Inositol phosphates from barley low-phytate grain mutants analysed by metal-dye detection HPLC and NMR

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Inositol phosphates from barley low-phytate grain mutants and their parent variety were analysed by metal-dye detection HPLC and NMR. Compound assignment was carried out by comparison of retention times using a chemical hydrolysate of phytate [Ins(1,2,3,4,5,6)P6] as a reference. Co-inoculating retention times indicated the presence of phytate, d/1-Ins(1,2,3,4,5)P5, Ins(1,2,3,4,6)P5, d/1-Ins(1,2,3,4,5,6)P6, d/1-Ins(1,2,3,4)P4, d/1-Ins(2,5,6)P3, and d/1-Ins(4,5,6)P3 in PLP1B mutants as well as the parent variety. In grain extracts from mutant lines PLP1A, PLP2A and PLP3A unusual accumulations of d/1-Ins(1,3,4,5)P4 were observed whereas phytate and the above-mentioned inositol phosphates were present in relatively small amounts. Assignment of d/1-Ins(1,3,4,5)P4 was corroborated by precise co-chromatography with a commercial Ins(1,3,4,5)P4 standard and by NMR spectroscopy. Analysis of inositol phosphates during grain development revealed accumulation of phytate and d/1-Ins(1,3,4,5)P4, which suggested the tetrakisphosphate compound to be an intermediate of phytate synthesis. This assumption was strengthened further by phytate degradation assays showing that d/1-Ins(1,3,4,5)P4 did not belong to the spectrum of degradation products generated by endogenous phytase activity. Metabolic scenarios leading to accumulation of d/1-Ins(1,3,4,5)P4 in barley low-phytate mutants are discussed.

Key words: grain filling, Hordeum vulgare, inositol phosphate kinase, phytase, seed-specific.

INTRODUCTION

Seed-specific accumulation of large amounts of phytate [Ins(1,2,3,4,5,6)P6] is a unique feature of inositol phosphate metabolism in the plant kingdom [Ins should be taken to mean myo-Ins with the numbering of the ρ-configuration unless the compound is a meso-compound or prefixes like d/1- are explicitly added. In this article, d/1- is used to indicate that metal-dye detection (MDD) HPLC and NMR do not discriminate between stereoisomers]. Being the storage compound for phosphorus, phytate can account for 50–80% of the total seed phosphorus in many plant species (reviewed in [1–4]). Another function of plant phytate is mineral storage, which is conveyed by the high density of negatively charged phosphate groups located around the myo-inositol ring. Thus phytate forms complexes with mineral cations (mostly K+, Mg2+ and Ca2+), which on electron micrographs appear as globular inclusions in protein bodies [5]. Depending on the plant species, phytate globoids are localized predominantly in the aleurone layer (wheat and barley) or in the embryo (maize) [6]. During germination, phytate is degraded continuously by phytases, providing growing seedlings with phosphate, mineral cations and myo-Ins (reviewed in [1–4]).

In spite of the numerous physiological investigations of phytate accumulation and storage there still remain a lot of questions regarding the biochemical arrangement of phytate metabolism in plants. How plants synthesize phytate and how phytate metabolism is co-ordinated with signalling mechanisms involving other inositol phosphates is not fully understood. The only completely described plant phytate synthesis pathway known at present was elucidated in the duckweed Spirodela polyrhiza [7]. Using an in vitro labelling approach, an order of phosphorylation steps was established which proceeds via Ins3P, Ins(3,4)P2, Ins(3,4,6)P2, Ins(3,4,5,6)P2, and Ins(1,3,4,5,6)P2 to phytate. However, investigations of inositol phosphate kinases from Arabidopsis thaliana [8] and soybean [9] indicated that phytate synthesis pathways operating in land plants may be quite different from the pathway in the aquatic species Spirodela. In both soybean and Arabidopsis, an Ins(3,4,5)P2/6-kinase activity was identified which yielded Ins(1,3,4,5)P4 as the major phosphorylation product. An additional kinase activity from soybean seeds utilized Ins(1,3,4,5)P4 to produce Ins(1,3,4,5,6)P5 which, together with a previously described Ins(1,3,4,5,6)P2 2-kinase [10], established a biosynthetic route to phytate. This hypothetical pathway bears some resemblance to the phytate synthesis pathway in fission yeast Schizosaccharomyces pombe [11], and the nucleus-associated pathway in the slime mould Dicystostelium discoideum [12]. In both pathways phytate synthesis proceeds via Ins(1,4,5)P3 which is phosphorylated to Ins(1,3,4,5,6)P5 (D. discoideum and S. pombe) and Ins(1,4,5,6)P5 (S. pombe only). As in Spirodela and soybean, Ins(1,3,4,5,6)P5 is the final intermediate in both slime mould and fission yeast.

