Nutritionally Relevant Parameters in Low-Phytate Barley (*Hordeum vulgare* L.) Grain Mutants

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Nutritionally relevant parameters in barley low-phytate mutant grains were analyzed in order to assess the potential value of these lines for future feeding trials. Phytate (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate) levels in grains from A- and B-type low-phytate mutants corresponded to 25% and 66% of those of the parent line content, repectively. These relative decreases in phytate were accompanied by proportional increases of inorganic phosphate amounts. Apart from phytate, A-type grains also contained substantial quantities of *myo*-inositol 1,3,4,5-tetrakisphosphate. Phytate levels in straw and root material from mutants were similar to parent line controls, indicating that low-phytate mutations were grain specific. Analysis of K, Mg, Ca, and Zn revealed normal or slightly increased mineral cation levels in grains from all low-phytate lines, suggesting that mutationally impaired phytate accumulation did not affect mineral storage capacity. Other nutritionally important parameters such as starch and protein contents were similar to parent line controls. Finally, dynamic changes in the phosphorus composition during kernel development suggested that A-type mutations directly affected phytate synthesis, whereas B-type mutations seemed to act on regulation of synthesis.

Keywords: *Phytate; phosphorus; phosphate; mutants; mineral cations; potassium; magnesium; calcium; zinc; phytase; barley*

INTRODUCTION

Phytate (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate) is the major phosphorus (P) storage compound in plant seeds and can account for up to 80% of seed total P (Loewus et al., 1990; Raboy, 1990). The remaining P is represented by soluble inorganic phosphate (P_i) and cellular P (i.e., P bound in nucleic acids, phosphorylated proteins, P-lipids, and P-sugars). Because of its high density of negatively charged phosphate groups, phytate forms mixed salts with mineral cations ("phytins") which are assumed to play an important role in mineral storage (Raboy, 1997). Phytins contain predominantly K and Mg, whereas other metals such as Ca, Zn, Fe, and Ba are found in much smaller amounts. As visualized by electron microscopy, globular phytin inclusions are typically located in protein bodies of the aleurone layer (Lott, 1984). In many plant species, such as barley and wheat, 90% of the phytin is localized in the aleurone and only 10% in the embryo. In maize, on the other hand, most of the phytin is found in the embryo and only a small fraction (ca. 10%) is found in the aleurone (O'Dell et al., 1972). During germination, phytin is degraded by the action of phytases, which provides the growing seedling with phosphate, mineral cations, and *myo*-inositol. Apart from its storage function, phytate has also been assumed to play an important role in P homeostasis, buffering cellular P levels (Raboy, 1997).

Phytate is nearly indigestible for monogastric animals such as swine and poultry. The phytate passes through the digestive system, none of the minerals are used by the animal, and the minerals accumulate in the soil with the breakdown of the animal feces. To date, nutritional availability of P is most commonly improved by supplying animal fodder with P_i and/or microbial phytases. Other strategies, however, aim at overexpression of phytases in transgenic cereals and on mutational breeding of low-phytate mutants (Hatzack et al., 1999).

Recent identification of such mutants has not only opened new approaches to improving the nutritional P-supply but has also led to a better understanding of phytate metabolism and genetics (Raboy and Gerbasi, 1996; Larson et al., 1998). Raboy and co-workers found that maize and barley (*Hordeum vulgare*, cv Harrington) low-phytate mutants fell into two phenotype categories, termed lpa-1 and lpa-2 (lpa, low phytic acid). Grains of lpa-1 lines contained 25%-50% of the wild-type phytate level with no unusual lower inositol phosphates present. In lpa-2 lines, on the other hand, 8%-15% of the total P was present in the form of lower inositol phosphates, whereas phytate corresponded to 25%-50% of the wild-type level. In both lpa-1 and lpa-2 mutants, relative reductions in phytate-P were accompanied by near-molar increases in P_i and/or lower inositol phosphates (Raboy, 1998).

