Biochemical and molecular characterization of two low-phytate pea lines

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by

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ABSTRACT

Phytate is the major storage form of phosphorus in crop seeds, but is not well digested by humans and non-ruminant animals. In addition, phytate chelates several essential micronutrients which are also excreted contributing to phosphorus pollution in the environment. This research was aimed at the biochemical and molecular characterization of two low phytate pea mutant lines, 1-150-81 and 1-2347-144 developed at the Crop Development Centre, University of Saskatchewan in collaboration with Dr. Victor Raboy, USDA, Idaho. Low phytic acid (*lpa*) crops are low in phytic acid and high in inorganic phosphorus (P_i). In Study I, two *lpa* pea genotypes, 1-150-81, 1-2347-144, and their progenitor CDC Bronco were evaluated in field trials for two years. The *lpa* genotypes did not significantly differ from CDC Bronco in all agronomic traits assessed except for lower seed weight and grain yield. The concentration of IP_6 at 14 DAF was not significantly different among CDC Bronco, 1-150-81 and 1-2347-144. However, the concentrations of IP_6 among CDC Bronco, 1-150-81 and 1-2347-144 started to differ significantly from 21 DAF onwards. The *lpa* genotypes 1-150-81 and 1-2347-144 showed 65% and 60% reduction in IP_6, respectively, when compared to their progenitor CDC Bronco at 49 DAF. The Pi concentrations between the *lpa* genotypes were similar and significantly higher than CDC Bronco from 21 DAF to 49 DAF. At 49 DAF, 1-150-81 and 1-2347-144 were 72 and 84% higher in Pi, respectively, than CDC Bronco. The total P concentration was similar in *lpa* genotypes and CDC Bronco throughout the seed development. This study elucidated the rate and accumulation of phosphorus compounds in *lpa* genotypes. In Study II, aiming at understanding the genetic basis of the *lpa* mutation in pea lines 1-150-81 and 1-2347-144, a 1530 bp open reading frame of *myo*-inositol phosphate synthase gene (MIPS) was amplified from CDC Bronco and the *lpa* genotypes. Sequencing results showed no difference in coding sequence in *MIPS* between CDC Bronco and *lpa* genotypes. Transcript levels of both *MIPS* and *myo*-inositol tetra*kis*phosphate1-kinase (*ITPK1)* were relatively lower at 49 DAF than at 14 DAF for CDC Bronco and *lpa* lines. There was no difference in expression level of both *MIPS* and *ITPK1* between CDC Bronco and the *lpa* genotypes at 49 DAF. The data demonstrated that mutation in *MIPS* was not responsible for *lpa* trait in pea. Study III was aimed at developing a single nucleotide polymorphism (SNP) based genetic linkage map and mapping genomic regions associated with phytic acid-phosphorus (PA-P) concentration using PR-15 recombinant inbred lines (RILs) derived from a cross between a low phytate (*lpa*) pea genotype, 1-2347-144 and

normal phytate pea cultivar CDC Meadow. A total of 163 RILs were genotyped using 1536 SNP markers in an Illumina GoldenGate array. Three hundred and sixty seven polymorphic SNP markers, ordered into 7 linkage groups (LGs), generated a linkage map with a total length of 437.2 cM. The phytic acid locus was mapped on to LG5. A quantitative trait locus (QTL) for iron bioavailability was mapped on to the same location in LG5 as phytic acid concentration. Potential benefits arising out of this research include improved bioavailability of phosphorus, iron and zinc in foods and feeds, less phosphorus excretion and environmental pollution and a saving in feed costs.

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LIST OF ABBREVIATIONS

ANOVA: analysis of variance

BLAST: Basic Local Alignment Search Tool

CDC: Crop Development Centre

cM: centiMorgan

CTAB: hexadecyltrimethylammonium bromide

DAF: days after flowering

DW: dry weight

ddH_2O: distilled deionized water

DNA: deoxyribose nucleic acid

FEBIO: iron bioavailability

FECON: total iron concentration

IP_3: *myo*-inositol-1,4,5-trisphosphate

IP_4: *myo*-inositol-1,4,5,6-tetra*kis*phosphate

IP_5: *myo*-inositol-1,3,4,5,6-penta*kis*phosphate

IP_6: phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate)

ITPK1: *myo*-inositol tetra*kis*phosphate 1-kinase

LG: linkage group

LOD: logarithm of the odds ratio

lpa: low-phytic acid

LSD: least significant difference

MIPS: *myo*-inositol-3-phosphate synthase

MQM: multiple QTL mapping

NCBI: National Center for Biotechnology

NS: not significant

ORF: open reading frame

P: phosphorus

PA-P: phytate-phosphorus

PCR: polymerase chain reaction

P_i: inorganic phosphorus concentration

ppm: parts per million

QTL: quantitative trait loci

RIL: recombinant inbred line

RT-PCR: reverse transcriptase-polymerase chain reaction

SD: standard deviation

SNPs: single nucleotide polymorphisms

sqRT-PCR: semi-quantitative reverse transcription PCR

UPGMA: Unweighted Pair Group Method with Arithmetic Mean

CHAPTER 1

1. Introduction

Field pea (*Pisum sativum* L.) belongs to the family of cool season legume crops (*Leguminosae*). Field pea contains high levels of the essential amino acids, lysine and tryptophan, which are usually low in cereal grains. Consequently, field pea can supplement the low amount of protein present in food and feed processed from cereal grains. Field pea flour is valued not only as a vegetable protein source but also, in part, due to its unique functional properties. Field pea seeds are low in fat and cholesterol free. The use of vegetable proteins as functional ingredients in the food industry is increasing and special attention has been given to the use of field peas because they are already an accepted part of the human diet throughout the world.

The high nutritional value of seeds including pea comes from the deposition of starch, proteins and essential minerals during seed development. In this process there is a corresponding accumulation of phytate, or inositol hexakisphosphate (IP_6), which typically comprises 1% of the dry weight and is responsible for approximately two thirds of total seed phosphorus (Raboy 2001). Researchers have identified phytic acid as a significant factor in phosphorous cycling in agricultural systems (Brinch-Pedersen et al. 2002). Natural variation in phytic acid and phosphorus levels in seeds has been demonstrated in *Arabidopsis thaliana* (Bentsink et al. 2003).

Negatively charged sites of phytic acid bind and form salts with K^{+}, Mg^{++}, Ca^{++}, Mn^{++}, Zn^{++}, Ba^{++}, and Fe^{+++}. In pea, phytate represents approximately 1.2% of the seed dry weight (Marquardt and Bell 1998), and therefore high concentrations of phosphorus and mineral cations bound to phytate are available in the seeds. Although the role of phytate reserves in seeds is poorly understood, its abundance in grain feed is known to cause nutritional and environmental problems. Monogastric animals are unable to digest phytate, which poses problems in release of phosphate, inositol, and essential minerals. High phytate, grain based diets are feared to exacerbate iron and zinc malnutrition. In countries where livestock is fed primarily grain-based feed, the excreted phytate contributes to environmental phosphorus pollution by washing into surface waters, where it accelerates eutrophication (Brinch-Pederson et al. 2002). Collectively, these problems have provided strong impetus to develop seeds with reduced phytate content.

Low phytate mutations have been induced in maize (*Zea mays* L.), soybean (*Glycine max* (L.) Merr.), barley (*Hordeum vulgare* L.), common bean (*Phaseolus vulgaris*) and rice (*Oryza sativa* L.), through chemical mutagenesis and this trait was successfully incorporated into commercial cultivars in these species (Larson et al. 2000; Raboy et al. 2000; Rasmussen and Hatzack 1998; Wilcox et al. 2000).

Plant seeds are a major source of nutrients for humans and animals. Adequate dietary phosphorus is essential for human health and optimal livestock production. Breeding programs require understanding of the major factors affecting total P and phytate accumulation and phytate-P to total P ratio in crops. Improvement in both quantity as well as quality of food is needed to cope with the increasing human population. The 'Green revolution' in cereals averted problems of starvation and has helped humanity to a great extent, but it did not address health problems related to deficiencies of vitamins and minerals. There exists a scope of improvement in quality of food (Guttieri et al. 2004).

Two low-phytate pea genotypes (1-2347-144 and 1-150-81) were isolated at the Crop Development Centre (CDC), University of Saskatchewan through chemical mutagenesis of cultivar CDC Bronco (Warkentin et al. 2012). Phytic acid phosphorus concentration in these genotypes was 60% lower than their progenitor CDC Bronco. These genotypes had consistently higher inorganic phosphorous concentration in the seeds over three generations in comparison to CDC Bronco. The agronomic performances of the low-phytate genotypes were similar to CDC Bronco, except for a slight delay in days to flowering and maturity, and slightly lower seed weight and grain yield (Warkentin et al. 2012). The low phytate trait in 1-150-81 and 1-2347-144 is controlled by a single gene (Rehman et al. 2012). These genotypes are characterized by higher iron bioavailability than CDC Bronco (Liu et al. 2014). The present research is aimed at characterization of these mutant genotypes at the biochemical and molecular levels. This research helped us to understand the nature of the low phytate mutation(s), develop genetic markers for the low phytate trait and set the stage for development of low phytate pea cultivars.

CHAPTER 2

2. Literature review

2.1 Phytic acid

Late 19th century chemists identified an abundant acid in several plant seeds that they termed as 'phytic acid' (Reddy et al. 1982). Subsequently shown to be *myo*-inositol (1, 2, 3, 4, 5, 6) hexakisphosphate (IP_6), this compound was first known as the storage form of phosphorus (P) in seeds (Cosgrove 1980). Phytic acid and its lower phosphorylated derivatives play a vital role in the regulation of many fundamental processes in plant and animal cells (Shears et al. 1996). Since a large fraction of nutrient P taken up by crop plants is ultimately packaged into seed phytic acid P, this single small molecule represents a major pool or bottleneck in the flux of P in the world's agricultural ecology. As an illustration, it was estimated that the global annual production of seed phytic acid P by crops represents a sum equivalent to more than 50% of the total elemental P applied world-wide in mineral fertilizers (Lott et al. 2000).

Applied interest in seed phytic acid primarily concerns its roles in human health and animal nutrition. It is a strong chelator of mineral cations such as calcium, iron and zinc forming mixed salts that are largely excreted by humans and other non-ruminant animals such as poultry, swine and fish (Erdman 1980, Sharply et al. 1994). Phytic acid-mineral complexes are insoluble and become unavailable for absorption to humans and non-ruminants, since they lack adequate amount of phytase enzyme in their intestine (Reddy et al. 1982, Krebs 2000). Phytic acid can also bind with negatively charged groups of proteins and starch molecules, which may result in a decrease of protein and starch digestibility and bioavailability (Cheryan 1980, Thompson 1993, Rickard et al. 1997). All these facts led some researchers to consider phytic acid to be an antinutrient. In addition, the undigested phytic acid-phosphorus excreted by non-ruminant animals, is an important component of phosphorus pollution in the environment (Raboy et al. 2001). Due to the nutritional and environmental concerns, development of cultivars with the low phytate trait has become an important breeding objective. However, studies have also shown the possible beneficial effects of phytic acid on human health. Phytic acid can lower serum cholesterol and triglycerides, offer protection against cardiovascular diseases, renal stone

formation, and several types of cancers (Zhou et al. 1995, Shamsuddin et al. 1997, Grases et al. 2000, Urbano et al. 2000, Graf 1983). Phytic acid is a molecule of great interest in several research areas such as human and animal nutrition, food processing, plant physiology and biochemistry, plant breeding and medical sciences (Feil 2001).

2.1.1 Structure of phytic acid

Hartig in 1855-1856 isolated small nonstarch grain particles from seeds, which were considered to be an essential source of nutrients for seed germination and plant growth (Rose 1912). Further characterization of those grains was performed by many researchers and subsequently names as 'inosite-phosphoric acid' (Reddy et al. 2002). The chemical nomenclature for phytic acid is *myo*-inositol (1, 2, 3, 4, 5, 6) hexakisphosphate. The molecular formula is $C_6H_{18}O_{24}P_6$ and molecular weight is 660.03. It is a compound in which all six hydroxyl groups of *myo*-inositol are esterified as phosphates. The hydroxyl group can be oriented in either an axial or equatorial manner, resulting in nine possible stereoisomers, the most common of which is the *myo* form (Schultz et al. 1996). Figure2.1 shows the numbering and structure of *myo*-inositol. The term 'phytate' or IP_6 refers to any salt of phytic acid. Phytate is either soluble or insoluble and can be in both dissolved and precipitated forms.

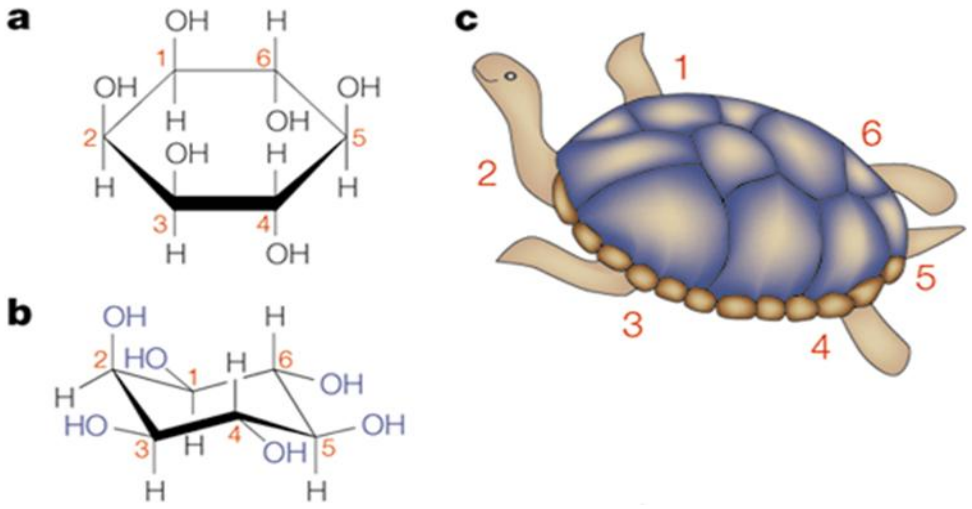

Fig.2.1. Structure of myo-inositol (a) *myo*-inositol as Haworth projection, (b) "chair" representation showing thermodynamically stable conformation of *myo*-inositol *in vivo*, (c)

Agranoff noted that if the hydrogens are ignored, the structure superficially resembles a turtle (Irvine et al. 2001)

At physiological pH, inositol and its phosphorylated derivatives adopt a thermodynamically stable, staggered chair conformation, with one axial and five equatorial groups (Barrientos and Murthy 1996), that has been said to resemble a turtle (Agranoff 1978). The numbering of carbons begins with the turtle's front right flipper and proceeds in an anticlockwise direction around the ring.

Apart from phytic acid, there are five other *myo*-inositol phosphates depending on the number of phosphate groups attached to the inositol ring (Murthy 1996). Table 2.1 describes the other *myo*-inositol phosphates and their accepted abbreviations.

Table 2.1. List of *myo*-inositol phosphates present in plants and their abbreviations (from Shears and Turner 2007).

Full name	**Number of Phosphate groups**	**IUPAC abbreviation**	**Common abbreviation**
myo-inositol	0	Ins	Ins
myo-inositol monophosphate	1	Ins P_1	IP_1
myo-inositol bisphosphate	2	Ins P_2	IP_2
myo-inositol trisphosphate	3	Ins P_3	IP_3
myo-inositol tetraphosphate	4	Ins P_4	IP_4
myo-inositol pentakisphosphate	5	Ins P_5	IP_5
myo-inositol hexakisphosphate	6	Ins P_6	IP_6

2.1.2 Occurrence and distribution of phytic acid

Phytic acid, which is abundantly present in seeds, also exists in roots, tubers, pollens and spores of many plant species (Feil 2001). The abundance of IP_6 in seeds, pollen and other plant tissues is probably the main reason why derivatives of this compound are the most abundant form of organic phosphorus in soils (Raboy 2003). Grain crops typically contain about 10 mg phytic acid per gram seed dry weight, representing about 65% to 85% of seed total P (Raboy et al. 1990, 1997). Total P concentration typically ranges from 3.0 to 4.0 mg per gram in seed produced by grain crops, with phytic acid P ranging from 2.0 to 3.0 mg per gram. Once synthesized, most phytic acid P is deposited as a mixed phytate or phytin salt of potassium and magnesium, although phytates also contain other mineral cations such as iron and zinc (Lott 1984). These salts are packaged into discrete, membrane-bound inclusions referred to as globoids. Phytic acid biosynthesis takes place in the tissue where it is eventually stored (Feil 2001). In cereal grains essentially all phytates is localized in the germ and aleurone tissues (O'Dell et al. 1972). In maize, greater than 80% is found in the germ with the remainder in the aleurone. In wheat, barley, rice and other small grains, the reverse is observed. In rice, the aleurone is composed of a high phytic acid-containing particle surrounded by a coat of protein and carbohydrates (Ogawa et al. 1975, 1977). In legumes, the globoids are present only in cotyledons and not in seed coats. These globoids are located within a proteinaceous matrix of protein bodies. In the case of pea, phytic acid is present in the form of water-soluble potassium phytate (Lott et al. 1985). In mature dry pea seeds, 99% of the phytic acid is present in cotyledons and 1% is present in the embryo axis. Sixty five percent of the total phosphorus in pea cotyledons and 10% of total phosphorus in the embryo axis arise from phytic acid phosphorus (Ferguson and Bollard 1976). More than 88% of phytic acid is present in pea cotyledons, and seed coats contain almost no phytic acid (Beal and Mehta 1985).

Formation of the lower inositol phosphates (Table 2.1) occurs during germination (Ayet et al. 1997), digestion in the intestinal tracts of humans and animals (Reddy et al. 2002) and during food processing (Reddy et al. 2002). Lower inositol phosphates can also be found in

mature seeds. IP_6 accounts for 87% and IP_5 for 13% of total inositol phosphates in mature lentil seeds (Ayet et al. 1997). In wheat, IP_5 was higher and ranged from 5 to 10 % of IP_6. Trace amounts of IP_3 and IP_4 were also detected in wheat bran (Reddy et al. 2002).

Phytic acid accumulates throughout the developing stages of many seeds and grains and reaches its highest level at seed maturity. In peas, the phytic acid content increases from 0.16 to 1.23% during maturation (Welch et al. 1974). Phytic acid content in soybean during maturation increased from 0.87 to 1.26% (Yao et al. 1983). In winged beans there was a proportional increase in phytic acid at four developmental stages of seed maturity (Kadam et al. 1982). The proportion of phytic acid phosphorus relative to total phosphorus in seeds increases up to maturity, since the total amount of P deposited into the seeds increases with time (Raboy and Dickinson 1987, Raboy 1997). The differences in phytic acid concentration among many seeds is a result of several conditions such as differences in climatic conditions, fertilizer applications, cultivar differences, moisture content of seeds, soil conditions, procedures to measure phytic acid and choice of years for seed production (Lott 2000). During germination, phytins are broken down by endogenous phytase enzymes, releasing their P, *myo*-inositol and minerals that are used by the growing seedling.

2.2 Phytic acid biosynthesis

The phytic acid biosynthesis pathway is widespread in many eukaryotic cells. The inositol molecule needed for phytic acid biosynthesis is provided *de novo* during seed development. The biochemical pathway to phytic acid can be summarized as consisting of two parts: the early Ins supply pathway (Fig. 2.2, steps 1 and 2), and the later Ins phosphate/Ptd Ins phosphate pathways that convert Ins to IP_6 (Fig. 2.2, steps 4, 5, 9, 10 and 11). Based on the *lpa* seed P and Ins P phenotype, it is hypothesized that *lpa* mutations are in genes important to either the Ins supply or the Ins/Ptd Ins P pathways, not to supply P to the developing seed. The sole known synthetic source of the Ins ring is the activity of the enzyme D-*myo*-Inositol (3) P_1 synthase (MIPS; E.C. 5.5.1.4), that converts glucose 6-P to D-Ins (3) P_1 (Fig. 2.2, step 1; Loewus and Murthy 2000). MIPS gene expression proximal to the site of phytic acid synthesis during grain development in rice was demonstrated (Yoshida et al. 1999). The product of MIPS activity, D-Ins (3) P_1, may be directly phosphorylated to yield IP_6, via one or more kinases (Fig. 2.2, steps 4 and 5), or may first be hydrolyzed via a specific monophosphatase (E.C. 3.1.3.25) to yield free

Ins and inorganic P (Fig. 2.2, step 2). If the pathway starts with Ins, not Ins (3) P_1, the first step may be phosphorylation of Ins back to D-Ins (3) P_1, a reaction catalyzed by the enzyme D-Ins 3-kinase (E.C. 2.7.1.64; Fig. 2.2, step 3; English et al. 1966, Loewus et al. 1982).

The latter part of the pathway to phytic acid in the seed may proceed via two alternative pathways that utilize different series of Ins phosphate intermediates. Phytic acid synthesis may proceed via the sequential phosphorylation of free uncombined Ins phosphates, proceeding through specific intermediates such as D-Ins (3, 4, 6) P_3 (Fig. 2.2, steps 4 and 5; Biswas et al. 1978, Brearley and Hanke 1996, Stephens and Irvine 1990). Alternatively the pathways may begin with the synthesis of phosphatidylinositol (PtdIns; Fig. 2.2, step 9), and proceed through PtdIns phosphate intermediates (Fig. 2.2, step 10), the release of D-Ins (1, 4, 5) P3 (Fig. 2.2, step 11), and its subsequent phosphorylation to yield phytic acid. Pyrophosphate-containing Ins phosphates may be intimately involved in seed phytic acid metabolism and accumulation (Fig. 2.2, steps 8 and 9), but little is known to date concerning these compounds and their metabolism in plant or seed tissue (Brearley and Hanke 1996). *Myo*-inositol 1,3,4,5,6-pentakisphosphate is the penultimate *myo*-inositol phosphate in the pathway to phytic acid, regardless of the precursor pathways. Its conversion is catalyzed by *myo*-inositol polyphosphate 2-kinase (Biswas et al. 1978).

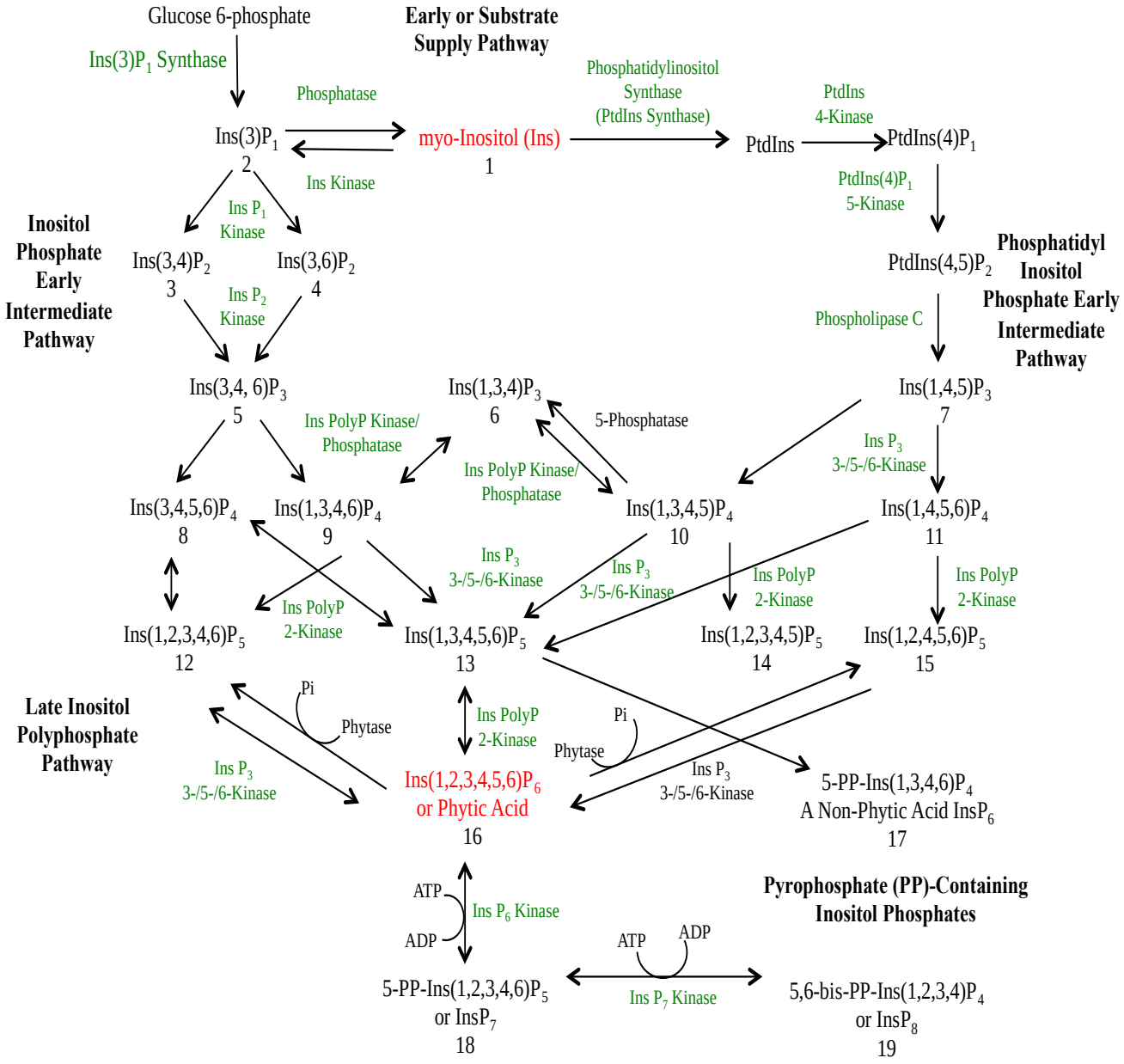

Fig. 2.2. Phytic acid biosynthesis from glucose 6-P to $Ins(3)P_1$ and eventually to IP_6 or "phytic acid". Enzymes catalyzing each step are shown in green. "Early or Substrate Supply Pathway" shows the formation of Ptd Ins from glucose 6-P. The "Inositol phosphate early intermediate pathway" proceeds through soluble inositol phosphates and "Phosphatidylinositol phosphate early intermediate through PtdInsP. The "Late Inositol Polyphosphate Pathway converts InsP3 to phytic acid and PP-Ins phosphates. (Raboy 2001).

