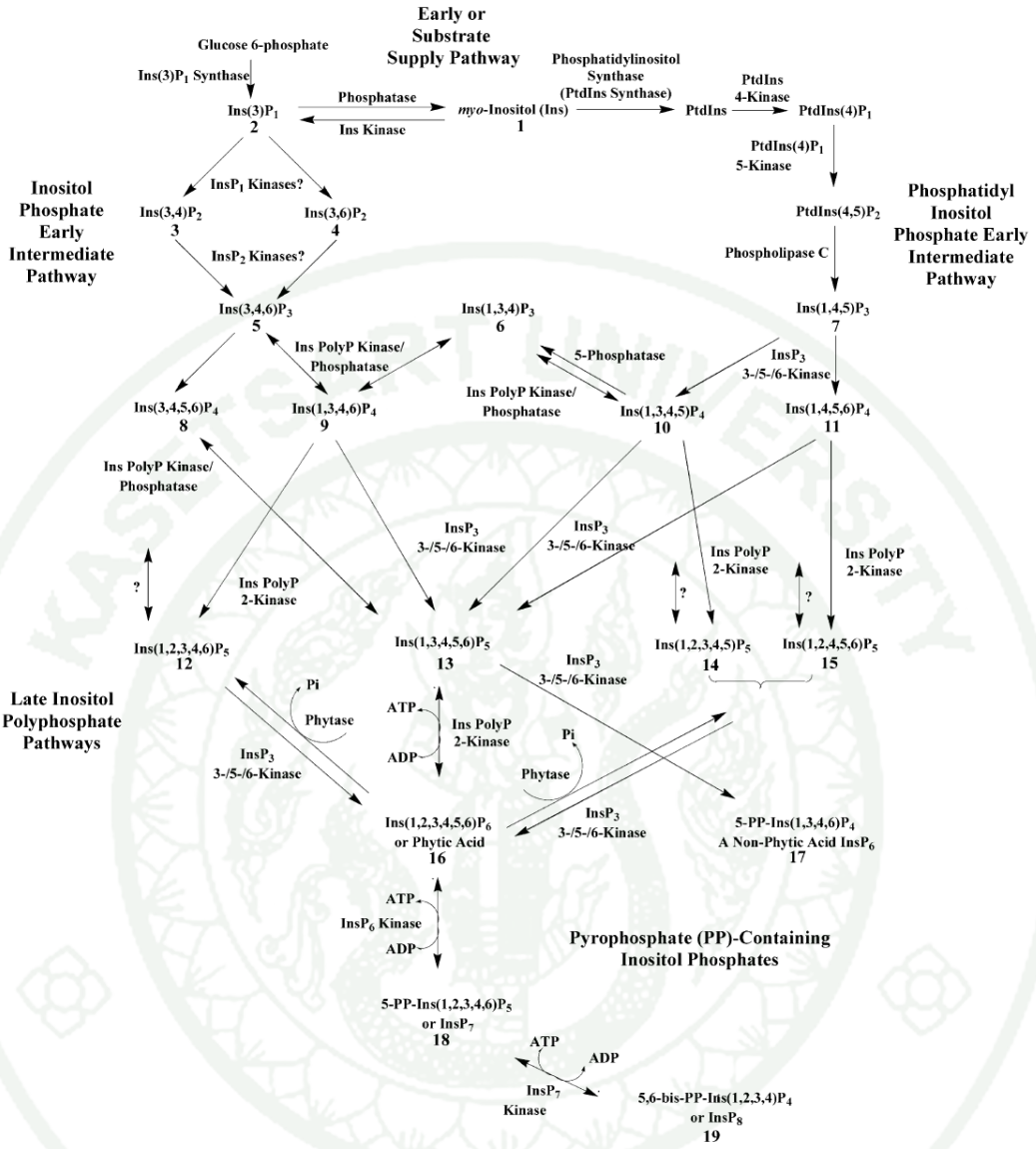



**Figure 5** Pathway of phytic acid synthesis

**Source**: Raboy (2003)

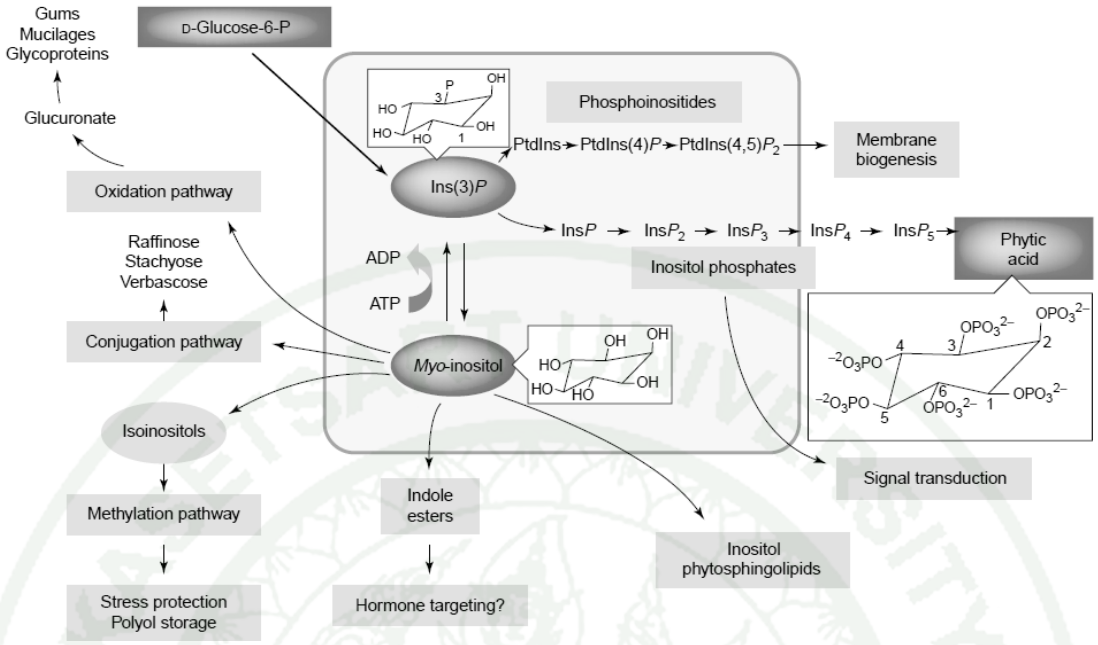



**Figure 6** Metabolism of *myo*-inositol and related compounds

**Source**: Pederson *et al*., 2002

## Mungbean

Mungbean (*Vigna radiata* (L.) Wilczek) is also known as green gram, golden gram, Oregon pea, chickasano pea, chiroko or simply mung (Akpapunam, 1996). It is synonymous with *Phaseolus aureus* Roxb. The crop originated in India and must have been derived from var. *sublobata* which occurs wild throughout India and Burma. From there, it has spread to South and East Asia, East and Central Africa, the West Indies and the USA. Mungbean group in a broad sense consists of some Asian traditional pulses, such as mungbean, azuki bean, rice bean, blackgram, moth bean, and over 10 wild species, which occur in subtropical and warm-temperate regions in Asia. Verdcourt (1970) reported that this group is homogeneous with highly specialized floral structures and thus treated as the subgenus *Ceratotropis* of the genus *Vigna*.

Mungbean is very similar to blackgram which is sometimes confusing. Some consider mungbean and blackgram to be conspecific but there are good reasons to consider the two as distinct although closely related species. Mungbean is a low altitude crop grown from sea level to approximately 2,000 m, usually as a dry land crop. It thrives best on a good loam soil with well distributed rainfall of 70-90 cm per year. It is drought resistant but susceptible to water-logging. The temperature range for optimum performance is between 20 and 45$^{o}$C. The plant itself may be short and erect in growth or suberect with a slight tendency to twine with the vine length of 1.2-1.5 m (Akpapunam, 1996). Like other food legumes, mungbean seed is a good source of proteins, vitamins, minerals and calories. The seed constitutes 55-60 % soluble carbohydrate, 3.3% crude fiber and 20-25 % protein content. Mungbean proteins are adequate in most essential amino acids with the exception of the sulphur amino acids, i.e. methionine and cystine which may be nutritionally limiting. Mungbean seed contains high level of P and K but low in Ca, Fe and Na. It also contains 4.0 mg.g$^{-1}$ of ascorbic acid and 2.5 mg.g$^{-1}$ of niacin.

Mungbean seed is an important food crop. In India, dry mungbean is boiled and eaten whole or after splitting into dhal (seed coat removed). The bean can be parched and ground into flour after removal of the testa. The flour is used in various Indian and Chinese dishes. The green pods are eaten as a vegetable. The stems, leaves, roots, pods and seeds contain high protein, even harvested at an advanced stage of maturity. They also play important roles as supplements of animal feeds. Mungbean can be grown as green manure, hay, cover crop and for forage. Mungbean meal can be mixed in feeds. Besides, it can fix $N_2$ from air to soil by *Rhizobium* bacteria in a similar manner to soybean. All parts of mungbean plant can help increasing organic N and improving soil chemical properties.

Another major use of mungbean is to be consumed as vegetable in form of mungbean sprout. Mungbean sprout is 2 - 4 days old seedling which is high in water content up to nearly 10 times of the original seed moisture. In comparison to seed, the sprout contains higher vitamin $B_1$, $B_2$ and C and free P, but lower in some minerals, phytic acid and trypsin inhibitor (Naivikul and Sotanasomboon, 1989).

**Phytic acid content in mungbean**

Chitra *et al.* (1995) analyzed phytic acid and total P contents in chickpea, pigeonpea, urd bean, mungbean and soybean and found that phytic acid content differed significantly among and within these species. Phytic acid content was the highest in soybean (36.4 $mg.g^{-1}$) followed by urd bean (13.7 $mg.g^{-1}$), pigeonpea (12.7 $mg.g^{-1}$), mungbean (12.0 $mg.g^{-1}$) and chickpea (9.6 $mg.g^{-1}$). On an average, phytic acid constituted 78.2 % of the total P content and this percentage figure was the highest in soybean and the lowest in mungbean. Naivikul and Sotanasomboon (1989) reported phytic acid content in seed (12.75 - 16.41 $mg.g^{-1}$) and seedling (7.99 - 10.76 $mg.g^{-1}$) of mungbean, while Sompong *et al*. (2007) found 5.94 - 17.87 $mg.g^{-1}$ phytic acid in seed.

**Reducing phytic acid content**

There are many ways to decrease the level of phytic acid in plants. Domestic processing and cooking methods including soaking, cooking and sprouting decreased phytic acid contents in amphidiploids (blackgram × mungbean) (Kataria *et al*., 1989). Germination reduced the phytic acid content of chickpea and pigeonpea seed by over 60% and that of mungbean, urd bean, and soybean by about 40%. Sulieman *et al*. (2007) evaluated changes during germination in phytic acid, phytase activity and extractible minerals of lentil cultivars. They found that phytic acid content decrease significantly with an increase in germination time. Fermentation reduced phytic acid content by 26-39% in legumes (Chitra *et al*., 1996). Ashton and Williams (1958) found that phytic acid P is broken down into inorganic P during the germination of oats and that no phytic acid P was present after 2 weeks of germination. Tabekhia and Luh (1980) studied the effects of germination, cooking and canning on the changes in total P, inorganic P and phytic acid retention in black-eyed, red kidney, mungbean, and pink beans. They found that soaking the dry beans in water for 12 hr at 24°C resulted in a slight decrease in phytic acid. After germination for 96 hr or longer there was a significant breakdown in phytic acid, and an increase in inorganic P. Cooking the dry beans at 100°C for 3 hr had little effect on phytic acid retention while heat

processing the dry beans with high pressure at 115.5°C for 3 hr in cans resulted in some increase in inorganic P and a reduction in phytic acid.

Adding commercial phytase extracted from fungi (*Aspergillus niger*), canola, alfafa, tobacco and *Arabidopsis* is costly. Phytase has been studied intensively in the past few years because of the great interest in using such enzymes for reducing the phytic acid content in animal feed and food for human consumption. High native phytase activities are present in microorganism, especially fungi, whereas lower activities have been reported for legume seeds (Viveros *et al*., 2000). Plant-expressed *PhyA* apoplastic phytase has been developed as a patented feed supplement in canola seeds (Phytaseed) and in alfalfa. Raghavendra and Halami (2009) screened and selected potent degradating lactic acid bacteria and found that two *Pediococcus pentosaceus* strains, CFR R38 and CFR R35, can degrade sodium phytate.

A transgenic strategy to seed production of active phytase enzyme is to aim for a direct reduction of phytic acid in feed grains. This approach can target phytic acid synthesis as in the RNAi knockdown of *Gm*MIPS*1* or the ABC (ATP-binding cassette) transporter (Nunes *et al*., 2006). Pen *et al.* (1993) demonstrated that transgenic tobacco seeds could be a source of *PhyA* phytase. Another popular transgenic strategy is to direct expression of a phytase during embryo development at the site of phytic acid synthesis or storage (Bilyeu *et al*., 2008).

Breeding of low phytic acid (*lpa*) crops has recently been considered as a potential way to increase nutritional quality of crop products. Most of them have been *lpa* mutants identified via phenotypic screening of mutagenized plants. However, a little research has been studied on phytic acid level in germplasm. Thavarajah *et al*. (2009) analyzed the phytic acid concentration in seeds of 19 lentil (*Lens culinaris* L.) genotypes grown at two locations for two years in Saskatchewan, Canada. The lentils were naturally low in phytic acid (phytic acid = 2.5 - 4.4 $mg.g^{-1}$; phytic acid P = 0.7 - 1.2 $mg.g^{-1}$), with concentrations lower than those reported for low phytic acid mutants of corn, wheat, common bean and soybean.

**Genetics and breeding of phytic acid and P contents in plants**

Analysis of natural variation for phytic acid and total P accumulation in seeds and leaves in a large number of accessions of *Arabidopsis thaliana*, using a novel method for phytic acid detection, revealed a wide range of variation in phytic acid varing from 7.0 mg to 23.1 $mg.g^{-1}$of seed. QTL analysis of phytic acid and total P levels in seeds and leaves, using RILs population revealed five genomic regions affecting the quantity of phytic acid and total P. One of them appears as a major locus in *Ler/Cvi* RIL population and was further fine-mapped to a 99-kb region, containing 13 open reading frames. The maternal inheritance of the QTL and the positive correlation between phytic acid and total P both in seeds and leaves were found (Bentsink *et al.*, 2003).

Zhao *et al*. (2008) studied in *Brassica rapa* using 5 segregating populations, involving 8 parental accessions representing different cultivar groups. They detected two QTL for seed phytic acid, two for seed phosphate, one for leaf phosphate and one major QTL for leaf phytic acid. Co-localization of QTL suggested single or linked loci involving in accumulation of phytic acid or phosphate in seeds or leaves.

The rice low phytic acid mutation, *lpa1,* results in 45% reduction in seed phytic acid with a molar equivalent increase in inorganic P by the result of a single recessive mutation. The *lpa1* locus was mapped using microsattelite or simple sequence repeat (SSR) markers in an RIL population. Fine mapping encompasses a region of 135 kb (Andaya and Tai, 2005). Transgressive segragation was found for phytic acid and total P, which had the same QTL and a significant positive correlation was detected between them (Stangoulis *et al*., 2006). Larson *et al*. (2000) reported that *lpa1* gene in non-lethal rice *low phytic acid 1* mutant is distinguishable from *MIPS*. Besides, at least four *lpa* mutations were identified mutually non-allelic from studying of 8 independent *lpa* rice mutant lines from both *indica* and *japonica* subspecies (Liu *et al*., 2007). There was a research on reducing phytic acid levels in rice seeds by manipulating the expression of the rice Ins(3)$P_1$ synthase gene *RINO1* using transgenic methods. *RINO1* cDNA was transformed into rice plants in the

antisense orientation under the control of the rice major storage protein glutelin GluB-1 promotor (Kuwano *et al*., 2006).

In Maize, three types of low phytic acid mutation, *lpa1*, *lpa2* and *lpa3*, were identified and characterized. The *lpa1* mutant caused a decrease in phytic acid P content, accompanied by mole equivalent increase of inorganic P. Pilu *et al*. (2005) hypothesized that *lpa1* affects the first committed step in phytic acid synthesis pathway related to *MIPS*. The locus *lpa1-1* causes a 55% to 65% decrease in whole grain phytic acid level, but an increase in level of inorganic P. Although Raboy *et al*. (2000) proposed that *lpa1-1* is a mutation in the first part of the phytic acid synthesis pathway. It is now known to be a mutation in one of the multidrug resistance-associated protein (MRP) genes, *ZmMRP3*, a gene encoding an ABC transporter (Shi *et al*., 2007). The *lpa2* mutant results in the reduction of phytic acid P, but is complemented by an increase in both inorganic P and the lower inositol phosphates, and may involve in inositol phosphate kinase gene (Shi *et al*., 2003). This supported the hypothesis of Raboy *et al*. (2000) that *lpa2-1* is a mutation in the later part of the phytic acid synthesis pathway. However, Lin *et al*. (2005) reported that *lpa2-1* showed a 50% decrease in phytic acid level with less inositol phosphates, but an increase in inorganic P. The *lpa3* mutant, on the other hand involves in the *myo*-inositol kinase (*MIK*) gene which is characterized by an increase in *myo*-inositol levels and a lack of significant amounts of other inositol phosphates in seed (Shi *et al*. 2005). Pilu *et al*. (2003) isolated a viable recessive mutant *lpa241* showing about 90% reduction in phytic acid and ten-fold increase in inorganic P content. Pilu *et al*. (2005) hypothesized the occurence of *lpa241* mutation in three possible ways: (i) the mutation is located in *MIPS1S* promoter, (ii) the mutation is in a closely linked gene controlling the *MIPS1S* expression level, and (iii) the mutation is epigenetic, i.e. phenotype changed by an alteration of methylation pattern and/or chromatin conformation of the genome region in which *MIPS1S* is located.

The wheat low phytic acid mutant, JS-12-LPA expressed less phytic acid concentration in bran by 43%, but more inorganic P concentration by nearly fourfold. The inheritance data of $F_2$ and $F_{4:6}$ families were inconsistent with a single-gene

mutation, suggested that there were two or more genes controlling the trait (Guttieri *et al.*, 2004). However, wheat grain contains higher phytase activity compared to other major crops such as rice, maize, and soybean, and the phytase presenting in wheat flour can be activated during fermentation, and lead to a reduction of phytic acid content by between 13% and 100% (Viveros *et al*., 2000).

In Barley, Dorsch *et al*. (2003) reported 4 non-lethal *low phytic acid* mutations, M 635, M 955 and carrying the alleles *lpa1-1* and *lpa2-1*. In seed of the homozygous genotypes for M 635 and M 955, phytic acid levels decrease about 75 % and 95%, respectively, while the lower inositol phosphates diminished, and inorganic P increase. The *lpa1-1* caused 45% reduction in phytic acid accompanied by increasing of inorganic P and inositol penta*kis*phosphate ($InsP_5$). For the barley lines carrying *lpa2-1*, seed phytic acid decreases but inorganic P and the lower inositol phosphates increase. Moreover, Hu *et al*. (2008) found that 24 *lpa* mutants represent alleles at a minimum of 6 *lpa* loci in a screening of high seed inorganic P in $M_2$ line.

