Theor Appl Genet (2009) 118:1211–1221
DOI 10.1007/s00122-009-0975-8

ORIGINAL PAPER

Isolation and characterisation of an *lpa* (low phytic acid) mutant in common bean (*Phaseolus vulgaris* L.)

Bruno Campion · Francesca Sparvoli · Enrico Doria · Giovanni Tagliabue · Incoronata Galasso · Marzia Fileppi · Roberto Bollini · Erik Nielsen

Received: 15 February 2008 / Accepted: 20 January 2009 / Published online: 18 February 2009

Abstract Phytic acid is considered as one of the major antinutritional compounds in cereal and legume seeds. The development of *lpa* (low phytic acid) grains, resulting in increased mineral cation availability, is considered a major goal in the improvement of the nutritional quality of seed crops, especially those largely consumed in developing countries. From a mutagenised population of common bean we isolated a homozygous *lpa* mutant line (*lpa*-280-10) showing, compared to wild type, a 90% reduction of phytic acid, a 25% reduction of raffinosaccharides and a much higher amount of free or weakly bound iron cations in the seed. Genetic analysis showed that the *lpa* character is due to a recessive mutation that segregates in a monogenic, Mendelian fashion. Germination tests performed using varying ageing or stress conditions, clearly showed that the bean line *lpa*-280-10 has a better germination response than the wild type. These data, together with those obtained from 2 years of agronomic trials showing that the mutant seed yield is close to that of its parents and other evidence, indicate that the new *lpa*-280-10 mutation might be the first devoid of visible macroscopic negative effects in plants, pods and seeds.

Communicated by D. Lightfoot.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-009-0975-8) contains supplementary material, which is available to authorized users.

B. Campion · M. Fileppi
CRA, Unità di Ricerca per l'Orticoltura,
Montanaso Lombardo, Lodi, Italy

F. Sparvoli · G. Tagliabue · I. Galasso · M. Fileppi · R. Bollini
Istituto di Biologia e Biotecnologia Agraria, CNR, Milan, Italy

E. Doria · E. Nielsen (✉)
Dipartimento di Genetica e Microbiologia, Università di Pavia,
Via Ferrata 1, Pavia, Italy
e-mail: nielsen@unipv.it; nielsen@ipvgen.unipv.it

Introduction

Iron (Fe) and zinc (Zn) deficiencies are common in humans, particularly in developing countries, and can severely limit the physical and intellectual capacity of people, adversely affecting their health and well-being (The world health report, Chap 4, 2002). Bioavailability of these minerals is a critical factor for humans since mineral absorption from plant foods is often low. Different approaches to increase Fe and Zn levels in crops include: the application of fertilizers containing the respective mineral, the introduction of high Fe, Zn or Ca (calcium) traits into high-yielding crops by plant breeding, and genetic engineering. The latter approach could be used to increase both level and bioavailability of Fe, Zn and Ca. Increasing mineral uptake by the roots and controlling the redistribution of minerals from the leaf to the edible plant parts via the phloem would seem obvious targets. However, while certain genes coding for key proteins involved in Fe and Zn uptake and transport have been identified and offer the possibility of genetic manipulation, more information needs to be obtained on mineral transport (Kim and Guerinot 2007). An alternative approach would be to introduce or augment Fe storage proteins such as phytoferritin into edible plant parts, assuming that enough mineral is present in the whole plant, to enable the storage protein to be filled with mineral (Goto et al. 1998; Vasconcelos et al. 2003).

Another way to improve seed nutritional quality is by lowering the content of phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate, InsP6). This compound, representing the

main phosphorus (P) storage form in the seed, is one of the major constraints to micronutrient bioavailability, since it binds mineral cations, such as Fe^{2+}, Zn^{2+} and Ca^{2+}, forming mixed salts (phytin) that are largely excreted by humans and non-ruminant animals that have no or limited phytase activity in their digestive apparatus. Excreted phytin in turn has a significant impact on water pollution (eutrophication) (Raboy 2001). The development of low phytic acid (*lpa*) grain crops is considered an important goal in plant breeding programs aimed at improving nutritional quality as well as at developing environment friendly and sustainable production (Raboy 2006). A number of *lpa* mutants have been or are being used in breeding programs after testing under field conditions (Raboy 2002; Liu et al. 2006). Preliminary nutritional studies on humans have demonstrated that Fe and Zn retention is significantly higher in subjects fed with meals prepared from the *lpa*1-1 mutant than from wild type maize (Mendoza et al. 1998; Hambidge et al. 2004, 2005).

Over the past decade, mutations that significantly reduce the levels of seed phytic acid have been identified in the major grain crops, such as maize (Raboy et al. 2000; Pilu et al. 2003; Shi et al. 2003, 2005, 2007), barley (Larson et al. 1998; Rasmussen and Hatzack 1998), rice (Liu et al. 2006), wheat (Guttieri et al. 2004) and soybean (Wilcox et al. 2000; Hitz et al. 2002; Yuan et al. 2007). These mutations fall substantially into the following classes: those characterised by decreased phytic acid matched by increased P_i level such as the soybean LR33 mutant (Hitz et al. 2002) affected in *myo*-inositol-3-phosphate synthase gene and the maize *lpa1* mutant affected in an ABC transporter gene (Shi et al. 2007) leading to a lack of transport of phytic acid into a storage compartment after its synthesis and to a consequent blocking of the phytic acid path; the *lpa3* type isolated in maize and mutated in *myo*-inositol kinase gene, characterised also by accumulation of *myo*-inositol; the *lpa2* type mutations leading to decreased phytic acid that is matched by increases in both P_i and hypophosphorylated inositols and therefore concerning one of the inositol kinases involved in the sequential phosphorylation steps of the $InsP_3$, or $InsP_4$ or $InsP_5$ intermediates in the late part of the biosynthetic pathway (Shi et al. 2003; Guttieri et al. 2004; Raboy 2006). Interestingly, for the LR33 mutant of soybean, the mutation leads also to a simultaneous decrease of raffinosaccharides accumulation because *myo*-inositol is among the precursors of these sugars (Hitz et al. 2002).

Unfortunately, all *lpa* mutants, but especially *lpa1* type, are associated with various negative effects regarding seed physiology and plant performance, such as compromised germination and emergence, stress tolerance or seed filling (Meis et al. 2003; Pilu et al. 2005; Bregitzer and Raboy 2006; Guttieri et al. 2006). Thus, the agronomic potential should be taken into account in projects aimed at obtaining *lpa* crops.