2.2.1 *myo*-inositol phosphate synthase (MIPS)

MIPS catalyzes the conversion of glucose 6-phosphate into inositol trisphosphate, which is the first step in phytic acid biosynthesis pathway. The maize genome contains up to seven loci containing sequences with high homology to the canonical MIPS gene (Larson and Raboy 1999). One of these is located on maize chromosome 1S, proximal to the maize low phytic acid 1 locus. Barley differs from maize in two ways (Larson and Raboy 1999). First, the barley genome contains only one MIPS-homologous sequence located on a segment of chromosome 4 H that is clearly orthologous to the maize chromosome 1S region containing a MIPS gene and *lpa*1 mutation. Second, the barley *lpa*1 mutation maps to a locus on chromosome 2H that is separate from the single copy MIPS gene on chromosome 4H. They have also reported the isolation, inheritance and seed P phenotype of the first non-lethal rice *lpa*1mutant, and compared its map position to that of a single-copy of rice MIPS gene developmentally upregulated at stages corresponding with seed phytic acid synthesis. These findings have contributed to the interest in MIPS as a target for manipulation to produce low phytate crops.

Low phytic acid (*lpa*) genotypes of *Arabidopsis*, potato, rice and soybean have been generated by down-regulation (antisense or RNA interference) or mutations in MIPS gene (Murphy et al. 2008, Keller et al. 1998, Xu et al. 2009, Nunes et al. 2006). Mutation in MIPS gene is often associated with lower seed yield, seed viability, increased susceptibility to pathogens and undesired morphology (Keller et al. 1998, Murphy et al. 2008, Yuan et al. 2007).

The spatial and temporal expression of MIPS has been characterized in *Glycine max* by Kumari et al. (2012). One of the two MIPS genes in rice is highly expressed in developing seeds (Suzuki et al. 2007). Similar expression was observed in one of the four MIPS genes in soybean (Chappell et al. 2006). Coelho et al. (2007) reported the variation in enzyme activity and gene expression of MIPS and phytate accumulation in seed development in common bean (*Phaseolus vulgaris* L.). MIPS enzymatic expression was higher in a high phytic acid wheat genotype than in a low phytic acid genotype (Ma et al. 2013).

Myo-inositol tetra *kis*phosphate 1-kinase (*ITPK1*; EC 2.7.1.159) phosphorylates IP_3 and IP_6 in the phytic acid biosynthesis pathway (Rasmussen et al. 2010). While many *myo*-inositol kinase enzymes have been identified in plants, *ITPK1* knockout studies in maize, generated mutants with 30% reduction in phytate content (Shi et al. 2003). Stevenson-Paulik et al. (2005)

generated phytate-free seeds in *Arabidopsis* through disruption of inositol polyphosphate kinases. These reports show that phytate synthesis has other regulation points than MIPS.

2.3 Role of phytic acid in plants

Phytic acid plays many physiological roles in plants. The possible functions of phytic acid in plant seeds include: storage of phosphorus, storage of energy, inducing dormancy when the seeds approach maturity by competing for ATP molecules, regulating the concentration of inorganic phosphorus in seeds and storage of mineral ions (Cosgrove 1980). This view was challenged when Lott et al. (1995) stated that the role of phytic acid in seeds is limited to phosphorus and mineral storage, and control of inorganic P homeostasis. They also pointed out that there is insufficient *myo*-inositol stored in phytic acid to support all cell wall synthesis until seedling establishment. Figure 2.3 explains the physiological role of myo-inositol in plant metabolism. Phytic acid plays a role in signal transduction in both plant and animal cells (Missiaen et al. 1996, Shears et al. 1996). Phytic acid prevents the production of aflatoxins in plants (Gupta and Venkitasubramanian 1975, Dayi et al. 1994). During seed dormancy it can act as an antioxidant (Graf et al. 1987, Graf and Eaton 1990).

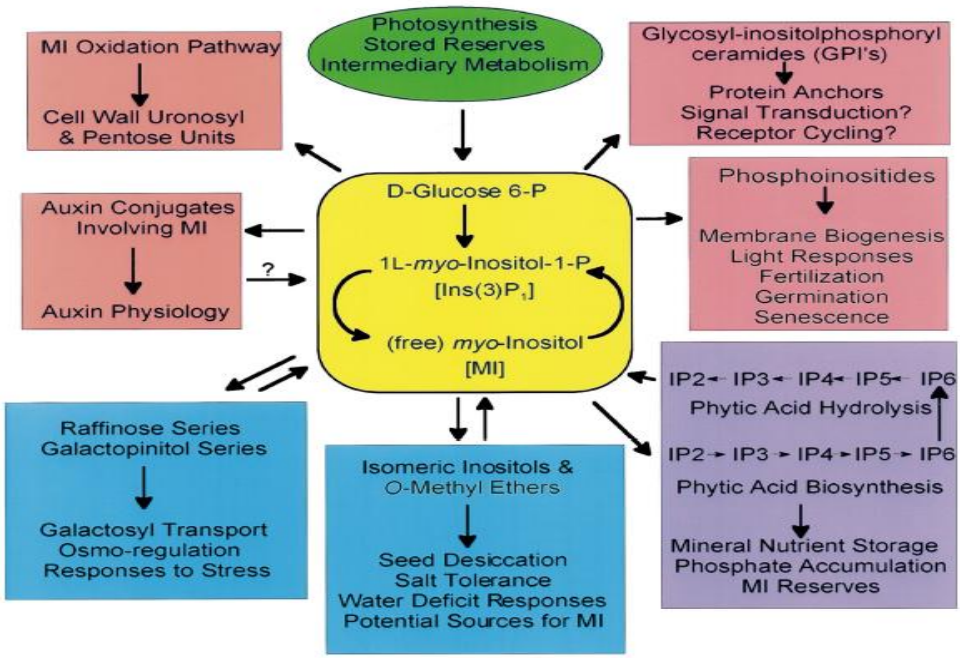

Fig. 2.3. Flow chart representing the physiological role of *myo*-inositol in plant metabolism (Loewus and Murthy 2000)

2.4 Nutritional impact of phytic acid

2.4.1 Micronutrient availability

Due to its high chelating potential, phytic acid can bind positively charged mineral ions in the intestinal tract of humans and animals (Feil 2001). This alters the digestion, solubility, and functionality of these essential mineral ions and makes them unavailable for absorption (Feil 2001). These mineral ions include zinc, iron, calcium, magnesium and copper. The order of the ability of mineral ions to form complexes with phytic acid is as follows: $Cu^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+}$ (Feil 2001). Zinc deficiency in humans was reported in 1963 among Egyptians, who consumed mainly beans and bread (Prasad et al. 1963). Since their food was abundant in phytic acid, which reduced zinc absorption, it resulted in dwarfism and hypogonadism (Oberleas 1983). An insoluble phytate-mineral ions complex is formed by dietary phytate. This complex is not absorbed or digested by the human gastro-intestinal tract, which lacks phytate-degrading enzyme. Brune et al. (1992) showed that phytate inhibited the absorption of non-heme iron in humans. Phytic acid reduces Ca^{2+} absorption but to a lesser extent than Zn^{2+} and $Fe^{2+/3+}$ reductions in humans (Feil 2001). When phytic acid was added to diets, it decreased the retention of copper in rats by 75% (Davies and Nightingale 1975). In contrast, Lee et al. (1988) demonstrated that phytic acid supplement enhanced the bioavailability of copper. They postulated that phytic acid increases the utilization of copper because it can bind with other mineral ions like zinc, which compete with copper at the site of absorption. Phytic acid supplements affected the absorption of manganese in rats (Davies and Nightingale 1975), but it had no effect on humans (Davidson et al. 1991). Phytic acid increased the selenium absorption in chickens with an adverse effect on their growth (Shan and Davis 1994). The factors affecting the solubility and stability of different phytate-mineral ion complexes include pH, the individual cation, phytate to cation molar ratio and the presence or absence of other compounds in the solution (Oberleas 1983).

2.4.2 Protein digestibility

The binding of phytic acid with proteins is dependent on pH (Cheryan 1980). When the pH value of proteins are lower than their isoelectric point, the phosphoric group of phytic acid binds with the cationic group of basic amino acid and forms a protein-phytate complex (Feil

2001). These complexes can be dissociated only at about pH 3 and below. The formation of protein-phytate complexes may affect the protein structures, protein solubility and digestibility by preventing enzymatic activity (Kumar et al. 2010).

When the pH is above the isoelectric point of the protein, both the protein and phytic acid are negatively charged. In such cases, the binding of protein with phytic acid is mediated by cations such as calcium and magnesium (Cheryan 1980). *In vitro* experiments have demonstrated the inhibitory activity of phytic acid on various enzymes such as pepsin, trypsin, chymotrypsin (Kumar et al. 2010).

2.4.3 Starch digestibility

Phytic acid reduces the blood glucose response or glycemic index in humans (Lee et al. 2006). Phytate binds with carbohydrates and affects the digestibility and absorption of glucose. Phytate either directly binds with starch through hydrogen bonding, or indirectly through proteins associated with starch (Rickard and Thompson 1997). It was proposed that phytate inhibits amylase activity by binding with Ca^{2+} ions (Selle et al. 2000). When bean flour was subjected to dephytation, the glycemic index in humans increased (Thompson et al. 1987). A reduction in phytate mediated hydrolysis of starch was demonstrated through *in vitro* studies by incubating human saliva with either wheat or bean starch spiked with Na phytate (Thompson et al. 1987, Yoon et al. 1983).

2.4.4 Lipid digestibility

Phytic acid binds with lipids and its derivatives along with other mineral nutrients to form “lipophytins” (Vohra and Satanarayana 2003). Calcium, phytate and lipid form metallic soaps in the gut lumen of chickens, which results in deprivation of energy utilization from lipid sources (Leeson 1993). When young chickens were fed diets containing fat and phytic acid, a large percentage of fat was excreted. However the results were not convincing enough to support the presence of fat-phytate complex in humans (Matyka et al. 1990).

From all these facts, it is evident that phytic acid can generally be regarded as an antinutrient for humans and non-ruminant animals, as it can cause micronutrient deficiency, and

reduced protein, starch and lipid digestibility. In contrast, some studies have shown beneficial health effects of phytic acid, as mentioned earlier in this section.

2.5 Environmental impact of phytic acid

The bioavailability of phytic acid-phosphorus to humans and non-ruminant animals is very low. Pigs utilize only 17% of the phosphorus in maize grain (Jongbloed and Kemme 1990). In barley, dry beans and soybean the digestibility of phosphorus ranged from 37-39%. Pea and wheat show higher rates of digestion with values of 45% and 47% respectively (Jongbloed and Kemme 1990). The unused phosphorus in food and feed passes through the intestinal tract and is excreted. Animal manures are applied as a fertilizer to crop production lands, thus accumulating phosphorus in soil. Phytate in soil can also come from decaying seeds, pollen and other plant parts. Soil erosion and run off processes transport phosphorus from soil to ponds and lakes. Input of phosphorus to surface water stimulates the production of aquatic biomass (Gilliam et al. 1985). Phytic acid-protein complexes are excreted, which eventually reduce the amount of protein retained by fish (Storebakken et al. 1998). This releases large amounts of unused protein contributing to eutrophication of seawater surrounding fish farms. Yano et al. (1999) reported an aggravated level of eutrophication when animal diets were supplemented with inorganic phosphorus to compensate the loss caused by phytic acid phosphorus. Figure 2.4 illustrates the nutritional and environmental impacts of phytic acid.

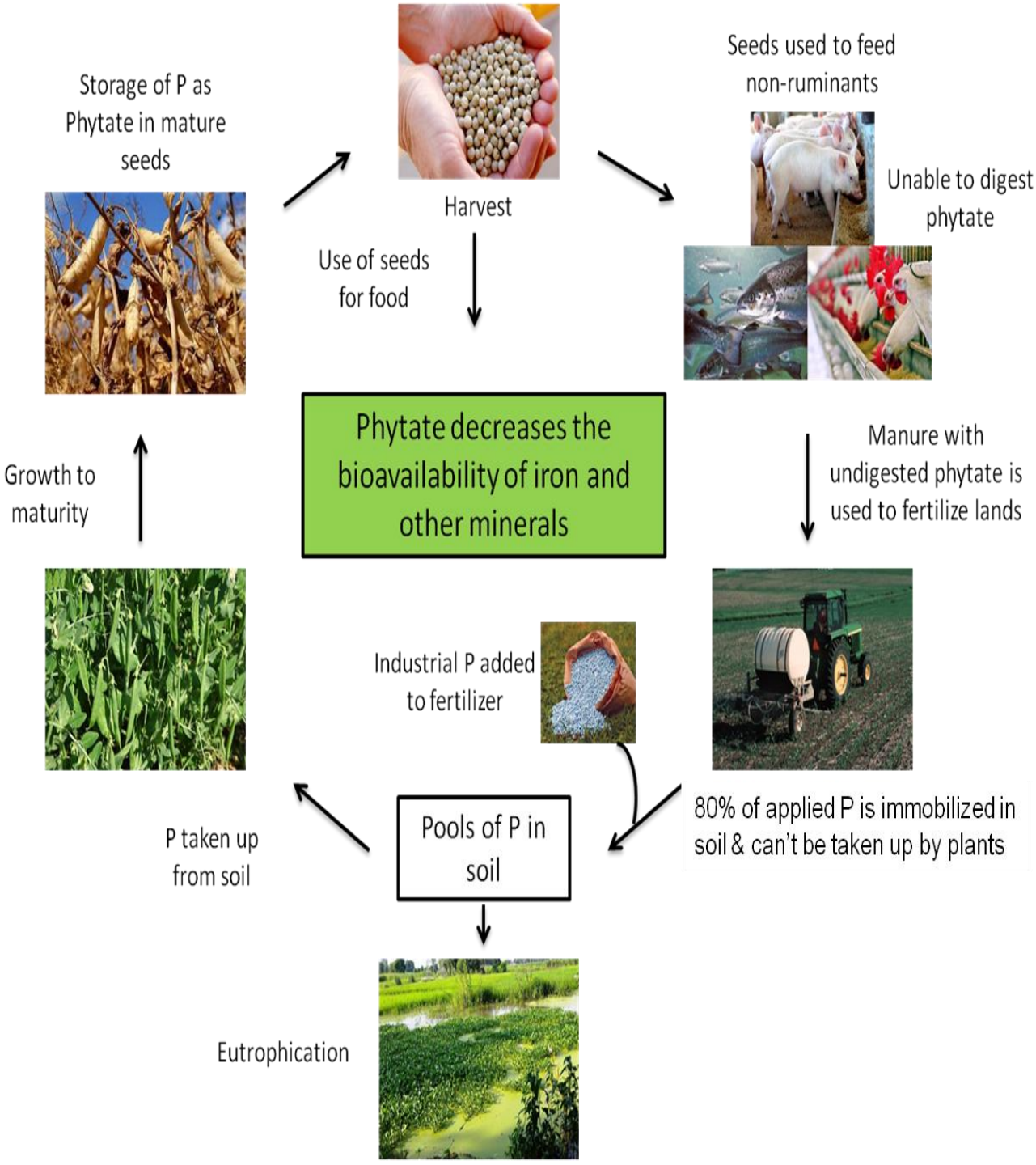

Fig. 2.4. Schematic representation of nutritional and environmental impact of phytic acid

(adapted and modified from Brinch-Pedersen et al. 2002)

2.6 Methods to reduce phytate in seeds

2.6.1 Soaking

Soaking of cereals and legume grains is widely used at industrial and household levels. Soaking makes the seed coats of grains permeable to water. Phytate is water-soluble and is discarded along with the soaked water. Soaking can last for short periods (15-20 min) or for very long periods (12-16 h). A significant amount of phytate hydrolysis takes place during soaking due to the activation of endogenous phytases and acid phosphatases, which are present naturally in the seeds (Reddy et al. 2002). Soaking of lentil seeds at room temperature for 12 hours resulted in a 27% reduction in phytate (Vidal-Valverde et al. 1994). A 56% reduction in phytate was observed in barley grains soaked in water for 24 hours at 55^0C (Fredlund et al. 1997).

2.6.2 Cooking

Phytate is a heat stable compound and is not easily degraded during cooking. However, the endogenous phytase being thermolabile gets inactivated at a higher temperature (Kumar et al. 2010). Reduction in phytate through cooking can occur only during the early part of cooking phase when the temperature is lower. Therefore in order to achieve a higher amount of phytate degradation, cooking is combined with certain other treatments like fermentation, soaking, or germination (Reddy et al. 2002).

2.6.3 Fermentation

Fermentation of grains is an effective method to reduce phytate content, improve starch and protein digestibility and bioavailability of mineral ions (Caulibaly et al. 2011). A 96-97% reduction in phytate was reported when oat and rye bran bread was made with 10% sourdough having a pH of 4.6 (Larsson and Sandberg 1992). Lactic acid fermentation when combined with germination can result in complete degradation of phytate in white sorghum and maize gruels (Svanberg et al. 1993). Tempeh, miso and koji are food products made from soybean fermentation. The organisms *Rhizopus oligosporus* and *Aspergillus oryzae* used in this type of fermentation produce intra- and extra-cellular phytate degrading activity (Fujita et al. 2003).

2.6.4 Germination

During germination, phytate is hydrolyzed by endogenous enzymes, which releases inorganic phosphorus (Reddy et al. 2002). Free *myo*-inositol and nutrients bound to it are released and used by the developing seed. Germination in pea (72-192 h, 25^{o}C) resulted in 20 to 80% reduction in phytate (Alonso et al. 1998, Belavady and Banerjee 1953, Honke et al. 1998). A 66% reduction in phytate was reported in rice germinated for 96 hours at 32^{o}C (Marero et al. 1991).

2.6.5 Addition of phytase to feed and food

Addition of commercially produced microbial phytases (exogenous) to animal feeds increases the availability of phytic acid phosphorus and also decreases phosphorus excretion at the same time (Bohn et al. 2008). Microbial phytase increased phosphorus availability by 38%, 12% and 15% in pigs fed with maize, wheat and triticale, respectively (Dungelhoef et al. 1994). Nahm (2002) reported a 60% reduction in manure phosphorus due to phytase addition. Supplementing chicken feed with exogenous phytase reduced the excretion of protein, calcium, sodium and phytate phosphorus (Cowieson et al. 2003, Nahm 2002). In aquaculture, a low cost plant based protein diet is desired over a meat based protein diet. Therefore dephytinisation is an important prerequisite in this industry. Some fish have a basic environment (pH 8) in their intestinal tract, which is not an optimal condition for phytase activity (Bohn et al. 2008). In such cases the acidic microbial phytases may not perform well (Cao et al. 2007). A transgenic pig that can constitutively secrete microbial phytase in their salivary glands had been generated. The animal required almost no inorganic phosphorus supplementation and showed up to a 75% reduction in phosphorus excretion (Golovan et al. 2001a, 2001b).

Phytase is used as an ingredient in bread making to decrease phytate content and to release calcium which promotes the activation of alpha-amylase activity (Haros et al. 2001). When wheat bread was treated with fungal phytase, the non-heme iron absorption in humans almost doubled when compared to untreated bread (Sandberg et al. 1996). Addition of phytase along with amylase to cereal porridges, increased iron absorption by 3-fold (Hurrell et al. 2003).

Bilyeu et al. (2008) demonstrated that soybean lines could be genetically engineered to express phytase enzyme in appropriate tissues of seeds. This blocked phytic acid accumulation and produced seeds with high levels of phytase.

2.7 Development of low phytic acid (*lpa*) crops

The low phytic acid (*lpa*) mutations have the potential to alleviate environmental and nutritional problems associated with phytic acid in animal feeds (Ertl et al. 1998). Low phytic acid crops may also offer improved nutrition for human populations that depend upon grains and legumes as staple foods. Moreover, these *lpa* mutants provide a valuable system to study grain phytic acid synthesis. Unlike the normal genetic and environmental affects that result in quantitative variation in seed total P, *lpa* mutants show large effects on the partitioning of P into phytic acid phosphorus, inorganic phosphorus and lower inositol phosphates. Low phytic acid crops have been generated through chemical mutagenesis of normal crops, which mutates one or more genes in the phytic acid biosynthesis pathway. *lpa* genotypes have been isolated in maize (*Zea mays*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*) rice (*Oryza sativa*), *Arabidopsis*, soybean (*Glycine max*), common bean (*Phaseolus vulgaris*) and field pea (*Pisum sativum*). These mutants have genetically reduced of phytic acid content ranging from 50% to 95%. The phytic acid reduction in these mutants corresponds to an increase in inorganic phosphorus content (Raboy 2001). Two types of loss-of-function mutations have been reported: low phytic acid 1-1 which is the first recessive allele of the *lpa*1 locus and low phytic acid 2-1, which is the first recessive allele of the *lpa*2 locus. *Lpa*1 mutations are caused by a change in the genes encoding enzymes early in the phytic acid biosynthesis pathway. Whereas *lpa*2 mutations occur in genes involved late in the phytic acid biosynthesis pathway. Since the mutations affect different parts of the pathway, they accumulate different inositol phosphates in the mutated crop variety. *Lpa*1 mutants do not accumulate inositol polyphosphates (Raboy et al. 2000). *Lpa*2 mutants accumulate other inositol phosphates such as IP_3 and IP_5 (Dorsch et al. 2003). Embryo-specific silencing of expression of an ATP binding cassette (ABC) transporter in maize produced seeds with low-phytic acid with no adverse effect on seed weight (Shi et al. 2007). When the *myo*-inositol methyltransferase (*IMT*) gene was transferred to *Brassica napus* through a transgenic approach, a 19 to 35% reduction in phytate was achieved without affecting the seed parameters (Dong et al. 2013). In addition, rice low-phytate mutants produced through RNAi

mediated seed-specific silencing of inositol penta*kis*phosphate 2-kinase (*IPK1*) gene, had no undesirable agronomic characters (Ali et al. 2013). *lpa* genotypes of *Arabidopsis*, potato, rice, soybean and canola have been generated by down-regulation (antisense, RNAi, or cosuppression) or mutations in *MIPS* gene (Keller et al. 1998, Nunes et al. 2006, Georges et al. 2006, Murphy et al. 2008, Xu et al. 2009).

The effect of low phytic acid mutants on mineral absorption had been studied. The absorption of zinc, iron and calcium in humans was significantly increased when they were fed with low phytic acid maize (Mendoza 2002). Low phytic acid mutants can also improve nutrient bioavailability in animal feed. In poultry industry, low phytate mutants increased bird weight by 16% when compared to a diet that contained normal phytate seeds. Increase in calcium absorption among birds was also observed (Mendoza 2002). Fecal waste phosphorus reduced up to 40%. Broiler chickens fed with low phytate pea had higher bone strength than chickens fed with normal phytate pea (Thacker et al. 2013). It also increased the bioavailability of inorganic phosphorus, which indirectly reduces phosphorus pollution in environment. These facts prove that the low phytate mutants could be used to improve phosphorus and essential minerals' bioavailability and also reduce environmental phosphorus pollution.

2.7.1 Maize *lpa* genotypes

Maize *lpa* genotype was the first ever *lpa* genotype to be developed (Raboy et al. 2000). The ethyl methanesulfanate (EMS) induced mutants showed a 50-66% reduction in phytic acid phosphorus without any alteration in seed total phosphorus. The gene mutated in maize *lpa*1-1 was a "multidrug resistance-associated protein", which is an ATP binding cassette (ABC) transporter with 11 exons (Shi et al. 2007). *Lpa*2 mutation in maize was associated with a mutation in Ins (1,3,4)P_3 5-/6-kinase gene that catalyzes the conversion of inositol trisphosphate intermediates to inositol pentakisphosphate in the phytic acid biosynthesis pathway (Shi et el. 2003). In 2005, Shi et al. identified *lpa*3 mutant in maize. The gene responsible for *lpa*3 encoded a *myo*-inositol kinase (*MIK*) and was expressed in developing embryos.

2.7.2 Barley *lpa* genotypes

Barley mutants (*lpa*1 and *lpa*2) reduce phytic acid levels by 35 to 50% (Larson et al. 1998, Dorsch et al. 2003).The *lpa*1 and *lpa*2 mutations were mapped to sites on barley

chromosome 2H and 7H, respectively. Ye et al. (2011) showed that the *lpa*1 mutant in barley is a result of a nonsense mutation in a putative sulphate transporter gene.