Ashton and Williams (1958) found no phytate in maturing oats during panicle emergence and up to the milk- ripe stage. However, at maturity approximately 60% of the P was in the form of phytate. They also reported significant difference in phytic acid levels as influenced by oat variety. Hall and Hodges (1966) were tracking P metabolism during the germination of oats and reported that during the first eight days of germination phytic acid P was converted entirely into inorganic P and appeared in the roots and shoots.

Numerous rapeseed products were shown to have relatively high levels of phytic acid. Rapeseed meal contains approximately 3-5% (Uppstrom and Svensson, 1980). Sesame seed contains the highest levels of phytic acid (5.18%) found in nature (de Boland *et al*., 1975). Saio *et al.* (1977) found that certain isolated protein bodies from sunflower seed were rich in P, which probably represented phytic acid storage sites.

Oltmans *et al*. (2005) found that phytic acid P in soybean lines homozygous for the recessive alleles *pha1* and *pha2* is reduced to about 25% of total P and with significantly lower mean seed yield than the normal-phytic acid lines in all populations. Moreover, Oltmans *et al*. (2004) discovered an epistatic interaction between the loci. A more detail study indicated that the *LR33* mutation of soybean is a change in the third base of the codon for amino acid residue 396, which decreases the specific activity of the seed-expressed *myo*-inositol 1-phosphate synthase by about 90% (Hitz *et al*., 2002). Yuan *et al*. (2007) isolated 2 *lpa* mutations, *Gm-lpa*-TW-1 had a phytic acid P reduction of 66.6% and a six-fold increase in inorganic P, and *Gm-lpa*-ZC-2 had a phytic acid P reduction of 46.3% and a 1.4-fold increase in inorganic P. The two *lpa* mutations were both inherited in a single recessive gene model but were non-allelic. Walker *et al*. (2006) mapped *lpa* loci in populations from a *lpa* mutant line, identified two loci on linkage group N and L associated with phytic acid and discovered an epistatic interaction between loci.

However, Dieckert *et al*. (1962) isolated one protein-rich fraction of peanuts that contained 0.5% phytic acid while an aleurone grain-containing protein-rich fraction contained 5.7 %. Chen and Pan (1977) found between 50 and 60% of the phytic acid in peas (*Pisum sativum*) was still present at five days after germination. Campion *et al*. (2009) isolated a homozygous *lpa* common bean line, *lpa*-280-10, from a mutagenised population. This line showed a 90% reduction of phytic acid and the *lpa* character is due to a recessive mutation segregating in a monogenic manner. Blair *et al*. (2009) evaluated QTL for seed P and phytic acid content in an inter-gene pool RIL population of common bean planted at medium and high level of soil P and found three QTLs for percentage or net seed phytic acid. Sompong *et al*. (2010) identified low and high seed phytic acid mungbean germplasms, V1658BBR and V1141BG. The high phytic acid P was controlled by dominant alleles at two independent loci of major genes showing duplicated recessive epistasis.

## Molecular breeding

Many commercial breeding companies and research institutes have used molecular (DNA) markers to increase the effectiveness of selection in breeding and to shorten the time required in development of new varieties. The potential of using genetic markers, linkage maps and indirect selection in plant breeding have been known for more than 80 years. However, it was not until the advent of DNA marker technology in 1980s (Peleman and Voort, 2003) that a large number of environmentally insensitive genetic markers could be adequately generated to follow the inheritance of important agronomic traits.

Many molecular markers are now available, each with different advantages and disadvantages. RFLP, restriction fragment length polymorphism, is the first widely used molecular marker. Its assays are performed by hybridizing a chemically or radioactively labeled DNA probe with transferred DNA fragment cutting from restriction enzyme. RAPD, random amplification of polymorphic DNA, is a PCR-based technique employing an oligonucleotide primer to amplify genomic fragments. AFLP, amplified fragment length polymorphism, is based on a combination of restriction digestion and PCR amplification. SSR is tandemly repeated mono-, di-, tri, tetra-, penta-, and hexa-nucleotide motif. It is based on the PCR amplification of tandem repeats using unique flanking DNA sequences as oligonucleotide primers. Polymorphism is caused by variation in number of repetitive units. The SSR markers are reproducible, easy, co-dominant, and do not require so pure DNA templates.

Most of quantitative traits in crop plants are controlled by polygenes. These gene loci are described as quantitative trait loci (QTL) which can be detected with molecular marker methods. The information need for analyzing QTL is phenotypic data of the trait of interest in the segregating population. The routine analytical steps require normality test of the phenotypic distribution, genotypic data and construction of a linkage map. To construct a linkage map, polymorphic molecular markers are scored on the random segregating populations. The distance and order of the markers

are determined on the basis of the frequency of genetic recombination between the markers in the population.

**Molecular breeding in mungbean**

Not many genetic markers were developed specifically for mungbean. Recently, SSR markers, a marker system of choice, have been developed from mungbean genomic DNA. The number of these SSRs is still very limited. However, SSRs from azuki bean, common bean and cowpea can be used in mungbean (Somta and Srinives, 2007). The most comprehensive mungbean linkage map consists of 255 loci with an average distance between adjacent markers of 3 cM, but no map has resolved 11 linkage groups, which is the haploid chromosome number of mungbean (Somta and Srinives, 2007). Quantitative trait loci (QTLs) for five important traits, including bruchid resistance, powdery mildew resistance, mungbean yellow mosaic virus (MYMV) resistance, seed weight and hardseededness, were mapped with molecular markers in mungbean (Somta and Srinives, 2007).

# MATERAILS AND METHODS

## Materials

### Materials for planting and harvesting

1. mungbean seeds
2. tags
3. paper bags
4. plastic bags
5. irrigation water
6. fertilizers
7. insecticides, fungicides and herbicides
8. cutters
9. bamboo
10. sticks

### Materials for crossing

1. forceps
2. tags
3. male and female flowers
4. ethanol ($C_2H_5OH$)

### Materials for collecting agronomic traits

1. balance (Mettler AE240)
2. vernier caliper
3. rulers

**Materials for germination**

1. mungbean seeds
2. trays
3. tissue papers
4. cutters
5. deionized water ($dH_2O$)

**Materials for sample preparation for P compound analysis**

1. hot air oven
2. blender (RT-02A)
3. 40-mesh sieve
4. desiccator

**Materials for phytic acid analysis**

1. equipment

1.1 centrifuge (5415 Eppendorf Centrifuge and Sorvall® RC-5B Refrigerated Superspeed Centrifuge)

1.2 microtitre plate reader (Dynex Technology MRXII)
1.3 refrigerator (Fisher Scientific Isotemp)
1.4 vortex (Vortex-2 genie VWR Scientific)
1.5 balance (Mettler AE240)
1.6 hot water bath (Listed UL® 543H)
1.7 shaker (Bello Biotechnology)
1.8 500- and 1000-ml volumetric flasks
1.9 500- and 1000-ml cylinders
1.10 1000-µl micropipette
1.11 50-ml beakers

1.12 1.5-ml microtubes and racks

1.13 microtitre plates

1.14 15-ml capped-conical tubes

1.15 parafilms

2. Chemicals

2.1 hydrochloric acid (HCl)

2.2 ammonium iron (III) sulfate ($FeNH_4(SO_4)_2.12H_2O$)

2.3 2,2' bipyridine ($(C_5H_4N)_2$)

2.4 thioglycolic acid ($HSCH_2COOH$)

2.5 sodium phytate ($C_6H_6Na_{12}O_{24}P_6$)

2.6 sodium sulfate ($Na_2SO_4$)

**Materials for inorganic P analysis**

1. equipment

1.1 microtitre plate reader (Dynex Technology MRXII)

1.2 centrifuge (5415 Eppendorf Centrifuge)

1.3 refrigerator (Fisher Scientific Isotemp)

1.4 vortex (Vortex-2 Genie VWR Scientific)

1.5 balance (Mettler AE240)

1.6 shaker (Bello Biotechnology)

1.7 200- and 1000-µl micropipettes

1.8 500- and 1000-ml cylinders

1.9 500-ml volumetric flasks

1.10 50-ml beakers

1.11 1.5-ml microtubes and racks

1.12 microtitre plates

2. Chemicals

2.1 trichloroacetic acid (TCA)
2.2 magnesium chloride ($MgCl_2$)
2.3 sulfuric acid ($H_2SO_4$)
2.4 ammonium molypdate ($(NH_4)_6Mo_7O_{24}.4H_2O$)
2.5 ascorbic acid (vitamin C)
2.6 potassium dihydrogen monophosphate ($KH_2PO_4$)
2.7 $dH_2O$

**Materials for total P analysis**

1. equipment

1.1 microtitre plate reader (Dynex Technology MRXII)
1.2 heating block (Thermo Electron Corporation)
1.3 balance (Mettler AE240)
1.4 15-ml tubes (Pyrex)
1.5 200- and 1000-µl micropipettes
1.6 microtitre plates
1.7 500- and 1000-ml cylinders
1.8 50-ml beakers
1.9 500- and 1000-ml volumetric flasks

2. Chemicals

2.1 sulfuric acid ($H_2SO_4$)
2.2 hydrogen peroxide ($H_2O_2$)
2.3 ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$)
2.4 ascorbic acid (vitamin C)
2.5 sodium hydroxide (NaOH)
2.6 $dH_2O$

2.7 potassium dihydrogen monophosphate ($KH_2PO_4$)

**Materials for DNA extraction**

1. equipment

1.1 autoclave (Sanyo MLS-3780)
1.2 -20$^{o}$C freezer (Sanyo)
1.3 water bath (Listed UL® 543H)
1.4 centrifuge (Universal 32R Hettich Zentrifugen)
1.5 incubator (Memmert)
1.6 1.5-ml microtubes and racks
1.7 mortars and pestles
1.8 spatulas
1.9 2-, 20-, 200- and 1000-µl micropipette
1.10 100-ml cylinders
1.11 500-ml volumetric flasks
1.12 50-ml screwed-cap tubes
1.13 ice boxes
1.14 1000-ml beakers
1.15 toothpicks

2. chemicals

2.1 2-amino-2-hydroxymethyl-propane-1,3-diol hydroxymethyl-aminomethane (Tris-base)

2.2 liquid nitrogen
2.3 sodium chloride (NaCl)
2.4 $C_2H_5OH$
2.5 RNAse A
2.6 polyvinylpyrrolidone (PVP)

2.7 Fe-ethylene diaminetetraacetic acid (Fe-EDTA)

2.8 chloroform ($CHCl_3$)

2.9 cetyl trimethyl ammonium bromide (CTAB)

2.10 isopropanol ($C_3H_7OH$)

2.11 $dH_2O$

2.12 β-mercaptoethanol ($HSC_2H_4OH$)

2.13 isoamyl alcohol ($(CH_3)_2CHC_2H_4OH$)

**Materials for DNA concentration checking**

1. equipment

1.1 agarose gel electrophoresis apparatus (Gelmate 2000)

1.2 balance (Mettler AE240)

1.3 microwave (Empire)

1.4 Gel Documentation (Vilber Lourmate TCX-20)

1.5 2-, 20-, and 200-µl micropipettes

1.6 parafilms

1.7 1.5-ml microtubes and racks

1.8 1000-ml beakers

1.9 1000-ml volumetric flasks

1.10 250-ml flasks

2. chemicals

2.1 agarose

2.2 Tris-base

2.3 boric acid ($B(OH)_3$)

2.4 Fe-EDTA

2.5 $dH_2O$

2.6 ethydium bromide (EtBr)

2.7 5-, 10-, 50-, 100- and 200-ng λ DNA markers

2.8 6x loading dye (0.25% bromophenol blue and 30% glycerol)

**Materials for PCR**

1. equipment

1.1 thermocycler (Gene Amp® PCR system 9700)
1.2 centrifuge (Universal 32R Hettich Zentrifugen)
1.3 -20$^{o}$C freezer (Sanyo)
1.4 2-, 20-, 200- and 1000-µl micropipette
1.5 PCR plates
1.6 ice boxes

2. chemicals

2.1 DNA extracts
2.2 SSR primers
2.3 dNTP
2.4 ultrapure water
2.5 $MgCl_2$
2.6 *Tag* DNA polymerase
2.7 ammonium sulfate ($(NH_4)_2SO_4$) buffer

**Materials for acrylamide gel electrophoresis**

1. equipment

1.1 acrylamide gel electrophoresis apparatus (Biorad Sequi-Gen®GT)
1.2 centrifuge (Universal 32R Hettich Zentrifugen)
1.3 magnetic bars and stirrers (AGE VELP®Scientifica)
1.4 shaker (Nuve SL350)
1.5 1000-ml cylinders

1.6 2500- and 5000-ml beakers

1.7 10-µl multichannel pipettes

1.8 basins

2. chemicals

2.1 $C_2H_5OH$

2.2 acetic acid ($CH_3COOH$)

2.3 clearview solution

2.4 acrylamide solution

2.5 urea ($(NH_2)_2CO$)

2.6 silver nitrate ($AgNO_3$)

2.7 Tris-base

2.8 $B(OH)_3$

2.9 tetramethyl ethylenediamine (TEMED)

2.10 Fe-EDTA

2.11 formaldehyde (HCHO)

2.12 ammonium persulfate ($(NH_4)_2(SO_4)_2$ or APS)

2.13 sodium carbonate ($Na_2CO_3$)

2.14 sodium thiosulfate ($Na_2S_2O_3$)

2.15 $dH_2O$

2.16 Φ174 DNA marker

2.17 sequencing dye (98 ml 98% formamide, 2 ml 10 mM Fe-EDTA, 0.02% bromophenol and 0.02% xylene cyanol)

## Methods

### Part I: Identification of germplasm with high and low seed phytic acid

Eleven cultivated and 11 wild mungbean accessions (Table 1) were used in this study. The cultivated ones were originated from major growing countries and deposited at AVRDC - The World Vegetable Center, Taiwan. Five of them were

previously identified as high and five were low in total P content (Sompong *et al.*, 2007), while Sukhothai may be suitable for developing a QTL population because it is highly different in manner from wild accession. All wild accessions are of Australian origin. All 22 accessions were grown in growth chambers in a glass house of Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand in a completely randomized design (CRD). Seed phytic acid content was assessed by modified colorimetric method from Haug and Lantzsch (1983). Firstly, mature seeds were dried for 72 hr at 60$^{o}$C, milled to pass though a 40 mesh (0.5 mm) screen, and stored in desiccators until analysis. Then, 0.05 g of ground seed was placed into a 1.5-ml microtube, to which 1 ml of 0.2 N HCl with 10% $Na_2SO_4$ were added. The solution was shaken mechanically at 4 $^{o}$C overnight, centrifuged at 10,000g for 15 min, and the supernatant was decanted. Each 250 µl extract was put into a 15-ml conical tube with 2.25 ml 0.2 N HCl with 10% $Na_2SO_4$ and 5 ml ferric solution (prepared by putting 0.2 g of $FeNH_4(SO_4)_2.12H_2O$ in 0.2N HCl with 10% $Na_2SO_4$), boiled for 30 min and cooled down to room temperature around 15 min. The tubes were closed with caps and centrifuged at 10,000g for 10 min. The supernatant was decanted, pipetted 100 µl into a microtitre plate and developed into a red solution by 150 µl HL solution. The solution was prepared by putting 1 g of 2,2' bipyridine with 1 ml of thioglycolic acid and adjust volume to 100 ml by $dH_2O$. The absorption of the solution was measured by a microtitre plate reader at 510 nm and the indirect phytic acid concentration was calculated using a standard curve of $C_6H_6Na_{12}O_{24}P_6$ at 0, 6.25, 12.5, 25, 50 and 75 µg P. Thus, phytic acid P in $mg.g^{-1}$ was calculated using the formula

$$PAP = \frac{0.001CV_e}{WV_S}$$

and $PA = 3.552PAP$.

Where PAP is phytic acid P, PA is phytic acid, C is phytic acid P concentration (µg/100 µl) from the standard curve, $V_e$ is the extracted volume (ml), W is the sample weight (g), and $V_s$ is the sample volume (µl).

**Table 1** Mungbean germplasm used in this study

| Cultivated accessions | Wild accessions |
|---|---|
| V1137BG | AusTRCF085148 |
| V1141BG | AusTRCF321924 |
| V1158AG | AusTRCF321925 |
| V1658BBR | AusTRCF321927 |
| V1687ABLM | AusTRCF321936 |
| V1722BG | AusTRCF321995 |
| V1724AG | AusTRCF322000 |
| V1725BG | AusTRCF322011 |
| V3092AG | AusTRCF322012 |
| V3251ABLM | AusTRCF322013 |
| Sukhothai | AusTRCF322015 |

**Part II: Estimation of narrow-sense heritability of P compound contents in seed**

Four $F_3$ populations were derived from crosses between two high and two low seed phytic acid accessions, V1725BG, Sukhothai, AusTRCF321925 and AusTRCF322012, respectively by selfing or full-sib mating in nested design following Figure 7. Two hundred $F_3$ plants from 40 $F_2$ families of four crosses were sown in a field of Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand in a completely randomized design (CRD) each plot had two rows of 5.0 m long with 75 cm between rows and 50 cm between hills.Three seeds were sown in each hill and the seedlings were thinned to one plant/hill at 7-14 days after germination.

Available P in the soil was taken a random and analyzed by Bray II method (Fixen and Grove, 1990). Sample preparation and phytic acid P determination were the same as the previous experiment. Total P and inorganic P contents were assessed by colorimetric method modified from Chen *et al*. (1956). For total P determination, 0.05 g of ground seed was put into a 15-ml tube, filled with 1 ml of concentrated

$H_2SO_4$, and digested in a heating block at around 200 $^oC$. A few drops of $H_2O_2$ were added every minute until the solution is clear without ash and colorless. The solution was cooled down to room temperature for 15 min and made up volume to 6.25 ml by $dH_2O$. Ten microliters of the extracts were pipetted into microtitre plates with 10 µl of 5N NaOH, 80 µl of $dH_2O$ and 100 µl of Chen's reagent (prepare from 1:1:1:2 of 6N $H_2SO_4$: 0.02 M $(NH_4)_6Mo_7O_{24}.4H_2O$: 10% vitamin C: $dH_2O$) at room temperature for 1.5 hr. Finally, the absorption of the blue solution was determined by a microtitre plate reader at 810 nm and total P content was calculated using the standard curve of $KH_2PO_4$ at 0, 155, 465, 930 and 1395 ng P. Total P in $mg.g^{-1}$ was calculated using the formula

$$TP = \frac{0.000001CV_e}{WV_s}.$$

Where TP is total P, C is total P concentration (ng/10 µl) from the standard curve, $V_e$ is the extracted volume (ml), W is the sample weight (g), and $V_s$ is the sample volume (µl).