Among major grain crops, common bean is almost entirely used for direct human consumption and is a very important source of nutrients for people worldwide (Beebe et al. 2000; The World Health Report 2002). However, his nutritional potential is limited by the presence of phytate as well as other heat stable antinutritional factors such as polyphenols and tannins (Aw and Swanson 1985; Wang et al. 2003), raffinosaccharides (flatulence agents), and of digestive enzyme inhibitors and lectins (Bender and Reaidi 1982; Welch and Graham 2004). In particular, it has been reported that, on average, because of the presence of phytic acid, only 2–3% of the 3–4 mg of iron contained in 100 g of beans is actually absorbed during digestion, while iron from veal muscle would have 20% absorbance (Martinez-Torres and Layrisse 1971). Moreover, mainly due to the difficulties met in the transformation of common bean, no transgenic plants are available in which a phytase-encoding gene has been introduced leading to enzyme accumulation in the dry seed, as achieved in soybean, canola, wheat and maize (Chiera et al. 2005; Ponstein et al. 2002; Brinch-Pedersen et al. 2006; Chen et al. 2008). Finally, no *lpa* mutants have been isolated in this species until now.

Here we present data on the isolation and initial characterisation of a bean *lpa* mutant line (*lpa*-280-10) obtained by chemical mutagenesis of a lectin-free bean line (Campion et al. 2008). We also provide evidence that, distinct from other *lpa* mutants, the bean line *lpa*-280-10 mutation does not cause macroscopic negative effects adversely influencing seed germination, plant growth, seed yield and other traits of agronomic relevance.

Materials and methods

Plant material

A scheme showing the origin of the common bean lines used in the present work and the related breeding flow chart is presented in Fig. 1 of the Electronic Supplementary Material (ESM). The F_3 bean breeding population "905", determinate semi-climbing type Ib (Singh 1982) plants producing black lectin-free seeds of around 155 mg, was used for mutagenesis and selection of *lpa* mutants. The lectin-free "905" was obtained from the crosses [♀BAT 881 × (♀A55 × G6388♂)] (Campion et al. 2008). The accessions A55 and BAT 881, producing lectin-containing seeds, were kindly provided by Dr. S. Singh, CIAT (Centro Internacional de Agricultura Tropical, Cali, Colombia) to improve plant architecture and fertility of Italian common bean genetic materials. The former was used as the best available combiner (general combining ability), the latter as a very good yield performer

in the Po valley environment. G6388 is a wild common bean accession also from CIAT that does not contain lectins (Sparvoli et al. 1994). The agronomic performance of the *lpa* line obtained in this work (*lpa*-280-10) was compared with that of the accessions A55 and BAT 881 and that of the advanced uniform and stable line 938, selected from 905, as the best performer. The line 905 from which the *lpa* mutant was obtained, was not tested in the field trial because of its high genetic variability (being an F_3 breeding population). Finally, in the framework of a breeding program aimed to develop "*lpa* + lectin-free" cultivable materials (started in summer 2006), the *lpa* trait of *lpa*-280-10 was introgressed into three groups of superior lectin-free (SLF) plants (Campion et al. 2008) distinguishable on the basis of their genetic background: climbing producing "borlotto seeds", climbing producing large white seeds, bush producing small white seeds. The *lpa* F_2 progenies coming from the "♀SLF plants × *lpa*-280-10♂" crosses were grown in 2007 under field conditions in a spatially isolated area in order to avoid possible undesired pollinations from foreign phytate-containing beans. Related *lpa* F_3 progenies have been evaluated in a field trial carried out in 2008.

Seed inorganic phosphate, phytic acid phosphate, and total phosphate quantitative analyses

Bulks of dry bean seeds were milled in a coffee-grinder and further ground to fine powdery flour in a mortar in which liquid nitrogen was poured. When it was necessary to analyse seeds to be eventually sown, small pieces of cotyledons were cut avoiding damage in the embryo and then ground directly in a mortar as described above and analysed. For P_i analysis, 20 mg of flour were extracted with 400 µl of a 12.5% TCA, 25 mM $MgCl_2$ solution for 20 min at room temperature and left overnight at 4°C. After centrifugation, an aliquot of 100 µl of the supernatant was added to 900 µl of a freshly prepared Chen's reagent (6 N H_2SO_4: 2.5% ammonium molybdate: 10% ascorbic acid: H_2O [1:1:1:2, v/v/v/v]) and incubated at 50°C for 1 h before reading absorbance at 650 nm (Chen et al. 1956). A Na_2HPO_4 solution was used as the phosphate standard. Total seed phosphate (P_{tot}) was determined following wet-ashing of flour aliquots (50–150 mg) and colorimetric assay of digested P (Chen et al. 1956). Phytic acid phosphate (PAP) fractions were determined by a modification of the ferric precipitation method (Raboy 1990), as described by Pilu et al. (2003). Each sample was extracted and analysed in three replicates.

Mutagenesis of a bean population and identification, reproduction and confirmation of a *lpa* mutant line

In May 2004, 7,000 seeds of the lectin-free breeding population 905 (Fig. 1 of ESM) were treated with a 48 mM EMS (ethyl methanesulfonate) water solution for 12 h at around 22°C as described by Motto et al. (1975). The ratio seed/solution used was 2/1 (w/v). After treatment, M_1 seeds were rinsed in demineralised water and sown in the open field. M_1 plants were grown to maturity and harvested singly. A first screening for high inorganic phosphorus (HIP) was performed on the 953 more productive M_2 progenies. In order to speed up, simplify and reduce substantially the screening work, for every M_2 progeny, analyses were carried out on the flour obtained by milling 15 M_2 pooled seeds from each M_1 plant. Although M_1 plants are expected to produce heterogeneous M_2 seeds, we assumed that bulks of 15 seeds were representative enough to allow the detection of the presence of mutated HIP seeds. In the second phase of the screening process, 42 seeds of the M_2 progeny showing the highest P_i/PAP ratio (line 280) were analysed singly to obtain confirmation of the presence of seeds displaying genuine HIP phenotype. Then, 180 seeds of the line 280 were sown and related M_2 plants grown in a heated glasshouse from March to June 2005 at a temperature not lower than 17°C. Sixteen seeds of each of 52 M_3 seed progenies, each collected from a single M_2 plant, were submitted singly to HIP analysis in order to identify the M_3 progenies which were uniform for the presence of *lpa* mutation in the seeds. The M_3 line "280-10", found to produce only *lpa* seeds, was multiplied for two generations and related M_5 seeds provided for further studies. HIP analyses were also performed on M_4 seed progenies of the *lpa*-280-10 mutant to confirm the homozygous condition of *lpa* mutation.

Backcross *lpa*-280-10 × wild type 905 and HIP analysis of the *lpa* trait in F_1 seed progenies

About 100 ♀*lpa*-280-10 × 905♂ crosses were made to produce F_1 seeds. Thirty four F_1 seeds were analysed for the free phosphate content in order to verify the dominant or recessive expression of the *lpa* mutation when present in heterozygous condition.