2.7.3 Wheat *lpa* genotypes

The wheat *lpa* genotype resulted in a 37% reduction in seed phytic acid and 5-fold increase in inorganic phosphorus. The total phosphorus levels remained the same between wild type and *lpa* mutant wheat (Guttieri et al. 2004). The mutation increased the phosphorus content of the endosperm and decreased the phosphorus content of the bran. Inheritance studies suggest two or more genes are responsible for the *lpa* trait.

2.7.4 Rice *lpa* genotypes

Rice *lpa* mutant isolated through gamma irradiation, showed 45% reduction in seed phytic acid, which was accompanied by a molar equivalent increase in inorganic phosphorus (Larson et al. 2000). *Lpa*1 mutation in rice was caused by a single recessive allele. *Lpa*1 gene was found associated with gene encoding 2-phosphoglycerate kinase (2-PGA kinase) (Kim et al. 2008). Ali et al. (2013) developed low phytate rice by RNAi mediated seed-specific silencing of the inositol 1,3,4,5,6-pentakisphosphate 2-kinase (*IPK1*) gene. *IPK1* gene catalyzes the last step of phytic acid biosynthesis in rice.

2.7.5 Soybean *lpa* genotypes

Lpa soybean genotype was generated through chemical mutagenesis using ethyl methanesulfonate (Wilcox et al. 2000). Mutation in MIPS gene resulted in *lpa* soy genotype with more than 50% reduction in seed phytic acid (Hitz et al. 2002). This mutation also reduced the raffinosaccharide content in the seeds. Nunes et al. (2006) proved that RNAi mediated silencing of the *myo*-inositol-1-phosphate synthase (*GmMIPS1*) gene in transgenic soybean inhibited seed development and reduced phytate content. An embryo specific silencing of ATP-binding cassette (ABC) transporter reduces phytic acid content in soybean seeds (Shi et al. 2007).

2.7.6 Common bean *lpa* genotype

An *lpa* mutant common bean genotype was generated through EMS chemical mutagenesis (Campion et al. 2009). It was characterized by a 90% reduction in phytic acid and a

25% reduction in raffinosaccharides when compared with the wild type common bean seed. The *lpa* mutant also had higher amount of free iron cations in the seed. Inheritance studies performed on these seeds showed that the *lpa* character is due to a mutation in a single recessive gene. The structural genes involved in the phytic acid biosynthesis pathway in common bean have been mapped on the common bean reference genetic map (Fileppi et al. 2010).

2.7.7 Field pea *lpa* genotypes

Two low phytate mutants of field pea have been isolated through chemical mutagenesis using EMS. The *lpa* genotypes, 1-150-81 and 1-2347-144 showed a 60% reduction in seed phytic acid with a compensating increase in inorganic phosphorus (Warkentin et al. 2012). When compared to their progenitor CDC Bronco, which is a normal phytate genotype, these genotypes performed similar in many agronomic characteristics. They were slightly lower in seed weight and grain yield and slower in days to flowering and maturity. Inheritance studies performed on these genotypes showed that the low phytate trait is controlled by a single recessive allele (Rehman et al. 2012).

2.8 Phosphorus fractions in developing seeds

As phytic acid represents a significant portion of total seed P, the accumulation of phytate and inositol phosphates has been studied in different plant species. Raboy and Dickinson (1987) recorded the accumulation of phytic acid, inorganic P, and total P in wild-type soybean lines. Raboy et al. (2000) did similar work with the maize *lpa*1–1, *lpa*2–1, and wild type lines. In both studies, phytate accumulated gradually during seed development. Inorganic P concentration decreased during seed development, and total P levels remained relatively consistent. The maize *lpa* genotypes had little to no increase in phytate, and inorganic P concentration was high and did not decrease during development. While the final levels and partitions of P forms in barley are well documented, it is unclear when in seed development the expression of the *lpa* trait is initiated and how seed phosphorus accumulates in developing seeds of different barley *lpa* genotypes (Bowen et al. 2006). Larson et al. (2000) reported that a reduction in seed phytate results in a molar equivalent increase in inorganic phosphorus in rice. The low phytic acid rice showed 45% reduction in phytic acid that was paired with a molar equivalent increase in P_i (Liu et al. 2004). The changes in seed phytic acid concentrations during soybean seed development of low phytic acid genotypes were compared with normal phytic acid genotypes (Israel et al. 2011).

IP_3 content was relatively low in all genotypes and decreased during seed maturation. The other inositol phosphates such as IP_4 and IP_5 were not detected. In chickpea, the maximum accumulation of phytic acid occurred between 28 and 42 days after flowering (DAF). The concentration of inorganic phosphorus decreased throughout the developmental stages. The total phosphorus content decreased rapidly between 14 and 28 DAF during seed development (Zhawar et al. 2011). Walker (1974) showed that 90% of total phytic acid in the cotyledons of common bean accumulated between days 24 and 30 of embryogeny. The inorganic phosphorus concentration in cotyledons continuously increased from days 16 to 28, which showed that phosphorus levels are unaffected by the steady synthesis of phytic acid. In fruit explants of common bean, inorganic phosphorus, sucrose, abscisic acid (ABA) and *myo*-inositol increased the phytic acid concentration. This showed that phytic acid content could be altered by culturing bean fruit explants using *in vitro* methods (Coelho et al. 2008). In castor bean, phytic acid phosphorus in the endosperm increased linearly after 25 days after pollination (DAP) until maturation at 40 DAP. Inorganic phosphorus increased linearly between 20 and 30 DAP and then declined rapidly until quiescence. A similar trend was observed for total phosphorus accumulation (Greenwood et al. 1984).

2.9 Pea genome mapping

2.9.1 Genetic composition of pea

The genome size of pea is 4.45 Gbp (Dolezel et al. 2010). Next generation sequencing data indicated that 50-60% of pea nuclear DNA is composed of moderate to highly repeated sequences (Macas et al. 2007, Novak et al. 2010). The entire genome is arranged in 7 pairs of chromosomes (2n=14).

2.9.2 Linkage mapping in pea

Linkage maps in organisms indicate the position and relative genetic distances between specific markers along chromosomes. McPhee (2007) provided a comprehensive review on genetic linkage maps in pea. Herbert Lamprecht in the 1920s initiated the construction of genetic linkage mapping in pea with his classical genetic studies (Blixt 1972). By analyzing two-point crosses the first genetic linkage maps of pea were established. These early studies placed more than 160 of the 400 morphological markers on one of the seven linkage groups (LG) of pea

(Blixt 1972). This early linkage map assigned genes to individual LGs. Isozymic markers in pea were placed on the framework map of Blixt (1974) (Mahmoud et al. 1984, Weeden and Marx 1984, 1987). In the early 1990s with the advent of restriction fragment length polymorphisms (RFLP), linkage maps with increased density were generated which further explained genetic linkage in pea (Ellis et al. 1992, Timmerman et al. 1993, Dirlewanger et al. 1994). The introduction of polymerase chain reaction (PCR) facilitated the improvement of genetic mapping in many crop species. Yu et al. (1995) and Laucou et al. (1998) individually reported genetic linkage maps with randomly amplified polymorphic DNA (RAPD) markers. Amplified fragment length polymorphism (AFLP) markers based in pea (Vos et al. 1995, Tar'an et al. 2003, 2004) produced linkage maps with increased marker density (Timmerman-Vaughn et al. 1996). The naturally occurring repeated sequences in pea offered sources of polymorphism and were used to construct genetic linkage maps (Hellens et al. 1993, Turner and Ellis 1997). Gilpin et al. (1997) mapped 29 ESTs (expressed sequence tags) on a RFLP, RAPD and AFLP pea linkage map. Eight RGA (resistance gene analog) markers were placed on a map based on three different crosses (Timmerman-Vaughn et al. 2000). Copia-like retrotransposon markers (RBIPs) were also used to create a linkage map in pea (Flavell et al. 1998). Loridon et al. (2005) generated the first pea genetic map based on sequence-tagged microsatellite (STMS) markers.

2.10 Single Nucleotide Polymorphism (SNP)

Single nucleotide polymorphisms (SNPs) are single nucleotide substitutions or small insertion-deletion polymorphisms among haplotypes. DNA is made up of four nucleotides: adenine (A), thymine (T), cytosine (C) and guanine (G). Any of these four bases may be present at a given position in any genome. SNP variation takes place when a single nucleotide base, e.g., "C" at a given position is replaced by any of the other three nucleotides. The variation is caused either by transition: purine-purine (A-G) or pyrimidine-pyrimidine (C-T) exchanges or by transversion: purine-pyrimidine or pyrimidine-purine exchanges (Vignal et al. 2002). SNPs stand out distinctively from many other types of mutations naturally occurring in genomes by their numbers per genome, their relatively low mutation rates, their even distribution across the genomes and their relative ease of detection (Weising et al. 2005). SNP markers can be used in a wide range of applications such as high-resolution genetic maps, genetic diagnostics, mapping traits, analysis of genetic structure of populations, and phylogenetic analysis (Rafalski 2002).

2.10.1 SNPs in plant genomes and SNP markers

The average SNP density in plant genomes is approximately one SNP per 200-500 bp, but it depends on the species investigated (Weising et al. 2005). The analysis of the maize genome using eight inbred populations revealed an extremely high prevalence of SNPs (one SNP per 83 bp). The high degree of polymorphism could be a consequence of open pollination in maize (Bhattramakki et al. 2002). The SNP density in wheat is only about two SNPs per kbp. The density could be higher in enzymes involved in the starch biosynthesis pathway in wheat (Bhattramakki et al. 2002). More than 37,000 SNPs are publicly available for *Arabidopsis* in the database of The Arabidopsis Information Resource (TAIR). SNPs can be discovered in plants through various methods that include re-sequencing PCR amplicons (involves Sanger sequencing), shotgun genomic libraries, and expressed sequence tag (EST) library construction (Rafalski 2002). With the advent of high-throughput multiplex assays, or chip based SNP discovery, thousands of SNP markers can be genotyped at one time in a crop species. A saturated genetic linkage map of chickpea was constructed using 697 SNP markers using GoldenGate genotyping technology from Illumina (Gaur et al. 2012). A genetic linkage map of soybean with 657 SNPs using SoyS-NP6K Illumina Infinium Beadchip Genotyping Array was generated (Akond et al. 2013). A linkage map using 1536 GoldenGate SNP assay was constructed and tested in diploid and tetraploid recombinant inbred lines (RIL) of peanut (Bertioli et al. 2013). ESTs derived from nine lentil accessions were used to develop an Illumina GoldenGate 1536-SNP assay (Sharpe et al. 2013). A highly multiplexed SNP genotyping assay for genetic mapping and germplasm diversity studies in pea was evaluated using a set of 384 SNP markers (Deulvot et al. 2010). Leonforte et al. (2013) developed a genetic linkage map in pea using SNP and SSR (single sequence repeat) markers. This linkage map permitted the identification of salinity tolerance quantitative trait loci (QTL) in pea. A consensus genetic map of pea has been developed using 2070 SNP markers, which showed high homology with Medicago chromosomes (Duarte et al. 2014). Recently, a genetic SNP consensus map with 5 different RIL populations using 1536 SNP markers in Illumina GoldenGate array has been developed (Sindhu et al. 2014).

2.11 Quantitative Trait Loci (QTLs)

Many agronomic traits such as plant height, days to flowering, disease resistance, quality and yield are controlled by several genes. Within the plant genome there are regions that contain

genes associated with a particular quantitative trait. These regions are termed as quantitative trait loci (QTLs) (Collard et al. 2005). The development of DNA based molecular markers opened doors towards QTLs and their identification. Linkage maps constructed using different DNA markers have been used to identify genes controlling simple traits (controlled by a single gene) and also QTLs. QTL mapping is the process of constructing genetic linkage maps and performing QTL analysis to identify genomic regions associated with a specific trait (Mohan et al. 1997). The markers that are linked to specific genes in the genomes could be used in marker-assisted selection (MAS) in plant breeding. MAS either substitutes or helps phenotypic selection and makes it more efficient, effective, reliable and cost-effective compared to conventional plant breeding methods (Collard et al. 2005). QTL mapping and association studies are gaining importance in dissecting the genetic determination of many economically important traits in plant breeding.

2.11.1 Mapping of loci associated with *lpa* trait and P compounds in seeds

The inheritance and linkage map positions of two low phytic acid barley mutations, *lpa*1-1 and *lpa*2-1 were described by Larson et al. (1998). These mutations were associated with low phytic acid content in barley. The MIPS gene was sequenced and mapped in maize and barley to determine if the MIPS position corresponded to the previously mapped *lpa*1 mutations in maize and barley. The results supported MIPS candidate gene hypothesis in maize *lpa*1 but rejected the hypothesis in barley *lpa*1 (Larson and Raboy 1999). Walker et al. (2006) identified two loci associated with low phytate phenotype in soybean. They also discovered an epistatic interaction between the identified loci. In *Brassica rapa*, 25 QTLs associated with phytate and phosphorus concentrations in seeds and leaves were detected (Zhao et al. 2008). Blair et al. (2009) identified QTL for seeds phosphorus and seed phytate content in recombinant inbred population of common bean. In barley, PCR-based DNA markers flanking three major *lpa* loci; *lpa*1-1, *lpa*2-1and a locus linked to the MIPS gene was developed (Oliver et al. 2009). Seven QTLs, two for phytic acid phosphorus, four for inorganic phosphorus and one for total phosphorus content were detected in seeds of mungbean. Seed phytic acid and total phosphorus content correlated with days to flowering and maturity in mungbean (Sompong et al. 2012). QTLs for phytic acid concentration under different nitrogen treatment were identified in maize (Liu et al. 2013).

2.12 Objectives

The specific objectives of this study were as follows,

1. To characterize the accumulation of phosphorus-containing compounds in developing seeds of low phytate pea (*Pisum sativum* L.) mutants.
2. To identify the mutation in coding sequences of *myo*-inositol phosphate synthase (*MIPS*) gene involved in phytic acid biosynthesis pathway.
3. To identify and map genomic regions associated with phytic acid-phosphorus concentration in pea recombinant inbred lines.

2.13 Hypotheses

1. Phytic acid accumulates at maturity during seed development in pea.
2. Mutation in coding sequences of *myo*-inositol phosphate synthase (*MIPS*) gene is responsible for the low-phytate trait in pea.
3. Genetic markers associated with phytic acid-phosphorus concentration can be mapped in the recombinant inbred lines developed.

CHAPTER 3

3. Accumulation of phosphorus-containing compounds in developing seeds of low-phytate pea *(Pisum sativum* L.) mutants

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"Arun S.K. Shunmugam, Cheryl Bock, Gene C. Arganosa, Fawzy Georges, Gordon R. Gray and Thomas D. Warkentin. Accumulation of phosphorus-containing compounds in developing seeds of low-phytate pea *(Pisum sativum* L.) mutants.

3.1 Abstract

Low phytic acid (*lpa*) crops are low in phytic acid and high in inorganic phosphorus (P_i). In this study, two *lpa* pea genotypes, 1-150-81, 1-2347-144, and their progenitor CDC Bronco were grown in field trials for two years. The *lpa* genotypes did not significantly differ from CDC Bronco in all agronomic traits assessed except for lower seed weight and grain yield. Phytic acid (IP_6), lower inositol phosphates, inorganic phosphorus (Pi) and total phosphorus (total P) concentrations were determined in developing seeds of CDC Bronco and *lpa* genotypes. The *lpa* genotypes were lower in IP_6 and higher in Pi when compared to CDC Bronco. The total P concentration was similar in *lpa* genotypes and CDC Bronco throughout the seed development. The action of *myo*-inositol phosphate synthase (MIPS) (EC 5.5.1.4) is the first and rate limiting step in phytic acid biosynthesis pathway. Aiming at understanding the genetic basis of the *lpa* mutation in pea, a 1530 bp open reading frame of *MIPS* was amplified from CDC Bronco and the *lpa* genotypes. Sequencing results showed no difference in coding sequence in *MIPS* between CDC Bronco and *lpa* genotypes. Transcription levels of both *MIPS* and *myo*-inositol tetra*kis*phosphate 1-kinase (*ITPK1*) were relatively lower at 49 DAF than at 14 DAF for CDC Bronco and *lpa* lines. This study elucidated the rate and accumulation of phosphorus compounds in *lpa* genotypes. The data also demonstrated that mutation in *MIPS* was not responsible for *lpa* trait in pea.

Keywords: D-*myo*-inositol phosphate synthase (MIPS) • D-*myo*-inositol tetra*kis*phosphate

1-kinase (ITPK1) • Phosphorus • Phytate biosynthesis • Phytic acid • *Pisum sativum* L.

3.2 Introduction

Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate; IP_6) is the major storage form of phosphorus (P) in most plant seeds (Cosgrove 1980). In cereal grains essentially all phytates are localized in the germ and aleurone tissues (O'Dell et al. 1972) whereas in dicotyledons, phytate is distributed throughout the cotyledon and present within subcellular protein inclusions (Lott et al. 1984). Endogenous phytase enzymes break down phytate during seed germination and release its phosphorus, *myo*-inositol and mineral contents for use by the growing seedling (Raboy 2001). IP_6 also accumulates in other plant tissues and organs that accumulate nutrient stores for subsequent redistribution, such as pollen, roots and tubers (Reddy et al. 2002).

Applied interest in seed IP_6 primarily concerns its roles in human health and animal nutrition. It is a strong chelator of mineral elements such as iron, zinc, calcium and potassium, forming mixed salts that are largely excreted by humans and other non-ruminant animals such as poultry, swine and fish (Erdman 1980, Sharply et al. 1994). Excretion of seed-derived IP_6 can contribute to dietary iron and zinc deficiencies, a major public health problem in the developing world. In addition, undigested phytate excreted by non-ruminant animals represents an important source of phosphorus pollution in the environment (Raboy et al. 2001). Due to nutritional and environmental concerns, the development of cultivars with a low-phytate trait has become an attractive breeding objective in many crop species. Chemically induced, non-lethal recessive mutants that decrease seed phytic acid content have been isolated and genetically mapped in maize (*Zea mays* L., Raboy and Gerbasi 1996, Raboy et al. 2000), barley (*Hordeum vulgare* L., Larson et al. 1998, Rasmussen and Hatzak 1998) and soybean (*Glycine max* L. Merr, Wilcox et al. 2000). Recently, Warkentin et al. (2012) developed and characterized two low-phytic acid mutants of field pea (*Pisum sativum* L.). The *low phytic acid* (*lpa*) mutations have the potential to alleviate the environmental and nutritional problems associated with phytic acid in animal feeds (Ertl et al. 1998). Moreover, *lpa* crops may also offer improved nutrition for human populations that depend upon grains and legumes as staple foods. In addition, these *lpa* mutants provide a valuable system to study seed phytic acid synthesis.

In plants, the six-carbon cyclitol *myo*-inositol gives rise to compounds with roles in such diverse functions as signal transduction, membrane biogenesis, stress tolerance and the generation of seed storage compounds including IP_6 (Loewus and Murthy 2000, Stevenson et al.

2000). The *de novo* synthesis of *myo*-inositol involves the conversion of glucose 6-phosphate to L-*myo*-inositol-1-phosphate (IP_1) which is subsequently dephosphorylated to release free *myo*-inositol. The former reaction is catalyzed by *myo*-inositol-1-phosphate synthase (MIPS; EC 5.5.1.4), the first and rate-limiting enzyme of the pathway (Loewus and Loewus 1983, Loewus 1990, Loewus and Murthy 2000, Stevenson et al. 2000). This makes *MIPS* an attractive target for manipulation to produce low-phytate crops. Previous studies have demonstrated that 50 to 95 % reductions in phytic acid can be obtained when this enzyme is targeted through mutagenesis or by genetic engineering methodologies (Pilu et al. 2003, Wilcox et al. 2000, Kuwano et al. 2006, 2009, Georges et al. 2006, Nunes et al. 2006, Panzeri et al. 2011). In soybean, mutations in *MIPS* coding sequences conferred a decreased phytic acid phenotype and an effective reduction in phytate content (90 to 95%) has been observed when *GmMIPS1* was silenced through a RNA interference (RNAi) approach (Hitz et al. 2002, Nunes et al. 2006). A *lpa* phenotype was also produced by manipulating the *MIPS* gene through an antisense approach in *Oryza sativa* L. (Kuwano et al. 2009). *myo*-inositol tetra *kis*phosphate 1-kinase (*ITPK1*; EC 2.7.1.159) phosphorylates IP_3 and IP_6 in the phytic acid biosynthesis pathway (Rasmussen et al. 2010). While many *myo*-inositol kinase enzymes have been identified in plants, *ITPK1* knockout studies in maize, generated mutants with 30% reduction in phytate content (Shi et al. 2003).

The objective of this study was to investigate the accumulation of phytic acid and other phosphorus compounds in developing seeds of normal and low-phytate genotypes of field pea (*Pisum sativum* L.). We also examined *MIPS* gene expression and analyzed the sequence at the nucleotide and protein levels to ascertain if variation in *MIPS* coding sequences was responsible for the *lpa* trait in pea. This will help us to understand the nature of the low phytate mutation(s), develop markers for low phytate trait furthering the development of low-phytate cultivars.

3.3 Materials and methods

3.3.1 Plant material and growth conditions

Seeds of two *lpa* field pea (*Pisum sativum* L.) genotypes (1-150-81 and 1-2347-144; Warkentin et al. 2012), and their progenitor, a normal phytate genotype (CDC Bronco; Warkentin et al. 2005) were obtained from the Crop Development Centre at the University of Saskatchewan. A four-replicate randomized complete block field trial was conducted at two locations in Saskatchewan (Rosthern and Saskatoon) in 2010 and 2011. Field trials were managed using standard techniques for field pea production in Saskatchewan and have been

described earlier (Warkentin et al. 2012). Seeding in both years was conducted between May 14 and May 18. Flowers were tagged at the time of flowering and developing seeds were taken 7 days after flowering (DAF) and every 7 d thereafter until maturity on day 49. Pea pods were collected in Ziploc® bags, transported to the laboratory on ice packs and stored at -80^{o}C until use. Final harvesting in both years was conducted between August 27 and September 17. Field plots were evaluated for several phenotypic parameters during the growing season in each year. These included percent emergence, days to flower, days to maturity, plant height, mycosphaerella blight score, lodging, grain yield and 1000 seed weight. The details of these determinations have been described previously (Warkentin et al. 2012).

For molecular studies, seeds of the aforementioned genotypes were sown in six inch plastic pots filled with Sunshine® Mix #3/LG3 (Sun Gro Horticulture Canada Ltd., Seba Beach, AB) and plants grown in a controlled environment chamber (PGR15; Conviron, Winnipeg, MB) set at 23/18°C (day/night) temperatures with 16 h day length. The chamber was illuminated with fluorescent lights (T5/HO/835; Sylvania) to provide a photosynthetic photon flux density (PPFD) of 400 µmol photons m^{-2} s^{-1}. Plants were regularly provided with a water soluble fertilizer (Plant-Prod® 20-20-20 Classic; Plant Products Co. Ltd., Brampton, ON) and irrigated with ddH_2O as required. The experiment consisted of four biological replicates for each genotype. Each replicate consisted of 4 pots with two plants per pot. Developing seeds were collected at the same time intervals described above and frozen in liquid nitrogen.

3.3.2 Extraction and detection of inositol phosphates

Seed coats were dissected from the cotyledons and each component was freeze dried (FreeZone 6 Liter Console Freeze Dry System; Labconco, Kansas City, MO) and stored at -80°C until use. Seed coat or cotyledons were ground using glass beads to a 0.5 mm diameter using a custom designed mill powered with an inverter drive (SM-PLUS Sub-Micro; Leeson Corporation, Grafton, WI). Samples were stored at -20°C until extraction. Inositol phosphates were extracted from the samples (100 mg) using the extraction method described by Talamond et al. (2000) with minor modifications (Thavarajah et al. 2009). Extracts were filtered using 0.45 µm Acrodisc® syringe filters (25 mm; Pall Corporation, Port Washington, NY) and used immediately for high-performance liquid chromatography (HPLC) analysis.

Detection of inositol phosphates was performed using anion-exchange HPLC on a Dionex ICS 3000 BioLC® system (Dionex, Sunnyvale, CA) and a protocol similar to those reported

previously (Talamond et al. 2000; Thavarajah et al. 2009). The system consisted of an AS50 Autosampler with a 100 µL injection loop, an AS50 Thermal Compartment (set at 30°C), a GP50 Gradient Pump and an ED50 Electrochemical Detector coupled with an Anion Self-Regenerating Suppressor (ASRS 300, 4-mm) running in external water mode and a current of 297 mA. Separation was achieved using an OmniPac PAX-100 analytical anion exchange column (Dionex; 8.5 µm, 4 x 250 mm) preceded by an OmniPac PAX-100 guard column (Dionex; 8.5 µm, 4 x 50 mm). Inositol phosphates were separated with a multi-step gradient using water purified by a Milli-Q Water System (Millipore, Milford, MA) to a resistance of ≥ 18 mΩ. Mobile phases were water (A), 200 mM NaOH (B), and water/isopropanol (50:50, v/v) (C). The total run time was 80 min, which included an equilibration to starting conditions. A column flow rate of 1.0 mL min^{-1} was maintained for the mobile phase flow, with a linear gradient profile consisting of solvent A with the following proportions (v/v) of solvent B or C: 0-13 min, B = 6% and C = 12%; 13-30 min, B = 30% and C = 2%; 30-43 min, B = 56% and C = 2%; 43-55 min, B = 56% and C = 2%; 55-65 min, B = 61% and C = 8%; 65-80 min, B = 6% and C = 2%. Chromeleon software (Dionex) was used to plot chromatograms and analyze the data. A standard solution was prepared for each of IP_6 and its lower isomeric forms IP_1, IP_3, IP_4, IP_5 (#P8810, #I1267, #I7012, #I5514, #I9261 respectively; all from Sigma-Aldrich, St. Louis, MO). This was used to establish retention times for these compounds, which were subsequently used to determine peak identity in the samples. Inositol phosphate quantification was afforded using external standard curves with R^2 values of 0.99 or greater for each compound. Concentrations for each of the inositol phosphate standards ranged from 1.25 to 25.0 ppm. Phytic acid phosphorus (P_{PhA}) was calculated as the number of moles of phytic acid/3.56 as described by Thavarajah et al. (2009), assuming all P_{PhA} comes from IP_6.