Barley mutant phenotypes similar to lpa-1 and lpa-2 were identified in an independent screening analysis conducted on mutagenized grains of the P01 near-isogenic line of cv Pallas (Rasmussen and Hatzack, 1998). Semiquantitative analysis by TLC indicated that grains from A-type mutants contained very high amounts of P_i, low levels of phytate, and traces of lower inositol phosphates. Subsequent analysis of A-type mutants by "metal-dye" detection HPLC identified substantial amounts of D/L-*myo*-inositol 1,3,4,5-tetrakisphosphate (unpublished data). TLC analysis of B-type grains, on

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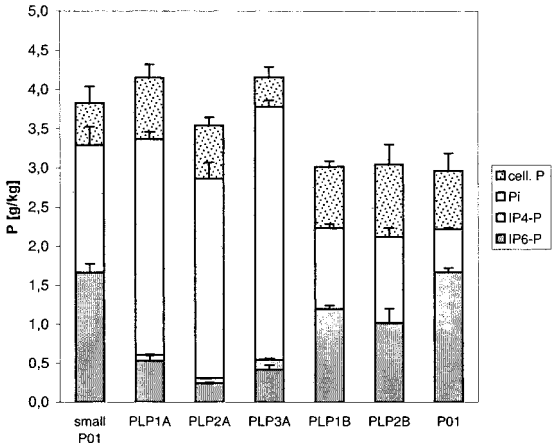

Figure 1. Phosphorus-containing compounds in low-phytate mutant grains. Phytate phosphorus (IP6-P) and phosphorus bound in inositol tetrakisphosphate (IP4-P) were calculated from "metal-dye" detection HPLC analysis of phytate and D/L-*myo*-inositol 1,3,4,5-tetrakisphosphate. Inorganic phosphate (P_i) was determined photometrically, and cellular P (cell. P) was calculated by subtracting phosphate, phytate-P, and tetrakisphosphate-P from total P. Pallas-P01 controls, consisting of small-size and normal-size grains, are designated "small P01" and "P01", respectively. Values are means ± SD, $n = 6$.

the other hand, showed moderate relative decreases in phytate and somewhat higher P_i levels than in the parent line (cv Pallas-P01) controls. Unusual inositol phosphates were not observed in B-types.

The major objective of this study was to assess the potential nutritional value of Pallas-P01 low-phytate (PLP) barley mutants by quantitative analysis of phytate-P, P_i, total P, mineral cations, total protein, starch, and phytase levels. Another objective was to characterize A- and B-type PLP mutants with respect to dynamic changes of P composition during kernel development.

MATERIALS AND METHODS

Chemicals. Dodecasodium phytate and D-*myo*-inositol 1,3,4,5-tetrakisphosphate were purchased from Sigma (St. Louis, MO). Standard chemicals were obtained from Merck (Germany).

Plant Material. Low-phytate mutant lines PLP1A, PLP2A, PLP3A, PLP1B, and PLP2B were generated by chemical mutagenesis of barley grain (*Hordeum vulgare* L. cv Pallas near-isogenic line P01). Mutagenesis, screening procedures, and TLC characterization of mutant lines are described in Rasmussen and Hatzack (1998). For analysis of P levels during grain development, plants were grown in a greenhouse facility with a 16-h photoperiod at 220 μE m^{-2} s^{-1}. At flowering, first spikes were tagged, and 10−15 grains from the spike middle sections were collected for analysis. Grain material from mature plants was from the 1998 harvest of Sejet Plantbreeding (Horsens, Denmark).

Quantification of Phosphorus and Phosphorus-Containing Compounds. Total phosphorus content was measured by the molybdate blue method (Murphy and Riley, 1962). Following digestion of dried (3 h, 70 °C), ground material (0.5-mm sieve, Culatti, Italy) in a solution of nitric and perchloric acids (4:1, v/v), the extracts were filtered (0.2-μm polycarbonate, Millipore), diluted 10-fold with distilled water, and analyzed on an Autoanalyzer II (Technicon, USA). For determination of inorganic phosphate, phytate-P, and inositol tetrakisphosphate-P, grains were ground and extracted with 10% (w/v) trichloroacetic acid (Rasmussen and Hatzack, 1998; Hatzack and Rasmussen, 1999). Soluble inorganic phosphate (P_i) was measured in diluted TCA extracts (1:5 in water), applying the method of Engelen et al. (1994). Phytate-P and P present in D/L-*myo*-inositol 1,3,4,5-tetrakisphosphate were quantified by HPLC analysis of inositol phosphates in grain extracts using the "metal-dye" detection (MDD) technique developed by Mayr (1990). Samples for HPLC analysis were prepared by TCA extraction (see above) followed by charcoal treatment (Mayr, 1990). Chemically inert HPLC equipment was from Shimadzu (Japan) and a column combination consisting of a 1-mL Resource Q and a Mono Q HR 5/20 column (both Pharmacia, Sweden) was employed.