Introgression of the *lpa* trait into three different bean genetic backgrounds and analysis of F_2 and F_3 progenies

Sixty SLF plants selected for the most important agronomical traits were grown from July to October 2006 in a glasshouse as reported above. These plants were chosen in equal numbers from each of three different groups of lectin-free lines (see "Plant material") and crossed with *lpa*-280-10 (used as the male parent) to produce F_1 hybrids which were then grown and selfed to obtain F_2 seeds segregating for the *lpa* trait. A conservative screening for the P_i content was carried out on 1,299 seeds of fifteen F_2 progenies so as to identify HIP seeds and to obtain data for defining the segregation

ratio of the HIP phenotype. For each one of the fifteen progenies analysed, the chi-square (χ^2) value was calculated, then all values were summed to find the "χ^2 Total". A χ^2 value was also calculated on the sum of the individual observations which were pooled across families to find the "χ^2 Pool". The homogeneity degree was estimated by calculating the "χ^2 Heterogeneity" = (χ^2 Total − χ^2 Pool) and related *P* level for (Total no. of progenies − 1) (Pool phenotypic classes − 1) degrees of freedom.

All the seeds displaying the HIP phenotype were sown and related F_2 plants singly grown in the open field from July to October 2007 to produce F_3 *lpa* seed progenies. Five of these F_3 *lpa* progenies, all exhibiting determinate growth habit, were cultivated in a field trial carried out in 2008 at Montanaso Lombardo to evaluate their agronomic performance. The female parent of one progeny was a lectin-free borlotto type bean (25/5x no. 33), whereas that of the other four (586/8x no. 61, 586/8x no. 87, 586/8x no. 147, 586/8x no. 160) was a lectin-free small white seeded bean.

Germination trials

The viability of M_5 seeds of the line *lpa*-280-10, was assessed by controlled germination test (CGT), accelerated ageing test (AAT) and stress integrated germination test (SIGT). The wild type line 905 was used as the control. Each test, replicated three times, was performed on samples of 50 seeds per line. For the AAT, seeds were incubated in a thermostatic chamber with 100% relative humidity at 45°C for 48 or 96 h and then, allowed to germinate under the CGT conditions as described below (Baskin 1977). In the CGT and SIGT, seeds were soaked in 70 ml water for 8 h at 25°C and then transferred to 70 ml of new water (CGT) or of a 0.2 and 0.4 M NaCl aqueous solution (SIGT) at 25°C for additional 12 h. At the end of the treatment, seeds were rinsed several times in distilled water, surface dried and distributed in two 20 cm diameter Petri dishes lined with filter papers soaked with distilled water to complete germination at 25°C in the dark. The choice of conditions for SIGT was based on previous experiments made on wheat and *Brassica* (Dell'Aquila and Di Turi 1996; Scialabba et al. 1999). Counts of germinating seeds were made twice a day, starting on the first day of root emergence and until the maximum of germination was achieved. The following germination parameters were estimated: germination percentage; time in hours at which 10% germination (T_{10}) was reached, determined by extrapolating the corresponding germination curves (Tadmor et al. 1969); mean germination time, that is the reciprocal of germination rate, expressed as MGT (h) = $\sum(hn)/\sum n$, where h is the number of the hours from the beginning of the germination test and n is the number of seeds germinating at hours h (Ellis and Roberts 1981). Data were statistically analysed by applying the Student's *T*-test.

Evaluation of *lpa*-280-10 agronomic performance

The mutant *lpa*-280-10 (at M_5 generation) and three wild type lines having a genetic background close to that of *lpa*-280-10 (see "Plant material") were evaluated in two field trials, one carried out in 2007 and the other in 2008, at the CRA-Research Unit of Montanaso Lombardo, Lodi, North Italy. The results of soil analysis showed the following main values: pH (H_2O) 5.18; sand 491, silt 369, clay 140 g kg^{-1}; active and total limestone 0 g kg^{-1}; available P 59 mg kg^{-1} (P_2O_5 according to Bray and Kurtz); exchangeable Ca 4.24 meq 100 g^{-1} (849.70 mg kg^{-1}); exchangeable Mg 0.67 meq 100 g^{-1} (81.47 mg kg^{-1}).

Two hundred and forty seeds were sown according to a randomised complete block design with four replications. In each experimental plot (2.50 × 0.80 m in 2007 and 3.00 × 0.60 m in 2008) of two rows, 60 seeds were sown (30 + 30). For each plot, the following parameters were examined: number and percent of emerged seedlings surveyed 26 days after sowing, dry seed yield (14% water content) expressed as t ha^{-1}, plant growth-period duration (no. of days from sowing date to harvest), average plant height, average seed weight determined on a pool of 500 seeds. After skewness and kurtosis tests made to check the normality condition of data distribution, data were submitted to ANOVA analysis followed by "Duncan's multiple range test" in order to rank yield means and compare their difference values at significance levels for $P \leq 0.05$ and $P \leq 0.01$.

Raffinosaccharide determination

Raffinosaccharides were determined by using the Raffinose/Galactose assay kit, purchased from Megazyme©. The principle of this assay kit is based on the following biochemical reactions: the α-galactosides are hydrolysed to D-galactose and sucrose by an α-galactosidase; D-galactose is then oxidised by NAD^+ to D-galactonic acid in the presence of the enzyme D-galactose dehydrogenase (β-GalDH) with the formation of reduced NADH; the amount of NADH formed in this reaction, measured by the increase in absorbance at 340 nm, is stoichiometric with the amount of D-galactose.

Iron extraction and detection

Extraction of total iron

About 300 mg of flour prepared from bean seeds of wild type 905 and M_5 *lpa*-280-10 were mixed thoroughly with 1.5 ml of 70% ultra-pure nitric acid and the sample digested

for 2 h at room temperature. A fraction of the digested sample (0.6 ml) was evaporated to dryness in a mineralizer at 100°C. The residue was dissolved in a 20 mM desferrioxamine (DFO) solution in 10 mM Tris-HCl buffer (pH 5) and processed for HPLC analysis as described farther on.

Differential extraction of iron at various HCl concentrations

Flour samples (5 g/sample, 5 replicates for each experimental material) were mixed with 15 ml of HCl at different concentrations (0.01; 0.03; 0.1; 0.4 N) in a test tube and shaken overnight at 4°C. After a 15 min centrifugation at 6,000×*g*, the pellets containing the unextracted iron were discarded and supernatants were transferred in a mineralizer at 100°C until complete evaporation. The residues were dissolved in ultra pure nitric acid, dried again in the mineralizer and processed with DFO as described in the following paragraph.

HPLC analysis

Iron was determined by HPLC analysis of the chelate complex ferrioxamine (FO) as described by Tesoro et al. (2005). This method is based on iron chelation with DFO, a potent iron chelator with little affinity for other metal ions. The dried samples prepared as described above were dissolved in 1 ml of 20 mM DFO freshly prepared in 10 mM Tris-HCl buffer (pH 5), incubated at room temperature in the dark for 3 h, and then centrifuged for 5 min at 7,500×*g*. Twenty microlitre of the clear supernatant were finally injected into the HPLC system (Kontron Instrument 420 system, equipped with a C18 column Zorbax ODS column 250 × 4.6 mm, 5 µm, Agilent Technologies). Data from three replications/sample were collected in two independent experiments.