3.3.3 Analysis of phosphorus levels

Inorganic phosphorus (P_i) levels were determined as described by Warkentin et al. (2012). Ground cotyledon samples (50 mg) were extracted overnight at 4°C in 1 mL of 0.4 M HCl followed by vigorous mixing. A 10 µL aliquot of the extract was aliquoted into a microtitre plate with 90 µL of ddH_2O and 100 µL of freshly prepared Chen's reagent. Chen's reagent contains 6 N H_2SO_4, 2.5% (w/v) $(NH_4)_2MoO_4$ (ammonium molybdate), 10% (w/v) ascorbic acid and ddH_2O (1:1:1:2) (Chen et al. 1956). The mixtures were incubated for two hours at room temperature before reading the A_{655} with a microplate absorbance spectrophotometer (xMark™;

Bio-Rad Laboratories, Hercules, CA) against a water blank. Standard curves of K_2HPO_4 were constructed ranging from 10 to 50 ppm with R^2 values of 0.95 or greater. Sample values were interpolated from these curves and expressed on a dry weight (DW) basis.

Total P in cotyledons was extracted by the wet ashing method (Raboy et al. 2000). Ground samples (50 mg) were incubated with 1 mL of concentrated (18.4 M) H_2SO_4 overnight at room temperature. Two hundred µL of 30% (v/v) H_2O_2 was added and the samples were incubated in a heating block between 220 and 250°C for 30 min. Samples were removed and allowed to cool at room temperature for 15 min. This cycle was repeated until the sample became clear. The volume of the samples was adjusted to 6.25 mL with ddH_2O and total extractable P was determined spectrophotometrically using the method of Chen et al. (1956) as described above.

3.3.4 Reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing

Total RNA was isolated from 75 mg of seed from 14 DAF samples with an RNeasy® Plant Mini Kit (Qiagen, Valencia, CA) using the RLC buffer according to the manufacturer's instructions. The RNA was eluted in 30 µL of RNase-free water. The samples were quantified (A_{260}) and purity (A_{260}:A_{280}) determined using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, Ottawa, ON). Gel electrophoresis on denaturing 1.2% (w/v) agarose gels containing formaldehyde was used to examine the quality of the RNA. This was assessed by the sharpness of the rRNA bands and 2:1 ratio of 28S rRNA to 18S rRNA. Gels were run in 1× MOPS buffer and stained with ethidium bromide (Sambrook and Russell 2001). The isolated RNA was stored at -80^{o}C until further use. cDNA was synthesized from 100 ng of total RNA using the QuantiTect® Reverse Transcription Kit (Qiagen) as described by the supplier.
The annotated Cool Season Food Legume Genome Database (http://www.coolseasonfoodlegume.org/; Main et al. 2013) was searched for *MIPS* and a contig in *Pisum sativum* identified (Pisum_sativum_v2_Contig5216) which contained the entire coding region of the *PsMIPS* gene, confirmed by translation and alignment with alfalfa (*Medicago sativa*) *MIPS* (GenBank Accession Number EF408869.1). Gene specific primers for *PsMIPS* were designed from this contig using the Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; Ye et al. 2012) at the National Center for Biotechnology Information (NCBI) to amplify a 1,602-bp fragment encompassing the 1,530-bp open reading frame (ORF). cDNA was amplified by polymerase chain reaction (PCR) using an

iCycler (Bio-Rad Laboratories) and a thermostable DNA polymerase (Q5 High-Fidelity; New England Biolabs, Whitby, ON). Forward (5′-ATGTTCATAGAGAGTTTCAAGGTTGAGAGT-3′) and reverse (5′-GCTTGTGTTGGATTGGCTCCAGA-3′) primers were used at a final concentration of 0.5 μM each and 2 μL of the cDNA reaction was used as a template in the 25 μL PCR reaction. The following cycling conditions were used: cDNA denaturation at 98°C for 30 s, then 35 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, followed by a final extension at 72°C for 2 min. PCR products were visualized on a 1.2% (w/v) agarose gel run in 1× TAE and stained with ethidium bromide (Sambrook and Russell 2001). The PCR product corresponding to 1,602-bp was excised from the gel and extracted using the QIAPrep Gel Extraction Kit (Qiagen®) according to the manufacturer's instructions. Extractions from multiple PCR reactions were pooled and directly sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) at the National Research Council of Canada (NRC; Saskatoon, SK).

3.3.5 Semi-quantitative reverse transcriptase-polymerase chain reaction (sqRT-PCR)

Transcript levels of *PsMIPS* and *PsITPK1* were examined using sqRT-PCR and performed using the Verso 1-Step RT-PCR ReddyMix™ Kit (Thermo Fisher Scientific) as recommended by the supplier. Targets were amplified using specific primers to *PsMIPS*, *PsITPK1* or pea *18S* small subunit nuclear rRNA. Gene-specific primers for *PsITPK1* were designed to a contig in *Pisum sativum* (Ps_contig_mira-and-tgicl-ass_franssen_35215) identified from the Cool Season Food Legume Genome Database as described above for *PsMIPS*. This contig contained the entire coding region of the *PsITPK1* gene, confirmed by translation and alignment with alfalfa (*Medicago sativa*) *ITPK1* (GenBank Accession Number XM003610595.1). Primers (forward primer, 5′- TCTACTCTCTGCAACACAACACA-3′; reverse primer, 5′-AACCACTGCCTAGCCAAGGA-3′) were designed to amplify a 1,554-bp fragment encompassing the 978-bp ORF of *ITPK1* from pea. Primers (forward primer, 5′-CATTGGAGGGCAAGTCTGGT-3′; reverse primer, 5′- CCAGCGGAGTCCTAAAAGCA-3′) for pea *18S* (GenBank accession number U43011.1) generated a 510-bp amplicon that was used as a reference gene (Ozga et al. 2003). RNA was isolated as described above and 100 ng was used as template in the 25 μL reactions. The following cycling conditions were used: cDNA synthesis at 50°C for 15 min, enzyme inactivation at 95°C for 2 min, then 40 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, followed

by a final extension at 72°C for 5 min. PCR products were visualized on a 1.2% (w/v) agarose gel run in 1× TAE and stained with ethidium bromide (Sambrook and Russell 2001).

3.3.6 Sequence analysis

Sequencing results were assembled using the BioEdit sequence alignment editor (v7.2.5, http://www.mbio.ncsu.edu/BioEdit/bioedit.html; Hall 1999). Deduced amino acid sequences were obtained using the Translate Tool on the ExPASy SIB Bioinformatics Resource Portal (http://web.expasy.org/translate/). MIPS nucleotide and protein sequences were obtained from GenBank (www.ncbi.nih.nlm.gov) at NCBI and aligned using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi, Altschul et al. 1997) and CLUSTALW2.1 at the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/msa/clustalw2/; Larkin et al. 2007). Domain analysis of MIPS amino acid sequences was performed using InterProScan (http://www.ebi.ac.uk/interpro/interproscan.html; Quevillon et al. 2005). Phylogeny analysis was performed using the MEGA6 program (http://www.megasoftware.net/; Tamura et al. 2013) and available amino acid sequences (NCBI protein sequence database; http://www.ncbi.nlm.nih.gov/nuccore).

3.3.7 Statistics

All statistical analyses were performed using SAS® 9.3 (SAS Institute Inc., Cary, NC). The experimental data presented are mean values from two locations over two years. Levene's test (Levene 1960) was conducted to analyse the homogeneity of variance and the results represent means ± standard error (SE), based on four replications. Significant differences were determined by a one-way analysis of variance (ANOVA), $P < 0.05$. Differences among the means were analyzed by a least significant difference (LSD) post-hoc test at $P < 0.05$.

3.4 Results

3.4.1 Agronomic characteristics of low-phytate pea genotypes

The agronomic characteristics of the *lpa* pea genotypes were similar to their normal phytate progenitor CDC Bronco except for 1000 seed weight and grain yield at maturity (Fig. 3.1; Table 3.1). CDC Bronco and genotypes 1-150-81 and 1-2347-144 did not differ in percent emergence, days to flowering, plant height, mycosphaerella blight score, lodging and days to maturity at all four site years (Table 3.1). CDC Bronco was higher in mean 1000 seed weight (219 g) than 1-150-81 and 1-2347-144 with 207 and 205 g respectively. CDC Bronco had the

highest grain yield (2.8 t ha^{-1}), significantly greater than genotypes 1-150-81 and 1-2347-144, each with 2.3 t ha^{-1}.

3.4.2 Accumulation of phosphorus and phosphorus-containing compounds during seed development

In seed coat extracts of CDC Bronco, 1-150-81 and 1-2347-144, there was no detectable amount of IP_6 or other inositol polyphosphates during seed development from 14 to 49 DAF (data not shown). In addition, no lower inositol phosphate intermediates (IP_1, IP_3, IP_4, IP_5) other than phytic acid were detected in cotyledons of CDC Bronco, 1-150-81 and 1-2347-144 in any of the developmental stages analyzed (data not shown).

The concentration of IP_6 at 14 DAF was not significantly different among CDC Bronco, 1-150-81 and 1-2347-144 (Fig. 3.2a). However, the concentrations of IP_6 among CDC Bronco, 1-150-81 and 1-2347-144 started to differ significantly from 21 DAF onwards (Fig. 3.2a). In CDC Bronco, the concentration of IP_6 ranged from 0.20 mg g^{-1} DW at 14 DAF and increased steadily to 1.86 mg g^{-1} DW at 49 DAF (Fig. 3.2a). A similar trend was observed for IP_6 in 1-150-81 and 1-2347-144. However, the *lpa* genotypes 1-150-81 and 1-2347-144 showed 60% and 65% reduction in IP_6, respectively, when compared to their progenitor CDC Bronco (Fig. 3.2a). In 1-150-81 IP_6 concentrations ranged from 0.11 to 0.65 mg g^{-1} DW while the range of IP_6 concentration in 1-2347-144 was 0.08 to 0.75 mg g^{-1} DW (Fig. 3.2a). Since it was assumed all P_{PhA} came from IP_6, these results mirrored those of phytic acid and absolute values are presented in Fig. S1.

Fig. 3.1. Developing seeds of pea (*Pisum sativum* L.). Indicated are CDC Bronco and low-phytate genotype 1-150-81. Representative photographs are shown from the Rosthern site in 2010. DAF, days after flowering

Table 3.1. Agronomic traits of pea (*Pisum sativum* L.) cultivar CDC Bronco and low-phytate genotypes 1-150-81 and 1-2347-144 assessed at Saskatoon and Rosthern, Saskatchewan in 2010 and 2011. Values represent means ± SE, n = 16. Different letters within a column indicate a significant difference at $P < 0.05$ based on LSD test

Genotype	Emergence Count (%)[d]	Days to Flower	Plant Height (cm)[e]	Mycosphaerella Blight Score (0-9 Scale)[f]	Lodging Score (1-9 Scale)[g]	Days to Mature	Grain Yield (t ha^{-1})[h]	1000 Seed Weight (g)
CDC Bronco	56 ± 3.0 [a]	58 ± 1.0 [a]	79 ± 1.0 [a]	5.3 ± 0.1 [a]	5.4 ± 0.2 [a]	100 ± 1.0[a]	2.83 ± 0.1 [a]	219 ± 2.2 [a]
1-150-81	55 ± 3.0 [a]	59 ± 1.0 [a]	78 ± 2.0 [a]	5.2 ± 0.1 [a]	5.3 ± 0.1 [a]	102 ± 1.0 [a]	2.36 ± 0.2 [b]	207 ± 1.6 [b]
1-2347-144	53 ± 3.0 [a]	59 ± 1.0 [a]	76 ± 2.0 [a]	5.3 ± 0.1 [a]	5.3 ± 0.1 [a]	101 ± 1.0 [a]	2.33 ± 0.2 [b]	205 ± 2.3 [b]

[d]Based on seedlings in a 1 m^2 section of each plot counted 5 weeks after planting

[e]Measured when the pod set was completed

[f]Assessed base on 0-9 scale, where 0 = no disease, 9 = completely blighted

[g]Assessed based on 1-9 scale, where 1 = erect, 9 = completely lodged

[h]Residual harvest weighed after sampling developing seeds (120 pods from each plot)

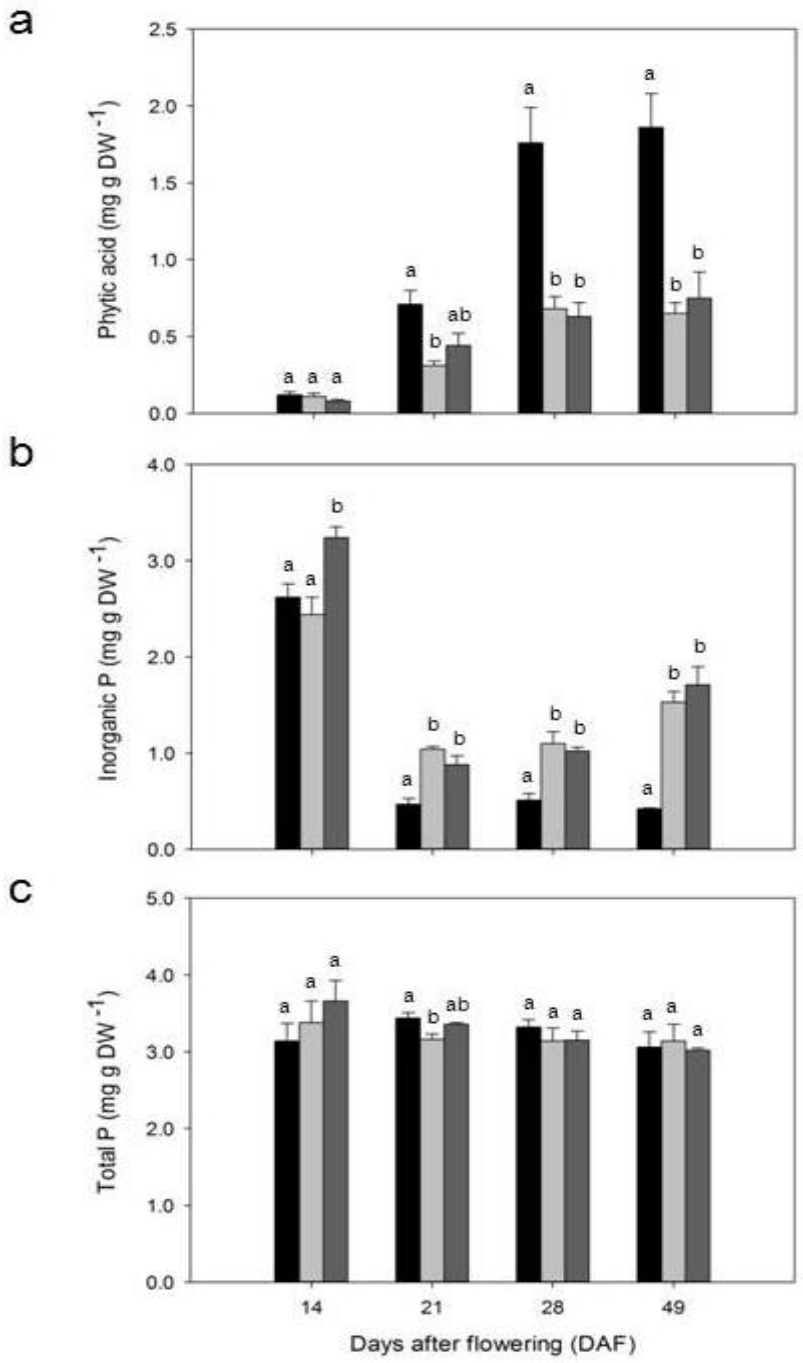

Fig. 3.2. Content of **a** phytic acid, **b** inorganic P (P_i) and **c** total P in developing seeds of pea (*Pisum sativum* L.) for CDC Bronco (black bars) and low-phytate genotypes 1-150-81 (light grey bars) and 1-2347-144 (dark grey bars). Values represent means ± SE, $n = 4$. Different letters associated with bars within each DAF are significantly different at $P < 0.05$. DAF, days after flowering; DW, dry weight; P, phosphorus.

P_i concentration at 14DAF was not significantly different for CDC Bronco and 1-150-81 with 2.62 and 2.44 mg g^{-1} DW, respectively (Fig. 3.2b). At 14 DAF, 1-2347-144 had more P_i (3.24 mg g^{-1} DW) than the other two genotypes. From 21 DAF to 49 DAF, P_i concentrations between the *lpa* genotypes were similar and significantly higher than CDC Bronco (Fig. 3.2b). At 49DAF, 1-150-81 and 1-2347-144 were 72 and 84% higher in P_i, respectively, than CDC Bronco.

The total P accumulation pattern was similar among CDC Bronco and the two *lpa* genotypes (Fig. 3.2c). The concentration of total P was not significantly different between CDC Bronco and the *lpa* genotypes except at 21DAF. CDC Bronco had 3.44 mg g^{-1} DW total P at 21 DAF which was significantly different from 1-150-81 and 1-2347-144 with 3.16 and 3.36 mg g^{-1}DW total P, respectively (Fig. 3.2c). The variations in IP_6 and P_i levels did not affect the total P concentration of these genotypes.

3.4.3 Characterization and bioinformatic analyses of *PsMIPS*

The *PsMIPS* primers amplified a single product of the correct size (1,602-bp) from 14 DAF seed samples of CDC Bronco, 1-150-81 and 1-2347-144 (data not shown). These fragments were excised and sequenced. The obtained cDNA sequence contained a 1,530-bp open reading frame (ORF), and encoded a protein of 510 amino acids with a molecular weight of 56.5 kD and pI of 5.35 (Fig. 3.3). The *PsMIPS* ORFs of CDC Bronco and the two *lpa* mutants, 1-150-81 and 1-2347-144 demonstrated a 100% homology (Fig. S2). There were no mutations observed in the form of nucleic acid substitutions between the three ORFs.

The deduced amino acid sequence of *Ps*MIPS was aligned along with four other plant MIPS and is shown in Fig. 4. The MIPS protein from *Pisum sativum* has a 97% identity with MIPS from *Medicago truncatula*, a 96% identity with *Cicer arietinum*, a 94% identity with *Glycine max* and a 92% identity with MIPS from *Phaseolus vulgaris* (Fig. 3.4). Also present in PsMIPS are four motifs that are highly conserved in all MIPS proteins: GWGGNNG (domain 1), LWTANTERY (domain 2), NGSPQNTFVPGL (domain 3) and SYNHLGNNDG (domain 4), all of which are involved in cofactor (NAD^+) binding and reaction catalysis of MIPS protein (Fig. 3.4; Majumder et al. 1997, 2003).

The *Ps*MIPS protein sequence of CDC Bronco was used in a phylogenetic analysis with 14 other MIPS sequences obtained from the NCBI database for a variety of plants.

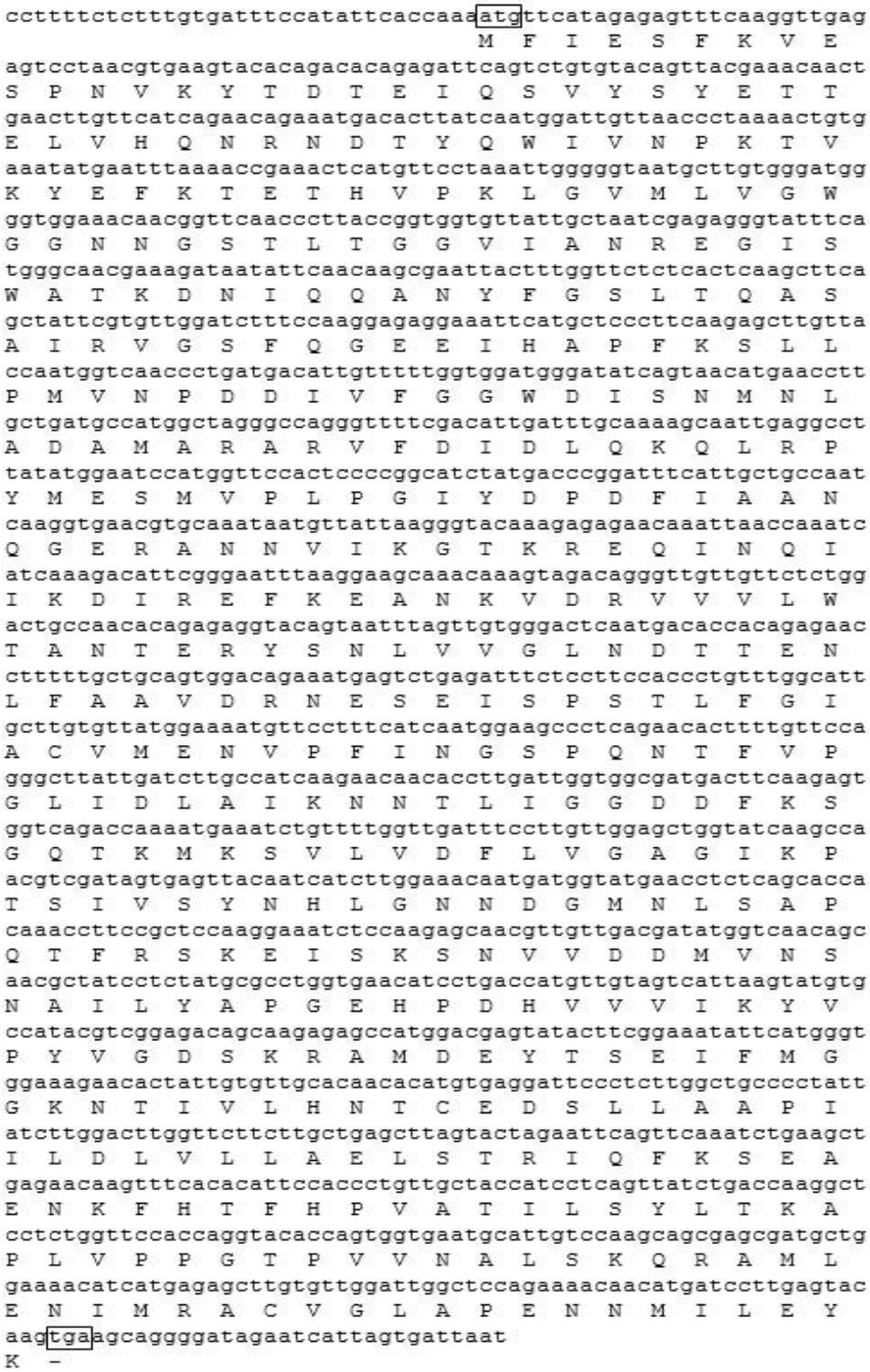

Fig. 3.3. *PsMIPS* cDNA and deduced amino acid sequence from *Pisum sativum* CDC Bronco. Initiation and termination codons are boxed and shown in bold text.