For inorganic P, 0.05 g of ground seed was placed into a 1.5-ml microtube, to which 1 ml of extraction buffer prepared by putting 12.5 g of TCA and 0.238 g of $MgCl_2$ in 100 ml of $dH_2O$ was added. It was shaken overnight at 4 $^oC$, centrifuged at 10,000g for 15 min, and the supernatant was decanted. Ten microliter of extract was mixed with 90 µl of $dH_2O$ and 100 µl of Chen's reagent at room temperature for 1.5 hr. Finally, the absorption of the blue solution at 810 nm was determined by a spectrophotometer and inorganic P content was calculated using the standard curve of $KH_2PO_4$ at 0, 155, 465, 930 and 1395 ng P. Inorganic P in $mg.g^{-1}$ was calculated using the formula

$$IP = \frac{0.000001CV_e}{WV_s}.$$

Where IP is inorganic P, C is total P concentration (ng/10 µl) from the standard curve, $V_e$ is the extracted volume (ml), W is the sample weight (g), and $V_s$ is the sample volume (µl)**.**

The data were analyzed for variation according to sources (ANOVA) following Table 2 and narrow-sense heritability ($h_n^2$) of seed phytic acid was calculated from the formula

$$h_n^2 = V_A/(V_A+V_E).$$

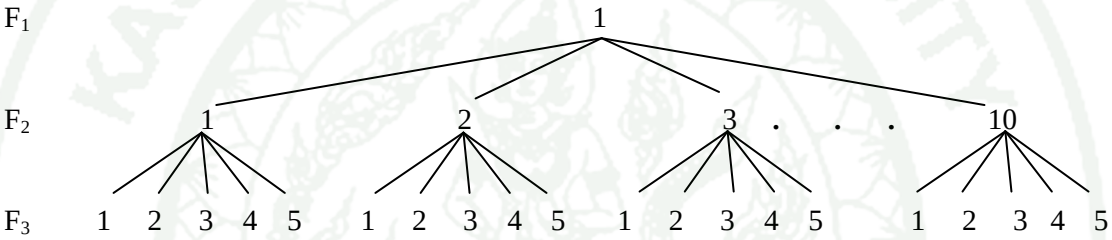



**Figure 7** Hierarchical structure of $F_3/F_2$ families in inbreeding series

**Table 2** Skeleton ANOVA for the analysis of $F_3/F_2$ families

| Source | *df* | *MS* | ems |
|---|---|---|---|
| Between $F_2$ families | n-1 | $MS_B$ | $\sigma^2_W + r\sigma^2_B$ |
| Between $F_3$'s within $F_2$ families | n(r-1) | $MS_W$ | $\sigma^2_w$ |

*df* = degrees of freedom, n = number of $F_2$ families, r = number of $F_3$ plants per $F_2$ families, MS = mean square, $MS_B$ = between $F_2$ families mean square, $MS_W$ = within $F_2$ families mean square, ems = expected mean square, $\sigma^2_B$ = between $F_2$ families variance component, $\sigma^2_W$ = within $F_2$ families variance component

Source: Kearsey and Pooni (1996)

The estimates of $\sigma^2_B$ and $\sigma^2_W$ are

$$\sigma^2_B = V_A + \frac{1}{4} V_D + V_{EC}$$

and $\sigma^2_W = \frac{1}{2} V_A + \frac{1}{2} V_D + V_E$ (Kearsey and Pooni, 1996).

Where $V_A$ is the additive variance component, $V_D$ is the dominant variance component, $V_E$ is the environmental variance component and $V_{EC}$ is the common environmental variance component. It is impossible to estimate four parameters, $V_A$, $V_D$, $V_{EC}$ and $V_E$ with two statistics, $\sigma^2_B$ and $\sigma^2_W$, so two parameters need to be ignored. $V_{EC}$ will be set to zero, while $V_D$ can also be ignored because the contribution of dominance effect in breeding of self-pollinating plants is generally small. Even though there is a complete dominance, Kearsey and Pooni (1996) prove that the contribution of $V_A$ is still eight-fold higher than $V_D$. Therefore, ignoring dominance will cause only little bias to the estimates. So, the estimates of $\sigma^2_B$ and $\sigma^2_W$ are

$$\sigma^2_B = V_A$$

and $\sigma^2_W = \frac{1}{2} V_A + V_E$.

Based on the ANOVA table (Table 2), $\sigma^2_B$ and $\sigma^2_W$ can be calculated from mean squares as follow:

$$\sigma^2_B = (MS_B - MS_W)/r$$

and $\sigma^2_W = MS_W$.

Therefore, $V_A$ and $V_E$ can be calculated from equations,

$$V_A = (MS_B - MS_W)/r$$

and $V_E = MS_W - \frac{1}{2r}(MS_B - MS_W)$.

Frequency distribution of each P compound content was plotted based on the number of classes as determined from the formula quoted by LeClerg *et al*. (1962) as follows:

$$N_C = 2.5\sqrt[4]{N_S}\ .$$

Where $N_C$ is the number of classes and $N_S$ is the number of $F_3$ plants.

**Part III: Changes in P compound contents during germination**

Seeds of AusTRCF322012, AusTRCF321925, V1725BG and Sukhothai were placed on wet tissue papers and watered daily. Germinating seeds were collected at 12, 24, 48, and 72 hr and kept frozen at -20$^{o}$C. The design of the experiment was a CRD with factorial treatments (4 accessions × 5 seedling ages). Samples were prepared to assess total P and inorganic P contents by colorimetric method modified from Chen *et al*. (1956), and assess phytic acid P content by modified colorimetric method from Haug and Lantzsch (1983) as the two previous experiments.

**Part IV: QTL analysis of P compound contents and major agronomic traits**

1. Genotypic analysis

An $F_2$ population was developed from a cross between a high (V1725BG, $P_1$) and a low (AusTRCF321925, $P_2$) phytic acid mungbean accessions. One $F_1$ plant was self-pollinated and 170 $F_2$ plants were used for the analysis. The population was grown in a glass house of Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand and genotyped with SSR markers.

1.1 DNA extraction

DNA of young leaves of the parents, $F_1$, and individual $F_2$ plants were extracted by using the CTAB method (Lodhi *et al.*, 1994) with slight modification. Approximately 0.1 g of the leaves was ground with liquid nitrogen in a mortar, put into 1.5-ml microtubes and kept in -80 $^{o}$C freezer until extraction. Seven hundred microliters of extraction buffer (100 mM Tris-HCl pH 8.0, 20 mM Fe-EDTA pH 8.0, 1.4 M NaCl, 3% CTAB, 2% PVP and 2 % β-mercaptoethanol) was put in the microtubes and incubated in 65$^{o}$C water bath for 30 min. Then, 700 µl of 24:1 of chloroform: isoamyl alcohol was added, inverted and centrifuged at 12,000g room temperature for 30 min. The upper layer liquid was decanted, added with 500 µl of the chloroform: isoamyl alcohol and centrifuged at 12,000g room temperature for 30 min. The upper layer liquid was decanted, added with 250 µl of 5 N NaCl and 500 µl of isopropanol, incubated at -20$^{o}$C for at least 20 min, and centrifuged at 12,000g room temperature for 15 min. The precipitate was washed for 3 times with 1 ml of 70 % ethanol and centrifuged at 12,000g room temperature for 2 min. The DNA precipitate was dried at 37$^{o}$C for 2 hr in a hot air oven, dissolved with 40 µl of TE buffer composed of 10 mM Tris-HCl pH 8.0 and 1 mM Fe-EDTA, and incubated with 20 µl of 1 mg.ml$^{-1}$ RNAse A at 37$^{o}$C for 30 min. The DNA extract was kept at 4$^{o}$C until used.

1.2 Evaluation of DNA concentration

DNA concentration was estimated by agarose gel electrophoresis. Firstly, 50 ml of 0.8 % agarose in 1x TBE buffer, diluted from 10x TBE buffer (108 g of Tris-base, 55 g of boric acid, 7.4 g of Fe-EDTA and adjust volume to 1,000 ml by $dH_2O$) was poured into trays of agarose gel electrophoresis apparatus. It was waited to set for 30 min. One microliter of DNA extract and different amount of λ DNA marker, such as 5, 10, 25, 50, 100 and 200 ng, were loaded into the gel. The gel was run at 100V for 30 min and stained with EtBr solution (a few drops of concentrated EtBr in $dH_2O$) for 5 min and washed with $dH_2O$ for a few seconds. The stained gel was visualized under UV light in a Gel Documentation and the DNA concentration

was estimated by comparing with the intensity of DNA markers. The DNA extracts were diluted to 1 ng/µl and kept until used.

1.3 Polymerase chain reaction (PCR)

A total of 991 SSR primers were screened for polymorphism between the parental DNA. They were designed from mungbean (Gwag *et al.* 2006; Somta *et al.*, 2008, 2009; Seehalak *et al.*, 2009; Thangphatsornruang *et al.*, 2009), azuki bean (Wang *et al.*, 2004a), cowpea (Li *et al.*, 2001), and common bean (*Phaseolus vulgaris* L.) (Gaitán-Solís *et al.*, 2002; Guerra-Sanz 2004; Buso *et al.*, 2006; Blair *et al.*, 2009; Benchimol *et al.*, 2007). Only 125 polymorphic primers were used in the population. The PCR was carried out following Somta *et al.* (2008). The composition of the reaction was pipetted to 96-well PCR plate and put in a thermocycler. In each total volume of 10 µl reaction, the mixture was composed of 2 µl of 1 ng. $\mu l^{-1}$ DNA template, 2 µl of 2.5 µM SSR primer, 2 µl of 1 mM dNTP, 2 µl of $dH_2O$, 1 µl of $NH_2SO_4$ buffer, 0.8 µl of 25 mM $MgCl_2$ and 0.2 µl of *Tag* DNA polymerase. The PCR conditions began from pre-denaturation at 94 $^oC$ for 2 min, denaturation at 94 $^oC$ for 30 min, annealing at 47-65 $^oC$ depending on base composition of each SSR primers for 30 sec, extension at 72 $^oC$ for 1 min, reprocessing from denaturation to extension for 35 cycles and post-extension at 72 $^oC$ for 10 min.

1.4 Acrylamide gel electrophoresis

An acrylamide gel electrophoresis apparatus was prepared by cleaning and assembling a glass and a chamber. Then, acrylamide gel was prepared from 80 ml of 5% acrylamide solution, 500 µl of 10% APS and 50 µl of TEMED and poured between the glass and the chamber. It was left for at least 1.5 hr, assembled with a chamber of 0.5x TBE buffer diluted from 10x TBE buffer and a power supply and pre-run at 80W for 30 min or around 45$^oC$. PCR products were added with 10 µl of a sequencing dye composed of 98 ml of 98% formamide, 2 ml of 10 mM Fe-EDTA, 0.02 g of bromophenol blue and 0.02 g of xylene cyanol, mixed well by centrifuging for a few minutes, and loaded 2 µl into the top of the cleaned gel equipped with a 96-

wells comb. Two microliters of 50 ng.µl $^{-1}$Φ174 DNA marker was prepared from 20 µl of 500 ng.µl$^{-1}$ Φ174 DNA marker stock, then 180 µl of the sequencing dye was loaded. The apparatus was run at 70W around 1-3 hr depending on sizes of PCR products ranged from 100 to 500 bp. The gel attached on the glass was taken out from the apparatus. The PCR products in the gel were fixed with soaking and shaking the gel in 2.5 l of 10% $CH_3COOH$ at 50 rpm for 20 min, washed twice with $dH_2O$ for 3 min, stained with 2.5 l of silver staining solution (prepared from 2.5 g of $AgNO_3$, 3.75 ml of 37% HCHO and $dH_2O$) for 30 min, washed with $dH_2O$ for a few sec, soaked in 2 l of developing solution, prepared from 30 g of $NaCO_3$, 1.5 ml of 37% HCHO, 200 µl of 10 mg.ml$^{-1}$ $Na_2S_2O_3$ and $dH_2O$, until DNA bands appeared. Then the reaction was terminated by soaking in 10% $CH_3COOH$ for a few minutes and washed by $dH_2O$. Finally, the DNA bands were visualized and scored.

2. Phenotypic analysis

Phenotypic data were collected for P compound contents in seed and seedling as well as major agronomic traits using the $F_{2:3}$ population grown in an experimental field with no replication at Kasetsart University, Kamphaeng Sean Campus, Nakhon Pathom, Thailand using CRD. Each $F_{2:3}$ line comprised at least 10 plants was sown in a two-row plot of 5.0 m long with 75 cm between rows and 25 cm between hills. Three seeds were sown in each hill and the seedlings were thinned to one plant/hill at 7-14 days after germination. Available P in the soil was taken a random and analyzed by Bray II method (Fixen and Grove, 1990). $F_4$ Seeds of each $F_3$ line were harvested in bulk and used for measurements of P compound contents, including phytic acid P, inorganic P and total P, in mature seeds and 3-day seedlings. Three agronomic traits viz. days to 50 % flowering, days to 50% maturity, and 100-seed weight were collected on $F_{2:3}$ plants.

3. Data analysis

3.1 Statistical analysis

Correlations between the contents and the traits, test for normal distribution, skewness, and kurtosis, as well as chi-square test for goodness of fit to the expected 3:1 (dominance) and 1:2:1 (co-dominance) ratio of segregating SSR markers in the 170 $F_{2:3}$ plants, all were analyzed by using the R program version 2.8.1 (R Development Core Team, 2006).

3.2 Phenotypic distribution

Frequency distribution of P compounds contents and agronomic traits in the 170 $F_{2:3}$ plants were done the same as other experiments that follow LeClerg *et al*., (1962).

3.3 Linkage map analysis

The genetic linkage map was constructed using the JoinMap3.0 software (van Ooijen and Voorips 2003) with the LOD threshold ≥ 8.0. Recombination values were transformed to centiMorgan (cM) by Kosambi's mapping function (Kosambi, 1944). SSR markers were assigned to a linkage group with a maximum map distance of 50 cM. The map was drawn by Mapchart2.2 software.

3.4 QTL analysis

QTL analysis was performed with the WinQTLCart2.5 software (Wang *et al.,* 2007) using the composite interval mapping (CIM) method. Model 6 of the CIM (forward and backward regression) with window size of 10 cM, background markers of 5, and 1-cM walkspeed was employed. Significant LOD score threshold at 0.01 probability level were determined to identify the QTL by running a 1,000 times of permutation test. The additive effects were also analyzed. The percentages of

phenotypic variation of the putative QTLs were calculated from simple and multiple regressions by using StatgraphicsPlus3.0 software. Linkage groups were named following azuki bean linkage groups (Han *et al.*, 2005). QTLs were named follow Somta *et al.* (2006).

# RESULTS AND DISSCUSSION

## Results

### Part I: Identification of germplasm with high and low seed phytic acid

All 22 accessions used in this study showed significant difference in seed phytic acid content. The accessions showing the highest seed phytic acid are V1137BG, V1725BG, AusTRCF321936 and Sukhothai (each with about 16.0 $mg.g^{-1}$), while the accessions with lowest content are AusTRCF085148 (8.27 $mg.g^{-1}$) and AusTRCF321925 (8.68 $mg.g^{-1}$) (Table 3). V1725BG and Sukhothai were chosen as the maternal lines in the later experiment due to their high in phytic acid content.

The male lines are the wild accessions, AusTRCF321925 and AusTRCF322012, showing the low seed phytic acid. AusTRCF085148 although possessed the lowest seed phytic acid content, was not chosen because it is not var. *sublobata* like others and thus it may not cross well with cultivated accessions.

### Part II: Estimation of narrow-sense heritability of P compound contents in seeds

Table 4 showed that phytic acid P contents are significantly different between $F_3/F_2$ families in three populations from three crosses including Sukhothai × AusTRCF321925, Sukhothai × AusTRCF322012 and V1725BG × AusTRCF321925, but not in the population from the cross of V1725BG and AusTRCF322012. All populations also showed significant difference in inorganic P content between the families, while two populations derived from V1725BG × AusTRCF321925, and V1725BG × AusTRCF322012 showed significant difference in total P content between the families.

**Table 3** Mean and standard deviation of seed phytic acid content in 22 mungbean accessions

| Accession name | Phytic acid[1/] ($mg.g^{-1}$) | Accession name | Phytic acid[1/] ($mg.g^{-1}$) |
|---|---|---|---|
| **Cultivated accessions** | | **Wild accessions** | |
| V1137BG | $15.98^{a} \pm 0.93$ | AusTRCF321995 | $13.87^{ab} \pm 0.60$ |
| V1141BG | $13.76^{ab} \pm 3.24$ | AusTRCF322000 | $12.32^{abc} \pm 1.06$ |
| V1158AG | $15.83^{a} \pm 0.00$ | AusTRCF322011 | $13.18^{ab} \pm 1.57$ |
| V1658BBR | $14.67^{ab} \pm 0.58$ | AusTRCF322012 | $10.35^{bc} \pm 0.97$ |
| V1687ABLM | $13.05^{ab} \pm 0.00$ | AusTRCF322015 | $15.21^{a} \pm 0.28$ |
| V1722BG | $15.11^{a} \pm 2.06$ | AusTRCF321936 | $16.09^{a} \pm 0.48$ |
| V1724AG | $15.57^{a} \pm 0.00$ | AusTRCF085148 | $8.27^{c} \pm 0.44$ |
| V1725BG | $16.08^{a} \pm 1.05$ | AusTRCF322013 | $14.91^{a} \pm 0.50$ |
| V3092AG | $14.48^{ab} \pm 0.33$ | AusTRCF321927 | $16.15^{a} \pm 0.32$ |
| V3251ABLM | $15.47^{a} \pm 0.00$ | AusTRCF321925 | $8.68^{c} \pm 6.08$ |
| Sukhothai | $15.90^{a} \pm 0.00$ | AusTRCF321924 | $13.93^{ab} \pm 0.55$ |

[1/]Means followed by the same letters are not significantly different as compared by Duncan's multiple range test (DMRT) at 0.05 probability level.

The estimated narrow-sense heritabillities of P compounds in seeds of the four $F_3$ populations are shown in Table 5. The estimates of phytic acid P were about 0.36, 0.21, 0.88 and 0.12 in the populations of Sukhothai × AusTRCF321925, Sukhothai × AusTRCF322012, V1725BG × AusTRCF321925 and V1725BG × AusTRCF322012, respectively. The estimates for inorganic P (0.54, 0.61, 0.30 and 0.60) were more similar among crosses than those of phytic acid P. For total P, the estimates from the populations Sukhothai × AusTRCF321925, and Sukhothai × AusTRCF322012 were both low (0.11 and 0.06), while those of V1725BG × AusTRCF321925 and V1725BG × AusTRCF322012 were similarly high (0.69 and 0.56).