Results

Screening for HIP phenotype in the mutagenised bean population

The first visible results of the mutagenic treatment on M_1 plants were a greatly reduced rate of seed germination (~50%), reduced plant size, plants with blind apices and plants with chlorophyll deficient sectors (chimeras). Eventually, only 2,028 out of 7,000 mutagenised seeds were able to growth into M_1 plants, 1,975 of which produced M_2 seeds. Identification of *lpa* mutants was carried out by screening these M_2 seeds for high levels of free phosphates (HIP phenotype) as previously done by Rasmussen and Hatzack (1998), Raboy et al. (2000), Pilu et al. (2003), and Liu et al. (2006).

Among the 953 M_2 seed families analysed, only eight, whose seed bulks showed a P_i level more than 30% higher than the general mean (0.42 mg P $g^{-1} \pm 0.08$) of wild type flours, were selected as candidate progenies putatively containing *lpa* seeds (Table 1). No major alteration in the amount of the total P stored in the seed was evident in these eight progenies when compared to the control (wild type 905), whereas they all showed a P_i/PAP ratio higher than that of the control, suggesting that a few M_2 seeds of these progenies could indeed carry the *lpa* character in their genome. We decided to focus further investigations exclusively on the line 280 because, among the eight candidates, it showed the highest P_i/PAP ratio.

Detection of *lpa* mutant seeds in the line 280

The analysis of P_i and PAP content carried out on an additional 42 M_2 seeds of the line 280 showed that two of them were endowed with a genuine HIP phenotype both containing about 6–7-fold more P_i (3.48 ± 0.183, 3.188 ± 0.121 mg P_i g^{-1}, respectively) and about 60–65% less PAP (0.75 ± 0.091, 0.63 ± 0.085 mg PAP g^{-1}, respectively) than the other 40 examined (mean P_i: 0.492 ± 0.124; mean PAP: 1.92 ± 0.28 mg g^{-1}) confirming that an *lpa* mutation had indeed occurred in this line.

Confirmation and segregation analysis of the *lpa* mutation

In two M_3 seed progenies (named *lpa*-280-10, *lpa*-280-36) out of the 52 screened for free P_i content, all the sixteen seeds analysed displayed the HIP phenotype, indicating that it was highly likely that the *lpa* mutation was present in a homozygous condition. Comparative analysis of the HIP phenotype subsequently made on M_4 seeds of *lpa*-280-10

Table 1 Free inorganic (P_i), phytic acid phosphorus (PAP) and total phosphorus amount detected in flours of eight M_2 common bean families selected for their higher P_i content and in the wild type 905 (first screening aimed to identify putative M_2 progenies having *lpa* seeds)

Sample	Free inorganic P (P_i) mg g^{-1}	Phytic acid P (PAP) mg g^{-1}	Total P mg g^{-1}	P_i/PAP
Wild type	0.42 ± 0.08	1.70 ± 0.05	3.25 ± 0.27	0.25 ± 0.06
280	**0.76 ± 0.03**	**1.38 ± 0.18**	**3.37 ± 0.27**	**0.55 ± 0.10**
447	0.69 ± 0.06	1.31 ± 0.08	3.79 ± 0.32	0.53 ± 0.07
514	0.70 ± 0.07	1.74 ± 0.02	3.75 ± 0.11	0.40 ± 0.04
639	0.73 ± 0.04	1.75 ± 0.15	3.15 ± 0.59	0.42 ± 0.09
652	0.66 ± 0.06	1.69 ± 0.04	3.03 ± 0.16	0.39 ± 0.05
657	0.69 ± 0.03	1.72 ± 0.18	3.48 ± 0.16	0.40 ± 0.10
868	0.74 ± 0.04	1.59 ± 0.03	3.56 ± 0.09	0.47 ± 0.03
916	0.84 ± 0.06	1.87 ± 0.08	3.71 ± 0.34	0.49 ± 0.07

Values are the means of five repeats. Values in bold are those of the family (280) selected for further studies

confirmed the homozygous condition of *lpa* mutation (Table 2).

The free P_i analysis carried out in 34 single F_1 seeds of the *lpa*-280-10 × 905 crosses revealed an average value (0.31 mg $g^{-1} \pm 0.14$) not significantly different from that (0.29 mg $g^{-1} \pm 0.06$) of wild type 905 seeds, indicating that the *lpa* trait is recessive. Meanwhile, the F_2 seeds obtained from the SLF lines × *lpa*-280-10 crosses were found to segregate for the HIP phenotype in a 1:3 HIP:wild type ratio (345HIP:954 wild type), respectively, confirming that the *lpa* trait behaves as a monogenic recessive character. In addition, the χ^2 value calculated on the pooled observations across progenies (χ^2 pool = 1.684) was not significant ($P = 0.1944$), further confirming the 1:3 segregation-ratio hypothesis. Moreover, the high P value (0.9187) of the χ^2 *heterogeneity* (7.389) showed that data are highly homogeneous, indicating that the number of examined samples was very consistent.

Seed germination tests of *lpa*-280-10 mutant

In order to test whether the *lpa* mutation had any deleterious effect on the plants, germination tests were carried out. No significant differences were shown between G and T_{10} values of wild type and *lpa* (Table 3), whereas significant differences were observed for MGT in both control and AAT germination tests. The MGT was slightly lower in *lpa*-280-10 seeds than in wild type seeds, indicating that the former are more vigorous than the wild type ones, although both reached 95–100% germination after 1 week of imbibition. Moreover, a stress treatment with 0.4 M NaCl imbibition for 12 h prior to radicle emergence phase showed that *lpa*-280-10 seeds have a MGT not differing from that of wild type seeds, confirming that our *lpa* mutation, even in adverse environmental conditions, apparently does not lead to an undesirably reduced rate of germination as has been reported for other *lpa* mutants (Raboy 2006).

Comparison of iron content and extractability in wild type and *lpa*-280-10 seeds

In order to verify whether the reduced phytic acid leads to an increase in the amount of non-phytin iron deposited in the seed, the content of iron was measured in both wild type and *lpa*-280-10 seeds by extracting flour samples with HCl solutions at different concentrations. Low-concentration HCl solutions (0.01/0.03 N) should extract only or mainly iron not complexed with phytic acid or other organic molecules and not sequestered by iron-storing protein, i.e. free or weakly bound organic iron (Chauhan and Mahjan 1988; Rakhi and Khetarpau 1995; Duhan et al. 2002; Engle-Stone et al. 2005). More concentrated HCl (0.4 N) solutions should extract both free or weakly bound organic iron and most phytin iron while total iron is extracted by mineralisation in 70% nitric acid. Besides demonstrating that the level

Table 2 Free inorganic (P_i), phytic acid phosphorus (PAP) and total phosphorus average content in flours of the common bean mutant line *lpa*-280-10 and in those of the wild type 905 from which the mutant was obtained