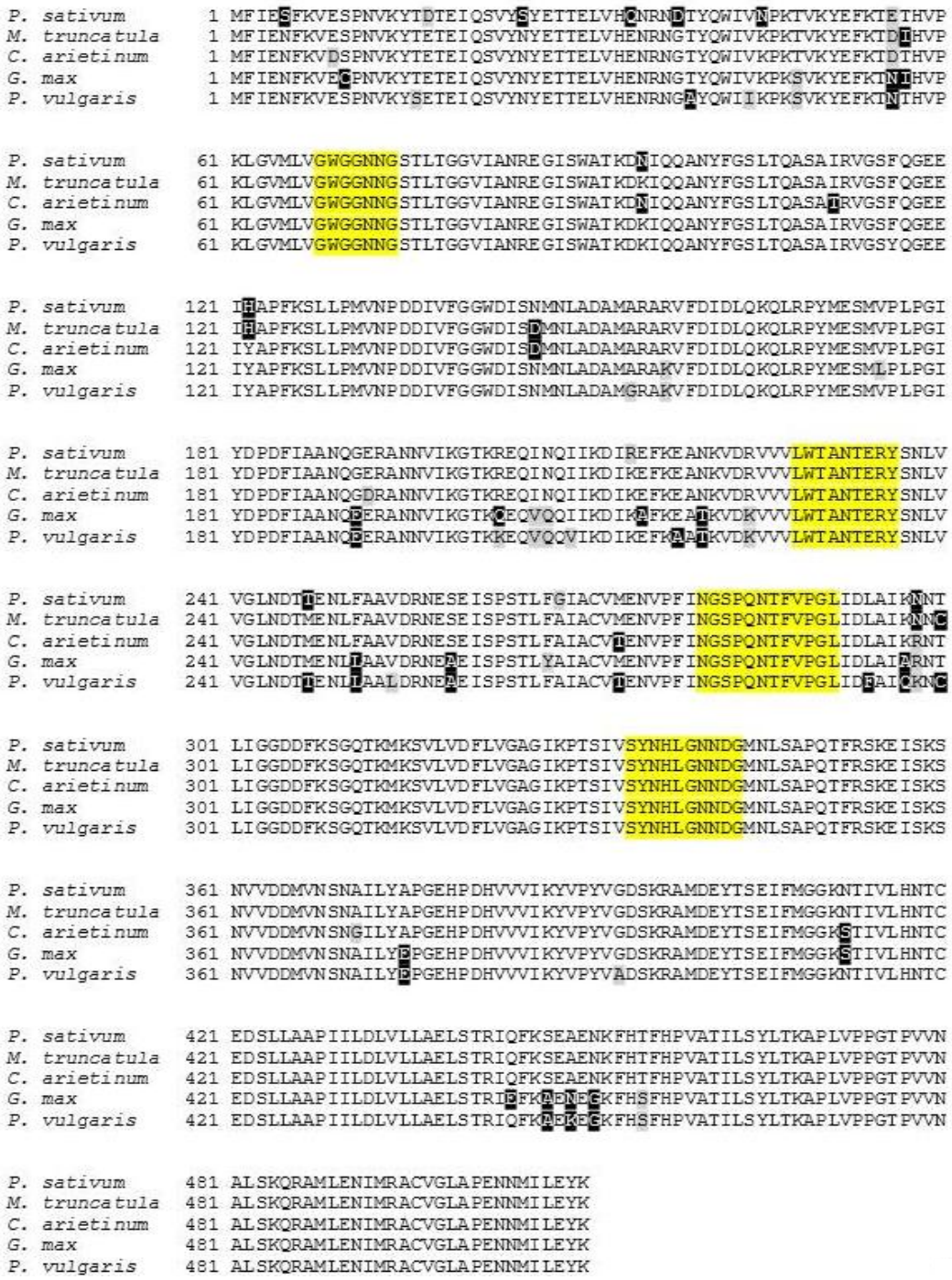

Fig. 3.4. Amino acid sequence alignment of *Ps*MIPS from *Pisum sativum* CDC Bronco with other plant MIPS sequences. Conserved domains are highlighted in yellow. Sequences were obtained from GenBank for *Medicago truncatula*, XP_003601987.1; *Cicer arietinum*, NP_001266035.1; *Glycine max*, ABC55420.1; and *Phaseolus vulgaris*, XP_007159720.1. Conserved and unconserved substitutions are indicated in black and grey boxes respectively.

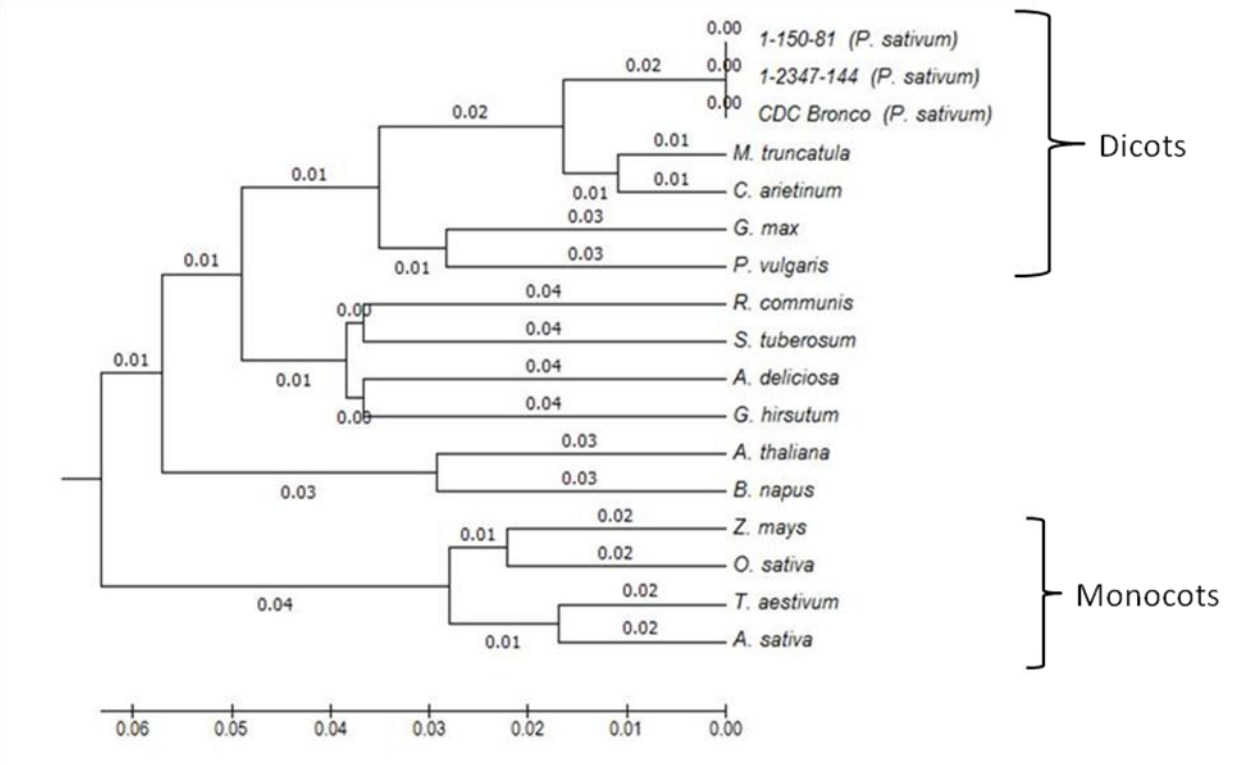

Fig. 3.5. Phylogenetic analysis of MIPS proteins from different plants including *Pisum sativum* L. (for CDC Bronco and low-phytate genotypes 1-150-81 and 1-2347-144). GenBank Accession Numbers include: *Medicago truncatula*, XP_003601987.1; *Glycine max*, ABC55420.1; *Cicer arietinum*, NP_001266035.1; *Phaseolus vulgaris*, XP_007159720.1; *Ricinus communis*, ACU30131.1; *Zea mays*, ACG33827.1; *Oryza sativa*, BAA25729.1;*Triticum aestivum*, AEQ61648.1; *Arabidopsis thaliana*, NP_179812.1; *Actinidia deliciosa*, AFV31635.1; *Brassica napus* ACJ65004.1; *Solanum tuberosum*, XP_006366474.1; *Gossypium hirsutum*, ACJ11714.1; *Avena sativa*, BAB40956.2. The scale bar represents 0.05 substitutions per amino acid site, reflected in the lengths of the branches. Bootstrap values from a 1000 iteration analyses are shown in italics.

The phylogenetic tree presented in Fig. 3.5 shows the evolutionary divergence among the plant MIPS sequences analysed. The analysis confirmed PsMIPS protein sequences of CDC Bronco and the two *lpa* genotypes, 1-150-81 and 1-2347-144 to be 100% identical and they cluster together. The monocots, *Z. mays, O. sativa, T. aestivum and A. sativa* clearly cluster together in one branch. Two distinct sub-branches were obtained for dicots. *P. sativum*, *M. truncatula* and *C. arietinum* clustered together in a branch while *G.max* and *P.vulgaris* formed the other branch. MIPS from *R. communis*, *S. tuberosum*, *A. deliciosa* and *G. hirsutum* were placed in a separate branch. *A. thaliana* and *B. napus* aligned together as an individual branch.

3.4.4 *PsMIPS* and *PsITPK1* gene expression

An examination of the *PsMIPS* transcript profile in developing seeds by sqPCR revealed decreased expression levels for CDC Bronco, 1-150-81 and 1-2347-144 at 49 DAF when compared to 14 DAF (Fig. 3.6). In comparison to CDC Bronco, *PsMIPS* expression at 14 DAF was somewhat decreased in 1-150-81 and elevated in 1-2347-144, while levels were essentially the same at 49 DAF (Fig. 3.6). A similar trend was observed for levels of *PsITPK1* transcripts (Fig. 3.6).

3.5 Discussion

3.5.1 Agronomic traits in low-phytate pea

Genes, alleles and environmental conditions affect the agronomic performance of all crops. It has been demonstrated that *lpa* crops can be generated without major compromises in plant performance (Gillman et al. 2009). The agronomic performance of 1-150-81 and 1-2347-144 did not significantly differ from CDC Bronco when assessed for traits such as percent emergence, plant height, lodging score, days to flowering and days to maturity. In soybean *lpa* genotypes a reduced seedling emergence has been observed (Anderson and Fehr 2008). The difference in temperature during seed filling was proposed to be reason for the reduced emergence in low phytate genotypes. A previous study by Warkentin et al. (2012) showed that 1-150-81 and 1-2347-144 were slightly different from CDC Bronco in days to flowering and days to maturity. They reported that 1-150-81 and 1-2347-144 flowered 3 days later and matured 2 to 3 days later than CDC Bronco. This was not observed in the present study. However, the grain yield at maturity was significantly different between 1-150-81, 1-2347-144 and CDC Bronco,

with 1-150-81 and 1-2347-144 showing 18 and 16% lower grain yields than CDC Bronco, respectively.

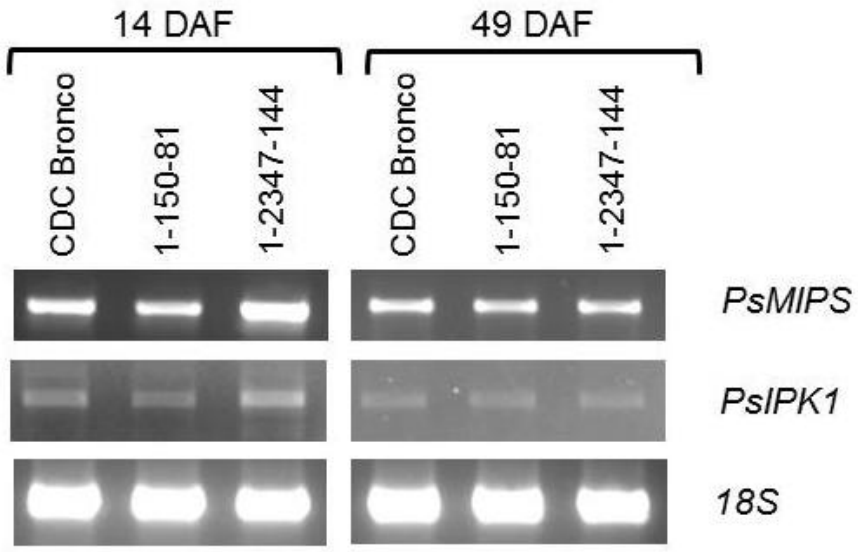

Fig. 3.6. Transcript abundance of *PsMIPS* and *PsITPK1* in developing seeds of pea (*Pisum sativum* L.) for CDC Bronco and low-phytate genotypes (1-150-81 and 1-2347-144) at 14 DAF and 49 DAF as indicated. A fragment of *18S* rRNA used as a loading control is also shown. Results are representative from a minimum of three independent experiments. DAF, days after flowering.

This is similar to differences in grain yield observed by Warkentin et al. (2012). Since the phytic acid biosynthesis pathway is active in most tissues of a plant, *lpa* mutations can also affect the vegetative processes apart from phytate accumulation (Raboy 2001). Lower seed weight might be attributed to a reduction in starch accumulation resulting from a defective inositol phosphate synthesis pathway (Bentsink et al. 2003). By targeting a specific gene or its expression to appropriate seed tissues, low-phytate crops can be produced restoring their seed weight and yield (Raboy 2001). Embryo-specific silencing of expression of an ATP binding cassette (ABC) transporter in maize produced seeds with low-phytic acid with no adverse effect on seed weight (Shi et al. 2007). When the *myo*-inositol methyltransferase (*IMT*) gene was transferred to *Brassica napus* through a transgenic approach, a 19 to 35% reduction in phytate was achieved without affecting the seed parameters (Dong et al. 2013). In addition, rice low-phytate mutants produced through RNAi mediated seed-specific silencing of inositol penta*kis*phosphate 2-kinase (*IPK1*) gene, had no undesirable agronomic characters (Ali et al. 2013). These studies provide evidence that low-phytate mutants can be produced in crop varieties with a compromise in agronomic traits like grain yield.

3.5.2 Redistribution of phosphorus in low-phytate pea during seed development

A large fraction of nutrient P taken up by crop plants is ultimately packaged into seed phytic acid and this single small molecule represents a major pool in the flux of P in the world's agricultural ecology. As phytic acid represents a significant portion of total seed P, the accumulation of phytate and inositol phosphates has been studied in different plant species. Grain crops typically contain about 10 mg g^{-1} phytic acid on a seed dry weight basis, representing about 65 to 85% of seed total P (Raboy 1990, 1997). Total P concentration typically ranges from 3 to 4 mg g^{-1} in seed produced by grain crops, with P_{PhA} ranging from 2 to 3 mg g^{-1}. In this study, the concentration of IP_6 and its lower isomeric forms (IP_1, IP_3, IP_4 and IP_5) were analyzed separately in seed coats and cotyledons. There was no traceable amount of inositol phosphates in seed coats. The accumulation of IP_6 was continuous and linear throughout the stages of seed development and the highest concentration was observed at 49 DAF. The other *myo*-inositol polyphosphates (IP_3, IP_4 and IP_5) and IP_1 were not present in traceable amounts. In soybean wild-types (Raboy and Dickinson, 1987) and maize *lpa1-1, lpa2-1* and wild types (Raboy et al. 2000), phytate was reported to accumulate gradually during seed development. P_i

concentration decreased during seed development in wild type, and total P levels remained relatively consistent. The maize *lpa* genotypes had little to no increase in phytate, and P_i concentration was high and did not decrease during development. In barley, the final levels and partitions of P forms are well documented. Phytic acid concentration in the *lpa* lines accumulated similar pattern to the normal phytate lines. Inorganic P concentration increased from 24 days till maturity in *lpa* lines. However, it is unclear when in seed development the expression of the *lpa* genotype is initiated and how seed P accumulates in developing seeds of different barley *lpa* genotypes (Bowen et al. 2006). Israel et al. (2011) compared the changes in seed phytic acid concentrations as well as *myo*-inositol phosphates during seed development between *lpa* and normal phytate genotypes and found that IP_3 content was relatively low in all genotypes and decreased during seed maturation. The other inositol phosphates such as IP_4 and IP_5 were not detected. Larson et al. (2000) reported that a reduction in seed phytate results in a molar equivalent increase in P_i in rice. In the present study, P_i concentration in the two *lpa* genotypes was 72 to 84% higher than in CDC Bronco. Between the two *lpa* genotypes, 1-2347-144 had 11% more P_i than 1-150-81. Throughout the developing stages, P_i concentration was higher at 14 DAF and decreased as IP_6 accumulation began. Thus, there appears to be an inverse relationship between P_i accumulation and phytic acid content. The total P concentration showed no significant difference between CDC Bronco, 1-150-81 and 1-2347-144 except at 21 DAF where CDC Bronco and 1-2347-144 had 8 and 6% more total P respectively than 1-150-81. This demonstrates that despite variations in IP_6 and P_i levels, the total P content in the seeds was unaffected. The significantly higher P_i concentration in *lpa* pea genotypes compared to CDC Bronco shows that the *lpa* genotypes tend to balance the total P levels within the seed to provide adequate supply of P required for normal seed development. Unlike the normal genetic and environmental effects that result in quantitative variation in seed total P, *lpa* mutants show large effects on the partitioning of P into P_{PhA}, P_i.

3.5.3 *myo*-inositol-3-phosphate synthase (*MIPS*) is identical in normal and low-phytate pea genotypes

Genetic mapping and comparing the position of *lpa* mutation with *MIPS* loci in rice have been reported by Larson et al. 2000. They mapped the rice *MIPS* gene on to a locus on chromosome 3, which was orthologous to the *MIPS* gene near maize *lpa1* on chromosome 1S. Previously, *MIPS* gene expression proximal to the site of phytic acid synthesis during grain development in rice was demonstrated (Yoshida et al.1999). Hitz et al. (2002) confirmed a

mutation in *MIPS* responsible for LR33 *lpa* mutation in soybean. Furthermore, higher *MIPS* expression was found in wheat genotypes with high phytic acid levels compared to a *lpa* genotype (Ma et al. 2013). These findings have contributed to the interest in *MIPS* as a target for manipulation to produce low phytate crops. *lpa* genotypes of *Arabidopsis*, potato, rice, soybean and canola have been generated by down-regulation (antisense, RNA interference, or cosuppression) or mutations in *MIPS* gene (Keller et al. 1998, Nunes et al. 2006, Georges et al. 2006, Murphy et al. 2008, Xu et al. 2009). The current study showed that there are no mutations in the *PsMIPS* nucleotide sequences between the *lpa* and normal phytate pea genotypes. This indicates that the low-phytate phenotype in pea is not due to mutations in coding regions of *MIPS* gene.

Our sqPCR analysis revealed that at 14DAF the expression of *PsMIPS* was higher in 1-2347-144 than CDC Bronco and 1-150-81. However, at 49 DAF, its expression was similar in both *lpa* genotypes and CDC Bronco. This further confirms that the reduction in phytate levels caused by *lpa* mutations is not directly controlled by changes in *MIPS* expression. Moreover, mutations affecting *MIPS* are often associated with lower seed yield, seed viability, increased susceptibility to pathogens and undesired morphology (Keller et al. 1998, Yuan et al. 2007, Murphy et al. 2008) and although the seed weight in *lpa* pea genotypes was lower, compared to their normal progenitor, the phytate reduction was not detrimental. The *lpa* genotypes were similar to CDC Bronco in all other agronomic traits including mycosphaerella blight resistance. Therefore, we suggest that the mutation could have occurred in the late steps of the phytate biosynthetic pathway, involving inositol kinases. For example, Shi et al. (2003) found that the maize *lpa*2 mutant is caused by a mutation in an inositol phosphate kinase gene. Stevenson-Paulik et al. (2005) generated phytate-free seeds in *Arabidopsis* through disruption of inositol polyphosphate kinases. Thus, to gain further insights into the mutation causing the low phytate phenotype in pea, it is necessary to analyse the other enzymes related to phytate biosynthesis. We examined the expression of *PsITPK1* gene through sqPCR and observed that its expression at 14 DAF was higher in 1-2347-144 compared to CDC Bronco and 1-150-81. At 49 DAF, there was no difference in expression level of *PsITPK1* between CDC Bronco and the *lpa* genotypes. This is similar to the trend we observed for *PsMIPS* expression in these genotypes. Mutation in inositol phosphate kinase gene in maize produced *lpa* mutants with 30% less IP_6 and 3-fold more P_i (Shi et al. 2003). Once the causative mutation is identified in these low-phytate pea genotypes,

it could then be mapped on to plant genomes and used in marker assisted breeding to select low-phytate genotypes.

In conclusion, this study describes the accumulation patterns of phosphorus compounds in developing seeds of two low-phytate pea genotypes. Based on the presented evidence, the possibility of a *MIPS* mutation being responsible for the low phytate trait in these pea genotypes is excluded. Interestingly, no accumulation of lower inositol polyphosphates was observed.

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CHAPTER 4

4. Mapping seed phytic acid concentration and iron bioavailability in a pea recombinant inbred line population

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"Arun S.K. Shunmugam, Xiaofei Liu, Robert Stonehouse, Bunyamin Tar'an, Kirstin E. Bett, Andrew Sharpe, Thomas D. Warkentin. Mapping seed phytic acid concentration and iron bioavailability in a pea recombinant inbred line population."

The work carried out in the above manuscript follows on previous work including;

1. Rehman, A.U., **Shunmugam, A**., Arganosa, G., Bett, K.E., Warkentin, T.D. 2012. Inheritance of the low-phytate trait in pea. *Crop Sci.* 52: 1-5.

2. Sindhu, A., Ramsay, L., Sanderson, L., Stonehouse, R., Li, R., Condie, J., **Shunmugam, A.S.K.,** Liu, Y., Jha, A.B., Diapari, M., Burstin, J., Aubert, G., Tar'an, B., Bett, K.E., Warkentin, T. D., and Sharpe, A. G., 2014. Gene-based SNP discovery and genetic mapping in pea. *Theoretical and Applied Genetics.* DOI: 10.1007/s00122-014-2375-y.

3. Jha, A.B., Tar'an, B., Diapari, M., Sindhu, A., **Shunmugam, A.S.K.,** Bett, K., Warkentin, T.D. 2014. Allele diversity analysis to identify SNPs associated with mycosphaerella blight resistance in pea. *Euphytica* (In press).

4.1 Abstract

Phytate, the storage form of phosphorus in seeds, is not well digested by monogastrics contributing to environmental pollution. This research was aimed at developing a single nucleotide polymorphism (SNP) based genetic linkage map and mapping genomic regions associated with phytic acid-phosphorus (PA-P) concentration using a recombinant inbred line (RIL) population (PR-15) derived from a cross between a low phytate (*lpa*) mutant pea genotype, 1-2347-144, and a normal phytate cultivar CDC Meadow. A total of 163 RILs were genotyped using 1536 SNP markers in an Illumina GoldenGate array. Three hundred and sixty seven polymorphic SNP markers ordered into 7 linkage groups (LGs) were used to generate a linkage map with a total length of 437.2 cM. PR-15 lines were grown in replicated field trails in Saskatoon and Rosthern, Saskatchewan in 2012 and 2013. Chi-square statistics confirmed the single gene inheritance of PA-P concentration in these RILs. Phytic acid-phosphorus (PA-P) phenotype was mapped on to LG5. Iron bioavailability (FEBIO) of PR-15 lines was negatively correlated with PA-P concentration. A quantitative trait locus (QTL) for FEBIO was mapped on to the same location in LG5 as phytic acid concentration. The QTL with a maximum LOD score of 15.1 explained 60.5 % of the phenotypic variation. The markers flanking this QTL region can be employed in marker-assisted selection to select pea lines with low phytate and greater iron bioavailability.

Key words: Phytic acid, Iron bioavailability, *lpa* mutants, Recombinant Inbred Lines, SNP Genotyping, Genetic Linkage map, QTLs.

Abbreviations: *lpa*, low phytic acid; RIL, recombinant inbred lines; LG, linkage group; SNP, single nucleotide polymorphism; QTL, quantitative trait locus; LOD, likelihood odds ratio; PA-P, phytic acid-phosphorus; FEBIO, iron bioavailability.

4.2 Introduction

myo-inositol (1,2,3,4,5,6) hexa*kis*phosphate (IP_6), termed as 'phytic acid' by 19^{th} century chemists was first known as the storage form of phosphorus (P) in seeds (Cosgrove, 1980). Phytic acid and its lower phosphorylated derivatives play a vital role in the regulation of many fundamental processes in plant and animal cells. The possible functions of phytic acid in plant seeds include: storage of phosphorus, storage of energy, inducing dormancy when the seeds approach maturity by competing for ATP molecules, regulating the concentration of inorganic phosphorus in seeds and storage of mineral ions (Cosgrove, 1980). As an illustration, it was estimated that the global annual production of seed phytic acid P by crops represents a sum equivalent to more than 50% of the total elemental P applied world-wide in mineral fertilizers (Lott et al., 2000).

Phytic acid has a negative effect on human health and animal nutrition. It chelates essential minerals such as iron, calcium, zinc, and potassium and forms insoluble phytate-mineral complexes, which are generally excreted by humans and non-ruminants, especially poultry, swine and fish (Sharply et al., 1994). These complexes become unavailable for absorption to humans and non-ruminants, since they lack adequate amount of phytase enzyme in their intestine (Reddy et al., 1982). Phytic acid can also bind with negatively charged groups of proteins and starch molecules, which may result in a decrease of protein and starch digestibility and bioavailability (Reddy et al., 1982). In addition, the undigested phytic acid-phosphorus excreted by non-ruminant animals is an important component of phosphorus pollution in the environment (Raboy et al., 2001). Due to the nutritional and environmental concerns, development of cultivars with the low phytate trait has become an important plant breeding objective. Low phytic acid (*lpa*) mutants have been isolated in maize (*Zea mays* L., Raboy and Gerbasi 1996; Raboy et al., 2000), barley (*Hordeum vulgare* L.; Larson et al., 1998; Rasmussen and Hatzak 1998) and soybean (*Glycine max* L. Merr, Wilcox et al., 2000). Two *lpa* genotypes of field pea (*Pisum sativum* L.) have been isolated through chemical mutagenesis using EMS. The *lpa* genotypes, 1-150-81 and 1-2347-144 showed 60% reduction in seed phytic acid with a compensating increase in inorganic phosphorus (Warkentin et al., 2012). When compared to their progenitor CDC Bronco, which is a normal phytate genotype, these genotypes performed

similar in many agronomic characteristics. They were significantly lower in seed weight, grain yield and slower in days to flowering and maturity. Inheritance studies performed with these genotypes showed that the low phytate trait is controlled by a single recessive allele (Rehman et al., 2012).

Iron deficiency is an important malnutrition risk, and approximately 2 billion people are affected by iron deficiency (WHO 2002). Since phytate chelates iron, this study also focussed on the potential benefits of *lpa* field pea in improving iron bioavailability. It was confirmed that foods containing more phytate have lower iron bioavailability (Glahn et al., 2002).