**Table 4** ANOVA of P compound contents of the $F_3/F_2$ families

| SOV | *Df* | MS | | | |
|---|---|---|---|---|---|
| | | Sukhothai × AusTRCF321925 | Sukhothai × AusTRCF322012 | V1725BG × AusTRCF321925 | V1725BG × AusTRCF322012 |
| **Phytic acid P** | | | | | |
| Between families | 9 | 0.1707* | 0.1768* | 0.3891** | 0.0737 $^{ns}$ |
| Within families | 40 | 0.0533 | 0.0815 | 0.0441 | 0.0454 |
| Total | 49 | - | - | - | - |
| **Inorganic P** | | | | | |
| Between families | 9 | 0.0192** | 0.0369** | 0.0113* | 0.0190** |
| Within families | 40 | 0.0041 | 0.0068 | 0.0040 | 0.0036 |
| Total | 49 | - | - | - | - |
| **Total P** | | | | | |
| Between families | 9 | 0.2402 $^{ns}$ | 0.0904 $^{ns}$ | 0.5465** | 0.4219* |
| Within families | 40 | 0.1532 | 0.1246 | 0.0880 | 0.0860 |
| Total | 49 | - | - | - | - |

SOV = source of variations, *df* = degrees of freedom, MS = mean squares, * and ** significant at 0.05 and 0.01 levels of probability, respectively, $^{ns}$ non significant

**Table 5** Estimation of narrow-sense heritabilities of P compound contents in the four $F_3$ populations

| Population | $\sigma^2_W$ | $\sigma^2_B$ ($V_A$) | $V_E$ | $h_n^2$ |
|---|---|---|---|---|
| **Phytic acid P** | | | | |
| Sukhothai × AusTRCF321925 | 0.0533 | 0.0235 | 0.0416 | 0.3609 |
| Sukhothai × AusTRCF322012 | 0.0815 | 0.0191 | 0.0719 | 0.2095 |
| V1725BG × AusTRCF321925 | 0.0441 | 0.0690 | 0.0096 | 0.8782 |
| V1725BG × AusTRCF322012 | 0.0454 | 0.0057 | 0.0425 | 0.1178 |
| **Inorganic P** | | | | |
| Sukhothai × AusTRCF321925 | 0.0041 | 0.0030 | 0.0026 | 0.5384 |
| Sukhothai × AusTRCF322012 | 0.0068 | 0.0060 | 0.0038 | 0.6108 |
| V1725BG × AusTRCF321925 | 0.0040 | 0.0015 | 0.0033 | 0.3045 |
| V1725BG × AusTRCF322012 | 0.0036 | 0.0031 | 0.0021 | 0.5962 |
| **Total P** | | | | |
| Sukhothai × AusTRCF321925 | 0.1532 | 0.0174 | 0.1445 | 0.1074 |
| Sukhothai × AusTRCF322012 | 0.1246 | 0.0068 | 0.1281 | 0.0565 |
| V1725BG × AusTRCF321925 | 0.0880 | 0.0917 | 0.0241 | 0.6854 |
| V1725BG × AusTRCF322012 | 0.0860 | 0.0672 | 0.0524 | 0.5619 |

$\sigma^2_B$ = between $F_2$ families variance component, $\sigma^2_W$ = within $F_2$ families variance component, $V_A$ = additive genetic variance component, $V_E$ = environmental variance component, $h_n^2$ = narrow-sense heritability

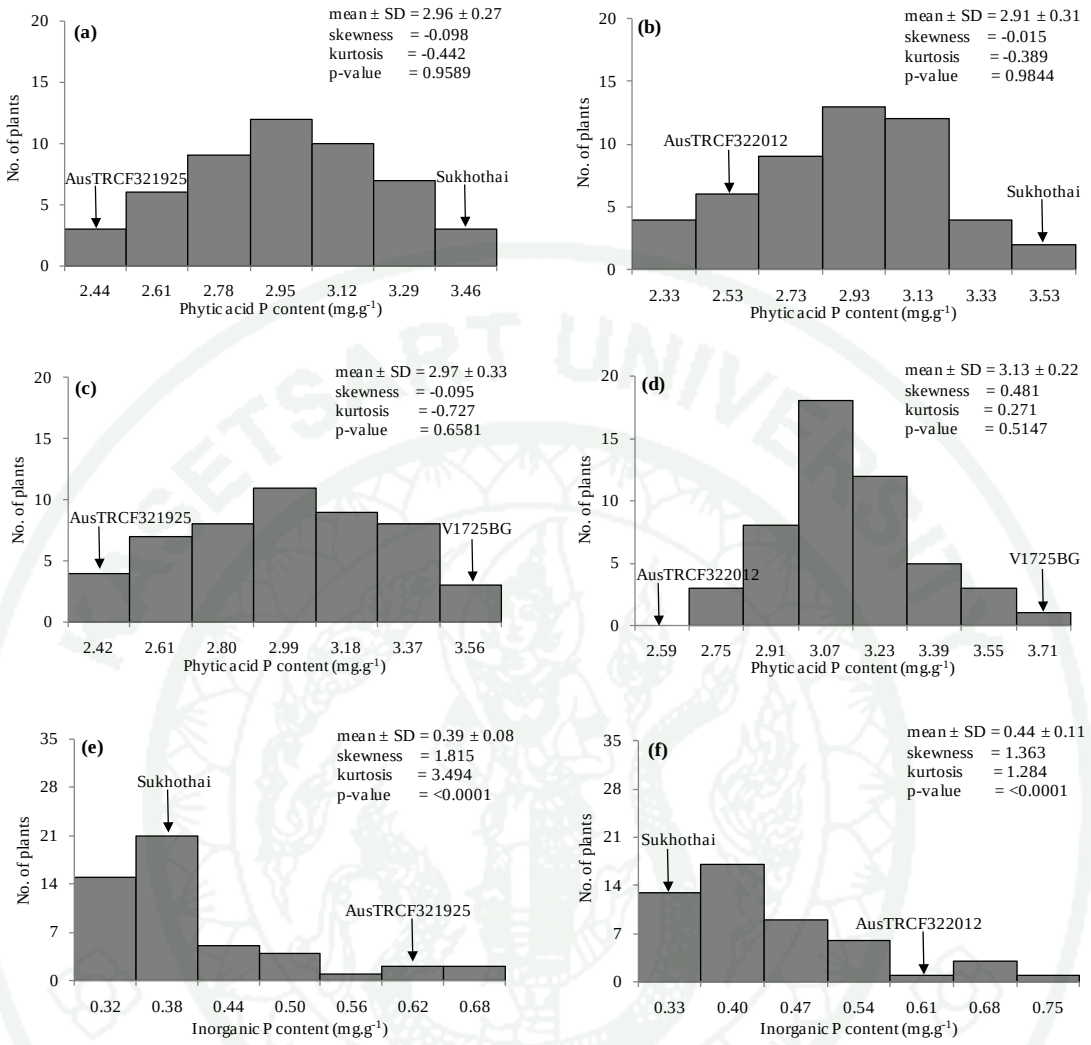



**Figure 8** Frequency distribution of P compound contents in $F_4$ seed of the four $F_3$ populations: (a) phytic acid P in Sukhothai × AusTRCF321925; (b) phytic acid P in Sukhothai × AusTRCF322012; (c) phytic acid P in V1725BG × AusTRCF321925; (d) phytic acid P in V1725BG × AusTRCF322012; (e) inorganic P in Sukhothai × AusTRCF321925; (f) inorganic P in Sukhothai × AusTRCF322012; (g) inorganic P in V1725BG × AusTRCF321925; (h) inorganic P in V1725BG × AusTRCF322012; (i) total P in Sukhothai × AusTRCF321925; (j) total P in Sukhothai × AusTRCF322012; (k) total P in V1725BG × AusTRCF321925; (l) total P in V1725BG × AusTRCF322012

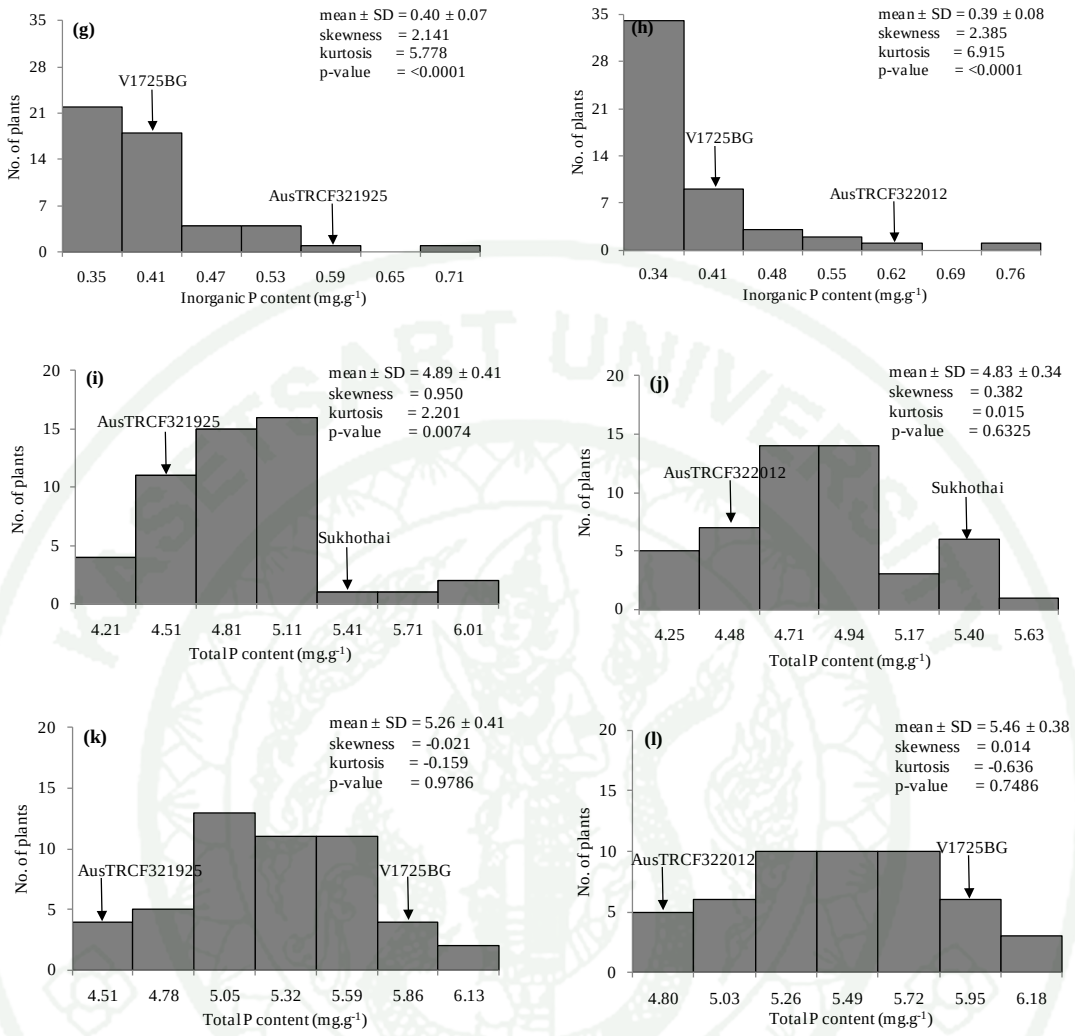



**Figure 8** (Continued)

**Table 6** Available P content in the soil grown to the four populations

| Soil grown to $F_3$ Population | Available P content[1/] ($mg.kg^{-1}$) |
|---|---|
| Sukhothai × AusTRCF321925 | 183.59[d] |
| Sukhothai × AusTRCF322012 | 205.40[c] |
| V1725BG × AusTRCF321925 | 301.55[a] |
| V1725BG × AusTRCF322012 | 227.72[b] |

[1/]Means followed by the same letters are not significantly different as compared by DMRT at 0.05 probability level.

All populations showed normal distribution in phytic acid P contents with *p*-value of 0.9589, 0.9844, 0.6581 and 0.5147 (Figure 8). Only the population of Sukhothai × AusTRCF322012 showed a little transgressive segregation. The population from the cross of V1725BG × AusTRCF322012 showed the contents that are all higher than the low P compound line, AusTRCF322012. P contents in the four populations skewed positively with the skewness of 1.815, 1.363, 2.141 and 2.385, and at the significant *p*-value<0.0001. All progenies showed transgressive segregation and not normally distributed. For distribution of total P content, the $F_2$ families of Sukhothai × AusTRCF322012, V1725BG × AusTRCF321925 and V1725BG × AusTRCF322012 showed normal distribution with *p*-value of 0.6325, 0.9786 and 0.7486, respectively, while only that of Sukhothai × AusTRCF321925 showed positive skewness (0.950) with the most data points falling on the left side of the curve (kurtosis = 2.201). Moreover, all populations showed transgressive segregation in total P content. The available P in soils was significantly different among 4 areas of four populations ($p$<0.0001) as Table 6.

**Part III: Changes in P compound contents during germination**

The accessions showing high phytic acid P content in seedlings at all ages were Sukhothai (4.10 $mg.g^{-1}$) and V1725BG (3.70 $mg.g^{-1}$), while the lowest accession was AusTRCF321925 (1.27 $mg.g^{-1}$) (Table 7).

For inorganic P content, the highest was V1725BG (2.7 $mg.g^{-1}$) and the lowest was Sukhothai (1.34 $mg.g^{-1}$). For seedling length, the high ones were Sukhothai and V1725BG, AusTRCF321925 and AusTRCF322012 were low (Table 7). The four accessions showed a dramatic increase in inorganic P and seedling length during germination, while phytic acid P content decreased and total P contents were rather stable (Table 8 and Figure 9).

**Table 7** Means of P compound contents and seedling length during 12-72 hr of germination in four mungbean accessions

| Accession name | Phytic acid P content ($mg.g^{-1}$) | Inorganic P content ($mg.g^{-1}$) | Total P content ($mg.g^{-1}$) | Seedling length (cm) |
|---|---|---|---|---|
| Sukhothai | $4.10^{a} \pm 0.38$ | $1.34^{b} \pm 0.49$ | $5.44^{ab} \pm 0.19$ | $2.70^{a} \pm 1.35$ |
| V1725BG | $3.70^{a} \pm 0.49$ | $2.70^{a} \pm 0.82$ | $6.40^{a} \pm 0.47$ | $2.60^{a} \pm 0.74$ |
| AusTRCF322012 | $2.53^{b} \pm 0.57$ | $1.99^{ab} \pm 0.90$ | $4.52^{bc} \pm 0.42$ | $1.88^{b} \pm 0.45$ |
| AusTRCF321925 | $1.27^{c} \pm 0.19$ | $2.46^{a} \pm 1.14$ | $3.73^{c} \pm 0.98$ | $1.66^{b} \pm 0.49$ |

In each character, means of accessions followed by the same letter are not significantly different as compared by DMRT at 0.05 probability level.

**Table 8** Means of P compound contents and seedling length of four mungbean accessions determined at different ages of germination

| Seedling age (hr) | Phytic acid P content ($mg.g^{-1}$) | Inorganic P content ($mg.g^{-1}$) | Total P content ($mg.g^{-1}$) | Seedling length (cm) |
|---|---|---|---|---|
| 0 | $4.70^{a} \pm 0.48$ | $0.24^{c} \pm 0.04$ | $4.94^{b} \pm 0.45$ | $0.00^{e} \pm 0.00$ |
| 12 | $4.22^{b} \pm 0.44$ | $0.47^{c} \pm 0.04$ | $4.69^{b} \pm 0.41$ | $0.41^{d} \pm 0.02$ |
| 24 | $4.14^{c} \pm 0.48$ | $0.72^{c} \pm 0.08$ | $4.87^{b} \pm 0.45$ | $1.15^{c} \pm 0.05$ |
| 48 | $2.81^{d} \pm 0.58$ | $2.81^{b} \pm 0.73$ | $5.63^{b} \pm 0.65$ | $2.70^{b} \pm 0.28$ |
| 72 | $1.13^{e} \pm 0.15$ | $6.09^{a} \pm 0.64$ | $7.22^{a} \pm 0.58$ | $6.70^{a} \pm 0.85$ |

In each character, means of seedling ages followed by the same letter are not significantly different as compared by DMRT at 0.05 probability level.

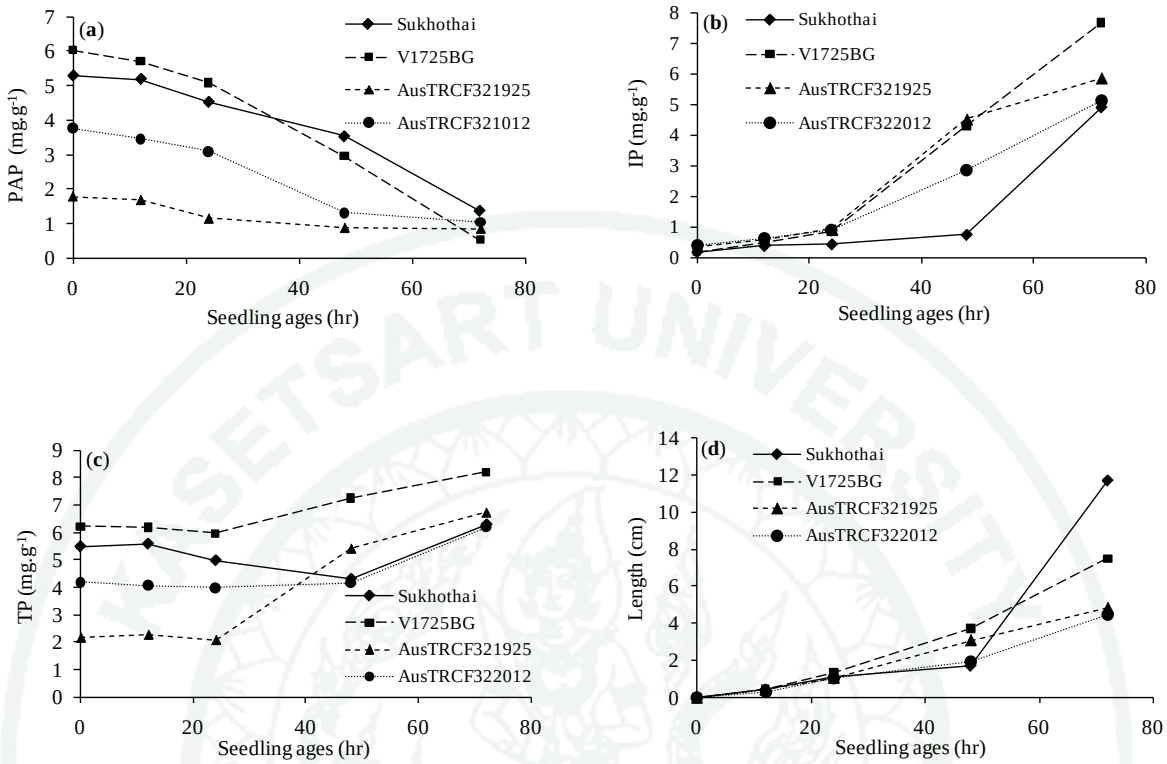



**Figure 9** Changes in phytic acid P (PAP), inorganic P (IP) and total P (TP) contents and seedling length during germination of four mungbean accessions (a) phytic acid P content change; (b) inorganic P content change; (c) total P content change; (d) seedling length change

**Part IV: QTL analysis of P contents and agronomic traits**

The available P in soil was not significantly different among three random areas ($p$=0.5376) and 3 replications ($p$=0.7871) as Table 9. V1725B and AusTRCF321925 were statistically different for total P, inorganic P and phytic acid P, in both seed and seedling. The means of V1725B and AusTRCF321925 for the traits in seed were 5.99 vs. 4.49, 0.37 vs. 0.63 and 3.64 vs. 2.47 and in seedling were 7.81 vs. 4.51, 1.26 vs. 2.85 and 2.95 vs. 2.12 mg $g^{-1}$, respectively (Table 10). For seed, total P, inorganic P and phytic acid P contents ranged from 3.72 to 6.53, 0.32 to 0.76 and 2.33 to 3.99 mg $g^{-1}$ in that order. The mean of those traits was 5.10±0.47, 0.45±0.07 and 3.11±0.28 mg $g^{-1}$, respectively. In case of seedling, total P, inorganic P and phytic acid P contents varied from 4.00 to 11.11, 0.71 to 2.99 and 1.48 to 3.44 mg $g^{-1}$, respectively. The mean of those traits was 6.05±1.00, 1.72±0.56 and 2.53±0.33 in that order. Seed size of V1725B and AusTRCF321925 was 4.3 fold difference. 100-seed weigh of V1725B was 4.92 g while that of AusTRCF321925 was 1.14 g. They were also different in earliness-related traits. The $F_{2:3}$ families were statistically different in seed size, days to flowering and days to maturity.