Line	Generation	P_i mg g^{-1}	PAP mg g^{-1}	Total P mg g^{-1}	[a]Raffinose mg g^{-1}
lpa-280-10	M_4	4.71 ± 0.57	0.52 ± 0.04	4.86 ± 0.49	6.00 ± 0.42
905 (wt)	F_7	0.29 ± 0.06	4.69 ± 0.73	5.57 ± 0.93	8.04 ± 0.43

[a] The raffinose content is the sum of free raffinose and the raffinose present in stachyose (raffinose + galactose)

Table 3 Comparison of germination response of *lpa*-280-10 and wild type 905 common bean seeds under different treatments

	G (%)		T_{10} (h)		MGT (h)	
	lpa	wt	*lpa*	wt	*lpa*	wt
Control	97 ± 0.60	96 ± 0.65	30	30	47.8 ± 4.23*	54.4 ± 4.64
AAT						
48 h	93 ± 0.67	88 ± 0.79	35	37	40.2 ± 2.02*	63.2 ± 4.14
96 h	39 ± 0.67*	26 ± 0.71	41	41	67.8 ± 6.14	67.7 ± 8.05
SIGT						
0.2 M NaCl	92 ± 0.62	89 ± 0.73	25	20	36.1 ± 2.10	36.2 ± 1.36
0.4 M NaCl	55 ± 0.60	47 ± 0.68	35	35	59.2 ± 3.98	61.2 ± 7.64

AAT accelerated ageing test, *SIGT* stress integrated germination test. G germination percentage, T_{10} time in hours at which 10% germination was reached, *MGT* mean germination time in hours, these were determined as described in "Materials and methods"

* Significant at $P < 0.05$ (Student's T-test)

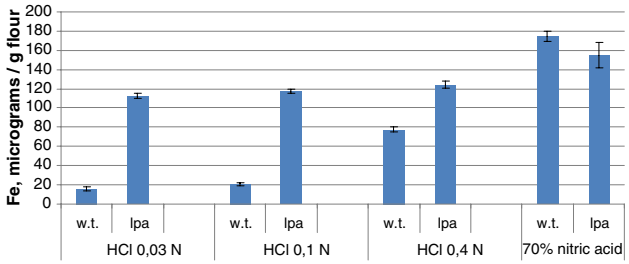

Fig. 1 Iron concentration in extracts from common bean *lpa*-280-10 or wild type 905 flours upon extraction with HCl at different concentrations or with 70% nitric acid

of total iron is not significantly lower in the *lpa*-280-10 mutant than in wild type seeds, the data obtained (Fig. 1), are consistent with the hypothesis that *lpa* mutant seeds contain a much higher level of free or weakly bound iron.

Agronomic performance of *lpa*-280-10

The plants of *lpa*-280-10 grown under field conditions displayed type Ib growth habit (Singh 1982) like the wild type 905, and showed normal development at all ontogenetic steps including seed maturation. The field trial performed in 2007 suffered from a damaging downpour which occurred during the germination phase. In spite of our intervention, focused on facilitating seed germination and seedling emergence, this rain storm led to the formation of a very compact soil crust which hampered seedling emergence. This explains the reduced "percentage of emerged seedlings" surveyed for the lines *lpa*-280-10, 938 and A55 and the absence of significant differences between mean values for the parameter "dry seed yield" (Table 4). The 2008 field trial, carried out in the same location, enjoyed better climatic conditions but had a slightly higher occurrence of soil diseases during seedling emergence compared to 2007 (data not shown). ANOVA analysis applied to 2008 data (Table 4) showed that *lpa*-280-10 dry seed yield value was equal (at $P \leq 0.05$ significance level) to that of all other lines except BAT 881. Plant emergence percentage of *lpa*-280-10 and A55 showed significantly higher values as compared to 938 and BAT 881. Highly significant differences were also observed for the parameters "average seed weight" and "plant height" in both growing seasons (Table 4).

Plant growth and fertility of new *lpa* bred materials

We crossed SLF plants with *lpa*-280-10 (see "Plant material") to check the feasibility and potentiality for introgressing the *lpa*-280-10 mutation in different genetic backgrounds. F_2 plant growth and yield appeared to be good or even very good in all three genetic groups. The first data on dry seed yield surveyed per single F_2 plant (semi-climbing type Ib growth habit) indicated that production in some cases exceeded the value of 300 g (distance plant to plant on the row = 35 cm; the potential dry seed production of bean climbing plants in Italy when cultivated single spaced, 30 cm on the row, ranges from 130 to 200 g). Also seed size and shape, surveyed on the F_3 generation, appeared normal. In a few *lpa* F_3 progenies in the climbing "borlotto" type genetic background, seed weight exceeded the value of 320 mg (double that of the *lpa*-280-10) indicating that the value of this parameter was increased already upon the first cross (the average seed weight of the "SLF borlotto" parents ranged from 550 to 700 mg). In the F_3 *lpa* progenies submitted to field evaluation at Montanaso

Table 4 Mean and standard deviation of five agronomic parameters surveyed in the common bean mutant *lpa*-280-10 and in three wild type lines genetically close to *lpa*-280-10, all submitted to field trial evaluation in 2007 and 2008

Year	Line or accession	Emerged seedlings (% ± SD)	Average seed weight (mg ± SD)	Plant height (cm ± SD)	Dry seed yield (t ha^{-1} ± SD)	Plant growth duration**
2007	BAT 881	84 ± 6.4aA	216 ± 8.9bB	62.0 ± 5.6bB	2.89 ± 0.39*	95
	A55	57 ± 9.5bB	243 ± 4.8aA	77.5 ± 2.9aA	1.98 ± 0.43*	96
	938	52 ± 8.8bcB	166 ± 8.7cC	50.0 ± 5.7cC	2.09 ± 0.73*	85
	lpa-280-10	43 ± 9.5cB	159 ± 4.9cC	78.0 ± 4.0aA	2.62 ± 0.68*	96
2008	BAT 881	70 ± 6.5cB	199 ± 3.9B	Not surveyed	5.28 ± 0.59aA	85
	A55	89 ± 2.9aA	237 ± 13.0A	Not surveyed	4.35 ± 0.21bAB	92
	938	76 ± 1.6bB	146 ± 6.7D	Not surveyed	3.84 ± 0.23bB	75
	lpa-280-10	92 ± 4.0aA	168 ± 6.2C	Not surveyed	4.39 ± 0.81bAB	93

Line 905: average seed weight = 155 mg. Accession G6388: average seed weight (plants grown in northern Italy greenhouse conditions) = 73 mg. Values not sharing a common letter are significantly different at $P \leq 0.05$ (small letters) and $P \leq 0.01$ (capital letters), respectively

SD standard deviation between replication values

* No statistical differences were detected with ANOVA analysis

** Number of days from sowing date to harvest, data not normally distributed

Lombardo in 2008, the percentage ± SD (four replicates) of emerged plants were as follows: line 25/5x no. 33 = 97.5% ± 2.9; 586/8x no. 61 = 75.8% ± 5.2; 586/8x no. 87 = 99.6% ± 0.9; 586/8x no. 147 = 99.2% ± 1.7; 586/8x no. 160 = 71.3% ± 5.3.