While a comprehensive analysis of phytate synthesis in a land-plant species is still lacking, detailed studies on phytate degradation have been carried out in wheat bran [13–15], barley aleurone layer [16], germinating mung bean [17,18], soybean seeds [19] and maize roots [20] as well as in lily pollen [21]. Although stereochemical similarities of phytate breakdown products can be recognized in closely related species such as wheat and barley [16], a general consensus of intermediates indicating conserved substrate processing by plant phytases does not appear to exist.

The identification of low-phytic acid mutants in maize [4] and in the barley varieties Harrington [22] and Pallas-P01 [23] has opened new opportunities to investigate the biochemistry and

Abbreviations used: PAR, 4-(2-pyridyldiazol)resorcinol; TOCSY, total correlations spectroscopy; TCA, trichloroacetic acid; MDD, metal-dye detection; MIPS, myo-inositol 1-phosphate synthase; PLP mutant, Pallas-P01 low-phytate mutant.

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genetics of plant phytic acid metabolism. Mutants found in maize and Harrington barley were categorized into two phenotypes (lpa1 and lpa2) and linkage mapping identified the chromosomal locations of lpa1 and lpa2 mutations [22]. Subsequent mapping studies indicated myo-inositol 1-phosphate synthase (MIPS) as a likely candidate gene for the lpa1 mutation [24]. Based on TLC analysis, Pallas-P01 low-phytate (PLP) barley mutants were initially divided into two distinct phenotype classes [23]. Grain extracts from A-type mutants contained extremely high levels of phosphate, low levels of phytate and traces of unidentified inositol phosphates, which were not observed in the parent line. Grains from B-types showed increased phosphate levels and moderately reduced phytate levels, while accumulations of other inositol phosphates were not detected. Genetic testing revealed that the A and B phenotypes are caused by recessive mutations in separate loci.

In this paper we present isomer-specific MDD-HPLC and NMR data on inositol phosphates isolated from grains of low-phytate mutants and their parent variety Pallas-P01. By evaluating inositol phosphate profiles from both mature and developing grains and by analysing patterns of enzymic phytate degradation we undertook a first step towards elucidating the phytate synthesis pathway in barley.

MATERIALS AND METHODS

Chemicals

Inositol phosphate standards [Ins(1,4,5)P₃, Ins(1,3,4)P₃, Ins-(1,3,4,5)P₄, and Ins(1,2,3,4,5,6)P₆] were obtained from Sigma (St. Louis, MO, U.S.A.). PAR [4-(2-pyridylazo)resorcinol] was purchased from Fluka (Buchs, Switzerland), yttrium(III) chloride hexahydrate from Acros (Geel, Belgium) and activated charcoal (Norit A) from Serva (Heidelberg, Germany). Triethanolamine was purchased from Fluka (Buchs, Switzerland), yttrium(III) chloride hexahydrate from Acros (Geel, Belgium) and activated charcoal (Norit A) from Serva (Heidelberg, Germany). Triethanolamine and ultrapure HCl used in MDD-HPLC as well as standard chemicals were obtained from Merck (Darmstadt, Germany). Water was purified using a filtration system (Millipore, Bedford, MA, U.S.A.).