Linkage maps in organisms indicate the position and relative genetic distances between specific markers along chromosomes. With the advent of high-throughput multiplex assays, or chip based SNP discovery, thousands of SNP markers can be genotyped at one time in a crop species. A saturated genetic linkage map of chickpea was constructed using 697 SNP markers using GoldenGate genotyping technology from Illumina (Gaur et al., 2012). A genetic linkage map of soybean with 657 SNPs using SoyS-NP6K Illumina Infinium Beadchip Genotyping Array was generated (Akond et al., 2013). A linkage map using 1536 GoldenGate SNP assay was constructed and tested in diploid and tetraploid recombinant inbred lines (RIL) of peanut (Bertioli et al., 2014). ESTs derived from nine lentil accessions were used to develop an Illumina GoldenGate 1536-SNP assay (Sharpe et al., 2013). A highly multiplexed SNP genotyping assay for genetic mapping and germplasm diversity studies in pea was evaluated using a set of 384 SNP markers (Deulvot et al., 2010). Leonforte et al., (2013) developed a genetic linkage map in pea using SNP and SSR markers. This linkage map permitted the identification of salinity tolerance quantitative trait loci (QTL) in pea. Recently, a comprehensive pea SNP map was generated using Illumina GoldenGate 1536-SNP assay (Sindhu et al., 2014). The PR-15 recombinant inbred line (RIL) population was developed by crossing one of the *lpa* genotypes (1-2347-144) and a normal phytate pea variety CDC Meadow.

The objective of the present study was to generate a SNP linkage map for PR-15 RILs, map the phytic acid-phosphorus phenotype and to identify quantitative trait loci (QTL) associated with iron bioavailability in PR-15 RILs. The development and use of molecular markers for selection for *lpa* trait in pea could facilitate breeding efforts aimed at developing *lpa* cultivars.

4.3 Materials and Methods

4.3.1 Plant material

PR-15 recombinant inbred lines (RILs) were developed from a cross between a *lpa* genotype 1-2347-144 (Warkentin et al. 2012) and CDC Meadow (Warkentin et al., 2007) under greenhouse conditions in November 2009 (Rehman et al. 2012). These lines were advanced to F_6 generation by single seed descent method. F_6 derived F_7 generation was derived by bulking F_6 in April 2011. A total of 277 RILs with the two parental cultivars were seeded in a field nursery near Saskatoon, Saskatchewan in May 2011. Young leaves were collected from RILs, frozen in liquid nitrogen and stored at -80˚C until DNA extraction using a modified CTAB method (Doyle and Doyle, 1990). One hundred sixty three lines were selected randomly from those 277 lines and were evaluated in field trials in 2012 and 2013 near Rosthern (Black soil zone) and Saskatoon (Dark brown soil zone), Saskatchewan. Experiments were laid out in a randomized complete block design with two replicates and plot size of 1 m^2 (micro-plots). Seventy five seeds were planted in four rows with 0.25 m between rows. Field experiments were managed using standard management practices for field pea production in Saskatchewan. Field plots were evaluated for percent emergence, days to flower, plant height, mycosphaerella blight score, lodging score, days to maturity and grain yield using methods previously described (Warkentin et al., 2012).

4.3.2 Estimation of phytate phosphorus (PA-P) concentration

Phytate phosphorus determinations were conducted using the Wade's reagent method (Gao et al., 2007). Briefly, 0.05 g of ground (Retsch Model ZM200, Newtown, PA, USA, 0.5 mm particle size) pea sample from each line was placed in a tube and 1 mL of 0.8N HCl: 10% Na_2SO_4 was added. The aliquots were put on a shaker for 16 hours, and then centrifuged at 3000 g for 20 minutes. Thirty µL of extract was placed in a new tube and 720 µL of double distilled water and 250 µL of Wade's Reagent (0.03% $FeCl_3$ $6H_2O$:0.3% sulfosalicylic acid) was added and the tube was vortexed for 10 seconds. A 200 µL aliquot was placed in microtitre plate wells, and the absorbance values were read at 490 nm using a microplate reader (xMark™; Bio-Rad Benchmark, Hercules, CA, U.S.A.). A stock solution containing 1 mg phytic acid phosphorus per

mL was prepared by dissolving 549.9 mg phytic acid dodecasodium salt hydrate (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) in 100 mL water. This stock solution was used to prepare standard solutions with 25, 50, 100, 200, 300, 400, 500 and 600 µL phytic acid phosphorus per mL .The standard curve was used to obtain the value for phytic acid phosphorus per 30 µL of the 1 mL total extract. This value was converted to mg of phytate phosphorus (PA-P) g-1 as follows:

$$\text{PA-P (ppm, µg/g in seed)} = \text{colorimeter reading (µg/mL in extract)} \times \frac{1 \text{ mL extract}}{0.05 \text{ g tissue}}$$

4.3.3 Estimation of iron bioavailability (FEBIO)

The FEBIO test was conducted at USDA-ARS, Ithaca, New York using an *in vitro* digestion/Caco-2 cell culture bioassay (Glahn, 2009). Due to the limited experimental capacity for the *in vitro* digestion/Caco-2 cell culture bioassay at USDA-ARS, Ithaca, New York, only a random selection of 80 out of 163 of the RILs from the 2012 Rosthern location with 2 replications was selected for evaluation of iron bioavailability. A 20 g sample of whole pea seeds was weighed and rinsed three times with 18 megaohm water, then 60 mL of 18 megaohm water was added to result in a ratio of 1:3 (sample:water) in a beaker. The beaker, which was covered with aluminium foil, was placed in an autoclave and cooked on liquid cycle for 30 min. After cooking, the samples were freeze dried (Labconco®, Kansas City, Missouri, USA), and then ground using a coffee grinder (Cuisinart®, Woodbridge, ON, Canada). For the *in vitro* digestion/Caco-2 cell culture bioassay, 0.5 g cooked ground sample was weighed into a 50 mL plastic centrifuge tube, then 10 ml of solution of 140 mM sodium chloride (NaCl) and 5 mM potassium chloride (KCl) was added, and the sample was mixed to simulate digestion conditions. After adjusting sample solution to pH 2 with 0.1 M HCl, 0.5 mL pepsin solution was added to digest samples and tubes were placed in a rocking incubator for 1 hour. After incubation, the pH of the sample solution was adjusted to 5.5-6.0 with 1.0 M sodium bicarbonate ($NaHCO_3$) and 2.5 ml of pancreatin-bile solution was added to digest samples, followed by adjusting the pH to 6.9-7.0 with 1.0 M $NaHCO_3$. Both pepsin solution and pancreatin-bile solution were purified by cation exchange resin (Chelex® 100, Bio-Rad Laboratories, Inc., CA, USA). After digestion, the Caco-2 cells were fed with 1.5 mL digested sample through a 15 kDa cut-off dialysis membrane. Then the cells were incubated to rock gently for 2 hours. Then the membrane was removed together with the digested sample solution, and the cells were placed back in the incubator

without rocking for 22 hours. After the 22-hour recovery, the cells were harvested and total protein was analyzed using a colorimetric assay (DC^{TM} Protein Assay, Bio-Rad Laboratories, Inc., CA, USA), and ferritin was analyzed using an enzyme immunoassay procedure (Spectro Ferritin, Ramco Laboratories Inc., Stafford, TX, USA). The protein assay was read using an Epoch microplate Spectrophotometer (BioTek®, Winooski, VT, USA). Iron bioavailability of samples (ng ferritin/mg protein) from different experiment runs were standardized by a standard lentil sample included in each run.

In the iron bioavailability test, due to the laboratory capacity to conduct the assay, the test was separated into 8 experimental runs consisting of 4 experimental runs for each replication in 2012 Rosthern. Since the activity of Caco-2 cells in each experimental run might be slightly different, line 1-2347-144 was repeated in each run as a standard. Thus, the final unit to represent iron bioavailability is described as the percentage of iron bioavailability in individual RILs compared to 1-2347-144 in this experiment (% to 1-2347-144), as is the typical procedure in the Glahn laboratory (Glahn, 2009).

4.3.4 SNP Genotyping

SNP genotyping of PR 15 was essentially done as described by Sindhu et al. (2014). Data clustering and allele calling were performed using GenomeStudio software 2010.3 (Illumina Inc., San Diego, CA) and as described in Sharpe et al. (2013). The markers in the RIL population were tested for deviations from segregation ratios through Chi-square analysis. In the present study, out of the 163 RILs genotyped, 4 RILs which showed poor allele calling were removed from the analysis leaving 159 lines for further linkage mapping construction and QTL analysis.

4.3.5 PR-15 linkage map construction

Carthagene 1.2.2 (De Keyser et al., 2010) was used to construct the linkage map with 159 PR-15 lines. The LOD (logarithm of odds ratio) set was 3.0, with a maximum distance less than 30 cM (Kosambi) between two markers. The obtained linkage groups were aligned to 7 pea chromosomes by comparing them with the pea consensus map generated by Sindhu et al. (2014).

4.3.6 Statistical analysis

Statistical analysis of phenotypic data was performed with Statistical Analysis Software (SAS® V9.3 (SAS Institute Inc. 2011, Cary, NC) using PROC MIXED. Genotypes, locations and years were considered as fixed effects and replications were considered as random effects. Levene's test (Levene 1960) was conducted to analyse the homogeneity of variance for all agronomic data and PA-P. PA-P concentration frequency distribution was carried out in MS Excel (Microsoft Canada Inc., Mississauga, ON, Canada).

4.3.7 QTL identification and mapping

MapQTL 5.0 (Van Ooijien., 2004) was used in QTL analysis with least square means of each trait analysed. MQM (multiple QTL mapping) method, with markers of highest LOD as cofactors was used to determine QTL for a particular trait.

4.4 Results

4.4.1 Analysis of agronomic traits of PR-15 lines

The Levene's test indicated that the variance of the agronomic traits assessed were all homogenous (data not shown) which allowed us to combine the years (2012 and 2013) and locations (Saskatoon and Rosthern) data. The analysis of variance (ANOVA) for several agronomic traits in PR-15 evaluated at Saskatoon and Rosthern in 2012 and 2013 is summarized in Table 4.1. PR-15 RILs differed significantly in plant height (HT), the third rating of mycosphaerella blight (MB3) and lodging score (LD). Genotypes did not differ significantly in days to flowering (DTF) and days to maturity (DTM). Year significantly affected all agronomic traits of PR-15 lines except for DTM. Location had a highly significant effect on DTF, HT, MB3, LD and DTM. The genotype-by-year interaction was significant only for LD and the genotype-by-location interaction only for DTF.

4.4.2 PA-P and FEBIO estimation

The variance of PA-P concentration in PR-15 lines in 2012 and 2013 were homogenous (data not shown). The combined analysis of variance for PA-P concentration in 163 PR-15 lines evaluated in 2012 and 2013 is shown in Table 4.2. Genotypes differed significantly in PA-P concentration as did locations and the genotype by location interaction. Meanwhile year and genotype by year did not have a significant effect on PA-P concentration. FEBIO was assessed in a sub-set of PR-15 lines from two replications grown in 2012 at Rosthern. The population median was 93.8 %, CDC Meadow was 84.1 % and 1-2347-144 was 100%. Genotype had a significant effect on FEBIO with F value 3.9 ($p \leq 0.001$). The correlation between the PA-P and FEBIO was significantly negative (-0.83; $p \leq 0.001$; Fig. 2).

4.4.3 Inheritance of PA-P

Fig. 4.1 shows the frequency distribution of the mean PA-P concentration over two years (2012 and 2013) and two locations (Saskatoon and Rosthern) for the 163 PR-15 RILs. As expected, mean PA-P concentration was higher in CDC Meadow (420 ppm) than 1-2347-144 (220 ppm). The mean PA-P concentration in RILs varied from 202 to 594 ppm. The PA-P frequency distribution followed a bimodal pattern. Chi-square analysis of PR-15 lines segregating for PA-P concentration is shown in Table 4.3. The lines were categorized as either low or normal phytate

based on cut-off values calculated by considering the PA-P concentrations of the parents. Heritability (h^2) of PA-P concentration in PR-15 lines calculated using heritability estimation methods of Singh et al., 1992, was 0.43.

Table 4.1: Analysis of variance with F values and significance levels for days to flowering (DTF), plant height (HT), mycosphaerella blight score (MB3), lodging score (LD) days to maturity (DTM) and grain yield in pea recombinant inbred line population (PR-15) derived from a cross between 1-2347-144 and CDC Meadow evaluated at Saskatoon and Rosthern in 2012 and 2013.

		F values					
Effect	**Num DF**	**DTF[a]**	**HT[b]**	**MB3[c]**	**LD[d]**	**DTM[e]**	**Grain Yield**
Genotype	164	1.06^{ns}	1.5**	1.77***	2.35***	0.99 ^{ns}	17.08***
Year	1	1111.07***	143.13***	6789.14***	4584.04***	0.0 ^{ns}	628.30***
Location	1	2601.02***	49.79***	911.4***	130.26***	30.16***	962.90***
Genotype x Year	164	1.12 ^{ns}	0.96 ^{ns}	1.05 ^{ns}	1.78***	0.99 ^{ns}	2.29***
Genotype x Location	164	1.22**	1.0 ^{ns}	1.05 ^{ns}	1.04 ^{ns}	0.98 ^{ns}	1.51***
Genotype x Year x Location	164	1.95***	1.13 ^{ns}	16.25***	1.23**	1.0 ^{ns}	3.48***

Note: ns, not significant; **, significant at $p \leq 0.05$; ***, significant at $p \leq 0.001$.

[a]DTF, days to flowering, assessed when at least 10% of plants had at least one open flower

[b]HT, plant height, measured when pod set was completed

[c]MB3, mycosphaerella blight score, the third rating when the RILs reached physiological maturity

[d]LD, lodging score, assessed at physiological maturity

[e]DTM, days to maturity, assessed when at least 80% of the pods turned to tan color

Table 4.2: Analysis of variance for phytic acid-phosphorus concentration in pea recombinant inbred line population (PR-15) derived from a cross between 1-2347-144 and CDC Meadow evaluated at Saskatoon and Rosthern in 2012 and 2013.

		F value
Effect	**Num DF**	**PA-P**
Genotype	164	5.22***
Year	1	0.65^{ns}
Location	1	132.92***
Genotype x Year	164	0.01^{ns}
Genotype x Location	164	1.63***
Genotype x Year x Location	164	0.01^{ns}

Notes: ns, not significant; ***, significant at $p \leq 0.001$. DF, degrees of freedom.

Table 4.3: Chi-square analysis for 1-2347-144 x CDC Meadow population segregating for phytate acid-phosphorus concentration based on single gene model.

Population	Generation	Year	Expected[a]		Observed		χ^{2bc}	P value
			Normal	Low	Normal	Low		
1-2347-144/CDC Meadow	$F_{6:9}$	2012	84	79	92	71	1.572	0.21
1-2347-144/CDC Meadow	$F_{6:10}$	2013	84	79	86	77	0.098	0.754

[a]Based on single gene model (1:1 ratio for normal:low)

[b]Any chi-square (χ^2) goodness of fit test value less than tabulated value for two classes (3.841) showed good fit between expected and observed frequencies for each class

[c]level of significance, $\alpha = 0.05$

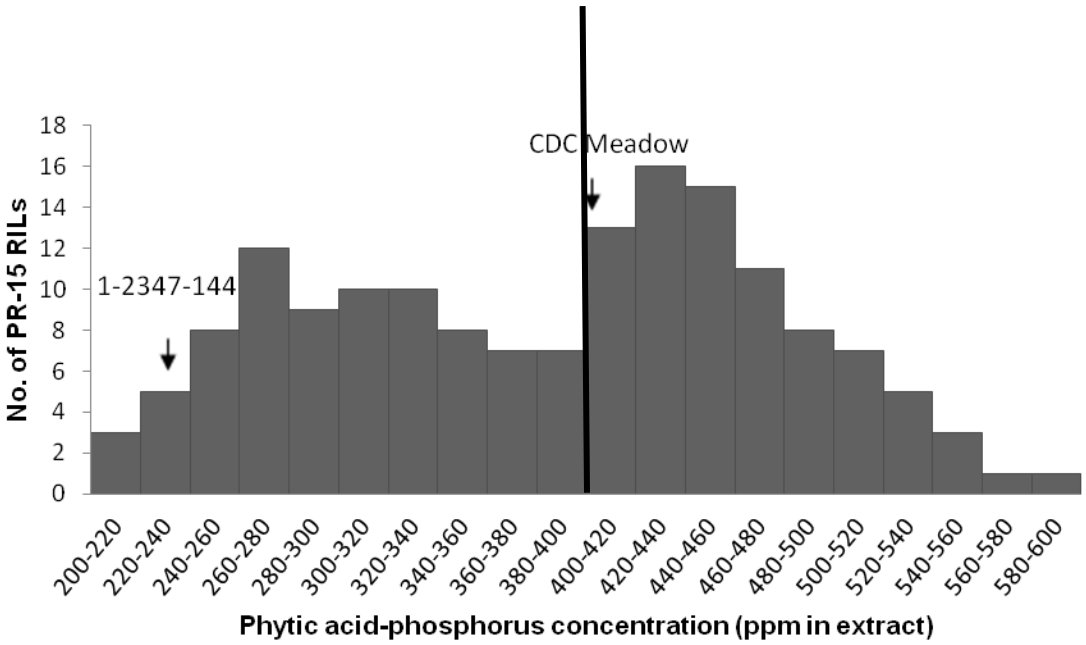

Fig. 4.1. Frequency distribution of the average phytic acid-phosphorus concentration over two years (2012 and 2013) and two locations (Saskatoon and Rosthern) for the 163 RILs of 1-2347-144 X CDC Meadow populations. The mean phenotypic values of the parental cultivars are shown with a black arrow. Black line in the distribution indicates the cut-off value division between the low and normal phytate lines

Lines with PA-P concentration lower than 400 ppm were categorized as low phytate and higher than 400 ppm were considered as normal phytate. In 2012, out of the 163 RILs, 92 were normal and 71 were low in PA-P concentration. In 2013, 86 were normal and 77 RILs were low in phytate (Table 4.3). The calculated critical value for $F_{6:9}$ (1.572) and $F_{6:10}$ (0.098) was significantly ($\alpha = 0.05$) less than the critical χ^2 value (3.841) indicating that the segregation ratios satisfactorily fit a 1 normal: 1 low phytate model in both generations. This supports single gene control of the low phytate trait in PR-15 lines.

4.4.4 Correlation between PA-P and FEBIO

Fig. 4.2 presents the scatterplot and the tendency of the correlation between the PA-P (ppm in extract) and FEBIO (% to 1-2347-144) of the subset RILs (n=80), with two replications in 2012 Rosthern. The correlation between the PA-P and FEBIO was significantly negative (-0.83; $p \leq 0.001$). PR-15 RILs with lower PA-P concentration tended to have higher FEBIO.

4.4.5 PR-15 linkage map construction and PA-P mapping

The PR-15 lines were genotyped using the Ps1536 GoldenGate Illumina array (Sindhu et al. 2014). Individual SNPs were viewed as GenoPlots in GenomeStudio and genotypes with similar signal profiles were aggregated in clusters. A total of 367 out of the 1536 SNP markers were polymorphic between the parental lines 1-2347-144 and CDC Meadow. From the inheritance of PA-P concentration in PR-15 (Table 4.3), it was noted that phytic acid phosphorus in these lines is controlled by a major single gene. With supporting inheritance results, PA-P concentration was mapped as a locus along with the other 367 SNP markers. To genotype the PA-P locus, PR-15 lines with low PA-P concentrations were designated as allele "a" (from 1-2347-144) and PR-15 lines with normal PA-P concentrations were designated as allele "b". The genetic linkage map of field pea developed from 367 SNP markers and the PA-P locus based on 159 RILs of the 1-2347-144 X CDC Meadow population is shown in Fig. 4.3. The 16 linkage groups (LGs) developed were assigned to their respective chromosomes based on the pea consensus map generated by Sindhu et al. (2014). LG I-A and I-B were assigned to LG I, LG II-A and II-B to LG II, LG III-A, III-B and III-C to LG III, LG IV-A, IV-B and IV-C to LG IV, LG V to LGV, LG VI-A, VI-B and VI-C to LG VI and LG VII-A and VII-B to LG VII of Sindhu et al. (2014) pea consensus map. Due to insufficient marker coverage, the assigned LGs indicated more than one

independent segment within a group. These segments are indicated by denoting the LGs followed by a letter (A, B or C) (Table 4.5).

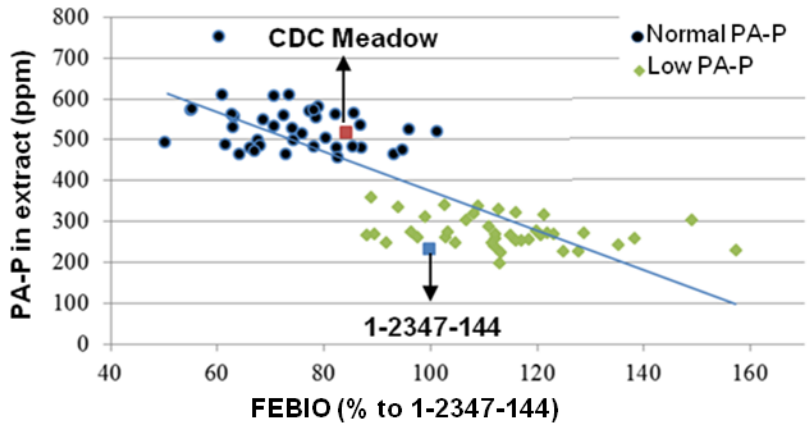

Fig. 4.2. Scatterplot between the PA-P (ppm in extract) and FEBIO (% to 1-2347-144) of the subset RILs (n=80), with two replications in 2012 Rosthern. Blue square - the low phytate parent (1-2347-144) and red square - the normal phytate parent (CDC Meadow).

Table 4.4: General features of the pea genetic map developed using single nucleotide polymorphic (SNP) markers based on 159 recombinant inbred lines of the 1-2347-144/CDC Meadow population.

Linkage groups	**Size (cM)**	**No. of mapped markers**	**Average marker density (cM)**
I-A	38.0	30	1.27
I-B	1.4	4	0.35
II-A	11.2	6	1.87
II-B	55.4	42	1.32
III-A	6.4	8	0.80
III-B	20.3	27	0.75
III-C	70.3	30	2.34
IV-A	3.9	9	0.43
IV-B	15.9	19	0.84
IV-C	10.4	13	0.80
V	56.0	53	1.06
VI-A	9.0	12	0.75
VI-B	17.5	27	0.65
VI-C	17.2	12	1.43
VII-A	70.9	59	1.20
VII-B	33.4	17	1.96
Average/Total	437.2	368	1.19

Note: cM = centiMorgan

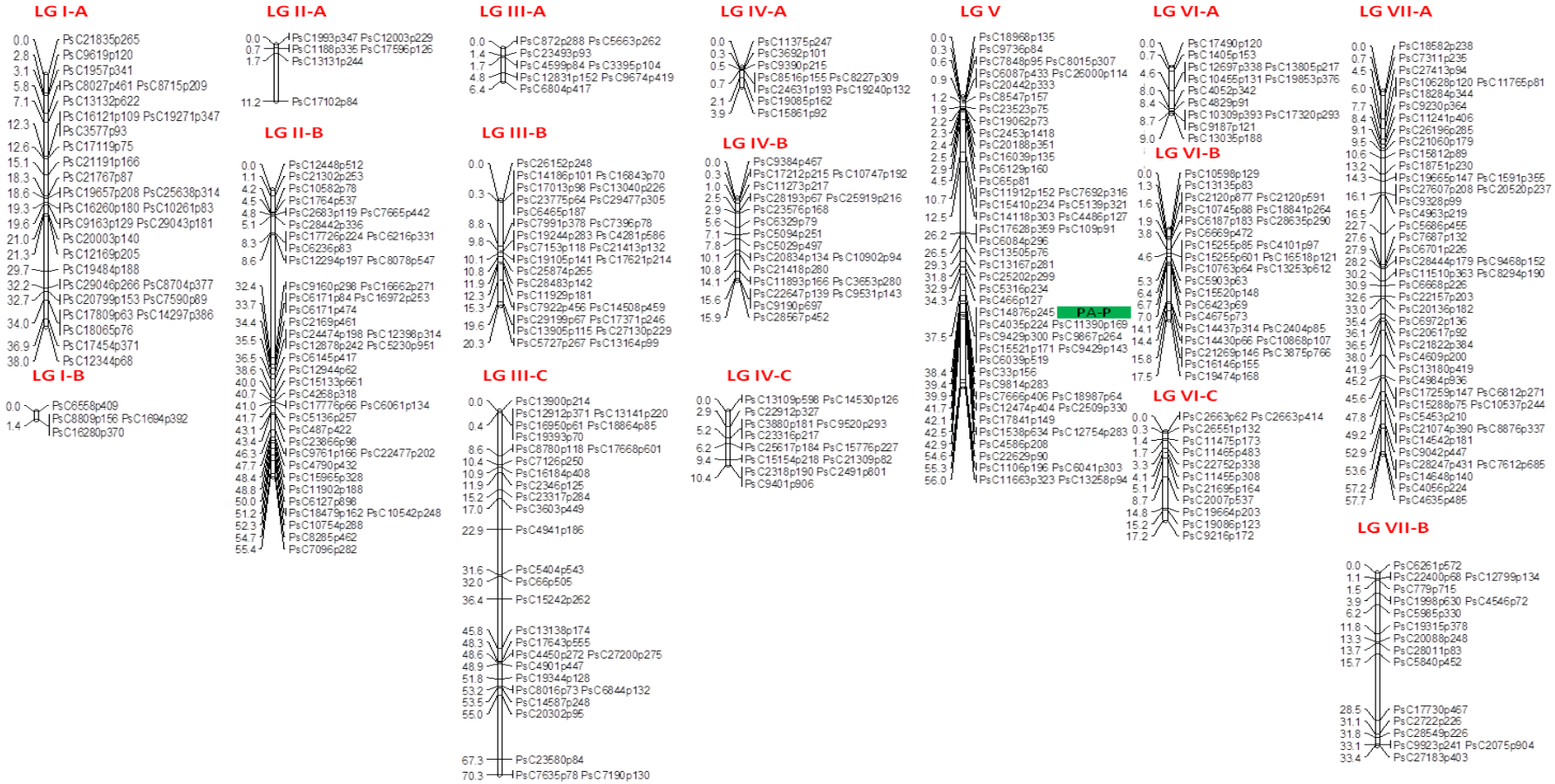

Fig. 4.3. Genetic linkage map of field pea (*Pisum sativum* L.) developed from 367 SNP markers and the PA-P locus based on 159 RILs of the 1-2347-144 X CDC Meadow population. LG I, LG II, LG III, LG IV, LG V, LG VI, and LG VII represent the linkage groups assigned to the seven chromosomes recognized in the pea genome based on pea consensus map by Sindhu et al. (2014). The PA-P locus is marked in green in LG V. The genetic distances calculated in centimorgans (cM) are indicated to the left of each LG.