Determination of Mineral Cations, Total Protein, Starch, and Phytase Activity. Potassium (K), calcium (Ca), magnesium (Mg), and Zinc (Zn) in the grain material were analyzed by atomic absorption spectroscopy (AAS) using standard procedures for the Perkin-Elmer (Wellesley, MA) model 2380. Total nitrogen was measured on a Carlo Erba EA 1110 (Italy) elemental analyzer as described in Jensen (1991). Enzymatic (amyloglucosidase) starch hydrolysis and subsequent photometric determination of D-glucose was carried out with a starch test kit from Boehringer Mannheim (Germany). Activity of endogenous phytase was determined in raw extracts obtained by suspending ground kernels in 10 volumes per weight of 100 mM sodium acetate, pH 5.5. Samples were agitated for 30 min at room temperature and centrifuged at 5000×g for 20 min. Phytase activity in supernatants was measured using a modification of the method of Engelen et al. (1994). Twenty microliters of sample was diluted with 80 μL of 100 mM sodium acetate, pH 5.5. To this dilution, 200 μl of substrate solution (9 mM phytate in 0.25 M sodium acetate, pH 5.5) was added, and the samples were incubated at 37 °C for 30 min. Reactions were stopped by the addition of 200 μl of molybdovanadate reagent and absorbance was measured at 405 nm using a PowerWave$_x$ microtiter plate reader (Bio-Tek, USA).

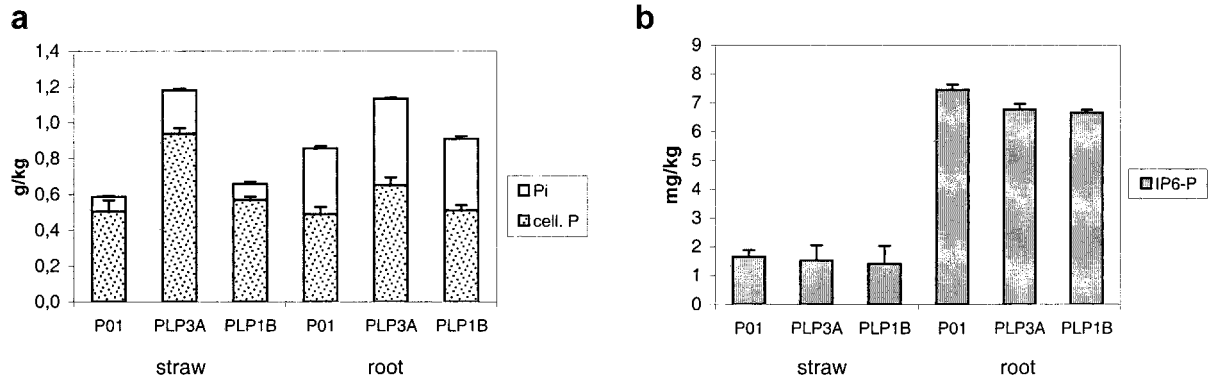

Figure 2. Analysis of phosphorus in root and straw material. Phosphorus compositions in vegetative tissues from mutant lines PLP3A and PLP1B were analyzed and compared with those of Pallas-P01 controls (P01). Inorganic P (P_i) and cellular P (cell. P) levels are displayed in (a), whereas the much lower phytate-P levels (IP6-P) are illustrated separately in (b). Values are means ± SD, $n = 4$.