Discussion

Common bean, the most consumed legume for human nutrition, supplies significant amounts of minerals to populations in Africa and Latin America and is one of the selected staple food crops targeted by the CGIAR (http://www.cgiar.org) for improving bioavailable iron and zinc (Welch et al. 2000). We have presented data on the isolation of a common bean *lpa* mutant. We showed that, compared to its wild type, the mutant seeds have improved nutritional characteristics. Beside showing that the amount of total iron is not significantly lower in *lpa*-280-10 mutant than in wild type seeds, the data we obtained show that our *lpa* mutant seeds contain a higher level of free or easily extractable iron and free phosphorus (seven and ten times more, respectively) and a 25% lower raffinosaccharide content (Table 2). Although several factors influence the rate of iron absorption by man and other mammals during passage through the digestive apparatus, we can reasonably assume that the large increment of iron not sequestered by phytic acid will translate itself into higher bioavailability. Even if, according to some authors (e.g. Welch 2002), the actual efficacy of a micronutrient-enriched seed line can only be definitively proved by feeding trials in test populations under free living conditions, our assumption is supported by previous research reporting the beneficial effects of cereal (maize, barley) *lpa* mutants in human and animal nutrition for mineral cations (iron, zinc and calcium) and phosphorus bioavailability (Mendoza et al. 1998; Adams et al. 2002; Hambidge et al. 2004, 2005; Linares et al. 2006; Mazariegos et al. 2006; Veum et al. 2007). Grain produced by *lpa* isohybrids of maize or isocultivars of barley are available in sufficient amounts to have allowed long-term feeding trials in communities that traditionally rely on such staple crops. However, similar evidence on *lpa* food legumes is still lacking (Raboy 2002). Large-scale field studies are, therefore, critical to confirm our results. We can now provide a food legume crop in a nutritionally improved genetic background, in the form of our *lpa* mutant, which could be used as a tool to determine the effect of dietary phytic acid on mineral and phosphorus nutrition.

Along with lectins, polyphenols and tannins, phytic acid is an antinutrient which impairs micronutrient bioavailability (Aw and Swanson 1985; Norton et al. 1985; Reddy et al. 1985; Welch 2002). The bean *lpa* mutant we isolated was obtained from an EMS mutagenised breeding population (905) that is also devoid of phytohemagglutinin, another characteristic that may improve iron bioavailability (Welch and Graham 2004), and of α-amylase inhibitor, a further anti-nutrient. Moreover, breeding is in progress to obtain lines that, in addition, will have reduced polyphenols and tannins content (F_3 seeds which are *lpa*, lectin-free and with low polyphenols and tannins content were already obtained in the summer of 2007).

The reduction in raffinosaccharides content is also a positive nutritional characteristic. In fact, stachyose and raffinose are one of the major causes of stomach discomfort associated with bean seed consumption, since they are not absorbed by the small intestine, but passed down to the colon.

Lpa mutations are often associated with lower seed yields and reductions of seed viability. These defects are not unexpected considering that *myo*-inositol and its phosphorylated derivatives play a central role in several metabolic processes and in signal transduction. The first evidence of this was from the work of Raboy et al. (2000), who reported an association between reduced seed phytic acid and reduced seed dry weight in maize *lpa-1* and *lpa-2* mutants. The maize *lpa1-1* is allelic to the *lpa1-241* mutant, however the latter displays additional defects such as stunted vegetative growth, impaired seed germination capacity and seed emergence delay. Interestingly, the severity of these traits is proportional to the increase in free P_i in the mutant seeds (Pilu et al. 2005). Similar results were obtained on four barley *lpa* mutants, where the agronomic performance is negatively correlated with phytate reduction (Bregitzer and Raboy 2006). We therefore ran field and germination trials to look for possible deleterious effects of the mutation in our *lpa*-280-10. The emergence response of *lpa*-280-10 seedlings during the first seed multiplication steps gave values which repeatedly approached 100% (data not shown). Very good emergence was also observed in the 2008 field trial, where mutant lines responded even better than the parent BAT 881 and the wild type line 938 (Table 4). A good response was again obtained in the F_3 *lpa* progenies coming from SLF plants × *lpa*-280-10 crosses, where seedling emergence rates reached values near 100% in two out of five cases. In 2008, the parameter "dry seed yield" in *lpa*-280-10 also reached a satisfactory level. Although it was slightly lower than that of BAT 881 at the $P \leq 0.05$ significance level, it was not significantly different from those of A55 and 938. In conclusion, the absence of macroscopic negative defects in the selected mutant *lpa*-280-10 and the very good emergence rate in the F_3 cross progenies indicate that the physiological expression of the two most critical parameters (seed yield and seedling emergence) is not hindered by the presence of the *lpa* mutation. The low seedling emergence in

2007 for *lpa*-280-10 should not be attributed to the *lpa* mutation, but to the particularly bad weather and soil conditions, which negatively influenced all the bean lines tested.

Meis et al. (2003) observed that homozygous *lpa* lines derived from the soybean LR33 mutant line had a field emergence percentage significantly lower than that of wild type lines and showed that AAT was effectively able to verify the field emergence potential. These authors also found a significant seed source effect on field emergence, which was lower for seeds produced in subtropical environments than for those from temperate areas. Although the common bean *lpa*-280-10 mutant displays a reduction in phytic acid content of about 90%, comparable only to that reported for the M955 mutant of barley and for the *lpa1-241* mutant of maize (Dorsch et al. 2003; Pilu et al. 2003), we found no significant negative macroscopic effects on seed germination capacity and seedling vigour and growth (Table 3). The results of the AAT showed that the *lpa*-280-10 seeds are much more vigorous than the wild type ones: after 48 h of treatment the *lpa* seeds had an MGT shorter than that of 905 seeds, and after a more severe treatment (96 h) they showed a higher germination percentage (39%) compared to the wild type (26%). In contrast, the AAT on *lpa* LR33 soybean seeds showed a strong reduction in germination percentages, which ranged from 7% (seeds produced in subtropical environment) to 21% (seeds produced in temperate environment), while the wild type seeds had germination values ranging from 86 to 90%. Finally, we found that the *lpa*-280-10 mutant seeds germinated slightly faster than those of wild type (MGT was 47.8 h compared to 54.4 h), and there were no differences in the SIGT.

To generalize, we observed no visible macroscopic defect in *lpa* plants and seeds during their growth and reproduction, either in growth chamber and greenhouse, or in the open field. Although extensive field trials have not yet been carried out, the cultivation under field conditions of *lpa* F_2 and F_3 progenies coming from the "SLF plants × *lpa*-280-10" crosses allowed us to verify for the first time the influence exerted by the *lpa*-280-10 mutation on seedling emergence, growth, and fertility of plants having genetic backgrounds different from that of the genetic materials used for mutagenesis and mutant selection. Once more, seedling emergence, plant growth and yield were satisfactory, based on qualitative observations.