Plant material

Barley (Hordeum vulgare L.) grains were from low-phytate mutant lines PLP1A, PLP2A, PLP3A and PLP1B and their parent variety Pallas-P01. Mutant lines were generated by chemical mutagenesis of Pallas-P01 grains and identified by screening techniques described in [23]. Immature seeds, used for analysis of inositol phosphate accumulation during grain development, were from first spikes of plants grown under greenhouse conditions. Following flowering, 10–15 kernels were collected at weekly intervals.

Trichloroacetic acid (TCA) extraction of inositol phosphates and diethyl ether treatment

Barley grains were dried in a dry-air oven at 70 °C for 2 h. Flour samples were prepared by crushing grains with a pair of pliers, followed by grinding in a mortar. Extraction was performed by suspending 40 mg portions of flour in a 10-fold excess of ice-cold TCA extraction buffer (10% w/v) TCA/5 mM NaF/5 mM EDTA) [25]. Samples were agitated for 1 h at 4 °C and centrifuged at 5000 g for 5 min at 4 °C. Pellets were resuspended in TCA extraction buffer and agitated for another 1.5 h as described above. Supernatants from both extraction rounds were pooled and TCA was largely removed by four consecutive ether extractions carried out as described in [25]. The final pH was 3.5.

Charcoal treatment and solid-phase extraction

In order to remove nucleotides, grain extracts were treated with activated charcoal as described in [26]. Subsequent solid-phase extraction was performed by adding diluted samples (1:50 with ice-cold water) to disposable columns (Bio-Rad, Hercules, CA, U.S.A.) that were filled with 1 ml of Q-Sepharose FF (Pharmacia, Uppsala, Sweden). Columns were washed twice with 2.5 ml of 2 mM HCl and inositol phosphates were eluted by 2×2.0 ml 1.5 M ammonium acetate [27]. Eluates were lyophilized and remaining ammonium acetate was removed by adding 2 ml of water, followed by another round of lyophilization. For MDD-HPLC analysis, samples were resuspended in a volume (in µl) equal to 20×(the weight of the flour sample in mg) of 5 mM sodium acetate/5 mM EDTA/1 mM NaF.

Preparation of a chemical phytate hydrolysate

Sodium phytate (50 mg) was dissolved in 5 ml of 6 M HCl and boiled at 100 °C for 2 h. The hydrolysate was divided into 25 µl portions that were dried in a speed-vac freeze-drier. For MDD-HPLC analysis hydrolysate portions were resuspended in 300 µl of 5 mM sodium acetate/1 mM NaF and 150 µl were injected. To facilitate assignment of inositol phosphates from barley grain samples, chromatographic profiles of the phytate hydrolysate were recorded before and immediately after HPLC analysis of respective grain samples.

In vitro degradation of phytate by endogenous barley phytase

Barley grains were crushed with a pair of pliers and ground with a mortar and pestle. Portions of 40 mg were suspended in 200 µl of 50 mM sodium acetate, pH 4.6, and incubated for 0, 5, 12.5, 25 and 50 min at 35 °C. Samples were vortexed briefly every 2 min and incubations were stopped by snap-freezing in liquid nitrogen. To each sample 200 µl of 20% (w/v) TCA/5 mM NaF/5 mM EDTA was added and inositol phosphate extraction was carried out as described above.

Preparative isolation of d,L-Ins(1,3,4,5)P₄

A suspension of 3 g of PLP3A grain flour in 30 ml of TCA extraction buffer was agitated for 1 h at 4 °C. Following centrifugation at 48000 g [10 min, 4 °C, SS34 rotor (Sorvall, Kendro Laboratory Products, Newtown, CT, U.S.A.)], the supernatant was filtered through cellulose (Whatman, Maidstone, Kent, U.K.) and 0.45 µm polycarbonate filters (Millipore). The pellet was resuspended in another volume of TCA extraction buffer and agitated for 1 h. Subsequent centrifugation and filtration were carried out as described above. Water-saturated diethyl ether (200 ml) was added to the pooled supernatants and the resulting mixture was agitated vigorously. The organic phase was removed after centrifugation at 48000 g (10 min, 4 °C). A total of three ether-extraction rounds were performed to bring the pH of the aqueous grain extract to 3.5. Starch was removed by adding ethanol to a final concentration of 40% (v/v), followed by an incubation at −20 °C (30 min) and a centrifugation at 48000 g (10 min, 4 °C). After two rounds of charcoal treatment [26], the grain extract was diluted with 1.3 litres of water and loaded on to a 70 ml Q-Sepharose FF column (1.6 cm × 35 cm), operated on an FPLC system (Pharmacia). The column was eluted by a linear gradient of ammonium acetate (0–1.5 M) at a flow rate of 13 ml min⁻¹ [28]. Fraction size was 21 ml and 1 ml of each fraction was lyophilized and resuspended in 5 µl of water prior to inositol phosphate analysis by TLC [23,29]. Fractions containing inositol tetrakisphosphates were analysed further by MDD-HPLC (acidic elution protocol) and the purest d/L-
Ins(1,3,4,5)P₄ fraction was lyophilized and subjected to NMR analysis.