The total coverage of the map was 437.2 cM with an average distance of 1.19 cM between the individual markers. The general features of the PR-15 linkage map are summarized in Table 4.4. LG 7-A represented the largest (70.9 cM) and most dense (59 markers) LG with an average marker density of 1.20 cM. LG 1-B was the smallest LG with only 4 markers and spanned 1.4 cM.

4.4.6 QTL analysis

To identify QTLs associated with FEBIO, the phenotypic estimates from a subset of PR-15 lines (n=80) grown in Rosthern 2012 was utilized. A QTL identified on LG 5 was associated with FEBIO and explained 60.5 % of the phenotypic variation (Table 4.5; Fig. 4.4). The LOD of the identified QTL was 15.12. The closest marker to FEBIO is the PA-P locus. The FEBIO QTL was at the same position where the PA-P locus was mapped (Fig. 4.4). A positive additive effect indicates that the phenotypic variation is increased by the low-phytate parent 1-2347-144. The agronomic traits, DTF, HT, MB3, LD and DTM assessed in PR-15 in 2012 and 2013 at Rosthern and Saskatoon were also subjected to QTL analysis. However, only one QTL which was associated with grain yield in Rosthern 2012 was identified on LG 6-C (Table 4.5). It explained 8.5 % of the phenotypic variation with a LOD value of 3.17. The closest marker to the grain yield QTL was PsC2663p62. The negative value of additive effect (-22.09) expressed by the QTL indicates that the phenotypic variation is decreased by 1-2347-144.

Table 4.5: QTLs identified for iron bioavailability and grain yield in pea recombinant inbred lines (PR-15) developed from a cross between 1-2347-144 and CDC Meadow in two locations over 2012 and 2013.

Trait[a]	Year	Location	Linkage group	Maximum LOD	Closest marker[b]	LOD[c]	R^{2d}	Additive effect[e]
FEBIO	2012	Rosthern	5	15.12	PA-P	3.0	60.5	22.15
Grain Yield	2012	Rosthern	6-C	3.17	PsC2663p62	3.0	8.9	-22.09

[a]FEBIO = iron bioavailability [b]Closest marker to the identified QTL with maximum LOD value

[c]Threshold level to declare a QTL significant was determined by performing 1000 permutation test

[d]Percentage of total variability explained by the QTL detected for the trait

[e]The value associated with 1-2347-144 allele. A negative value means that the 1-2347-144 allele decreases the value of the trait

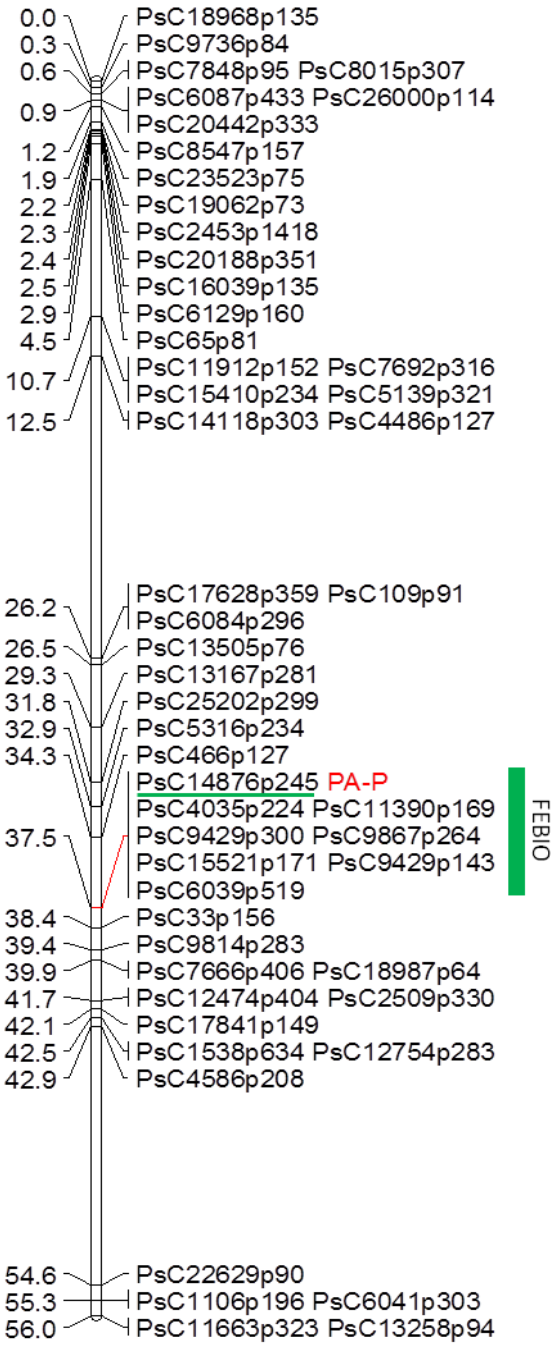

Fig. 4.4. Iron bioavailability (FEBIO) QTL mapping with significant QTL in linkage group 5. Shown in red is the phytic acid phosphorus (PA-P) locus. Underlined in green is the SNP locus PsC14876p245 associated with FEBIO that co-localizes with PA-P locus. The genetic distances calculated in centimorgans (cM) are indicated to the left of the LG.

4.5 Discussion

4.5.1 PA-P phenotype is controlled by a single gene

The present study provides confirmation to the single gene model of the inheritance of the low phytate trait as earlier reported by Rehman et al. (2012) where the *lpa* trait in pea genotypes, 1-150-81 and 1-2347-144 was controlled by a single recessive allele based on evaluating the F_1-F_3 and BC generations. The *lpa 1-1* mutation derived from chemical mutagenesis in barley lines followed a single gene inheritance pattern (Larson et al., 1998). The *lpa* trait in soybean which also caused a reduction in raffinosaccharide concentration was controlled by a single recessive gene (Hitz et al., 2002). However, Oltmans et al. (2004) in CX1834 soybean line showed that the *lpa* trait was controlled by two recessive genes with duplicate dominant epistasis. Pilu et al. (2003) observed a 3:1 segregation ratio in F_2 and F_3 generations of *lpa* maize that indicated a monogenic recessive basis of the *lpa* trait. Sompong et al. (2012) observed that PA-P in mung bean seed was normally distributed and inherited in a quantitative fashion. Whereas, in this study, PA-P concentration in PR-15 lines followed a bi-modal distribution pattern (Fig. 4.1). If inheritance of the low phytate trait in these lines was polygenic, a normally distributed frequency distribution would be expected. The PA-P phenotype is clearly divided into two groups (low and normal) that are evident from the distribution. A possible transgressive segregation in PR-15 lines can be explained by the fact that the range of PA-P concentration among the RILs is broader than the mean differences between 1-2347-144 and CDC Meadow. The Chi-square statistics (Table 4.4) performed with F_9 and F_{10} generations indicates that the low-phytate trait in these lines follow a single gene model.

4.5.2 Mapping PA-P phenotype in PR-15 linkage map

Since PA-P concentration in PR-15 lines was found to inherit in a Mendelian manner and the alleles formed qualitatively distinct phenotypes, a “major gene” approach was followed in mapping PA-P concentration. Recently, a genetic SNP consensus map based on 5 different RIL populations using 1536 SNP markers in Illumina GoldenGate array has been developed (Sindhu et al., 2014). The LGs in PR-15 linkage maps were assigned to their respective pea chromosomes by comparing with this consensus map. The PA-P locus was mapped on to LG 5, which co-localizes with several other SNP markers in that location (at 37.5 cM) (Fig. 4.4). These markers

can be used in marker-assisted selection to select low phytate cultivars. While the major gene approach has been used in the present study, other researchers have considered the inheritance of low phytate trait to be quantitative and mapped the QTLs associated with it. Walker et al., (2006) identified two loci associated with low phytate phenotype in soybean. They also discovered an epistatic interaction between the identified loci. In *Brassica rapa*, 25 QTLs associated with phytate and phosphorus concentrations in seeds and leaves were detected (Zhao et al., 2008). Blair et al. (2009) identified a total of nine QTL for seed phosphorus and seed phytate content in a RIL population of common bean. A compromise in agronomic performance is often associated with the low phytate trait. In *lpa* maize, up to 20% reduction in seed weight was observed as compared with the non mutant control (Raboy et al., 2000). Field emergence percentage was significantly lower in soybean LR33 mutant than that of wild type lines (Meis et al., 2003). Seed phytic acid and total phosphorus content correlated with days to flowering and maturity in mungbean (Sompong et al., 2012). In the present study an attempt was made to identify QTLs associated with agronomic traits such as days to flower, plant height, mycosphaerella blight, lodging and days to mature in PR-15 lines. A single QTL associated with grain yield in Rosthern 2012 was identified (Table 4.5).

4.5.3 FEBIO QTL co-localizes with PA-P locus

In the PR-15 RILs FEBIO was highly negatively correlated with PA-P. Thus, FEBIO in the PR-15 RILs might also be mainly controlled by pleiotropic effects of the same gene following Mendelian inheritance. The two parents (CDC Meadow and 1-2347-144) were found to be relatively close in FEBIO, but still to differ significantly (data not shown). Additionally, there were four lines that had more than 132 % FEBIO compared with the low phytate parent (1-2347-144), thus showing transgressive segregation. These results indicate that the other parent, CDC Meadow, might also have minor genes that enhanced FEBIO in the offspring. Lung'aho et al. (2011) reported ten QTLs for FEBIO in maize which explained 54% of the variance. In this study, a QTL explaining 60.5% phenotypic variation of FEBIO was identified (Table 4.5). The phytic acid locus was used as a cofactor in multiple QTL mapping (MQM) to identify the FEBIO QTL. Interestingly, this particular QTL co-localizes with the PA-P locus in LG 5. This acts as strong evidence that FEBIO is dependent on phytic acid concentration in PR-15.

In summary, a SNP marker based genetic linkage map was constructed for the PR-15 lines and PA-P locus was mapped on LG5. Interestingly, the phytic acid-phosphorus locus coincides with the FEBIO QTL. It is possible that the major gene controlling PA-P concentration also controls the FEBIO in these lines. However, before arriving at this conclusion, it is important to note that the FEBIO QTL presented here resulted only from one particular location and year. Further research on FEBIO would aid in understanding the inheritance of this trait and to develop a molecular breeding tool to address iron deficiency.

Acknowledgements

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CHAPTER 5

5. General discussion, conclusions and future research

Phosphorus is stored as phytic acid in the seeds of most plants. Phytate is abundantly distributed in the endosperm layer of cereals and cotyledons of legumes. Phytic acid plays a major role in P homeostasis, mineral storage, and signal transduction in plants. Phytic acid is not fully broken down by humans and non-ruminants animals and is excreted into the environment. Phytate chelates with essential mineral ions including iron, calcium, magnesium, and zinc to form phytate-mineral complexes. Humans and non-ruminants lack sufficient phytase enzyme in the gut microflora to break the phytate-mineral complex. This often results in P and micronutrient deficiencies. Phytate binds with cationic group of amino acids and forms protein-phytate complexes, eventually affecting protein digestibility. Similarly, phytate binds with starch through hydrogen bonding and affects its digestibility. Also, the waste water and sewage runoff from livestock farms that contain undigested phosphorus in the form of phytic acid contributes to environmental phosphorus pollution. This excess P initiates the process of eutrophication of fresh water bodies. Algal blooms have potentials to extinguish aquatic life and cause environment related health problems in humans. To address these problems, it is necessary to reduce the phytic acid concentration in plant seeds. Traditional methods to reduce phytate in seeds include soaking, cooking, fermentation and germination. These processes are usually time consuming and might not be suitable for large-scale operations in the food and feed industries. Addition of commercially available phytase enzyme can improve phytate degradation and reduce phosphorus excretion. Production of crop varieties with low phytate trait is a promising method to offer improved nutrition for human populations that depend on legumes and grains as staple foods. *lpa* genotypes have been isolated in maize (*Zea mays*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), rice (*Oryza sativa*), *Arabidopsis*, soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*).

At the Crop Development Centre (CDC), University of Saskatchewan, two low phytate pea lines were developed by Warkentin et al. (2012) through chemical mutagenesis of CDC Bronco. The objective of this thesis research was to characterize these two low phytate lines at the biochemical and molecular levels. The low phytate lines were shown to exhibit up to 60%

reduction in phytate concentration that was accompanied with a significant increase in inorganic phosphorus concentration. Though the phytic acid concentration in these low phytate lines are known, the timing of expression of the *lpa* trait during seed development was not known. Objective I of the research was to characterize the pattern of phosphorus compounds accumulation in developing seeds of low phytate pea lines. As phytic acid represents the significant portion of total seed P, the accumulation of this compound and other inositol phosphates had been studied in different plant species. In cereals, phytates are concentrated in the germ and aleurone tissues (O'Dell et al. 1972). In legumes, they are present in the form of globoids in cotyledons. In objective I, two low phytate lines 1-150-81, 1-2347-144 and their progenitor CDC Bronco were grown in replicated field trials in two locations in 2010 and 2011. Inositol phosphates (IP_1, IP_3, IP_4, IP_5 and IP_6) concentrations were estimated in seed coats and cotyledons separately in developing seeds. No inositol phosphates were detected in seed coats of both normal and low phytate lines. This agrees with the report of Beal and Mehta (1985). In their study the seed coat fractions contained little or no phytate. No other inositol phosphates other than IP_6 were traceable in both low and normal phytate lines in this research. The *lpa* mutation that caused changes in phytate accumulation may affect other physiological processes within the seed. The lower inositol phosphates could have acted as precursors to cell wall biosynthesis or oligosaccharide production. It is important to study the changes in seed P partitioning in these lines to understand the *lpa* trait. From my results, it is evident that the *lpa* pea genotypes tend to balance the total P levels within the seed to provide adequate supply of P required for normal seed development, and unlike genetic and environmental effects which often result in quantitative variation in seed total P, *lpa* mutants show large effects in partitioning P into phytic acid, inorganic P. Several conclusions were reached from this study; phytate reduction resulted in an increase in inorganic phosphorus concentration in *lpa* lines, total P concentration was not significantly different from normal phytate variety CDC Bronco, there were no inositol phosphates in seed coats, the *lpa* trait in the low phytate lines, 1-150-81 and 1-2347-144 is expressed throughout seed development, it is possible to develop *lpa* cultivars without significant loss of agronomic performance.

The expression of genes encoding enzymes involved in phytic acid biosynthesis pathway during seed development leads to phytic acid production. This production is sometimes continuous and may lead to an excess amount of phytic acid in seeds that eventually acts as an

anti-nutrient to humans and monogastric animals. It is necessary to identify the genes involved in phytic acid biosynthesis pathway to manipulate the phytate content in seeds through molecular plant breeding. Mutations in related genes reduce phytic acid content. In our "forward" genetics approach, we had isolated pea mutants with the low phytate trait. However, the mutated gene that produced the *lpa* trait in pea was unknown. To address this question, objective II of this research was aimed at sequencing the *myo*-inositol phosphate synthase (*MIPS*) gene involved in phytic acid biosynthesis pathway since it catalyzes the first and rate limiting step in the pathway. The open reading frames (ORF) of *MIPS* from 1-150-81, 1-2347-144 and CDC Bronco were isolated and compared. There was no mutation in the coding region among the identified *MIPS* sequences. Low phytic acid (*lpa*) genotypes of several crops have been generated by down-regulation (antisense or RNAi) or mutations in MIPS gene. Mutation in MIPS gene is often associated with lower seed yield, seed viability, increased susceptibility to pathogens and undesired morphology. Identical *MIPS* sequences between the low and normal phytate pea lines could have resulted in similar agronomic traits in them. The broad conclusions reached from the results of this study are, mutation in MIPS is not responsible for the low phytate trait in 1-150-81 and 1-2347-144, and mutations in genes involved in late steps of phytate biosynthesis pathway could be a responsible for the *lpa* trait.

Crop breeding programs employ genetic markers to track loci and genome regions associated with particular traits in crops. SNP markers occur either naturally or through induced mutation techniques such as chemical mutagenesis. There are several advantages of SNP markers; they occur at high frequency in any genome and are highly inherited. A recombinant inbred line population, PR-15, that segregated for phytate phosphorus concentration was generated by crossing 1-2347-144 and CDC Meadow (Rehman et al. 2012). Objective III of this research was aimed at mapping the phytic acid phenotype in PR-15 lines. A PR-15 linkage map was constructed using the 1536 SNP Illumina GoldenGate genotyping assay. The inheritance of phytic acid concentration in PR-15 lines followed a single gene model. Based on the Mendelian inheritance followed by phytic acid phosphorus concentration in PR-15 lines, a "major" gene approach was used to map the phytic acid phosphorus locus (PA-P). Other researchers have considered PAP as a quantitative trait and used QTL approach to map it on to the plant genome. The PAP locus was mapped on pea LG 5 and overlapped with several other SNP markers in the same location. By QTL analysis, a QTL explaining 60.5% of FEBIO phenotypic variation was

obtained. The key results from this study are, phytic acid phosphorus is a single gene inherited trait, phytic acid phosphorus can be mapped as a "major gene" in the PR-15 linkage map, FEBIO concentration QTL overlaps with PAP locus, and FEBIO might be controlled by the PAP locus.

The *lpa* trait in field pea can be an important breeding objective. *lpa* pea genotypes are superior in terms of P and mineral nutrients availability in foods than their progenitor CDC Bronco and other field pea cultivars in production internationally. When used as a feed to livestock, *lpa* pea can reduce phosphorus waste in the environment and save feed costs that involve the addition of phytase enzymes.

Finally, the concluding remarks on the three hypotheses tested in this thesis are:

1. Accepted the first hypothesis. Phytate accumulates at later stages in seed development.

2. Rejected the second hypothesis. Mutation in *myo*-inositol phosphate synthase (*MIPS*) gene is not responsible for the *lpa* trait in 1-150-81 and 1-2347-144.

3. Accepted the third hypothesis. Phytic acid phosphorus phenotype can be mapped in the recombinant inbred line population produced from a cross between 1-2347-144 and CDC Meadow.

Suggested future research related to this project includes the following:

1. Estimation of micronutrients in seeds during seed development and their spatial localization within the seeds to explore the potential of the *lpa* mutation in mineral availability.
2. Characterizing the pattern of raffinose family oligosaccharides (RFOs) accumulation in developing seeds of 1-150-81 and 1-2347-144, in comparison to CDC Bronco, to understand the relationship between phytic acid and sugars during seed development in *lpa* lines.
3. Estimation of raffinose family oligosaccharides (RFOs) in mature seeds of CDC Bronco, 1-150-81 and 1-2347-144 to evaluate the difference in sugar levels between a normal and low phytate line.

4. Enzyme activity assay for *MIPS* gene to correlate with *MIPS* gene expression and phytate metabolite accumulation in low and normal phytate lines.
5. Southern blot gel analysis to investigate the copy number of *MIPS* in *Pisum sativum* and to verify seed-specific expression of *MIPS*.
6. Sequencing the coding region of *ITPK1* gene in low and normal phytate lines to identify if a mutation in *ITPK1* was responsible for the *lpa* trait.
7. Gene expression and enzyme activity analysis for other phytic acid biosynthesis pathway genes such as *IPK1*, *MIK* and *2PGK* to find the mutation responsible for *lpa* trait (if there was no mutation found in *ITPK1*).
8. KASP validation assay to validate *lpa* mutation and QTL obtained for iron bioavailability in different accessions.

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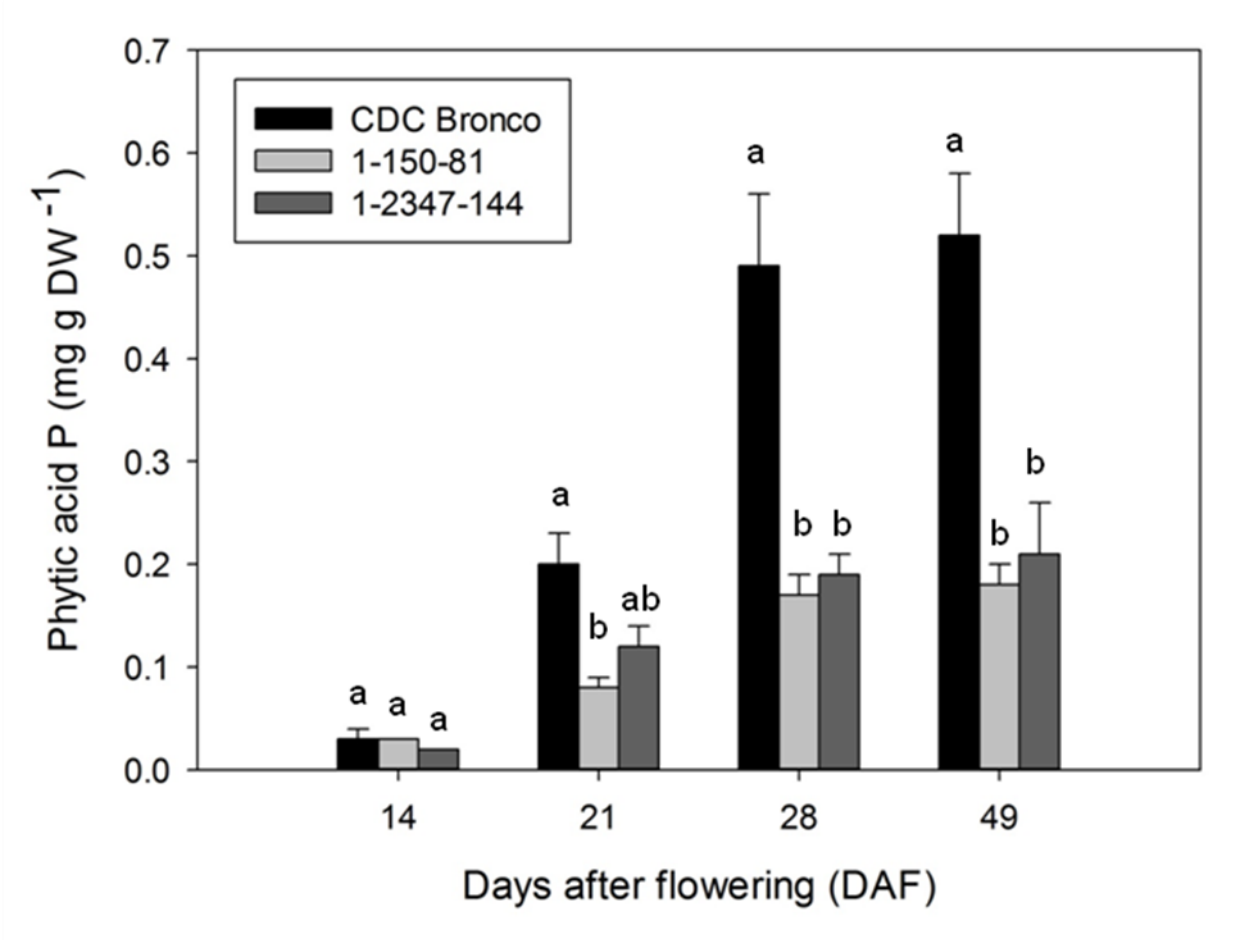
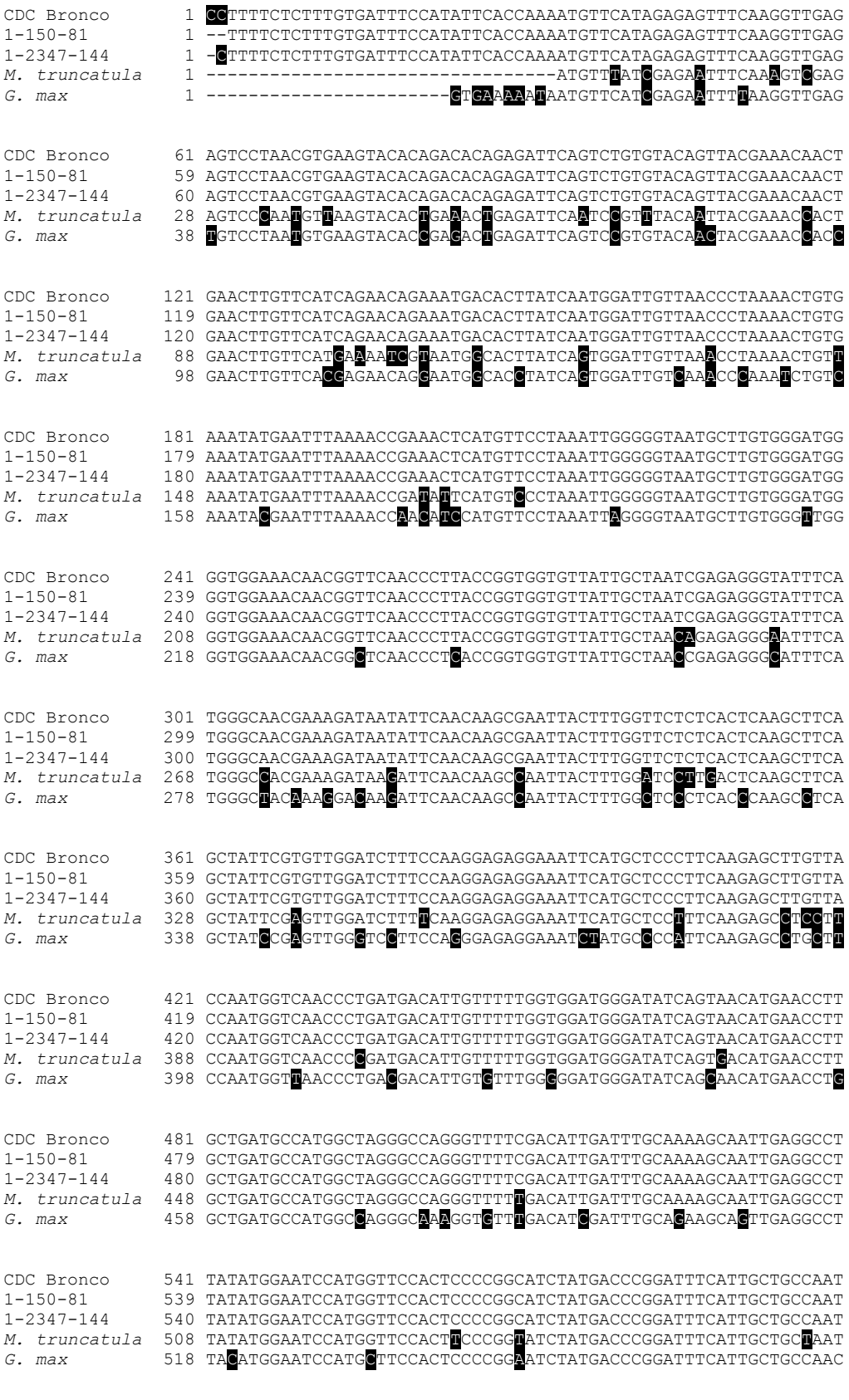
APPENDICES