In conclusion, although biochemical and molecular studies along with gene mapping are required to find the molecular lesion which is the basis of our *lpa*-280-10 bean, we are confident that we have produced the *lpa* common bean mutant long sought, endowed with putatively improved nutritional properties, i.e. increased free phosphate, increased free or weakly bound iron and decreased raffinosaccharides, and in which a "strong" *lpa* phenotype is not associated to macroscopic defects. Moreover, the mutation was selected in a common bean breeding line producing grains devoid of major bean antimetabolites (phytohemagglutinin and α-amylase inhibitor) (Campion et al. 2008) and has also been introgressed into low tannin and polyphenol lines. Therefore, we hope that this mutant may open the way for improving the common bean's potential for meeting the nutritional requirements of many people around the world.

Acknowledgments We are grateful to Dr. Sonia Mazzamurro for the screening work she carried out during her thesis stage in the E.N. laboratory, to Dr. Antonio Dell'Aquila for his help in germination test analysis and for his comments to the manuscript, and to Dr. Roberto Pilu for helpful discussions. Technical support by Mrs. Gloria Daminati and Mr. Rommel Ocampo Romero Ivan are acknowledged. Dr. Marzia Fileppi was supported by a research grant from CRA. This research was partially supported by Ministry of Agricultural Alimentary and Forest Politics with funds released by C.I.P.E (Resolution 17/2003) to B.C. and F.S. and by Ministry for University and Research (PRIN 2006) to E.N.

References

Adams CL, Hambidge M, Raboy V, Dorsch JA, Sian L, Westcott JL, Krebs NF (2002) Zinc absorption from a low-phytic acid maize. Am J Clin Nutr 76:556–559

Aw TL, Swanson BG (1985) Influence of tannin on *Phaseolus vulgaris* protein digestibility and quality. J Food Sci 50(1):67–71

Baskin CC (1977) Vigour test methods—accelerated aging. AOSA Newsl 51:42–55

Beebe S, Gonzalez AV, Rengifo J (2000) Research on trace minerals in the common bean. Food Nutr Bull 21:387–391

Bender AE, Reaidi GB (1982) Toxicity of kidney beans (*Phaseolus vulgaris*) with particular reference to lectins. J Plant Foods 4(1):15–22

Bregitzer PP, Raboy V (2006) Effects of four independent low-phytate mutations on barley (*Hordeum vulgare* L.) agronomic performances. Crop Sci 46:1318–1322

Brinch-Pedersen H, Hatzack F, Stöger E, Arcalis E, Pontopidan K, Holm PB (2006) Heat-stable phytases in transgenic wheat (*Triticum aestivum* L.): deposition, pattern, thermostability, and phytate hydrolysis. J Agric Food Chem 54(13):4624–4632

Campion B, Perrone D, Galasso I, Bollini R (2008) Common bean (*Phaseolus vulgaris* L.) lines devoid of major lectin proteins. Plant Breed. doi:10.1111/j.1439-0523.2008.01569.x

Chauhan BM, Mahjan L (1988) Effect of natural fermentation on the extractability of minerals from pearl millet flour. J Food Sci 53:1576–1577

Chen PS, Toribara TY, Warner H (1956) Microdetermination of phosphorus. Anal Chem 28:1756–1758

Chen R, Xue G, Chen P, Yao B, Yang W, Ma Q, Fan Y, Zhao Z, Tarczynski MC, Shi J (2008) Transgenic maize plants expressing a fungal phytase gene. Transgenic Res 17(4):633–643

Chiera JM, Finer JJ, Grabau EA (2005) Ectopic expression of a soybean phytase in developing seeds of *Glycine max* to improve phosphorus availability. Plant Mol Biol 56:895–904

Dell'Aquila A, Di Turi M (1996) The germination response to heat and salt stress in evaluating vigour loss in aged wheat seeds. Seed Sci Technol 24:341–346

Dorsch JA, Cook A, Young KA, Anderson JM, Bauman AT, Volkmann CJ, Murthy PP, Raboy V (2003) Seed phosphorus and inositol phosphate phenotype of barley low phytic acid genotypes. Phytochemistry 62(5):691–706

Duhan A, Khetarpaul N, Bishnoi S (2002) Content of phytic acid and HCl-extractability of calcium, phosphorus and iron as affected by various domestic processing and cooking methods. Food Chem 78:9–14

Ellis RH, Roberts EH (1981) The quantification of ageing and survival in orthodox seeds. Seed Sci Technol 9:373–409

Engle-Stone R, Yeung A, Welch R, Glahn R (2005) Meat and ascorbic acid can promote Fe availability from Fe-phytate but not from Fe-Tannic acid complexes. J Agric Food Chem 53:10276–10284

Goto F, Yoshihara T, Saiki H (1998) Iron accumulation in tobacco plant expressing soybean ferritin gene. Transgenic Res 7:173–180

Guttieri MJ, Bowen D, Dorsch JA, Raboy V, Souza E (2004) Identification and characterization of a low phytic acid wheat. Crop Sci 44:418–424

Guttieri MJ, Peterson KM, Souza E (2006) Agronomic performance of a low phytic acid wheat. Crop Sci 46:2623–2629

Hambidge KM, Huffer JW, Raboy V, Grunwald GK, Westcott JL, Sian L, Miller LV, Dorsch JA, Krebs NF (2004) Zinc absorption from low-phytate hybrids of maize and their wild-type isohybrids. Am J Clin Nutr 79:1053–1059

Hambidge KM, Krebs NF, Westcott JL, Sian L, Miller LV, Peterson KL, Raboy V (2005) Absorption of calcium from tortilla meals prepared from low phytate maize. Am J Clin Nutr 82:84–87

Hitz WD, Carlson TJ, Kerr PS, Sebastian SA (2002) Biochemical and molecular characterization of a mutation that confers a decreased raffinosaccharide and phytic acid phenotype on soybean seeds. Plant Physiol 128:650–660

Kim SA, Guerinot ML (2007) Mining iron; iron uptake and transport in plants. FEBS Lett 581:2273–2280

Larson SR, Young KA, Cook A, Blake TK, Raboy V (1998) Linkage mapping of two mutations that reduce phytic acid content of barley grain. Theor Appl Genet 97:141–146

Linares LB, Broomhead JN, Guaiume EA, Ledoux DR, Veum TL, Raboy V (2006) Effects of low phytate barley (*Hordeum vulgare* L.) on zinc utilization in young broiler chicks. Poult Sci 86:299–308

Liu QL, Xu XH, Ren XL, Fu HW, Wu DX, Shu QY (2006) Generation and characterisation of low phytic acid germplasm in rice (*Oryza sativa* L.). Theor Appl Genet 114:803–814