**MDD-HPLC analysis of inositol phosphates**

Inositol phosphate analysis by MDD-HPLC [26,30] was carried out as follows: acidic elution was performed on a chemically inert HPLC system (10Aᵦᵦ series, Shimadzu, Kyoto, Japan) equipped with a Resource Q (1 ml)/Mono Q HR 5/20 column configuration (both Pharmacia). Samples (usually 150 μl) were applied automatically and a LC-10A pump delivered solvent A (0.2 mM HCl/15 μM YCl₃) and solvent B (0.5 M HCl/22.5 μM YCl₃) at 1 ml·min⁻¹. Inositol phosphates were separated on a concave gradient running for 63 min: 0 min, 10 %; B: 10 min, 14.5 %; B; 23 min, 28 %; B; 31 min, 44 %; B; 35 min, 60 %; B; 40 min, 90 %; B; 43 min, 100 % B. Detection of inositol phosphates was performed by mixing PAR reagent (300 μM PAR/1.6 M triethanolamine, pH 9.0, with HCl) at 0.55 ml·min⁻¹ into the eluent. Compound detection at 546 nm was performed with a photodiode-array detector (SPD-M10Aᵦᵦ). Absorbance measurement at 250 nm was used to monitor potential nucleotide contaminations.

The alkaline elution protocol was performed on a Beckman (Fullerton, CA, U.S.A.) System Gold HPLC model equipped with a self-packed column [Source 15 Q (Pharmacia), 4.6 mm × 250 mm]. Elution was carried out by delivering a gradient of solvent A (20 mM triethanolamine in water) and solvent B (20 mM triethanolamine/0.6 M KCl) at a flow-rate of 0.75 ml·min⁻¹. 0 % B (0–5 min); from 0–25 % B (5–30 min); from 30–65 % B (30–90 min); and 100 % B (90–95 min), for a total run time of 95 min. The eluent was mixed with an YCl₃/PAR reagent (200 μM PAR/30 μM YCl₃/2 mM acetic acid) delivered at 0.4 ml·min⁻¹. The decrease in absorbance was monitored at 520 nm using a UV/visible detector (System Gold detector 166). Absorbance measurement at 249 nm was additionally integrated into the system to monitor potential nucleotide contamination.

**NMR analysis of d/L-Ins(1,3,4,5)P₄**

All NMR data were recorded on a Varian UNITY-INOVA 600 MHz spectrometer (Varian, Sunnyvale, CA, U.S.A.) equipped with a 5 mm (¹H, ¹³C, ¹⁵N) triple-resonance gradient probe at 25 ºC. The purified compound (≈ 250 μg) was dissolved in 0.5 ml of H₂O and pipetted into a 5 mm NMR tube. The pH of the sample was 7.01. The ¹H chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) via the solvent signal. In order to detect proton scalar couplings, phase-sensitive clean TOCSY (total correlations spectroscopy) spectra [31,32] were collected (512 t₁ × 2048 t₂) with a spin-lock time of 8 ms. A spectral width of 2.0 kHz was used in the t₁ and t₂ dimensions. The 90° pulse width was 8.9 μs (35.4 μs for spin-lock sequence). A total of 56 scans were collected for each block with a repetition time of 2.1 s. The total recording time for the two-dimensional spectrum was 17 h.