Appendix 1. Concentration of phytic acid P (P_{PA}) in developing seeds of pea (*Pisum sativum* L.) cultivar CDC Bronco (black bars) and low-phytate lines 1-150-81 (light grey bars) and 1-2347-144 (dark grey bars). Values represent means ± SE, $n = 4$. Different letters associated with bars within each DAF are significantly different at $P < 0.05$.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1-150-81 1 --TTTTCTCTTTGTGATTTCCATATTCACCAAAATGTTCATAGAGAGTTTCAAGGTTGAG
1-2347-144 1 -CTTTTCTCTTTGTGATTTCCATATTCACCAAAATGTTCATAGAGAGTTTCAAGGTTGAG
M. truncatula 1 ----------ATGTTTATCGAGAATTTCAAAGTCGAG
G. max 1 ----------GTGAAAAATAATGTTCATCGAGAATTTTAAGGTTGAG
CDC Bronco 61 AGTCCTAACGTGAAGTACACAGACACAGAGATTCAGTCTGTGTACAGTTACGAAACAACT
1-150-81 59 AGTCCTAACGTGAAGTACACAGACACAGAGATTCAGTCTGTGTACAGTTACGAAACAACT
1-2347-144 60 AGTCCTAACGTGAAGTACACAGACACAGAGATTCAGTCTGTGTACAGTTACGAAACAACT
M. truncatula 28 AGTCCCAATGTTAAGTACACTGAAACTGAGATTCAATCCGTTTACAATTACGAAACCACT
G. max 38 TGTCCTAATGTGAAGTACACCGAGACTGAGATTCAGTCCGTGTACAAGTACGAAACCACC
CDC Bronco 121 GAACTTGTTCATCAGAACAGAAATGACACTTATCAATGGATTGTTAACCCTAAAACTGTG
1-150-81 119 GAACTTGTTCATCAGAACAGAAATGACACTTATCAATGGATTGTTAACCCTAAAACTGTG
1-2347-144 120 GAACTTGTTCATCAGAACAGAAATGACACTTATCAATGGATTGTTAACCCTAAAACTGTG
M. truncatula 88 GAACTTGTTCATCAAAATCGTAATGCCACTTATCACGTGGATTGTTAAACCTAAAACTGTT
G. max 98 GAACTTGTTCACCAGAACAGCAATGGCACCTATCACGTGGATTGTCAAACCCAAATCTGTC
CDC Bronco 181 AAATATGAATTTAAAACCGAAACTCATGTTCCTAAATTGGGGGTAATGCTTGTGGGATGG
1-150-81 179 AAATATGAATTTAAAACCGAAACTCATGTTCCTAAATTGGGGGTAATGCTTGTGGGATGG
1-2347-144 180 AAATATGAATTTAAAACCGAAACTCATGTTCCTAAATTGGGGGTAATGCTTGTGGGATGG
M. truncatula 148 AAATATGAATTTAAAACCGATATTCATGTCCCTAAATTGGGGGTAATGCTTGTGGGATGG
G. max 158 AAATACGAATTTAAAACCAACATCCATGTTCCTAAATTAGGGGTAATGCTTGTGGGTTGG
CDC Bronco 241 GGTGGAAACAACGGTTCAACCCTTACCGGTGGTGTTATTGCTAATCGAGAGGGTATTTCA
1-150-81 239 GGTGGAAACAACGGTTCAACCCTTACCGGTGGTGTTATTGCTAATCGAGAGGGTATTTCA
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M. truncatula 208 GGTGGAAACAACGGTTCAACCCTTACCGGTGGTGTTATTGCTAACAGAGAGGGAATTTCA
G. max 218 GGTGGAAACAACGGCTCAACCCTCACCGGTGGTGTTATTGCTAACCGAGAGGGCATTTCA
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1-150-81 299 TGGGCAACGAAAGATAATATTCAACAAGCGAATTACTTTGGTTCTCTCACTCAAGCTTCA
1-2347-144 300 TGGGCAACGAAAGATAATATTCAACAAGCGAATTACTTTGGTTCTCTCACTCAAGCTTCA
M. truncatula 268 TGGGCCACGAAAGATAAGATTCAACAAGCCAATTACTTTGGATCCTTGACTCAAGCTTCA
G. max 278 TGGGCTACAAAGGACAAGATTCAACAAGCCAATTACTTTGGCTCCTCACCCAAGCCTCA
CDC Bronco 361 GCTATTCGTGTTGGATCTTTCCAAGGAGAGGAAATTCATGCTCCCTTCAAGAGCTTGTTA
1-150-81 359 GCTATTCGTGTTGGATCTTTCCAAGGAGAGGAAATTCATGCTCCCTTCAAGAGCTTGTTA
1-2347-144 360 GCTATTCGTGTTGGATCTTTCCAAGGAGAGGAAATTCATGCTCCCTTCAAGAGCTTGTTA
M. truncatula 328 GCTATTCGAGTTGGATCTTTTCAAGGAGAGGAAATTCATGCTCCTTTCAAGAGCCTCCTT
G. max 338 GCTATCCGAGTTGGCTCCTTCCAGGGAGAGGAAATCTATGCCCCATTCAAGAGCCTGCTT
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1-150-81 419 CCAATGGTCAACCCTGATGACATTGTTTTTGGTGGATGGGATATCAGTAACATGAACCTT
1-2347-144 420 CCAATGGTCAACCCTGATGACATTGTTTTTGGTGGATGGGATATCAGTAACATGAACCTT
M. truncatula 388 CCAATGGTCAACCCCGATGACATTGTTTTTGGTGGATGGGATATCAGTCACATGAACCTT
G. max 398 CCAATGGTTAACCCTGACGACATTGTGTTTGGCGGATGGGATATCAGCAACATGAACCTG
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1-150-81 479 GCTGATGCCATGGCTAGGGCCAGGGTTTTCGACATTGATTTGCAAAAGCAATTGAGGCCT
1-2347-144 480 GCTGATGCCATGGCTAGGGCCAGGGTTTTCGACATTGATTTGCAAAAGCAATTGAGGCCT
M. truncatula 448 GCTGATGCCATGGCTAGGGCCAGGGTTTTTGACATTGATTTGCAAAAGCAATTGAGGCCT
G. max 458 GCTGATGCCATGGCCAGGGCAAAGGTGTTTGACATCGATTTGCAGAAGCAGTTGAGGCCT
CDC Bronco 541 TATATGGAATCCATGGTTCCACTCCCCGGCATCTATGACCCGGATTTCATTGCTGCCAAT
1-150-81 539 TATATGGAATCCATGGTTCCACTCCCCGGCATCTATGACCCGGATTTCATTGCTGCCAAT
1-2347-144 540 TATATGGAATCCATGGTTCCACTCCCCGGCATCTATGACCCGGATTTCATTGCTGCCAAT
M. truncatula 508 TATATGGAATCCATGGTTCCACTTCCCCGGTATCTATGACCCGGATTTCATTGCTGCTAAT
G. max 518 TACATGGAATCCATGCTTCCACTCCCCGGAATCTATGACCCGGATTTCATTGCTGCCAAC

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CDC Bronco      601 CAAGGTGAACGTGCAAATAATGTTATTAAGGGTACAAAGAGAGAACAAATTAACCAAATC
1-150-81        599 CAAGGTGAACGTGCAAATAATGTTATTAAGGGTACAAAGAGAGAACAAATTAACCAAATC
1-2347-144      600 CAAGGTGAACGTGCAAATAATGTTATTAAGGGTACAAAGAGAGAACAAATTAACCAAATC
M. truncatula   568 CAAGGAGAACGTGCGAATAACGTTATCAAGGGTACAAAGAGAGAACAAATCAACCAAATC
G. max          578 CAAGAGGAGCGTGCCAACAACGTCATCAAGGGCACAAAGCAAGAGCAAGTTCAACAAATC

CDC Bronco      661 ATCAAAGACATTCGGGAATTTAAGGAAGCAAACAAAGTAGACAGGGTTGTTGTTCTCTGG
1-150-81        659 ATCAAAGACATTCGGGAATTTAAGGAAGCAAACAAAGTAGACAGGGTTGTTGTTCTCTGG
1-2347-144      660 ATCAAAGACATTCGGGAATTTAAGGAAGCAAACAAAGTAGACAGGGTTGTTGTTCTCTGG
M. truncatula   628 ATCAAAGACATTAAGGAATTTAAGGAAGCAAACAAAGTTGACAGGGTTGTTGTACTCTGG
G. max          638 ATCAAAGACATCAAGGCGTTTAAGGAAGCCACCAAAGTGGACAAGGTCGTTGTACTCTGG

CDC Bronco      721 ACTGCCAACACAGAGAGGTACAGTAATTTAGTTGTGGGACTCAATGACACCACAGAGAAC
1-150-81        719 ACTGCCAACACAGAGAGGTACAGTAATTTAGTTGTGGGACTCAATGACACCACAGAGAAC
1-2347-144      720 ACTGCCAACACAGAGAGGTACAGTAATTTAGTTGTGGGACTCAATGACACCACAGAGAAC
M. truncatula   688 ACTGCCAACACAGAGAGGTACAGTAACTTAGTTGTAGGACTCAATGACACCATGGAGAAC
G. max          698 ACTGCCAACACAGAGAGGTACAGTAATTTGGTTGTGGGCCTTAATGACACCATGGAGAAT

CDC Bronco      781 CTTTTTGCTGCAGTGGACAGAAATGAGTCTGAGATTTCTCCTTCCACCCTGTTTGGCATT
1-150-81        779 CTTTTTGCTGCAGTGGACAGAAATGAGTCTGAGATTTCTCCTTCCACCCTGTTTGGCATT
1-2347-144      780 CTTTTTGCTGCAGTGGACAGAAATGAGTCTGAGATTTCTCCTTCCACCCTGTTTGGCATT
M. truncatula   748 CTTTTTGCTGCTGTGGACAGAAATGAGTCTGAGATTTCACCTTCCACCCTGTTTGCCATT
G. max          758 CTCTTGGCTGCTGTGGACAGAAATGAGCCTGAGATTTCTCCTTCCACCTTGTATGCCATT

CDC Bronco      841 GCTTGTGTTATGGAAAATGTTCCTTTCATCAATGGAAGCCCTCAGAACACTTTTGTTCCA
1-150-81        839 GCTTGTGTTATGGAAAATGTTCCTTTCATCAATGGAAGCCCTCAGAACACTTTTGTTCCA
1-2347-144      840 GCTTGTGTTATGGAAAATGTTCCTTTCATCAATGGAAGCCCTCAGAACACTTTTGTTCCA
M. truncatula   808 GCTTGTGTTATGGAGAATGTTCCTTTCATCAATGGAAGTCCTCAGAACACTTTTGTTCCA
G. max          818 GCTTGTGTTATGGAAAATGTTCCTTTCATTAATGGAAGCCCTCAGAACACTTTTGTACCA

CDC Bronco      901 GGGCTTATTGATCTTGCCATCAAGAACAACACCTTGATTGGTGGCGATGACTTCAAGAGT
1-150-81        899 GGGCTTATTGATCTTGCCATCAAGAACAACACCTTGATTGGTGGCGATGACTTCAAGAGT
1-2347-144      900 GGGCTTATTGATCTTGCCATCAAGAACAACACCTTGATTGGTGGCGATGACTTCAAGAGT
M. truncatula   868 GGGCTTATTGATCTTGCCATCAAGAACAACTGTTTTGATTGGTGGTGATGATTTCAAAAGT
G. max          878 GGGCTGATTGATCTTGCCATCGCGAGGAACACTTTGATTGGTGGAGATGACTTCAAGAGT

CDC Bronco      961 GGTCAGACCAAAATGAAATCTGTTTTGGTTGATTTCCTTGTTGGAGCTGGTATCAAGCCA
1-150-81        959 GGTCAGACCAAAATGAAATCTGTTTTGGTTGATTTCCTTGTTGGAGCTGGTATCAAGCCA
1-2347-144      960 GGTCAGACCAAAATGAAATCTGTTTTGGTTGATTTCCTTGTTGGAGCTGGTATCAAGCCA
M. truncatula   928 GGTCAGACCAAAATGAAATCTGTTTTGGTGGATTTCCTTGTAGGAGCTGGTATCAAGCCA
G. max          938 GGTCAGACCAAAATGAAATCTGTGTTGGTTGATTTCCTTGTGGGGCTGGTATCAAGCCA

CDC Bronco     1021 ACGTCGATAGTGAGTTACAATCATCTTGGAAACAATGATGGTATGAACCTCTCAGCACCA
1-150-81       1019 ACGTCGATAGTGAGTTACAATCATCTTGGAAACAATGATGGTATGAACCTCTCAGCACCA
1-2347-144     1020 ACGTCGATAGTGAGTTACAATCATCTTGGAAACAATGATGGTATGAACCTCTCAGCACCA
M. truncatula   988 ACATCAATAGTGAGTTACAATCATCTTGGAAACAATGATGGTATGAACCTCTCAGCCCCA
G. max          998 ACATCTTATAGTCAGTTACAACCATCTGGGAAACAATGATGGTATGAATCTTTCGGCTCCA

CDC Bronco     1081 CAAACCTTCCGCTCCAAGGAAATCTCCAAGAGCAACGTTGTTGACGATATGGTCAACAGC
1-150-81       1079 CAAACCTTCCGCTCCAAGGAAATCTCCAAGAGCAACGTTGTTGACGATATGGTCAACAGC
1-2347-144     1080 CAAACCTTCCGCTCCAAGGAAATCTCCAAGAGCAACGTTGTTGACGATATGGTCAACAGC
M. truncatula  1048 CAAACCTTCCGCTCCAAGGAAATTTCCAAGAGCAACGTTGTTGACGATATGGTCAACAGC
G. max         1058 CAAACTTTCCGTTCCAAGGAAATCTCCAAGAGCAACGTTGTTGATGATATGGTCAACAGC

CDC Bronco     1141 AACGCTATCCTCTATGCGCCTGGTGAACATCCTGACCATGTTGTAGTCATTAAGTATGTG
1-150-81       1139 AACGCTATCCTCTATGCGCCTGGTGAACATCCTGACCATGTTGTAGTCATTAAGTATGTG
1-2347-144     1140 AACGCTATCCTCTATGCGCCTGGTGAACATCCTGACCATGTTGTAGTCATTAAGTATGTG
M. truncatula  1108 AATGCCATCCTCTATGCACCTGGCGAACATCCTGATCATGTTGTAGTCATTAAGTATGTG
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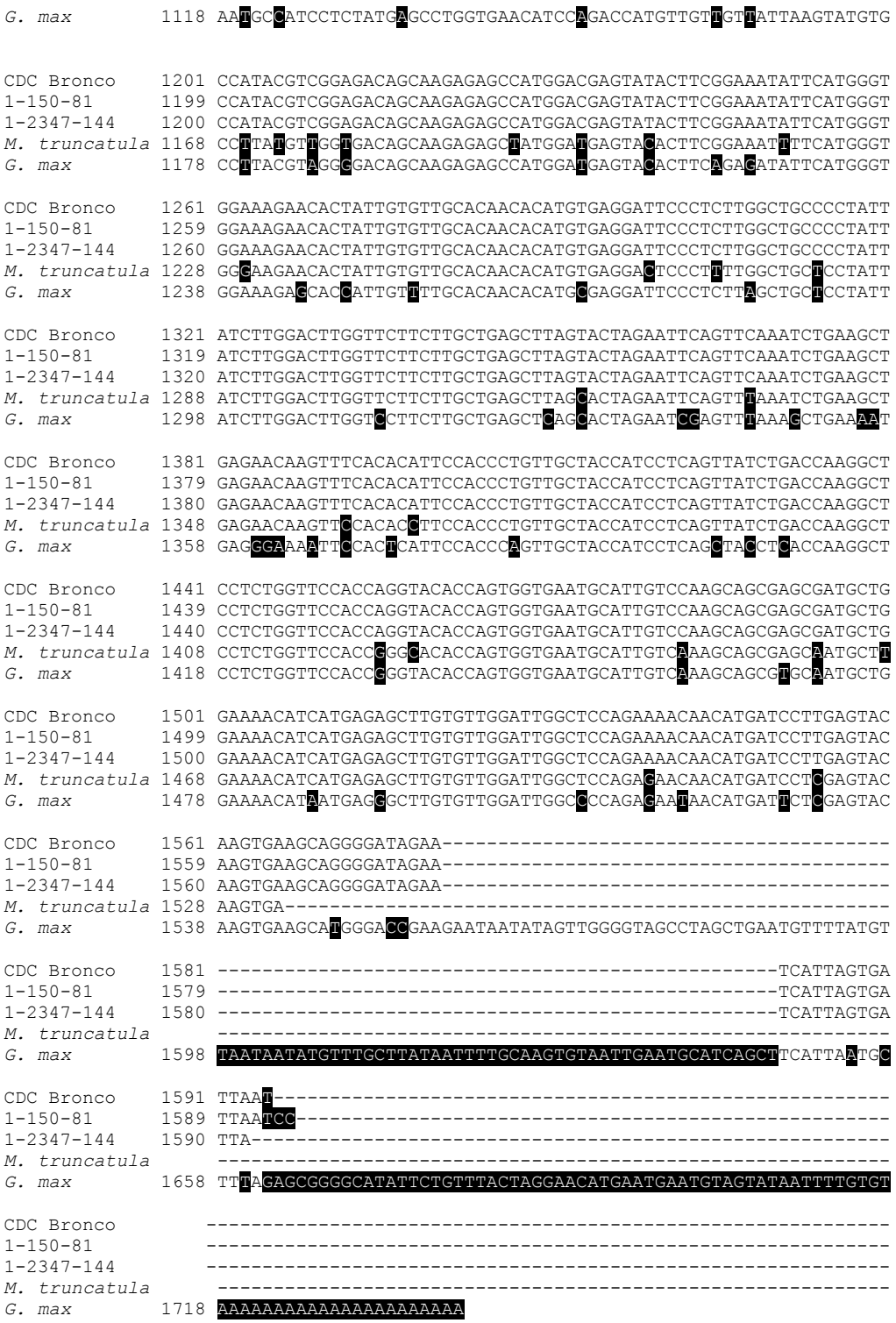
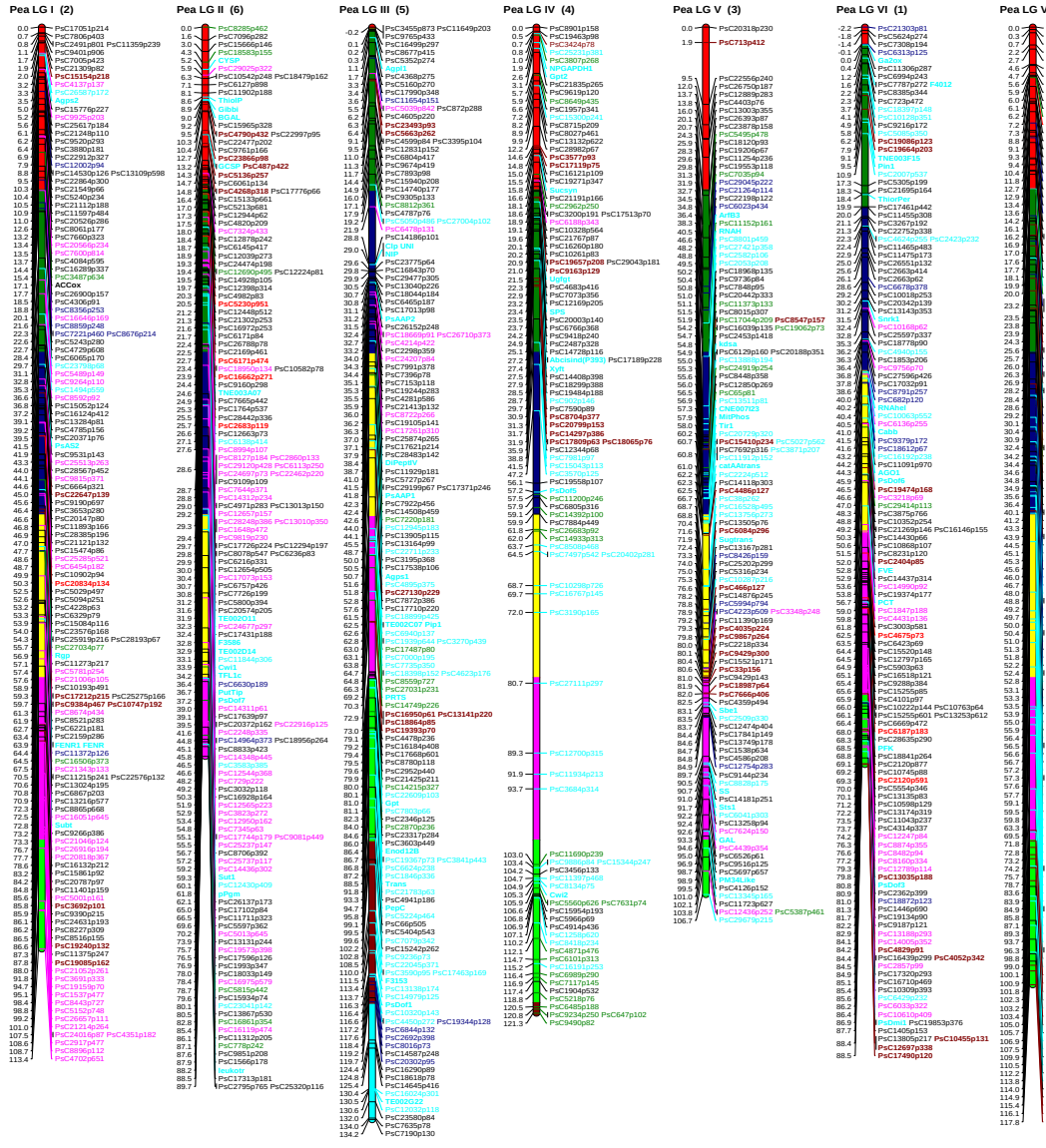


Appendix 2. Nucleotide sequence alignment of *PsMIPS* for CDC Bronco and low-phytate lines 1-150-81 and 1-2347-144. Also included for comparison are nucleotide sequences for *MIPS*

obtained from GenBank for *Medicago truncatula* (XM_003601939.1) and *Glycine max* (AY038802.1). Nucleotide differences are indicated in black boxes.



Appendix 3. Consensus SNP linkage map of *Pisum sativum* generated Sindhu et al. (2014) by using five RIL mapping populations. The seven linkage groups (LG I-VII) representing 7 chromosomes (given in parenthesis). Anchor markers identifying the linkage groups are shown black bold. The SNP markers common to all five RILs are shown bold red. SNP markers unique to individual RILs PR-02 (green), PR-07 (dark blue), PR-15 (brown), PR-19 (pink) and Pop-9 (light blue) are shown. Black SNP markers represent those markers shared by two or more of the

RIL populations. A total of 945 loci are represented in the consensus map. Linkage groups divided into BINs are shown color coded.