**Table 9** Available P content in the soil grown to the $F_{2:3}$ population

| Random area | Available P content (mg.$kg^{-1}$) |
|---|---|
| 1 | 226.41±3.38 |
| 2 | 217.67±5.65 |
| 3 | 222.10±11.58 |

**Table 10** Means and ranges for P compound contents in seed and seedling and agronomic traits in the parents and $F_{2:3}$ population

| Trait | Mean±SD | | | Population range (% of population mean) |
|---|---|---|---|---|
| | V1725BG | AusTRCF321925 | $F_{2:3}$ Population | |
| Seed phytic acid P (mg $g^{-1}$) | 3.64±0.08 | 2.47±0.28 | 3.11±0.28 | 2.33 - 3.99 (75 - 128) |
| Seed inorganic P (mg $g^{-1}$) | 0.37±0.05 | 0.63±0.07 | 0.45±0.07 | 0.32 - 0.76 (71 - 169) |
| Seed total P (mg $g^{-1}$) | 5.99±0.09 | 4.49±0.46 | 5.10±0.47 | 3.72 - 6.53 (73 - 128) |
| Seedling phytic acid P (mg $g^{-1}$) | 2.95±0.01 | 2.12±0.00 | 2.53±0.33 | 1.48 - 3.44 (58 - 136) |
| Seedling inorganic P (mg $g^{-1}$) | 1.26±0.15 | 2.85±0.00 | 1.72±0.56 | 0.71 - 2.99 (41 - 174) |
| Seedling total P (mg $g^{-1}$) | 7.81±0.06 | 4.51±0.00 | 6.05±1.00 | 4.00 - 11.11 (66 - 184) |
| 100-seed weight (g) | 4.92±0.09 | 1.14±0.17 | 2.72±0.56 | 1.61 - 5.40 (59 - 198) |
| 50% flowering (days) | 34.0±0.00 | 44.0±0.00 | 35.38±4.58 | 28.0 - 57.0 (79 - 161) |
| 50% maturity (days) | 46.0±0.00 | 66.0±0.00 | 50.90±4.89 | 45.0 - 69.0 (88 - 135) |

**Table 11** Correlations among P compound contents in seed and seedling and agronomic traits of the $F_{2:3}$ population

| | SDPAP | SLPAP | SDIP | SLIP | SDTP | SLTP | DFL | DMT |
|---|---|---|---|---|---|---|---|---|
| SLPAP | 0.321*** | | | | | | | |
| SDIP | 0.080 | 0.002 | | | | | | |
| SLIP | 0.199** | -0.030 | 0.051 | | | | | |
| SDTP | 0.534*** | 0.273*** | 0.181* | 0.386*** | | | | |
| SLTP | 0.303*** | 0.298*** | 0.188* | 0.647*** | 0.518*** | | | |
| DFL | 0.279** | 0.034 | 0.052 | 0.210** | 0.219** | 0.300** | | |
| DMT | 0.297** | 0.039 | 0.075 | 0.184* | 0.230** | 0.282** | 0.762** | |
| SD100WT | -0.053 | 0.201** | 0.059 | -0.223** | -0.142 | -0.085 | -0.056 | -0.018 |

SDTP = seed total P, SDPAP = seed phytic acid P, SDIP = seed inorganic P, SLDTP = seedling total P, SLPAP = seedling phytic acid P, SLIP = seedling inorganic P, SD100WT = 100-seed weight, DFL = days to 50% flowering, DMT = days to 50% maturity, *,** and *** = significant at 0.05, 0.01 and 0.001 probability level, respectively

Correlation coefficient (*r*) between traits is summarized in Table 11. Total P in seed showed positive correlation with inorganic P and phytic acid P in seed, total P, inorganic P and phytic acid P in seedling and days to flowering and maturity. Inorganic P in seeds only showed positive correlation with total P in seed and seedling. Positive correlations between phytic acid P in seed with total P in seeds, total P, inorganic P and phytic acid P in seedling, and days to flowering and maturity were observed. Total P in seedlings showed positive correlation with all other traits measured except seed weight. Inorganic P in seedlings is positively correlated with total P in seeding, total P and phytic acid P in seed, days to flowering and maturity, but negatively correlated with seed weight. Phytic acid P in seedling is positively correlated with total P and phytic acid P in seed, total P in seedling, and seed weight.

Distributions in P compound contents are shown in Figure 10. Only phytic acid P and total P contents in seed showed normal distribution with *p*-value of 0.7578 and 0.5117, respectively. Inorganic P content in seed and seedling and total P content in seedling skewed positively with skewness of 1.58, 0.373 and 1.215, respectively, while only phytic acid P content in seedling skewed negatively with a skewness of -0.591. Phytic acid P contents in seedling, inorganic P content in seed and total P content in seedling were leptokurtic (1.097, 3.986 and 3.195, respectively), while that of inorganic P content in seedling was platykurtic (-0.697). All the characters showed transgressive segregation. Frequency distributions of agronomic traits of the $F_{2:3}$ population are shown in Figure 10. Days to 50% flowering, days to 50% maturity and 100-seed weight showed positive skewness of 1.308, 1.828 and 0.921, respectively. All were leptokurtic (2.282, 3.343 and 2.260, respectively). Transgressive segregations were found in all agronomic traits.

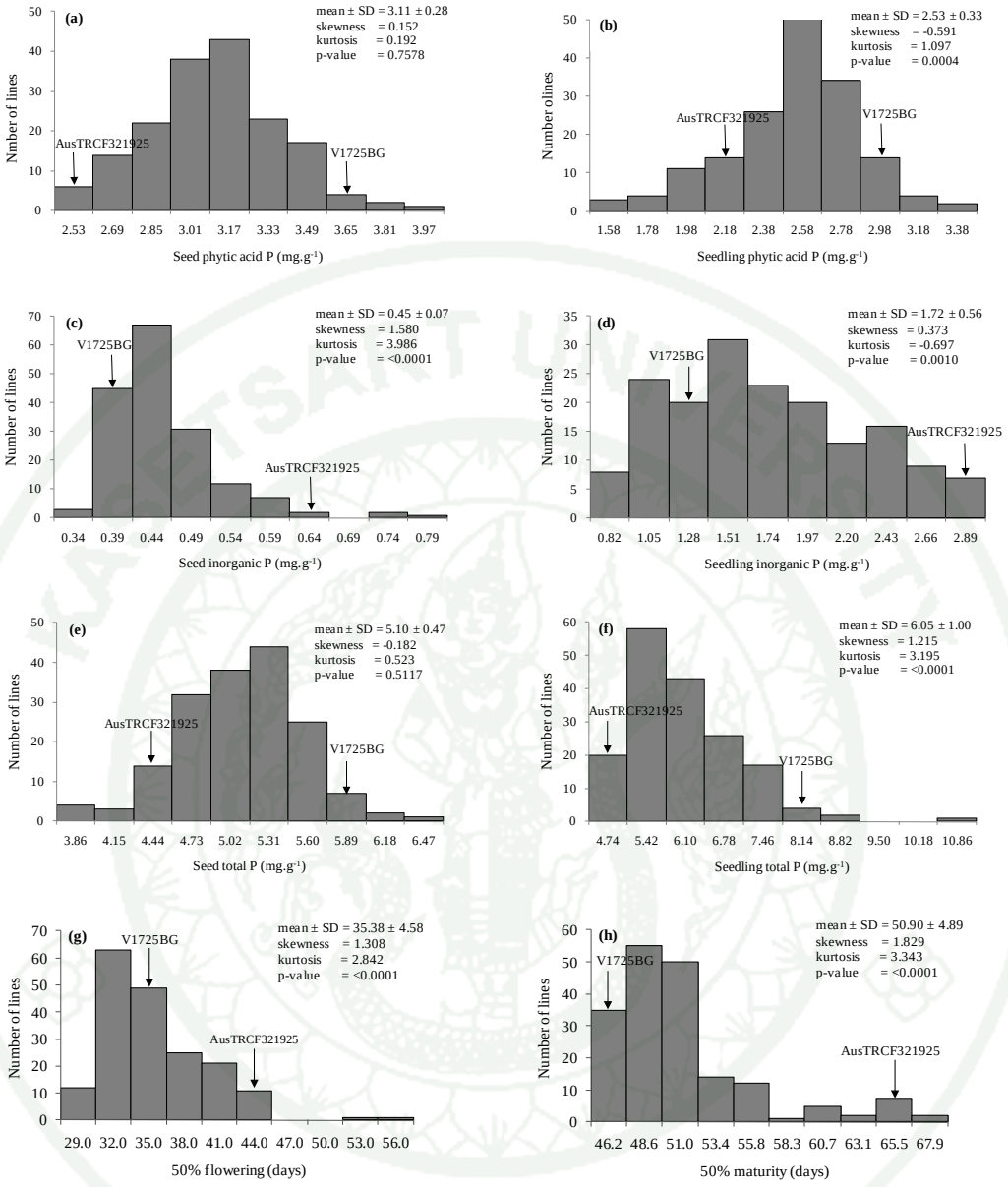



**Figure 10** Frequency distribution for (a) phytic acid P in seed, (b) phytic acid P in seedling, (c) inorganic P in seed, (d) inorganic P in seedling, (e) total P in seed, (f) total P in seedling, (g) days to 50% flowering, (h) days to 50% maturity and (i) 100-seed weight in $F_{2:3}$ population from the cross V1725BG × AusTRCF321925

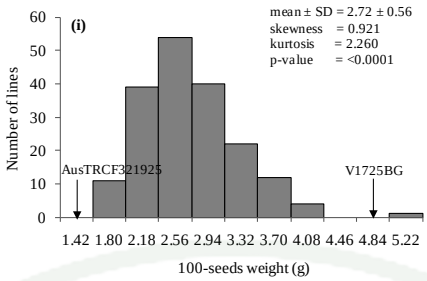



**Figure 10** (Continued)

**Table 12** Amplification and polymorphism of SSR markers in the mapping parents, V1725BG and AusTRCF321925, of the $F_2$ population

| SSR marker sources | Number of SSR markers | | |
|---|---|---|---|
| | screened | amplified (%) | polymorphic (%) |
| Mungbean | 546 | 437 (80.04) | 52 (11.90) |
| Azuki bean | 142 | 112 (78.87) | 43 (38.39) |
| Cowpea | 189 | 154 (81.48) | 20 (12.99) |
| Common bean | 114 | 60 (52.63) | 10 (16.67) |
| Total | 991 | 763 (76.99) | 125 (16.38) |

Screened 763 out of 991 SSR markers were amplifiable (Table 12). Amplification rate of SSRs from mungbean, azuki bean, cowpea and common bean were 80.04%, 78.87%, 81.48%, and 52.63%, respectively. One hundred and twenty-five polymorphic SSR markers showed polymorphism between the parents. Azuki bean SSRs were the highest percentage of polymorphic markers because azuki bean is conspecific with mungbean and developing method of SSR marker in each source is different (Appendix table 1). Polymorphic markers were subsequently used in the construction of the mungbean genetic linkage map. The map consisted of 13 linkage groups (LGs) and spanned a total length of 855.8 cM (Figure 11). The length of the LGs ranged from 19.8 to 104.6 cM. LG01 was the longest LG with a length of 104.6

cM, whereas LG04B was the shortest LG with a length of 19.8 cM. The number of markers per LGs ranged from 2 (LG 11B) to 16 (LG 1). The average distance between adjacent markers varied from 5.5 to 33.1 cM. LG09 and LG11B had gap more than 20 (Table 13).

Results of QTL analysis of P compound contents in seed and seedling, and three agronomic traits was summarized in Table 14. In total, 23 QTLs were found for the nine traits. Of the 13 QTLs identified for P compound contents in seed and seedling.

**Table 13** Characteristics of the linkage map

| Linkage group | Number of markers | Average interval (cM) | Length (cM) |
|---|---|---|---|
| 1 | 16 | 6.97 | 104.6 |
| 2 | 16 | 5.55 | 83.2 |
| 3 | 9 | 8.13 | 65.1 |
| 4A | 9 | 11.31 | 90.5 |
| 4B | 3 | 9.89 | 19.8 |
| 5 | 6 | 13.32 | 66.6 |
| 6 | 11 | 9.63 | 98.3 |
| 7 | 7 | 11.61 | 69.7 |
| 8 | 8 | 9.16 | 64.1 |
| 9 | 3 | 25.28 | 50.6 |
| 10 | 5 | 10.95 | 43.8 |
| 11A | 6 | 13.29 | 66.4 |
| 11B | 2 | 33.12 | 33.1 |
| Sum | 101 | - | 855.8 |
| Mean ± SE | 7.77±1.24 | 12.94±2.14 | 65.83±6.77 |

Two, four and one QTLs distributed on five linkage groups were found for phytic acid P, inorganic P and total P in seed in that order (Table 14 and Figure 11). The phenotypic variance explained (PVE) by these QTLs ranged from 3.43% to 11.24% of the trait variation. At QTLs *SDIP1.1, SDIP8.1* and *SDIP11.1*, the alleles from AusTRCF321925 decreased trait values. QTLs *SDPAP4.1* and *SDTP4.1* were overlapped.

For seedling QTLs, three QTLs were detected for phytic acid P, while two were identified for total P and one for inorganic P (Table 14 and Figure 11). These QTLs were on four linkage groups. The PVE of the QTLs varied between 1.38% and 7.56%, 0.35% and 7.49%, and 6.59% and 13.82% of the trait variation in that orders. Alleles of AusTRCF321925 at QTLs *SLPAP7.1, SLPAP8.1* and *SLTP4.1* reduced the value of respective P compound. QTLs *SLIP4.1* and *SLTP4.1* were co-localized to the marker interval CEDG088-CEDG091, whereas QTLs *SLPAP7.2* and *SLTP7.1* were both mapped to marker interval IAC98-Bmd26.

Three QTLs for days to flowering and two QTLs for maturity were detected, while five QTLs were identified for seed weight (Table 14 and Figure 11). The PVE of days to flowering QTLs ranged from 5.01% to 33.38%, wherase that of days to maturity varied from 7.24% to 25.67%. The PVE of the seed weight QTLs were between 6.83% and 18.41%. Location of the QTLs *DFL4.1* and *DMT4.1*, and *DFL7.1* and *DMT7.1* were overlapped.

Twenty-four ungrouped SSR markers were analyzed by using simple regression as Table 15. Some markers were significant and quite high %PVE in some trait. These of cp06039, ISSR886, and VR248 are 9.46%, 12.14% and 11.16% in seedling IP, 100-seed weight and days to 50% flowering, respectively. If these markers are grouped in the linkage map, it will be possible to get closer SSR markers to QTLs for the nine traits.

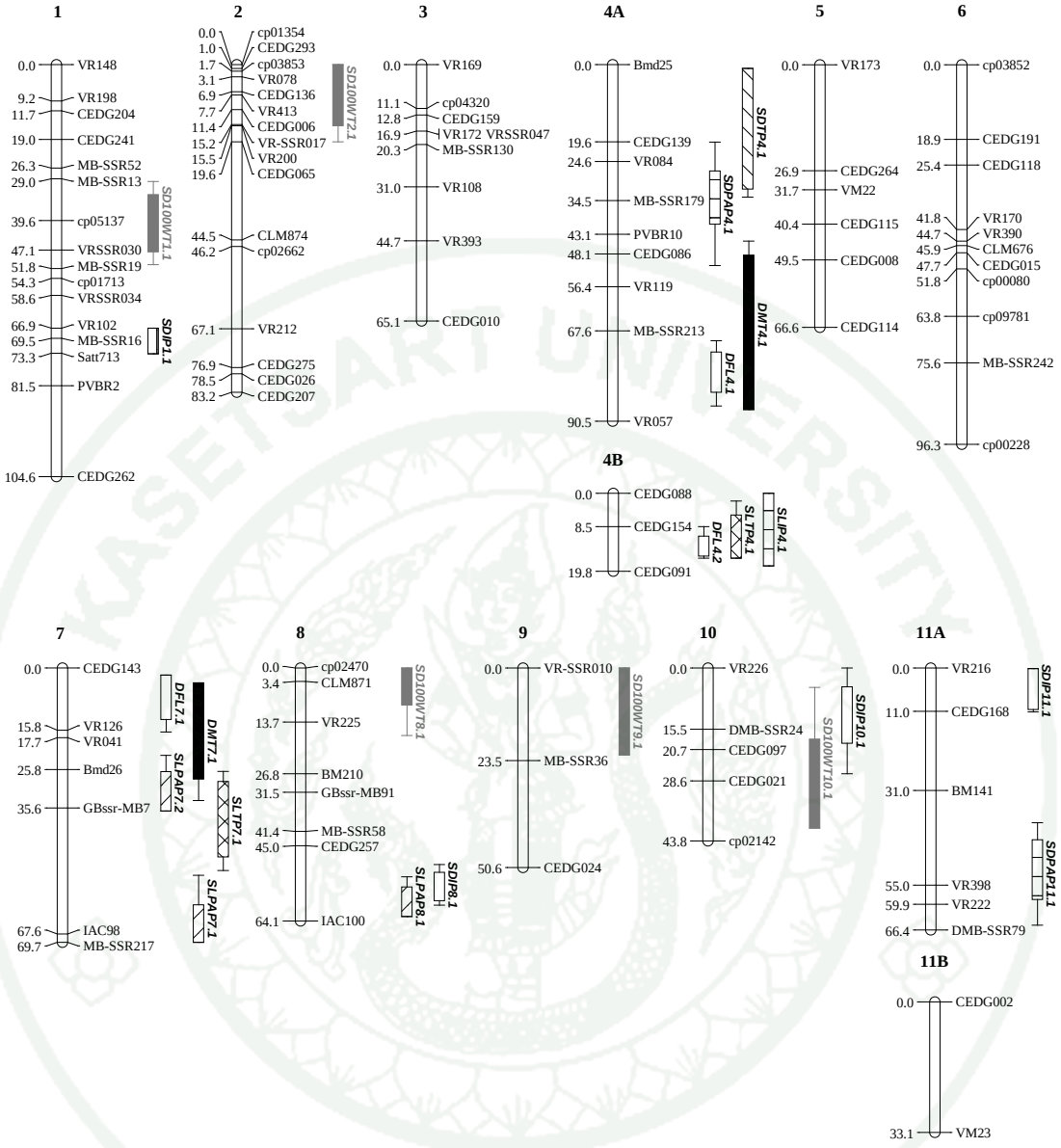



**Figure 11** An SSR-based linkage map of the $F_2$ population and genomic regions associated with P compound contents in seed and seedling and agronomic traits, SDTP = seed total P, SDPAP = seed phytic acid P, SDIP = seed inorganic P, SLDTP = seedling total P, SLPAP = seedling phytic acid P, SLIP = seedling inorganic P, SD100WT = 100-seed weight, DFL = days to 50% flowering, and DMT = days to 50% maturity