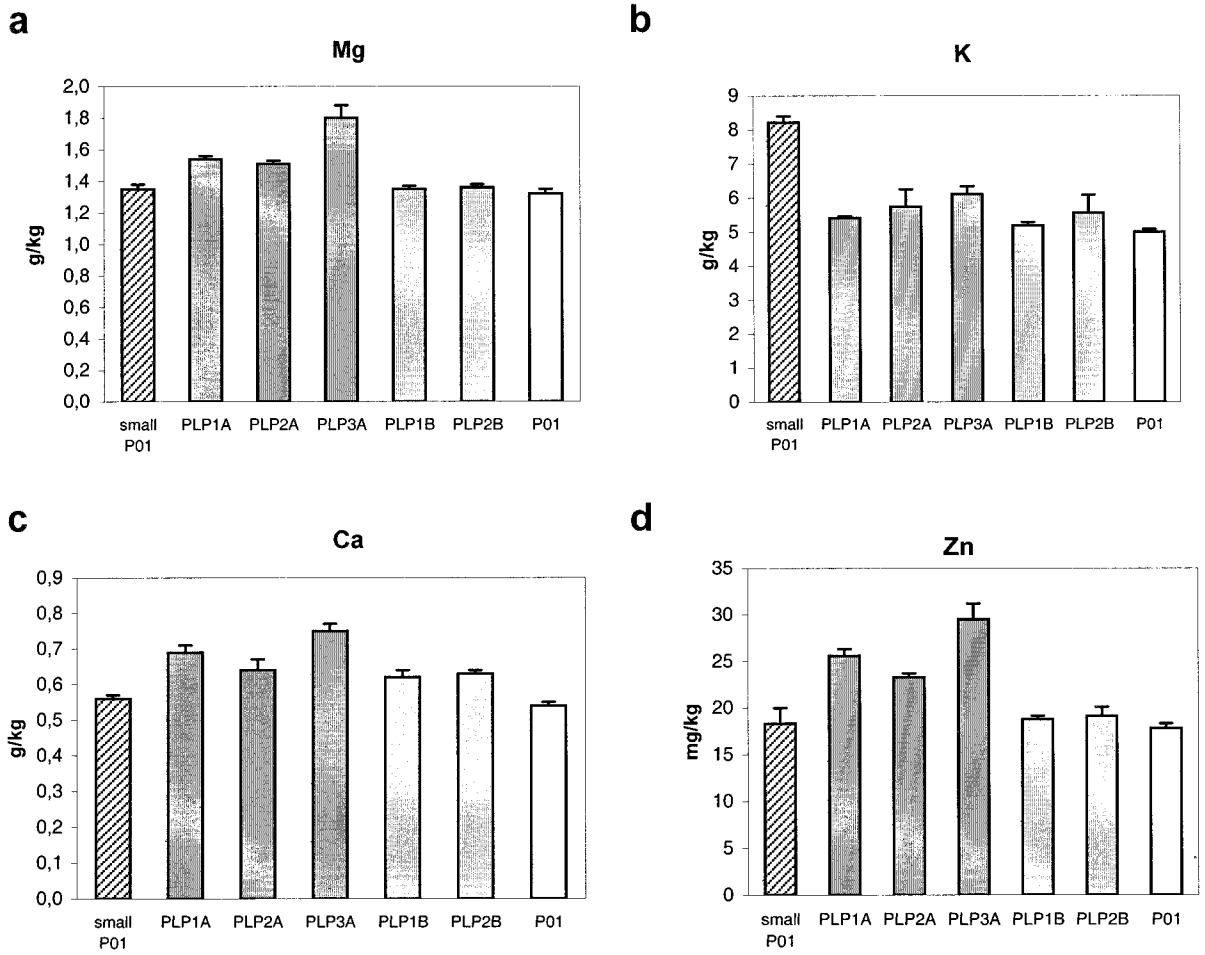

Figure 3. Mineral cation composition in grains. Quantification of magnesium (a), potassium (b), calcium (c), and zinc (d) was carried out by atomic absorption spectroscopy. Grain material from low-phytate mutant lines is compared with small-size (small-P01) and normal-size Pallas-P01 grains (P01). Values are means ± SD, $n = 4$.

RESULTS AND DISCUSSION

Phosphorus Composition in Grains. For comparison of P-composition in Pallas-P01 and mutant grains (Figure 1), two different parent-line control groups were selected to account for the different average kernel weights of A-type (23.5 mg) and B-type lines (34.1 mg). The first control set consisted of 300 individually selected small Pallas-P01 grains which had an average weight of 22.5 mg. This small-size grain material served as a reference for the relatively small A-type (PLP1A, PLP2A, and PLP3A) kernels. The second control set, which was used for comparison with B-type (PLP1B and PLP2B) grains, consisted of 10 × 100 randomly selected Pallas-P01 kernels to obtain a representative distribution of small and large grains, having a mean weight of 36.5 mg.

A-type and small-size Pallas-P01 grains contained higher total phosphorus amounts (3.5−4.2 g/kg) than B-type and normal-size controls (both 3.0 g/kg). This difference was most likely due to the higher ratio of P-rich aleurone mass over endosperm mass, which is a general feature of small grains. With regard to phytate-P, however, similar levels were found in small-size (1.66 ± 0.11 g/kg) and normal-size (1.67 ± 0.05 g/kg) controls. Phytate-P levels in A-type grains were significantly ($P < 0.05$, *t*-test) lower than in both Pallas-P01 control groups. With an average phytate-P value of 0.39 ± 0.05 g/kg, these lines contained just 24% of the phytate-P amount present in the parent line controls.