**RESULTS**

**Inositol phosphate profiles from low-phytate mutant barley grains**

MDD-HPLC analysis of grain extracts from mutant lines (PLP1A, PLP2A, PLP3A and PLP1B) and their parent variety Pallas-P01 (Figure 1) was performed by a strongly acidic elution protocol. Unknown inositol phosphates were assigned by comparing their retention times with those of NMR-identified [26] inositol phosphates present in a chemical phytate hydrolysate.

Inositol phosphates from barley low-phytate mutants

TCA extracts (150 μl each) from barley mutant (PLP1A, PLP2A, PLP3A and PLP1B) and Pallas-P01 grain extracts were resolved on a Mono Q HR 5/20 column using a strongly acidic HCl gradient. Inositol phosphates were detected by MDD, a post-column detection procedure in which the organic dye PAR is mixed with the eluent containing Y³⁻. The complex formation between Y³⁻ and PAR, resulting in a steady absorbance, is measured at 546 nm. Inositol phosphates and P_i outcompete PAR from Y³⁻–PAR complexes, yielding free PAR which has a lower absorption than Y³⁻–PAR [26,30]. For better comparison, HPLC profiles were mirrored and stacked. Peaks in profiles from grain extracts (indicated by letters) are assigned by identifying corresponding peaks in the InsP₄ hydrolysate trace. 1, InsP₄ and P_i; 2, d/L-Ins(1,4)P₄; 3, Ins(3,5)P₃; 4, d/L-Ins(3,4,5)P₃; 5, d/L-Ins(1,4,5)P₃; 6, d/L-Ins(1,5,6)P₃; 7, Ins(1,5,6)P₃; 8, d/L-Ins(1,3,5,6)P₄; 9, d/L-Ins(1,2,5,6)P₄; 10, d/L-Ins(1,2,4,5,6)P₅; 11, d/L-Ins(1,3,4,5,6)P₆; 12, d/L-Ins(1,2,4,5,6)P₆; 13, Ins(2,4,5,6)P₄; 14, d/L-Ins(1,4,5,6)P₅/Ins(1,2,3,4,6)P₅ (peak f), d/L-Ins(1,3,4,5,6)P₆; 15, d/L-Ins(1,2,3,4,5,6)P₆, d/L-Ins(1,2,4,5,6)P₆; 17, Ins(1,3,4,5,6)P₆; 18, InsP₀. The elution order listed is according to Mayr [26] and in the case of inositol mono-, bis- and tris-phosphate peaks only one isomer is noted per peak (see text).

The MDD-HPLC profile of Pallas-P01 grain extract revealed nine peaks (a–i) with retention times identical or very similar to peaks 1, 2, 5, 9, 12, 14, 15, 16 and 18 in the profile of the phytate hydrolysate. Since the strongly acidic elution protocol is best suited for the separation of inositol tetrakis-, pentakis- and hexakis-phosphates, peaks d–i could be assigned as d/L-Ins(1,2,3,4)P₄ (peak d), d/L-Ins(1,2,5,6)P₄ (peak e), d/L-Ins(1,4,5,6)P₅/Ins(1,2,3,4,6)P₅ (peak f), d/L-Ins(1,3,4,5,6)P₆ (peak g), d/L-Ins(1,2,4,5,6)P₆ (peak h) and InsP₀ (peak i). Assignment of peaks a–c, on the other hand, was more difficult since various inositol bis- and tris-phosphates elute groupwise and with very similar retention times on the acidic gradient. Peak a contained InsP₄ and P_i, peak b represented d/L-Ins(1,4)P₃ and other bisphosphate isomers whereas peak c corresponded to peak 5 containing d/L-Ins(1,4,5)P₅ and d/L-Ins(1,2,6)P₆ [26].

The elution profile of the PLP1B grain extract was very similar to the profile of Pallas-P01, indicating the presence of the same InsP isomers as in the parent line. The major difference between
the two profiles were relatively smaller peaks f, g and h in the PLPIB profile which corresponded to the smaller amounts of phytate in PLPIB grains (60–75% of the Pallas-P01 level).