**Table 14** QTLs detected for P compound contents in seed and seedling and agronomic traits of the $F_{2:3}$ population

| Trait | QTL | LG | Interval marker | LOD | %PVE from regression | | Additive effect | Dominant effect |
|---|---|---|---|---|---|---|---|---|
| | | | | | Simple | Multiple | | |
| Seed phytic acid P | *SDPAP4.1* | 4A | CEDG139-MB-SSR179 | 3.78 | 11.24 | 14.39 | 0.0406 | 0.1172 |
| | *SDPAP11.1* | 11A | BM141-VR222 | 4.00 | 6.06 | | 0.0670 | 0.0835 |
| Seed inorganic P | *SDIP1.1* | 1 | Satt713-VR102 | 4.33 | 5.25 | 16.97 | -0.0333 | 0.0129 |
| | *SDIP8.1* | 8 | CEDG257-IAC100 | 7.20 | 3.43 | | 0.0737 | -0.0083 |
| | *SDIP10.1* | 10 | VR226-CEDG097 | 3.45 | 5.59 | | -0.0006 | -0.0289 |
| | *SDIP11.1* | 11A | VR216-CEDG168 | 2.96 | 5.54 | | 0.0285 | -0.0208 |
| Seed total P | *SDTP4.1* | 4A | Bmd25-MB-SSR179 | 2.64 | 10.58 | | 0.1349 | 0.0007 |
| Seedling phytic acid P | *SLPAP7.1* | 7 | MB-SSR217-GBssr-MB7 | 5.35 | 7.56 | 8.11 | -0.3350 | 0.0504 |
| | *SLPAP7.2* | 7 | IAC98-Bmd26 | 4.13 | 7.57 | | 0.1458 | 0.0163 |
| | *SLPAP8.1* | 8 | MB-SSR58-IAC100 | 3.66 | 1.38 | | -0.5518 | 0.0823 |

*SDTP* = seed total P, *SDPAP* = seed phytic acid P, *SDIP* = seed inorganic P, *SLDTP* = seedling total P, *SLPAP* = seedling phytic acid P, *SLIP* = seedling inorganic P, *SD100WT* = 100-seed weight, *DFL* = days to 50% flowering, and *DMT* = days to 50% maturity, LG = linkage group, LOD = logarithm of the odds , PVE = phenotypic variance explain, positive values indicate that allelic contribution is from the cultivated accession V1725BG and negative from the wild accession AusTRCF321925 in additive and dominant effect

**Table 14** (Continued)

| Trait | QTL | LG | Interval marker | LOD | %PVE from regression | | Additive effect | Dominant effect |
|---|---|---|---|---|---|---|---|---|
| | | | | | Simple | Multiple | | |
| Seedling inorganic P | *SLPAP4.1* | 4B | CEDG088-CEDG091 | 2.65 | 7.49 | | 0.2413 | 0.0861 |
| Seedling total P | *SLTP4.1* | 4B | CEDG088-CEDG091 | 5.65 | 13.82 | 21.16 | -0.5252 | 0.1575 |
| | *SLTP7.1* | 7 | IAC98-Bmd26 | 4.62 | 6.59 | | 0.9745 | -0.2661 |
| Days to 50% flowering | *DFL4.1* | 4A | MB-SSR213-VR057 | 5.77 | 33.38 | 37.89 | 6.0000 | -1.3649 |
| | *DFL4.2* | 4B | CEDG154-CEDG091 | 8.35 | 25.70 | | -1.3533 | -1.0129 |
| | *DFL7.1* | 7 | VR126-CEDG143 | 4.43 | 5.01 | | -0.8575 | -0.8492 |
| Days to 50% maturity | *DMT4.1* | 4A | CEDG086-VR057 | 4.89 | 25.67 | 29.47 | 4.3978 | -0.9284 |
| | *DMT7.1* | 7 | GBssr-MB7-CEDG143 | 2.64 | 7.24 | | -1.2200 | 0.1603 |
| 100-Seed weight | *SD100WT1.1* | 1 | CP1713-CP5137 | 6.52 | 18.41 | 48.03 | -0.1465 | -0.2592 |
| | *SD100WT2.1* | 2 | VR078-CEDG136 | 2.60 | 6.80 | | -0.0618 | -0.1783 |
| | *SD100WT8.1* | 8 | CP2470-CLM871 | 2.51 | 11.70 | | -0.1873 | 0.0335 |
| | *SD100WT9.1* | 9 | VRSSR010-MB-SSR36 | 2.90 | 17.88 | | -0.2141 | 0.0046 |
| | *SD100WT10.1* | 10 | CEDG097-CP2142 | 3.02 | 12.38 | | -0.2313 | 0.2186 |

**Table 15** Single marker analysis of 24 ungrouped SSR markers by simple regression

| Marker | %PVE from simple regression | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | SDPAP | SLPAP | SDTP | SLTP | SDIP | SLIP | DFL | DMT | SD100WT |
| BM212 | 2.93* | ns | 4.42** | 2.82* | ns | ns | ns | ns | ns |
| CEDAAG001 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| CEDG020 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| CEDG043 | ns | ns | ns | 2.42* | ns | 2.89* | 3.32* | 4.64** | 5.02** |
| CEDG044 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| CEDG050 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| CEDG100 | ns | 6.45** | ns | ns | ns | ns | ns | ns | ns |
| CEDG108 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| CEDG149 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| CEDG156 | ns | ns | ns | ns | ns | ns | ns | ns | 6.14** |
| CEDG268 | ns | ns | ns | ns | ns | ns | ns | ns | 4.68** |

SDTP = seed total P, SDPAP = seed phytic acid P, SDIP = seed inorganic P, SLDTP = seedling total P, SLPAP = seedling phytic acid P, SLIP = seedling inorganic P, SD100WT = 100-seed weight, DFL = days to 50% flowering, and DMT = days to 50% maturity, PVE = phenotypic variance explain, *,** and *** = significant at 0.05, 0.01 and 0.001 probability level, respectively, ns = not significant

**Table 15** (Continued)

| Marker | %PVE from simple regression | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | SDPAP | SLPAP | SDTP | SLTP | SDIP | SLIP | DFL | DMT | SD100WT |
| CEDG269 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| CEDG305 | ns | ns | ns | ns | ns | ns | 2.82* | ns | ns |
| CLM636 | ns | ns | ns | ns | ns | 2.94* | ns | 4.01* | ns |
| cp10549 | ns | ns | ns | ns | 6.67** | ns | 2.85* | ns | 7.39** |
| cp06039 | ns | ns | ns | ns | 9.46** | ns | ns | 2.90* | 4.98** |
| ISSR886 | ns | ns | ns | ns | ns | ns | ns | ns | 12.14** |
| MB-SSR164 | 4.17* | ns | ns | ns | ns | ns | ns | ns | ns |
| MB-SSR24 | 3.08* | 5.65** | ns | ns | ns | ns | ns | ns | 2.76* |
| SS28 | ns | ns | ns | ns | 3.11* | ns | ns | ns | ns |
| VR044 | 2.94* | ns | ns | ns | ns | ns | ns | ns | ns |
| VR248 | ns | ns | ns | 5.05** | ns | ns | 11.16** | 6.38** | 4.91** |
| VR357 | 3.96** | 2.71* | ns | ns | 5.47** | ns | ns | ns | ns |
| VR62 | 4.85** | ns | ns | ns | ns | ns | 4.09* | ns | ns |

# Discussion

## Part I: Identification of germplasm with high and low seed phytic acid

This study reports phytic acid content in seed of 22 mungbean accessions to range from 8.27 to 16.08 $mg.g^{-1}$ (Table 3). Similarly, Chitra *et al.* (1995) analyzed phytic acid and total P contents in chickpea, pigeonpea, urd bean (black gram), mungbean and soybean, and found that phytic acid content differed significantly among and within these species. Phytic acid content ($mg.g^{-1}$) was the highest in soybean, followed by urd bean, pigeonpea, mungbean and chickpea. They reported the average phytic acid content in seed of three mungbean genotypes to be 12 $mg.g^{-1}$. However, Naivikul and Sotanasomboon (1989) and Sompong *et al*. (2007) found higher seed phytic acid content than the present study. Naivikul and Sotanasomboon (1989) studied in four mungbean varieties and found seed phytic acid content ranging from 12.75 - 16.41 $mg.g^{-1}$. While Sompong *et al*. (2007) analyzed seed phytic acid content of the same ten cultivated accessions to the present study, five of them were high and five of them were low in total P, ranging from 5.94 to 17.87 $mg.g^{-1}$. The factors affecting difference in seed phytic acid contents among the same accessions include genetic and environmental variation, especially location, soil type and moisture, year and fertilizer application (Dost and Tokul, 2006). Environmental factors influence the available P supply while the genetic factors influence the P uptake.

## Part II: Estimation of narrow-sense heritability of P compound contents in seeds

The normal distribution of phytic acid P and total P (Figure 8) showed quantitative trait inheritance. Similar explanation can be given to the estimated heritabilities in Table 5. The distribution in inorganic P in all populations skewed positively, implying that there was a dominant effect from maternal lines. The averaged heritability estimate was around 0.50, revealing similar magnitude of genetics and environment contributing to this trait. Transgressive segregation of

contents in many populations confirmed that there are also non-additve the genetic effect conditioning certain traits.

The estimated narrow-sense heritabilities of P compound contents in seeds of each population were not similar (Table 5), because (1) we used different parental lines, so additive effects and point mutations affected to phytic acid content were also different, (2) some populations used in this study did not segregate well in the contents (Table 4), and (3) The available P in soil of 4 populations (Table 6) were significantly different but they were higher than 90 $mg.kg^{-1}$, the maximum amount of test of P requirement of mungbean by Gunawardena *et al*. (1992). Phytic acid P content distribution in the cross of V1725BG × AusTRCF322012 that is not different between the families meant sampling was not good enough, so it effected to the low estimated heritability (0.12). Moreover, it was the same reason to the estimated heritabilities of total P content in two populations of the crosses of Sukhothai × AusTRCF321925 and Sukhothai × AusTRCF322012 that show the low estimated heritabilities (0.11 and 0.06, respectively). The heritability of phytic acid P content in the population of the cross of V1725BG was 0.88 that are closed to a report from Mebrahtu *et al*. (1997). They found the broad-sense heritability of phytic acid of vegetable soybean seed was 0.81 that was similar to Sompong *et al*. (2010) reported the broad-sense heritability estimate of phytic acid content in mungbean seed was 0.81 and closed to Wardyn and Russell (2004) who found the broad-sense heritability of maize grain among $S_1$ families mean was 0.82. However, Lorenz *et al*. (2008) found that broad-sense heritability was relatively low (0.60) in 90 $S_1$ families from the BS31 population of maize evaluated at two locations. Similary, the other estimated heritabilities in this study were also relatively lower than others previous studies because other were broad-sense heritabilities estimated genetic effect from both of dominant and additive effects, while ours were narrow-sense heritabilities estimated genetic effect from only additive effect.

**Part III: Changes in P compound contents during germination**

All four accessions showed a dramatic increase in inorganic P content following the seedling ages, with a tendency to increase further, while phytic acid P content decreased and tended to be stable at a similar level (Figure 9). Three day-old (72 hr) seedlings which are considered as commercial mungbean sprout are suitable for studying phytic acid content but not inorganic P content. Seedling of Sukhothai increased in length quicker than the other accessions during 48 - 72 hr. The change in seedling length seems to correlate with the pattern of change inorganic P content in all accession with the strongest relationship in Sukhothai. Sathe *et al*. (1983) found an increase in phytase following seedling ages in common bean. Phytase catalyses phytic acid molecule and releases free inorganic P to produce ATP to use in process of seedling elongation. The decrease in phytic acid P content in seedling was reported earlier by Naivikul and Sotanasomboon (1989) in four mungbean accessions. They reported that seed phytic acid ranged from 12.75 - 16.41 $mg.g^{-1}$, while seedling phytic acid ranged from 7.99 - 10.76 $mg.g^{-1}$. Chitra *et al*. (1996) also reported that germination reduced the phytic acid content of chickpea and pigeonpea seed by over 60%, and that of mungbean, urd bean, and soybean by about 40%. Total P contents of seedlings in the four accessions in this study were stable during the first 24 hr, but increased in the seedlings of V1725BG and AusTRCF321925, slightly decreased in those of Sukhothai and continued stable in AusTRCF322012 during the following 24 hr. During the 3$^{rd}$ day (the last 24 hr), seedlings of all accessions showed an increase in total P content. Finally, seedlings of all accessions at 72 hr showed similar total P contents, which were higher than the content in seed. These results are similar to Uttatam (2001) who studied in phytic acid, total P, protein and mineral contents in seed, seedling and other products of 2 soybean cultivars. She found that total P contents in seedlings at 48 and 72 hr were higher than that in seed.

**Part IV: QTL analysis of P compound contents and agronomic traits**

Similar to other seed crops, reducing the phytic acid content in seed and seedling of mungbean is becoming a major objective of mungbean improvement. Yet

unlike other crops which mutants with low phytic acid seed have been generated as genetic stocks for breeding low phytic acid cultivars, only naturally occurring germplasm that possesses low phytic acid have been identified recently (Sompong *et al.*, 2010). One of such mungbean germplasm was used as a mapping parent in this study.

Blair *et al*. (2009) evaluated QTLs for seed weight, P and phytic acid contents in seed of an RIL population of common bean planted in medium and high level of soil P and found a positive skewness in seed weight, similar to our skewness found in 100-seed weight. Continuous distribution for phytic acid P, inorganic P and total P in both seed and seedling of the $F_2$ population demonstrated the quantitative nature of these traits. This is in line with the quantitative inheritance of phytic acid and total P in soybean (Walker *et al.*, 2006; Gao *et al.*, 2008), common bean (Blair *et al.*, 2009; Cichy *et al.*, 2009), rice (Stangoulis *et al.*, 2006) and *Brassica rapa* (Zhao *et al.*, 2008). Transgressive segregation for phytic acid P, inorganic P and total P in both seed and seedling is of particular interesting. The transgressive segregants for low phytic acid P and high inorganic P were possibly contributed by the combination of alleles at multiple genes from both AusTRCF321925 and V1725BG. Transgressive segregation for seed phytic acid P and/or seed total P have been reported in soybean, common bean and rice (*Oryza sativa* L.). Above 70% of $F_{2:5}$ families of CX1834-1-6 (low- phytic acid line) × V99-3337 possessed seed phytic acid than V99-3337 (Gao *et al.*, 2008). More than 95% of the double haploid rice progeny from the cross IR64 × Azucena had seed phytic acid higher than their parents, although IR64 and Azucena showed very small different in seed phytic acid (Stangoulis *et al.*, 2006). While in common bean, nearly or more than half of segregant progenies of the G2333 × G19839 had lower seed phytic acid than the low- phytic acid parents (Blair *et al.*, 2009). However, transgressive segregations in all traits revealed that non-additive gene actions also play role in controlling the traits.

Positive correlation between seed total P and phytic acid concentrations is found commonly in crops including mungbean (Sompong *et al.*, 2007), soybean (Raboy *et al.*, 1984), common bean (Blair *et al.*, 2009) and rice (Stangoulis *et al.*,

2006). This is also the case in our current study. Regardless of the methods of determination and degree of freedom, the correlation value found in mungbean in this study ($r$ = 0.53) is higher than that of reported in common bean by Blair *et al.* (2009) ($r$ = 0.30-0.37), but far less than to the reported in mungbean ($r$ = 0.98), soybean ($r$ = 0.94) by Raboy *et al.* (1984) and in rice ($r$ = 0.99) by Stangoulis *et al.* (2006). Furthermore, in seedling, total P and phytic acid P concentrations also showed positive correlation ($r$ = 0.30). Seedling phytic acid P and seed phytic acid P or and seed total P also showed positive correlation ($r$ = 0.32 and $r$ = 0.27, respectively). As discussed by Blair *et al.* (2009), the positive correlation between total P and phytic acid is expected because determination of total P by acid digestion would also include phytic acid and defection of the phytic acid synthesis in low phytic acid mutant crops results in increased levels of inorganic P. The positive correlation between seed phytic acid P and seedling inorganic P ($r$ = 0.20) can be explained by Sathe *et al.* (1983). They studied in phytase in 5-day common bean seedling and found that 12 times increase from that of seed. They explained that seed phytic acid content was high, seedling inorganic P content was high and higher following seedling age because of high phytase in seedling.

In this study seed weight showed no significant correlation with any seed P compound components, although seed weight has been shown to be positively correlated with seed P and phytic acid in common bean (Blair *et al.*, 2009). Nonetheless, the seed weight showed positive correlation with seedling phytic acid P ($r$ = 0.20) and negative correlation with seedling inorganic P ($r$ = -0.22). Days to 50% flowering and days to 50% maturity are relatively highly correlated ($r$ = 0.76). Both of them showed positive correlation with seed phytic acid P, seedling inorganic P, seed total P and seedling total P (Table 2). The correlation between flowering and maturity times with P compounds is not expected. This may relate to duration or rate of uptake and/or translocation of P, and synthesis of P compounds in seed. Nearly all the P translocates to developing soybean seeds is incorporated into phytic acid from the third week of flowering to maturity (Raboy *et al.*, 1984). Thus the phytic acid is possibly affected by plant phenology. Although negative relationship between seed phytic acid and seed inorganic P has been reported in soybean (Wilcox *et al.*, 2000),

in our study there was no significant correlation between such traits. Nonetheless weak but significant correlation ($r$ = 0.18) between seed total P and inorganic P was found.

Not many QTL mapping studies have been conducted in mungbean. All are related to insect and disease resistance with an exception to seed size (review in Somta and Srinives, 2007). This is the first report for phytic acid and/or other P compounds in seed and seedling in mungbean. The number of QTLs detected for seed phytic acid and seed total P in this study is comparable to that of reported in other crops. While two QTLs for seed phytic acid were detected in this study; two QTLs were also identified for this trait in common bean (Blair *et al.,* 2009), soybean (Walker *et al.,* 2006; Gao *et al.,* 2008) and rice (Stangoulis *et al.,* 2006), and three QTLs were found in *Brassica rapa* (Zhao *et al.,* 2008). Effect of the QTLs detected in mungbean is markedly low than those reported in those crops. However, the number of QTLs detected for seed phytic acid in this our present study is in agreement with the results of Sompong *et al.* (2010) who reported that two major genes control seed phytic acid in mungbean. In this study one QTL was identified for seed total P, whereas one and two QTLs were found respectively in rice and common bean (Stangoulis *et al.,* 2006; Blair *et al.,* 2009). Four QTLs found for seed inorganic P in our study is higher than previous reports in other crops which one or two QTLs were reported for this trait (Stangoulis *et al.,* 2006; Walker *et al.,* 2006). Although it has been shown in soybean that low phytic acid QTLs were identified successfully by using inorganic P data (Walker *et al.,* 2006) and these QTLs were later validated using phytic acid data (Gao *et al.,* 2008). None of seed inorganic P QTLs co-localized or overlapped with seed phytic acid P QTL and seed total P QTLs in our study (Figure 11). Our results is in accordance with Gao *et al.* (2008)'s argument that direct and indirect phytic acid measurements can give some differences in the phytic acid - marker association analysis.