The two B-type lines had a mean phytate-P level of 1.11 ± 0.11 g/kg, which corresponded to 66% of parent line phytate-P. Compared with normal-size Pallas-P01 grains, relative decreases of phytate-P in B-types appeared together with molar-equivalent increases of P_i. In A-types, on the other hand, relative decreases in phytate-P were accompanied by proportional increases of both P_i and P bound in D/L-*myo*-inositol 1,3,4,5-tetrakisphosphate. Phosphorus present in this tetrakisphosphate accounted for ca. 15% of the inositol-bound P.

Proportional relative decreases in phytate-P and increases in P_i resulted into virtually unchanged cellular P levels in mutants. Taken together, these observations suggested that A- and B-type mutants were phenotypically similar to the lpa-2 and lpa-1 grain mutants identified by Raboy and co-workers (Raboy, 1998). Therefore, one of the most important conclusions drawn from the analysis of those mutants also applies for A- and B-type mutants: High concentrations of phytate (as found in Pallas-P01 grains) do not seem to be essential for P homeostasis − a conclusion supporting the notion that the primary function of phytate is to provide homeostasis for the germination seeds.

Phosphorus Composition in Vegetative Tissues. To investigate whether A- and B-type mutations affected the P compositions of vegetative tissues, straw and root material from PLP3A and PLP1B plants were analyzed (Figure 2). In comparison to B-type and Pallas-