Martinez-Torres C, Layrisse M (1971) Iron absorption from veal muscle. Am J Clin Nutr 24:531–540

Mazariegos M, Hambidge KM, Krebs NF, Westcott JE, Lei S, Grunwald GK, Campos R, Barahona B, Raboy V, Solomons NW (2006) Zinc absorption in Guatemalan schoolchildren fed normal or low-phytate maize. Am J Clin Nutr 83:59–64

Meis SH, Fehr WR, Schnebly SR (2003) Seed source effect on filed emergence of soybean lines with reduced phytate and raffinose saccharides. Crop Sci 43:1336–1339

Mendoza C, Viteri FE, Lonnerdal B, Young KA, Raboy V, Brown KH (1998) Effect of genetically modified, low-phytic acid maize on absorption of iron from tortillas. Am J Clin Nutr 68:1123–1128

Motto M, Soressi GP, Salamini F (1975) Mutation frequencies and chimeric formation in *Phaseolus vulgaris* after EMS treatment of dormant seeds. Radiat Bot 15:291–299

Norton G, Bliston FA, Bressani R (1985) Biochemical and nutritional attributes of grain legumes. In: Summerfield RJ, Roberts EH (eds) Grain legume crops. Collins, London, pp 73–114

Pilu R, Panzeri D, Gavazzi G, Rasmussen SK, Consonni G, Nielsen E (2003) Phenotypic, genetic and molecular characterization of a maize low phytic acid mutant (*lpa241*). Theor Appl Genet 107:980–987

Pilu R, Landoni M, Cassani E, Doria E, Nielsen E (2005) The maize l*pa241* mutation causes a remarkable variability of expression and some pleiotropic effects. Crop Sci 45:2096–2105

Ponstein AS, Bade JB, Verwoerd TC, Molendijk L, Storms J, Beudeker RF, Pen J (2002) Stable expression of phytase (*phyA*) in canola (*Brassica napus*) seeds: towards a commercial product. Mol Breed 14:31–44

Raboy V (1990) The biochemistry and genetic of phytic acid synthesis. In: Morre DJ, Boss W, Loewus FA (eds) Inositol metabolism in plants. Alan R. Liss, New York, pp 52–73

Raboy V (2001) Seeds for a better future: "low phytate" grains help to overcome malnutrition and reduce pollution. Trends Plant Sci 6:458–462

Raboy V (2002) Progress in breeding low phytate crops. J Nutr 132:503S–505S

Raboy V (2006) Seed phosphorus and the development of low-phytate crops. In: Turner BL, Richardson AE, Mullaney EJ (eds) Inositol phosphates: linking agriculture and environment. CAB International, Wallingford, pp 111–132

Raboy V, Gerbasi PF, Young KA, Stoneberg SD, Pickett SG, Bauman AT, Murthy PP, Sheridan WF, Ertl DS (2000) Origin and seed phenotype of maize low phytic acid 1-1 and low phytic acid 2-1. Plant Physiol 124:355–368

Rakhi G, Khetarpau N (1995) Effect of fermentation on HCl-extractability of minerals from rice-defatted soy flour blend. Food Chem 50:419–422

Rasmussen SK, Hatzack F (1998) Identification of two low-phytate barley (*Hordeum vulgare* L.) grain mutants by TLC and genetic analyses. Hereditas 129:355–368

Reddy NR, Pierson MD, Sathe SK, Salunkhe DK (1985) Dry bean tannins: a review of nutritional implications. J Am Oil Chem Soc 62(3):541–549

Scialabba A, Di Liberto C, Dell'Aquila A (1999) Salt-treatment integrated germination test in the evaluation of *Brassica villosa* subsp. *Drepanensis* seed quality. Seed Sci Technol 26:865–870

Shi JR, Wang HY, Wu YS, Hazebroek J, Meeley RB, Ertl DS (2003) The maize low phytic acid mutant lpa2 is caused by mutation in an inositol phosphate kinase gene. Plant Physiol 131:507–515

Shi JR, Wang H, Hazebroek J, Ertl DS, Harp T (2005) The maize low-phytic acid 3 encodes a myo-inositol kinase that plays a role in phytic acid biosynthesis in developing seeds. Plant J 42(5):708–719

Shi JR, Wang HY, Schellin K, Li BL, Faller M, Stoop JM, Meeley RB, Ertl DS, Ranch JP, Glassman K (2007) Embryo-specific silencing of a transporter reduces phytic acid content of maize and soybean seeds. Nat Biotech 8:930–937

Singh SP (1982) A key for identification of different growth habits of *Phaseolus vulgaris* L. Annu Rep Bean Improv Coop 25:92–95

Sparvoli F, Daminati MG, Bollini R (1994) Biochemical and molecular characterisation of a *Phaseolus vulgaris* mutant lacking the major lectin related seed proteins. Annu Rep Bean Improv Coop 37:110

The World Health Report (2002) Quantifying selected major risks to health. Chap 4, p 4. http://www.who.int/whr/2002/chapter4/en/index3.html

Tadmor NH, Cohen Y, Harpaz Y (1969) Interactive effects of temperature and osmotic potential on the germination of range plants. Crop Sci 9:771–774

Tesoro A, Novakovic J, Thiessen JJ, Spino M (2005) Validated HPLC assay for iron determination in biological matrices based on ferrioxamine formation. J Chromatogr B Analyt Technol Biomed Life Sci 823:177–183

Vasconcelos M, Datta K, Oliva N, Khalekuzzaman M, Torrizo L, Krishnan S, Oliveira M, Goto F, Datta SK (2003) Enhanced iron and zinc accumulation in transgenic rice with the ferritin gene. Plant Sci 64:371–378

Veum TL, Ledoux DR, Raboy V (2007) Low-phytate barley cultivars improve the utilization of phosphorus, calcium, nitrogen, energy, and dry matter in diets fed to young swine. J Anim Sci 85:961–971

Wang TL, Domoney C, Hedley CL, Casey R, Grusak MA (2003) Can we improve the nutritional quality of legume seeds? Plant Physiol 131:886–891

Welch RM (2002) Breeding strategies for biofortified staple plant foods to reduce micronutrient malnutrition globally. J Nutr 132:495S–499S

Welch RM, Graham RD (2004) Breeding for micronutrients in staple food crops from a human nutrition perspective. J Exp Bot 55:353–364

Welch RM, House WA, Beebe S, Cheng Z (2000) Genetic selection for enhanced bioavailable levels of iron in bean (*Phaseolus vulgaris* L.) seeds. J Agr Food Chem 48:3576–3580

Wilcox JR, Premachandra GS, Young KA, Raboy V (2000) Isolation of high inorganic P, low-phytate soybean mutants. Crop Sci 40:1601–1605

Yuan FJ, Zhao HJ, Ren XL, Zhu SL, Fu XJ, Shu QY (2007) Generation and characterisation of two novel low phytate mutations in soybean (*Glycine max* L. Merr.). Theor Appl Genet 115:945–957