Mungbean sprout is probably the most popular sprouts consumed in the world. Germination or sprouting of seeds effectively reduces phytic acid in mungbean (Mubarak, 2005; Naivikul and Sotanasomboon, 1989). Sprouting of mungbean seeds

for 72 hours can results in up to 45% reduction in phytic acid (Naivikul and Sotanasomboon, 1989). Loss of phytic acid during sprouting is due to the increased phytase activity. There are many gene/QTL mapping reports on seed phytic acid in crops but none on seedling phytic acid. This is the first report on QTLs on phytic acid in seedling. Among the six QTLs identified for the P compounds in seedling, only one (*SLPAP8.1*) shows co-localization with the QTLs for P compounds in seed (*SDIP8.1*) (Figure 11). However, *SLPAP8.1* had very small effect. This is expected because seed development requires P compounds formation while seedling development (germination) involves P compounds degradation. Co-location of seedling total P QTL (*SLTP7.1*) and seedling phytic acid QTL (*SLPAP7.1*), and of seedling total P QTL (*SLTP4.1*) and seedling inorganic P QTL (*SLPAP7.1*) (Figure 11) supports their significant correlation (Table 11). Therefore, breeding for phytic acid in seed and phytic acid in seedling must select for different QTLs and can be independently conducted.

Five seed weight QTLs were detected. The number is close to seed size QTLs that found in closely related *Vigna* species. Isemura *et al.* (2007) found six seed size QTLs in azuki bean and Isemura *et al.* (2010) found seven seed size QTLs in rice bean [*Vigna umbellata* (Thunb. Ohwi and Ohashi]. Mungbean, azuki bean and rice bean belong to the subgenus *Ceratotropis* (Asian *Vigna*), the comparative genome mapping reveal high genome conservation among species in this taxon (Chaitieng *et al.*, 2006; Isemura *et al.*, 2010). Comparison of common SSR markers revealed that seed weight QTLs detected on LGs 1 and 2 of mungbean were conserved in azuki bean (Isemura *et al.*, 2007; Kaga *et al.*, 2008) and rice bean (Isemura *et al.*, 2010), while the QTLs on LG 9 was conserved in azuki bean (Isemura *et al.*, 2007).

Three of seed weight QTLs were mapped to the same linkage groups in which seed inorganic P QTLs localized onto, although no significant correlation between the two traits. QTLs *SD100WT10.1* and *SDIP10.1* were overlapped. Similar results was reported by Blair *et al.* (2009) who found that all the three QTLs detected for seed weight in common bean were on the same linkage with seed P (net P content in the their paper) QTLs in which one seed weight QTL was overlapped with seed P QTLs.

Although there is a significant correlation between seedling phytic acid and seed weight, only minor QTL for seedling phytic acid P (*SLPAP8.1*) was mapped distantly to the same linkage group with seed weight QTL (*SD100WT8.1*) (Figure 11). Therefore, selection for low seed and seedling phytic acid can be achieved without or with a minimum effect of seed size. A similar conclusion was reported for common bean (Blair *et al.*, 2009).

Three and two QTLs were detected respectively for flowering and maturity times. Both flowering QTLs co-located with maturity QTLs (LGs 4A and 7) confirming high and significant correlation between the traits. Again, based on common marker comparison, QTLs for flowering on LG 4A appeared to be conserved in azuki bean (Kaga *et al.*, 2008; Isemura *et al.*, 2007) and rice bean (Isemura *et al.*, 2010), while QTL for maturity the same LG was conserved in only azuki bean (Kaga *et al.*, 2008). The QTLs on LG 4 for flowering and maturity in all the three crop species are major effect QTL. These further confirm high genome conservation in the Asian *Vigna* group.

On LG 4, maturity QTL overlapped with seed phytic acid P QTL, and flowering QTL moderately linked with the seed phytic acid P QTL. These support their significant correlation. Since at these major QTLs, the cultivated parent V1725BG provided the alleles for increased seed phytic acid P, flowering and maturity; thus selection for alternative QTLs alleles would result in low seed phytic acid genotypes accompanying by early flowering and maturity. Similarly, on LG 7 maturity QTL overlapped with one of the two QTLs, *SLPAP7.2*, for seedling phytic acid P. In addition *SLPAP7.2* located not far from flowering QTL. Again, at these QTLs V1725BG's alleles contributed higher seedling phytic acid P, but not time to flowering and maturity. Therefore selection for low seedling phytic acid together with early flowering and maturity mungbean lines is impossible to use the overlapped QTLs.

This thesis is the first to report QTL mapping for phytic acid P and P compound contents in seed and seedling of mungbean. They were analyzed from an

experimental field grown in one location and one season without replication, so application of the QTLs in marker-assisted selection (MAS) for improvement of mungbean variety in the contents should be confirmed.

# CONCLUSION AND RECOMMENDATION

## Conclusion

From the experimental results of this study, the conclusion can be drawn as follow:

1. V1137BG, V1725BG, AusTRCF321936, and Sukhothai were the highest phytic acid P in seed, while AusTRCF321925 was the lowest.

2. The estimates of narrow-sense heritability of phytic acid P content in seeds of the four populations ranged from 0.12 to 0.88, that of inorganic P ranged from 0.30 to 0.61, and that of total P varied from 0.11 to 0.69.

3. Inorganic P content increased and phytic acid P content decreased during germination, while total P content were rather stable.

4. The linkage map comprising 13 linkage groups were constructed from 101 polymorphic SSR markers.

5. QTL analysis for P compound contents and agronomic traits revealed that several markers associated with the traits and some QTLs were co-located.

## Recommendation

Selection for low seedling phytic acid together with early maturity mungbean lines can be obtained by developing an $F_2$ population and using the QTLs of the traits on LG7 (*DMT7.1* and *SLPAP7.2*) to select for plants showing repulsion phase of the markers linking to the traits.

# LITERATURE CITED


Akpapunam, M. 1996. Mungbean (*Vigna radiata* (L.) Wilczek), pp. 209-215. *In* E. Nwokolo and J. Smartt, eds. **Food and Feed from Legumes and Oil Seeds**. Chapman & Hall. England.

Andaya, C.B. and T.H. Tai. 2005. Fine mapping of the rice phytic acid (*LPA1*) locus. **Theor. Appl. Genet.** 111(3): 489-495.

Ashton, W.M. and P.C. Williams. 1958. The phosphorus compounds of oats. I. The content of phytate phosphorus. **J. Sci. Food Agric.** 9: 505-511.

Auesukaree, C., H. Tochio, M. Shirakawas, Y. Kaneko and S. Harashima. 2005. Plc1p, Agr82p, and Kcs1P, enzymes involved in inositol pyrophosphate synthesis, are essential for phosphate regulation and polyphosphate accumulation in *Saccharomyces cerevisiae*. **J. Biochem.** 280(26): 25127-25133.

Barrientos, L.G. and P.P.N. Murthy. 1996. Conformational studies of *myo*-inositol phosphates. **Carbohydrate Res.** 296: 39-54.

Benchimol, L.L., T. de Campos, S.A.M. Carbonell, C.A. Colombo, A.F. Chioratto, E.F. Formighieri, L.R.L. Gouvea and A.P. de Souza. 2007. Structure of genetic diversity among common bean (*Phaseolus vulgaris* L.) varieties of Mesoamerican and Andean origins using new developed microsatellite markers. **Genet. Resour. Crop Evol.** 54: 1747-1762.

Bilyeu, K.D., P. Zeng, P. Coello, Z.J. Zhang, H.B. Krishnan, A. Bailey, P.R. Beuselinck and J.C. Polacco. 2008. Quantitative conversion of phytate to inorganic phosphorus in soybean seeds expressing a bacterial phytase. **Plant Physiol.** 146: 468-477.

Bentsink, L., K. Yuan, M. Koornneef and D. Vreugdenhil. 2003. The genetics of phytate and phosphate accumulation in seeds and leaves of *Arabidopsis thaliana*, using natural variation. **Theor. Appl. Genet.** 106: 1234-1243.

Blair, M. W., T.A. Sandoval, G.V. Caldas, S.E. Beebe and M.I. Paez. 2009. Quantitative trait locus analysis of seed phosphorus and seed phytate content in a recombinant inbred line population of common bean. **Crop Sci.** 49: 237-246.

Brooks, J.R. and C.V. Morr. 1984. Phosphorus and phytate content of soybean protein components. **J. Agric. Food Chem.** 32: 674-676.

Buso, G.S.C., Z.P.S. Amaral, R.P.V. Brondani and ME. Ferreira 2006. Microsatellite markers for the common bean *Phaseolus vulgaris*. **Mol. Ecol. Notes** 6: 252-254.

Campion, B., F. Sparvoli, E. Doria, G. Tagliabue, I. Galasso, M. Fileppi, R. Bollini and E. Nielsen. 2009. Isolation and characterization of an *lpa* (low phytic acid) mutant in commom bean (*Phaseolus vulgaris* L.). **Theor. Appl. Genet.** 118: 1211-1221.

Cichy K.A., G.V. Caldas, S.S. Snapp and M.W. Blair. 2009. QTL analysis of seed iron, zinc, and phosphorus levels in an Andean bean population. **Crop Sci.** 49: 1742-1750.

Chen, P.S., I.T.Y. Toribara and H. Warner. 1956. Microdetermination of phosphorus. **Anal Chem.** 28: 1756 - 1758.

Chen, L. H. and S. H. Pan. 1977. Decrease of phytate during germination of pea seeds (*Pisum sativum*). **Nutr. Rep. Int.** 16: 125- 131.

Chaitieng, B., A. Kaga, N. Tomooka, T. Isemura, Y. Kuroda and D.A. Vaughan. 2006. Development of a black gram (*Vigna mungo* (L.) Hepper) linkage map

and its comparison with an azuki bean (*Vigna angularis* (Willd.) Ohwi and Ohashi) linkage map. **Theor. Appl. Genet.** 113: 1261-1269.

Chitra, U., U. Singh and P.V. Rao. 1996. Phytic acid, *in vitro* digestibility, dietary fiber, and minerals of pulses as influenced by processing methods. **Plant Foods Hum. Nutr.** 49(4): 307-316.

_____ V. Vimala and P. Geervani. 1995. Variability in phytic acid content and protein digestibility of grain legumes. **Plant Foods Hum. Nutr.** 47(2): 163-172.

Coelho, C.M.M., J.C.P. Santos, S.M. Tsai and V.A. Vitorello. 2002. Seed phytate content and phosphorus uptake and distribution in dry bean genotypes. **Braz. J. Plant** 14(1): 623-628.

de Boland, A.R., G.B. Garner and B.L. O'Dell. 1975. Identification and properties of phytate in cereal grains and oilseed products. **J. Agric. Food Chem.** 23: 1186-1189.

Dieckert, J.W., J.E. Snowden, Jr., A.T. Moore, D.C. Heinzelman and A.M. Altschul. 1962. Composition of some subcellular fractions from seeds of arachis hypogaea. **J. Food Sci.** 27: 321-325.

Dorsch, J.A., A. Cook, K.A. Young, J.M. Anderson, A.T. Bauman, C.J. Volkmann, P.P.N. Murthy and V. Raboy. 2003. Seed phosphorus and inositol phosphate phenotype of barley low phytic acid genotypes. **Phytochem.** 62: 691-706.

Dost, K. and O. Tokul. 2006. Determination of phytic acid in wheat and wheat products by reverse phase high performance liquid chromatography. **Anal. Chim. Acta.** 558: 22-27.

Fixen, P.E., and I.A. Grove. 1990. Testing soils for phosphorus. pp. 141-180. *In* Westerman, R.L., eds. **Soil Testing and Plant Analysis**. SSSA, Inc., Madison, WI.

Gaitán-Solís, E., M.C. Duque, K.J. Edwards, and J. Tohme. 2002. Microsatellite repeats in common bean (*Phaseolus vulgaris*): isolation, characterization, and cross-species amplification in *Phaseolus* ssp. **Crop Sci.** 42: 2128-2136.

Gao, Y., R.M. Biyashew, M.A. Saghai Maroof, N.M. Glover, D.M. Tucker and G.R. Buss. 2008. Validation of low - phytate QTLs and evaluation of seedling emergence of low - phytate soybeans. **Crop Sci.** 48: 1355-1364.

Graf, E., K.L. Empson and J.W. Eaton. 1987. Phytic acid a natural antioxidant. **J. Biol. Chem.** 262: 11647-11650.

Grases, F., J. Perello, P. Sanchis, B. Isern, R.M. Prieto, A. Costa-Bauza, C. Santiago, M.L. Ferragut and G. Frontera. 2009. Anticalculus effect of a triclosan mouthwash containing phytate: a double-blind, randomized, three-period crossover trial. **J. Periodont. Res.** 44: 616-621.

Guerra-Sanz, J.M. 2004. New SSR markers of *Phaseolus vulgaris* from sequence databases. **Plant Breeding** 123: 87-89.

Guttieri, M., D. Bowen, J. A., Dorsch, V. Raboy and E. Souza. 2004. Identification and characterization of a low phytic acid wheat. **Crop Sci**. 44: 418-424.

Gunawardena, S.F.B.N., S.K.A. Denso and F. Zapata. 1992. Phosphorus requirements and nitrogen accumulating by three mungbean (*Vigna radiata (*L.) Wilczek) cultivars. **Plant and Soil** 147: 267-274.

Gwag, J.G., W.K. Chung, H.K. Chung, J.H. Lee, K.H. Ma, A. Dixit, Y.J. Park, E.G. Cho, T.S. Kim and S.H. Lee. 2006. Characterization of new microsatellite markers in mungbean, *Vigna radiata* (L.). **Mol. Ecol. Notes** 6: 1132-1134.

Hall, J.R. and T.K. Hodges. 1966. Phosphorus metabolism of germinating oat seeds. **Plant Physiol.** 41: 1459-1464.

Han, O.K., A. Kaga, T. Isemura, X.W. Wang, N. Tomooka and D.A. Vaughan. 2005. A genetic linkage map for azuki bean (*Vigna angularis* (Willd.) Ohwi & Ohashi). **Theor. Appl. Genet.** 111: 1278-1287.

Hanakahi, L.A., M.B. Jones, C. Chappell, D. Pappin and S.C. West. 2000. Binding of inositol phosphate to DNA-PK and stimulation of double-strand break repair. **Cell** 102: 721-729.

Hatzack, F., F. Hubel, W. Zhang, P.E. Hansen and S.K. Rasmussen. 2001. Inositol phosphates from barley low-phytate grain mutants analysed by metal-dye detection HPLC and NMR. **Biochem. J**. 354: 473-480.

Harland, B.F. and D. Oberleas. 1987. Phytate in foods. **World Rev. Nutr. Diet.** 52: 235-259.

Haug, W. and H.I. Lantzsch. 1983. A sensitive method for rapid determination of phytate in cereals and cereal products. **J. Sci. Food Agric.** 34: 1423-1426.

Hitz, W.D., T.J. Carlson, P.S. Kerr and S.A. Sebastian. 2002. Biochemical and molecular characterization of a mutation that confers a decreased raffinosaccharide and phytic acid phenotype on soybean seeds. **Plant Physiol.** 128: 650-660.

Hix, D.K., C.F. Klopfenstein and C.E. Walker. 1997. Physical and chemical attributes and consumer acceptance of sugar-snap cookies containing naturally occurring antioxidants. **Cereal Chem.** 74(3): 281-283.

Hu, G., K. Peterson, H. Saneoka, P. Bregitzer, N. Wisniewski and V. Raboy. 2008. A screen for high seed inorganic P mutations in barley (*Hordeum vulgare* L.) reveals at least six low phytic acid loci and one high seed total P locus. **Theor. Appl. Genet.** 117: 19-28.

Isemura, T., A. Kaga, N. Tomooka, T. Shimizu and D.A. Vaughan. 2010. The genetics of domestication of rice bean, *Vigna umbellata*. **Ann. Bot.** 106: 927-944.

Isemura, T., A. Kaga, S. Konishi, T. Ando, N. Tomooka, O.K. Han and D.A. Vaughan. 2007. Genome dissection of traits related to domestication in azuki bean (*Vigna angularis*) and comparison with other warm-season legumes. **Ann. Bot.** 100(5): 1053-1071.

Kaga, A., T. Isemura, N. Tomooka and D.A. Vaughan. 2008. The genetics of domestication of azuki bean (*Vigna angularis*). **Genetics** 178: 1013-1036.

Kataria, A., B.M. Chauhan and D. Punia. 1989. Antinutrients in amphidiploids (blackgram × mungbean): varietal differences and effect of domestic processing and cooking. **Plant Foods Hum. Nutr.** 39: 257-266.

Kearsey, M.J. and H.S. Pooni. 1996. **The genetical analysis of quantitative traits.** Chapman&Hall. England.

Kosambi, D.D. 1944. The estimation of map distance from recombination values. **Ann. Eugen.** 12:172-175.

Kuwano, M., A. Ohyama, Y. Tanaka, T. Mimura, F. Takaiwa and K.T. Yoshida. 2006. Molecular breeding for transgenic rice with low-phytic acid phenotype through manipulating *myo*-inositol 3-phosphate synthase gene. **Mol. Breed.** 18(3): 263-272.

Larson, S.R., J.N. Rutger, K.A. Young and V. Raboy. 2000. Isolation and genetic mapping of a non-lethal rice (*Oryza sativa* L.) *low phytic acid 1* mutation. **Crop Sci.** 40: 1397-1405.

LeClerg, E.L., W.H. Leonard and A.G. Clark. 1962. **Field plot technique**, 2nd ed. Burgess Publishing Co., MN

Lemtiri-Chlieh, F., E.A.C. MacRobbie and C.A. Brearley. 2000. Inositol hexa*kis*phosphate is a physiological signal regulating the K1-inward rectifying conductance in guard cells. **Proc. Natl. Acad. Sci. U.S.A.** 97(15): 8687-8692.

Li, C.D., C.A. Fatokun, B. Ubi, B.B. Singh and G.J. Scoles. 2001. Determining genetic similarities and relationships among cowpea breeding lines and cultivars by microsatellite markers. **Crop Sci.** 41: 189-197.

Lin, L., I. Okenden and J.N.A. Lott. 2005. The concentration and distribution of phytic acid-phosphorus and other mineral nutrients in wild-type and *low phytic acid* 1-1 (*lpa1-1*) corn (*Zea mays* L.) grains and grain parts. **Can. J. Bot.** 83: 131-141.

Liu, Q.L., X.H. Xu, X.L. Ren, H.W. Fu, D.X. Wu and Q.Y. Shu. 2007. Generation and characterization of low phytic acid germplasm in rice (*Oryza sativa* L.). **Theor. Appl. Genet.** 114: 803-814.