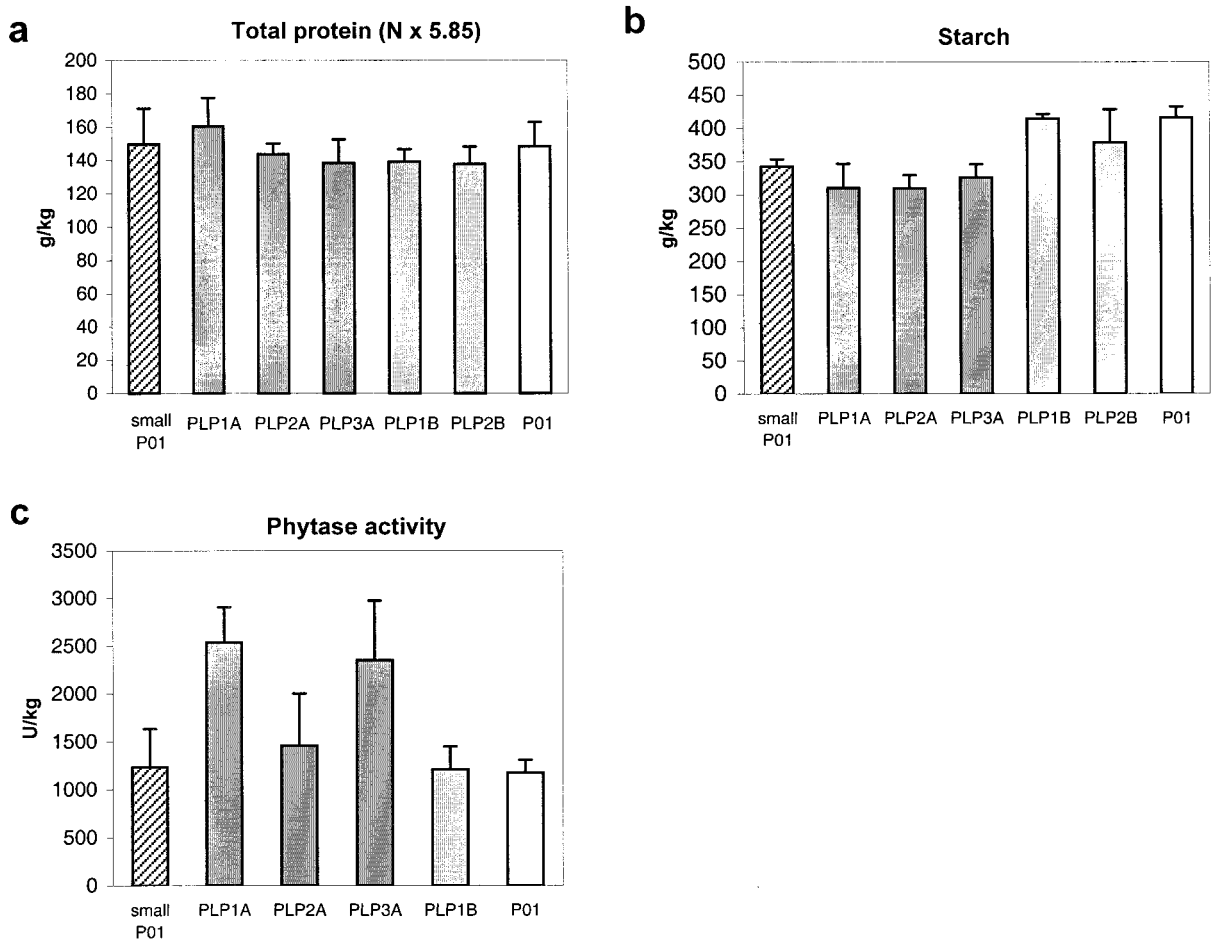

Figure 4. Quantification of total protein, starch, and phytase activity. Total protein amounts (a) were calculated by multiplying kernel total nitrogen (N) with 5.85. Starch content (b) was quantified by hydrolysis with amyloglucosidase and subsequent photometric determination of the resulting D-glucose. Endogenous phytase activity (c) was assayed by measuring the release of phosphate during phytate degradation (1 U = 1 μmol P_i released per minute). Values are means ± SD, $n = 5$.

P01, straw as well as root samples from PLP3A showed enhanced cellular P and P_i levels (Figure 2a). These relative increases were most pronounced in straw, which contained three times as much P_i and almost two times as much cellular P than PLP1B or the parent line material. However, with regard to phytate-P (which was found in much smaller amounts than in grains) similar levels were observed in straw and root samples from all three analyzed lines (Figure 2b). This finding indicated unperturbed phytate synthesis in vegetative tissues of mutants, which consequently suggested that A- and B-type mutations were tissue specific (i.e., affecting exclusively grain-localized phytate synthesis). Thus, the relatively high P levels found in PLP3A root and straw samples were most likely the consequence of enhanced transport of P to the grain during growth and maturation.

Mineral Cations in Low-Phytate Mutant Grains. Quantification of mineral cations (Figure 3) revealed normal levels in B-type grains, whereas slightly higher amounts (10−25% relative to Pallas-P01) were measured in grains from A-type lines. These relative increases suggested that the very high amounts of P_i present in A-type grains acted as an effective cation sink. Because all analyzed cations showed similar relative increases, it could furthermore be assumed that cation-sequestration by P_i is a rather nonselective mechanism. Thus, low levels of phytate did not result in quantitative losses of certain cations such as K^+ and Mg^{2+}, which are typically associated with phytate in "phytin" salts. In conclusion, the data indicate that low-phytate mutations did not have adverse effects on the mineral storage capacity of grains.

A remarkably high level of K, found in small-size Pallas-P01 kernels (Figure 3b), represented a somewhat unexpected finding. If this high level of K had been caused by high amounts of P_i (sequestering K^+) then one would have expected comparably high levels of K in A-type grains as well. As this was not observed, an alternative explanation may be suggested: considering that small grains are typically located in the tip region of barley spikes, it could be assumed that high K^+ levels in small Pallas-P01 grains were the result of differential cation distribution patterns along the spike.

Starch, Protein, and Phytase Levels. Data on starch, protein, and phytase levels are presented in Figure 4. With respect to total protein (N × 5.85), similar amounts were measured in all samples analyzed (Figure 4a). Starch content, on the other hand, was somewhat lower in A-type and small-size control grains as compared to B-type and normal-size controls (Figure 4b). Because starch is the major constituent of the voluminous endosperm, the minor reductions in starch content were most likely due to the relatively increased ratio of kernel surface mass over endosperm mass − a general feature of all small grains.