Lodhi, M.A., G.N. Ye, N.F. Weeden, and B.I. Reisch. 1994. A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. **Plant Mol. Biol. Rep.** 12: 6-13.

Lorenz, A.J., M.P. Scott and K.R. Ramkey. 2008. Genetic variation and breeding potential of phytate and inorganic phosphorus in a maize population. **Crop Sci.** 48: 79-84.

Mebrahtu, T., A. Mohamed and A. Elmi. 1997. Accumulation of phytate in vegetable soybean genotypes harvested at four developmental stages. **Plant Food Hum. Nutr.** 50: 179-187.

Miller, W.E., T.E. Maloney and J.C Greene. 1974. Algal productivity in 49 lake waters as determined by algal assays. **Water Res.** 8: 667-679.

Morton, R.K. and J.K. Raison. 1963. A complete intracellular unit for incorporation of amino-acid into storage protein utilizing adenosine triphosphate generated from phytate. **Nature** 200(4905): 429-433.

Mubarak, A.E. 2005. Nutritional composition and antinutritional factors of mung bean seeds (*Phaseolus aureus*) as affected by some home traditional processes. **Food Chem.** 89: 489-495.

Naivikul, O. and P. Sotanasomboon. 1989. Nutrient contents and antinutritional factors of mungbean sprouts. **J. Agri. Sci.** 23: 188-197. (in Thai with English abstract)

Nelson, D.E., G. Rammesmayer, H.J. Bohnert. 1998. Regulation of cell-specific inositol metabolism and transport in plant salinity response. **Plant Cell** 10: 753-764.

Nunes, A.C.S., G.R. Vianna, F. Cuneo, J.F. Amaya, G.D. Capdeville, E.L. Rech and F.J.L. Aragao. 2006. RNAi-mediated silencing of the *myo*-inositol-1-phosphate synthase gene (*GmMIPS1*) in transgenic soybean inhibited seed development and reduced phytate content. **Planta.** 224: 125-132.

Oatway, L, T. Vasanthan and J. Helm. 2001. Phytic acid. **Food Rev. Int.** 17: 419-431.

Oltmans, S.E., W.R. Fehr, G.A. Welke and S.R. Cianzio. 2004. Inheritance of low-phytate phosphorus in soybean. **Crop Sci.** 44: 433-435.

_____, V. Raboy and K.L. Peterson. 2005. Agronomic and seed traits of soybean lines with low-phytate phosphorus. **Crop Sci.** 45: 593-598.

Pederson, H.B., L.D. Sorensen and P.B. Holm. 2002. Engineering crop plants: Getting a handle on phosphate. **Trend Plant Sci.** 7(3): 118-125.

Peleman, J.D. and J.R.V.D. Voort. 2003. Breeding by design. **Trends Plant Sci.** 8(7): 330-334.

Pen, J., T.C. Verwoerd, P.A.V. Paridon, R.F. Beudeker, P.J.M.V. Elzen, K. Geerse, J.D.V. Klis, H.A.J. Versteegh, A.J.J.V. Ooyen and A. Hoekema. 1993. Phytase-containing transgenic seeds as a novel feed additive for improved phosphorus utilization. **Biotechnol.** 11: 811-814.

Pilu, R., M. Landoni, E. Cassani, E. Doria and E. Nielsen. 2005. The maize *lpa241* mutation causes a remarkable variability of expression and some pleiotropic effects. **Crop Sci.** 45: 2096-2105.

_____, D. Panzeri, G. Gavazzi, S.K. Rasmussen, G. Consoni and E. Nielsen. 2003. Phenotypic, genetic and molecular characterization of a maize low phytic acid mutant (*lpa241*). **Theor. Appl. Genet.** 107: 980-987.

R Development Core Team. 2008. **R: a language and environment for statistical computing**. R Foundation for Statistical Computing, Vienna, Austria. ISBN: 3-900051-07-0. Available Source: http://R-project.org, April 10, 2009.

Raboy, V. 2001. Seeds for a better future: 'low phytate' grains help to overcome malnutrition and reduce pollution. **Trend Plant Sci.** 6(10): 458-462.

_____. 2002. Progress in breeding low phytate crops. **J. Nutr.** 132(3): 503-505.

_____. 2003. Molecules of interest: *myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate. **Phytochem.** 64: 1033-1043.

_____. 2009. Approaches and challenges to engineering seed phytate and total phosphorus. **Plant Sci.** 177: 281-296.

_____, D.B. Dickinson, and F.E. Below. 1984. Variation in seed total phosphorus, phytic acid, zinc, calcium, magnesium, and protein among lines of *Glycine max* and *G. soja*. **Crop Sci.** 24: 431-434.

_____, P.F. Gerbasi, K.A. Young, S.D. Stoneberg, S.G. Pickett, A.T. Bauman, P.P.N. Murphy, W.F. Sheridan and D.S. Ertl. 2000. Origin and seed phenotype of maize *low phytic acid 1-1* and *low phytic acid 2-1*. **Plant Physiol.** 124: 355-368.

Raghavendra, P and P.M. Halami. 2009. Screening, selection and characterization of phytic acid degrading lactic acid bacteria from chicken intestine. **Inter. J. Food Microbiol.** 133: 129-134.

Safrany, S.T., J.J. Cafrey, X. Yang and S.B. Shears. 1999. Diphosphoinositol polyphosphates: the final frontier for inositide research? **Biol. Chem.** 380: 945-951.

Saiardi, A., C. Sciambi, J.M. McCaffery, B. Wendland and S.H. Snyder. 2002. Inositol pyrophosphates regulate endocytic trafficking. **PNAS.** 99(22): 14206-14211.

Saio, K., D. Gallant and L. Petit. 1977. Electron microscope research on sunflower protein bodies. **Cereal Chem.** 54: 1171-1181.

Sasakawa, N., M. Sharif and M.R. Hanley. 1995. Metabolism and biological activities of inositol penta*kis*phosphate and inositol hexa*kis*phosphate. **Biochem. Pharmacal.** 50(2): 137-146.

Sathe, S.K., S.S. Deshpande, N.R. Reddy, D.E. Goll and D.K. Salunkhe. 1983. Effects of germination on proteins, raffinose oligosaccharides and antinutritional factors in the great northern bean (*Phaseolus vulgaris* L.). **J. Food Sci.** 48: 1796-1800.

Seehalak, W., P. Somta, W. Musch, P. Srinives. 2009. Microsatellite markers for mungbean developed from sequence database. **Mol. Ecol. Resour.** 9: 862-864.

Sharpley, A.N., S.C. Charpa, R. Wedepohl, J.Y. Sims, T.C. Daniel, and K.R. Reddy. 1994. Managing agricultural phosphorus for protection of surface waters: issues and options. **J. Envi. Qual.** 23: 437-451.

Shi, J., H. Wang, J. Hazebroek, D.S. Ertl and T. Harp. 2005. The maize low-phytic acid 3 encodes a *myo*-inositol kinase that plays a role in phytic acid biosynthesis in developing seeds. **Plant J.** 42: 708-719.

_____, K. Schellin, B. Li, M. Faller, J.M. Stoop, R.B. Meeley, D.S. Ertl, J.P. Ranch and K. Glassman. 2007. Embryo-specific silencing of a transporter reduces phytic acid content of maize and soybean seeds. **Nature Biotech.** 25: 930-937.

_____, Y. Wu, J. Hazebroek, R.B. Meeley and D.S. Ertl. 2003. The maize low-phytic acid mutant *lpa2* is caused by mutation in an inositol phosphate kinase gene. **Plant Physiol.** 131: 507-515.

Sompong, U., S. Nakasathien, C. Kaewprasit and P. Srinives. 2007. Variation in phytic acid content in seed and correlation with agronomic traits of mungbean (*Vigna radiata* (L.) Wilczek). **Agri. Sci. J.** 38: 243-250. (in Thai with English abstract)

_____. 2010. Inheritance of seed phytate in mungbean (*Vigna radiata* (L.) Wilczek). **Euphytica** 171(3): 389-396.

Somta, P., A. Kaga, N. Tomooka, K. Kashiwaba, T. Isemura, B. Chaitieng, P. Srinives, and D. A. Vaughan. 2006. Development of an interspecific *Vigna* linkage map between *Vigna umbellata* (Thunb.) Ohwi & Ohashi and *V. nakashimae* (Ohwi) Ohwi & Ohashi and its use in analysis of bruchid resistance and comparative genomics. **Plant Breed.** 125: 77-84.

Somta, P. and P. Srinives. 2007. Genome research in mungbean [*Vigna radiata* (L.)Wilczek] and blackgram [*V. mungo* (L.) Hepper]. **SciAsia** 33: 69-74.

Somta, P., W. Musch, B. Kongsamai, S. Chanprame, S. Nakasathien, T. Toojinda, W. Sorajjapinun, W. Seehaluk, S. Tragoonrung, and P. Srinives. 2008. New microsatellite markers isolated from mungbean (*Vigna radiata* (L.) Wilczek). **Mol . Ecol. Resour.** 8: 1155-1157.

Stangoulis, J.C.R., B.L. Huynh, R.M. Welch, E.Y. Choi and R.D. Graham. 2006. Quantitative trait loci for phytate in rice grain and their relationship with grain micronutreint content. **Euphytica** 154: 289-294.

Sulieman, M.A., M.M. Eltyeb, M.A. Abbass, E.E.A. Ibrahim, E.E. Babiker and A.H. Eltinay. 2007. Changes in chemical composition, phytate, phytase activity and minerals extractability of sprouted lentil cultivars. **J. Biol. Sci.** 7(5): 776-780.

Sutton, A.L., S. Radciffe and B.C. Joren. 2004. **Overview of phosphorus issues in swine feeding**. Available Source: http://www.livestocktrail.uiuc.edu/sowm/paperdisplay.cfm?contentid=6509, January 19, 2010.

Tabekhia, M.M. and B.S. Luh. 1980. Effect of germination, cooking, and canning on phosphorus and phytate retention in dry beans. **J. food Sci.** 45(2): 406-408.

Talamond, P., S. Doulbeau, I. Rochette, J. P. Guyot and S. Treche. 2002. Anion-exchange high-performance liquid chromatography with conductivity: detection for the analysis of phytic acid in food. **J. Chrom. A.** 871: 7-12.

Thangphatsornruang, S., P. Somta, P. Uthaipaisanwong, J. Chanprasert, D. Sangsrakru, W. Seehalak, W. Sommanas, S. Tragoonrung, and P. Srinives. 2009. Characterization of microsatellites and gene contents from genome shotgun sequences of mungbean (*Vigna radiata* (L.) Wilczek). **BMC Plant Biol.** 9: 137.

Thavarajah, P., D. Thavarajah and A.Vandenberg. 2009. Low phytic acid lentils (*Lens culinaris* L.): a potential solution for increased micronutrient bioavailability. **J. Agric. Food Chem.** 57: 9044-9049.

Thompson, L.U. and L. Zhang. 1991. Phytic acid and minerals: effect on early markers of risk for mammary and coloncarcinogenesis. **Carcinogenesis** 12: 2041-2045.

Uppstrom, B. and R. Svensson. 1980. Determination of phytic acid in rapeseed meal. **J. Sci. Food Agric.** 31: 651–656.

Urbano, G., M. López-Jurado, P. Aranda, C. Vidal-Valverde, E. Tenorio and J. Porres. 2000. The role of phytic acid in legumes: antinutrient or beneficial function? **J. Physiol. Biochem.** 56(3): 283-294.

Uttatam, A. 2001. **Phytic acid, protein, iron and calcium in seeds and products of two soybean cultivars, KUSL20004 and Chakkapan 1**. M.S. thesis, Kasetsart University. (in Thai with English abstract)

van Ooijen, J.W. and R.E. Voorrips. 2003. **JoinMap®3.0: software for the calculation of genetic linkage maps.** Plant Research International, Wageningen

Verdcourt, B. 1970. Studies in the leguminosae-papilionoidae for the flora of tropical East Africa, **IV. Kew Bull.** 24: 507-569.

Veum, T.L., D.R. Ledoux, D.W. Bollinger, V. Raboy and A. Cook. 2002. Low-phytic acid barley improves calcium and phosphorus utilization and growth performance in growing pigs. **J. Anim. Sci.** 80: 2663-2670.

Viveros, A., C. Centeno, A. Brenes, R. Canales and A. Lozano. 2000. Phytase and acid phosphatase activities in plant feed stuffs. **J. Agri. Food Chem.** 48: 4009-4013.

Walker, D.R., A.M. Scaboo, V.R. Pantalone, J.R. Wilcox and H.R. Boerma. 2006. Genetic mapping of loci associated with seed phytic acid content in CX1834-1-2 soybean. **Crop Sci.** 46: 390-397.

Wang, S., C.J. Basten, and Z.B. Zeng. 2007. **Window QTL Cartographer 2.5**. Department of Statistics, North Carolina State University, Raleigh, NC, USA. Available Source: http://statgen.ncsu.edu/qtlcart/WQTLCart.htm, October 5, 2009

Wang, X.W., A. Kaga, N. Tomooka, and D.A. Vaughan. 2004a. The development of SSR markers by a new method in plants and their application to gene flow studies in azuki bean [*Vigna angularis* (Willd.) Ohwi and Ohashi]. **Theor. Appl. Genet.** 109: 352-360.

Wang, Y., C. Ribot, E. Rezzonico and Y. Poirier. 2004b. Structure and expression profile of the Arabidopsis *PHO1* gene family indicates a broad role in inorganic phosphate homeostasis. **Plant Physiol.** 135: 400-411.

Wardyn, B.M. and W.K. Russell. 2004. Resource allocation in a breeding program for phosphorus concentration in maize grain. **Crop Sci.** 44: 753-757.

Wilcox, J.R., G.S. Premachadra, K.A. Young, and V. Raboy. 2000. Isolation of high seed inorganic P, low-phytate soybean mutatnts. **Crop Sci.** 40: 1601-1605.

York, J.D., A.R. Odom, R. Murphy, E.B. Ives and S.R. Wente. 1999. A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. **Science** 285: 96-100.

Yuan, F.J., H.J. Zhao, X.L. Ren, S.L. Zhu, X.J. Fu and Q.Y. Shu. 2007. Generation and characterization of two novel low phytate mutations in soybean (*Glycine max* L. Merr.). **Theor. Appl. Genet.** 115: 945-957.

Zhao, J., D.C.L. Jamar, P. Lou, Y. Wang. J. Wu, X. Wang, G. Bonnema, M. Koornneef and D. Vreugdenhil. 2008. Quantitative trait loci analysis of phytate and phosphate concentrations in seeds and leaves of *Brassica rapa*. **Plant Cell Envi.** 31: 887-900.

Zhou, J.R. and J.W. Erdman Jr. 1995. Phytic acid in health and disease. **Crit. Rev. Food Sci. Nutr.** 35: 495-508.

# APPENDIX

**Appendix Table 1** Sources of SSR markers used in checking polymorphism among the mapping parents, V1725BG and AusTRCF321925

| Sources | Marker name | Methods of development | Number of markers | | |
|---|---|---|---|---|---|
| | | | screened | amplified | polymorphic |
| Mungbean | DMB-SSR | Data mining | 251 | 207 | 1 |
| | GBssr-MB | Enrichment | 5 | 5 | 2 |
| | MB-SSR | Enrichment | 171 | 109 | 16 |
| | MSR | Data mining | 22 | 19 | 0 |
| | VR | Pyrosequencing | 48 | 48 | 28 |
| | VR-SSR | Pyrosequencing | 49 | 49 | 5 |
| Azuki bean | CEDG | Enrichment | 142 | 112 | 43 |
| Cowpea | CLM | Data mining | 51 | 46 | 4 |
| | cp | Data mining | 118 | 93 | 14 |
| | VM | Enrichment | 20 | 15 | 2 |
| Common bean | BM | Enrichment | 3 | 3 | 3 |
| | Bmd | Data mining | 78 | 37 | 3 |
| | IAC | Enrichment | 24 | 18 | 2 |
| | PVBR | Data mining | 9 | 2 | 2 |
| | | Total | 991 | 763 | 125 |

**Appendix Table 2** Special thanksgiving for help

| Organization | Name |
|---|---|
| Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen | The Laboratory of Plant Molecular Biology and Transformation<br>The Laboratory of Plant Physiol-Molecular Biology |
| Faculty of Agriculture at Kamphaeng Saen | The Laboratory of Department of Soil Science |
| The Project on Biotechnology for Varietal Development of Thai Mungbean | Dr. Worapa Seehaluk<br>Mrs. Warunee Musch<br>Mr. Sompong Chankaew<br>Ms. Rattanakorn Kritsanachandee<br>Mr. Tanaporn Kajornpol<br>Ms. Jidapa Moongkanna<br>Dr. Sukumaporn Sriphadej<br>Dr. Chontira Sangsiri<br>Mr. Worawit Sorajjapinun<br>Mr. Prayoon Prathet<br>Ms. Malee Panngam<br>Ms. Tarika Yimram<br>Mrs. Suchada Rangkasiwit |
| Myanmar Agriculture Service Union of Myanmar | Mr. Nyi Nyi |
| Maejo University | Assist. Prof. Dr. Ruangchai Juwattanasamran<br>Dr. Pornpan Pooprompan<br>Dr. Kassinee Sitthiwong |
| Idaho State University | Assist. Prof. Dr. Debra Shain |
| University of Idaho | Assist. Prof. Dr. Jianli Chen |

**Appendix Table 2** (Continued)

| Organization | Name |
|---|---|
| Agricultural Research Service - United State Department of Agriculture (ARS-USDA), Aberdeen, Idaho, USA | Dr. Mike Bonman<br>Dr. Rebekah Ellen Oliver<br>Dr. Blair Goates<br>Dr. Kashun Liu<br>Dr. Gongshe Hu<br>Dr. Jonathan Whitworth<br>Ms. Amanda Lewis<br>Mr. Michael Woolman<br>Mrs. Kathy Satterfield<br>Mrs. Rose Wride<br>Mrs. Glenda Rutger |

# CIRRICULUM VITAE

**NAME** : Ms. Utumporn Sompong

**BIRTH DATE** : December 21, 1980

**BIRTH PLACE**: Bangkok, Thailand

**EDUCATION** :

| **YEAR** | **INSTITUTE** | **DEGREE/DIPLOMA** |
|---|---|---|
| 2000 | Chulalongkorn Univ. | B.Sc. (1st Class Honours) (Biochemistry) |
| 2006 | Kasetsart Univ. | M.S. (Agricultural Biotechnology) |

**POSITION/TITLE** : -

**WORK PLACE** : -

**SCHOLARSHIPS/AWARDS** :

1. Faculty of Science, Chulalongkorn Univ. Undergraduate Student - Support Scholarship 1998

2. Chumpot-Pantip Scholarship, Suan Pak Kard Palace, Bangkok, Thailand 1999-2000

3. Research Assistant Scholarship, Center for Agricultural Biotechnology, Kasetsart Univ. 2003-2004

4. A Scholarship for Supporting Thesis of Graduate School of Kasetsart Univ. 2005

5. The Royal Golden Jubilee (RGJ) Ph.D. Program Scholarship of the Thailand Research Fund 2007-2009