With respect to phytase, high levels of activity were observed in PLP1A and PLP3A but not in PLP2A and

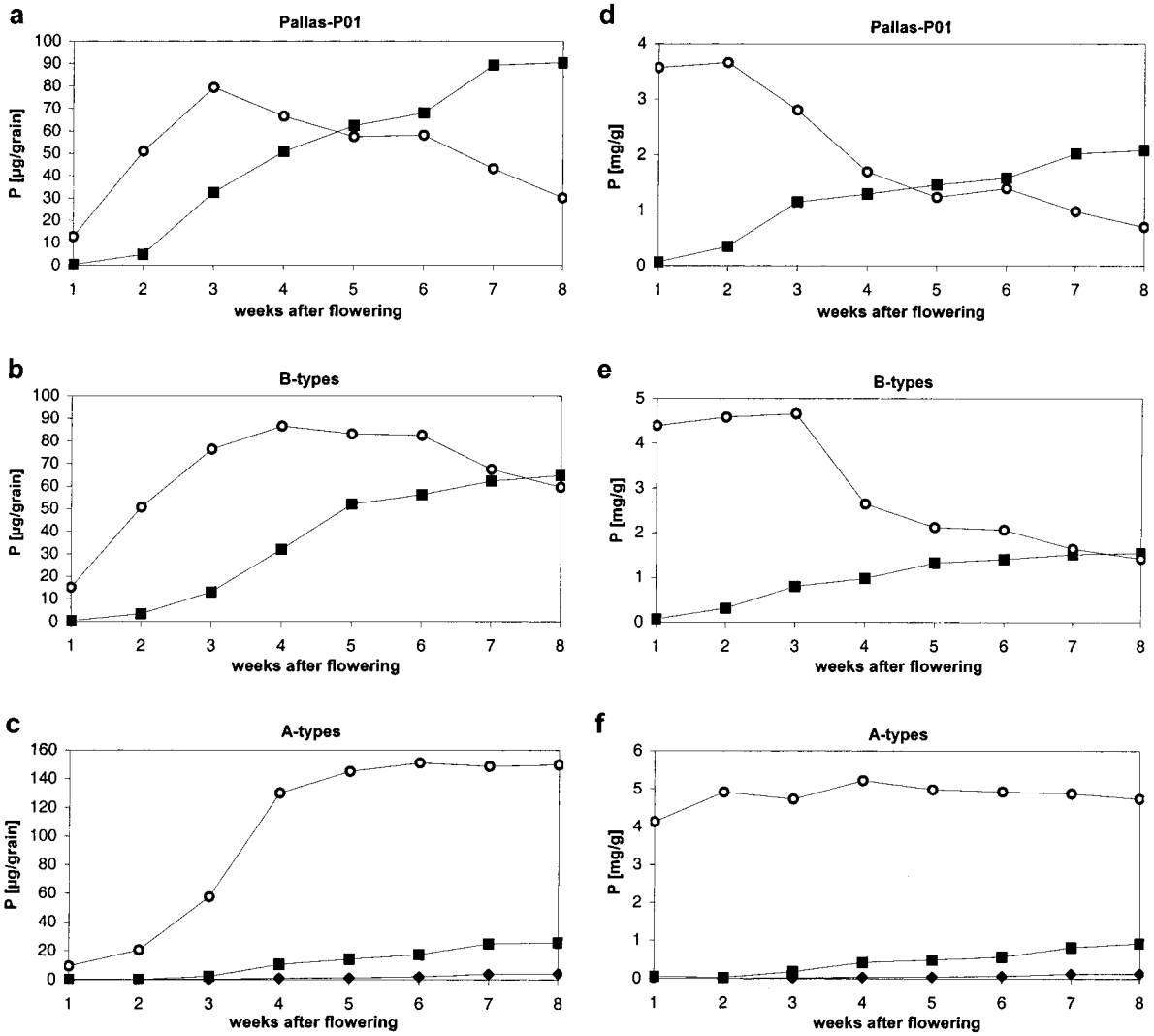

Figure 5. Changes in P composition during grain development. P_i (−○−), phytate-P (−■−) and P bound in D/L-*myo*-inositol 1,3,4,5-tetrakisphosphate (−◆−) are plotted on a per grain basis (µg P/grain) in (a−c). Diagrams d−f depict corresponding data expressed in milligram phosphorus per gram grain tissue (mg P/g). In the case of Pallas-P01 each data point represents the mean of triplicate determinations (a and d). Values in (b) and (e) were calculated from means of single determinations in two lines (PLP1B and PLP2B). Data points in (c) and (f) represent means of single measurements carried out in three A-type lines (PLP1A, PLP2A, and PLP3A). Time points of sample collection are given in weeks after flowering (waf).

PLP1B (Figure 4c). Considering that enhanced phytase activity is likely to increase nutritional P availability, it may be expected that PLP1A and PLP3A will prove particularly valuable in future feeding trials.

Accumulation of Phytate and Inorganic Phosphate in Developing Grains. Dynamic changes of phosphorus composition during development were analyzed at weekly intervals and plotted on a per grain (Figures 5a-c) as well as on a per gram basis (Figures 5d-f). The data indicated a two-phase developmental pattern in Pallas-P01 grains: the first 3 weeks after flowering comprised a growth phase, during which both P_i and phytate-P accumulated in grains. The second phase, which lasted for the remaining 5 weeks, had the characteristics of a maturation phase. During this period, P_i levels per grain decreased steadily while phytate-P continued to increase (Figure 5a). This indicated that P_i allocation to the grain had ceased while ongoing phytate synthesis depleted remaining P_i pools. Similar two-phase patterns had been previously observed in developing maize grains (Earley and DeTurk, 1944) and soybean seeds (Raboy and Dickinson, 1987).

B-type grains showed a slower rate of phytate-P accumulation than Pallas-P01 and the beginning of the maturation phase (week 6) was delayed (Figure 5b). The slowed-down synthesis rate observed in B-type grains suggested that B-type mutations affected genes involved in regulatory processes rather than phytate synthesis itself.

Dynamic changes observed in A-types were remarkably different from the changes observed in Pallas-P01 or B-types (Figures 5c and 5f). In A-types P_i levels per gram remained on a very high level (4−5 mg P/g) during the entire eight-week development period whereas phytate-P accumulated extremely slowly (Figure 5f). Expressed on a per-grain basis, a steep increase of P_i during the first four weeks was followed by a steady-state period during which P_i levels remained on a very high level (ca. 150 µg P/grain, Figure 5c). Extremely slow phytate synthesis in conjunction with accumulation of D/L-*myo*-inositol 1,3,4,5-tetrakisphosphate indicated that A-type mutations affected a gene essential for phytate synthesis. In this context, recent identification of D-*myo*-inositol 1,3,4,5-tetrakisphosphate 6-kinase in

soybean seeds (Phillippy, 1998) suggests that A-type mutants may result from mutations in a gene encoding the corresponding barley tetrakisphosphate kinase.

CONCLUSIONS

Analysis of nutritionally relevant parameters in Pallas-P01 low-phytate (PLP) barley mutants showed that these lines have the potential to significantly improve bio-availability of phosphorus. In comparison to the parent line Pallas-P01, which had an elemental composition similar to other Danish varieties (Sørensen and Truelsen, 1985), PLP mutants contained low amounts of the nearly indigestible phytate and high levels of nutritionally available P_i. Furthermore, the finding that other nutritionally important components such as K, Ca, Mg, Zn, protein, and starch levels were not severely affected by low-phytate mutations, clearly encourages the continuation of breeding work involving these mutants. An important objective in future breeding projects will be to outcross unfavorable traits such as small grain size. Small grain size, as found in A-type lines, is assumed to be caused by pleiotropic effects of low-phytate mutations and/or by different mutations, unrelated to phytic acid metabolism.

At present, limited amounts of PLP barley grain material preclude large-scale feeding trials such as the ones performed with low-phytate maize (Huff et al., 1998; Mendoza, 1998; Ertl et al., 1998; Sugiura et al., 1999). However, a recently conducted pilot study with 20 rats showed increased bio-availability of P and Zn when animals were fed with A-type grains (Poulsen et al., 2000). Follow-up studies will now have to show whether particular A-type lines, like the ones with enhanced phytase activity (PLP1A and PLP3A), offer an improved nutritional value. Taken together, our current knowledge of PLP mutants confirms that low-phytate crops represent a promising strategy to improve P-utilization and to reduce the environmental impact of phytate-rich manure.

One of the still-open questions surrounding low-phytate mutants is how mutant grains manage to store such large quantities of phosphate. A first attempt to answer this question has recently been undertaken by investigating starch phosphorylation patterns in PLP mutants (Blennow et al., 2000). The results of these physicochemical studies do not indicate any differences between mutant and wild-type barley grains: both contained nonphosphorylated starch. Thus, it seems more likely that the large amounts of P_i found in PLP mutant grains are stored in vacuolar compartments of the aleurone.

ABBREVIATIONS USED

TCA, trichloroacetic acid; TLC, thin-layer chromatography; cv, cultivar: waf, weeks after flowering.

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