A very small peak or shoulder eluting immediately after peak h was detected in some but not all Pallas-P01 and PLPIB profiles. This peak possibly represented Ins(1,3,4,5,6)P₆ since it had a similar retention time to peak 17. The small size of this peak and its occasional appearance precluded a reliable assignment.

Chromatographic profiles of grain extracts from PLPIA, PLP2A and PLP3A were quite similar to each other but showed many differences in comparison with the Pallas-P01 and PLPIB traces. Phytate (peak i) and peaks f, g and h were considerably smaller, indicating a phytate content of 15–25% relative to the Pallas-P01 level. The most striking feature of the three profiles from A-type mutants was the presence of peak l, which in correspondence with peak 11 was assigned as d/l-Ins(1,3,4,5)P₄. These substantial accumulations of d/l-Ins(1,3,4,5)P₄ appeared together with minor accumulations of inositol bis- and tris-phosphates (peaks j and k), which were not observed in Pallas-P01 and PLPIB profiles. In the case of peak j, an assignment of an isomeric identity was not possible since a corresponding peak was lacking in the profile of the phytate hydrolysate. Peak k however, corresponded to peak 4, which contained d/l-Ins(1,2,3)P₃, d/l-Ins(1,2,4)P₃, d/l-Ins(1,3,4)P₃ and d/l-Ins(1,3,5)P₃ [26].

Isomer assignment by co-chromatography

Co-chromatography experiments (Figure 2) were performed in order to verify the assignment of peak l as d/l-Ins(1,3,4,5)P₄ and to assign the inositol trisphosphate isomers present in peaks k and c (Figure 1). A commercial standard of Ins(1,3,4,5)P₄ was added to a PLPIA mutant grain extract and the mixture was resolved using the acidic MDD-HPLC protocol (Figure 2b). The standard compound co-eluted precisely with peak l, corroborating the assignment of peak l as d/l-Ins(1,3,4,5)P₄.

With regard to peaks k and c, co-chromatography experiments were carried out with another HPLC instrument on which an alkaline elution protocol was performed [26]. Compared with the acidic elution protocol, alkaline elution offers improved separation capacity in the inositol bis- and tris-phosphate range. When separation of a PLPIA mutant grain extract was performed under alkaline conditions, peaks k and c eluted after 62.5 and 63.5 min (Figure 2c), which corresponded to the retention times of d/l-Ins(1,3,4)P₄ and d/l-Ins(1,4,5)P₄ present in the profile of the phytate hydrolysate (results not shown). Subsequent analysis of PLPIA extract spiked with Ins(1,3,4)P₄ (Figure 2d) and Ins(1,4,5)P₄ (Figure 2e) standards strengthened the assumption that d/l-Ins(1,3,4)P₄ as well as d/l-Ins(1,4,5)P₄ were present in the mutant grain extract.

Preparative isolation and NMR analysis of d/l-Ins(1,3,4,5)P₄

Preparative isolation of d/l-Ins(1,3,4,5)P₄ was carried out in order to perform NMR characterization of this compound (Figure 3). Purification was achieved by TCA extraction of PLP3A flour, followed by anion-exchange chromatography on Q-Sepharose FF. TLC analysis of chromatographic fractions (Figure 3a) indicated the bulk of tetrakisphosphates eluting in fractions 48 and 49. These fractions were analysed further by MDD-HPLC employing the acidic elution protocol (results not shown). Fraction 48 contained d/l-Ins(1,3,4,5)P₄ with a purity of ≈ 99% while minor amounts of d/l-Ins(1,2,5,6)P₄ (≈ 5%) were present in fraction 49.

The ¹H-NMR one-dimensional reference and the clean TOCSY spectra recorded from fraction 48 are shown in Figures 3(b) and 3(c). The ¹H chemical shifts and proton–proton coupling patterns in the one-dimensional spectrum (Figure 3b) were consistent with what has been reported for Ins(1,3,4,5)P₄ [28–33,35]. The scalar coupling pattern in the two-dimensional clean TOCSY spectrum (Figure 3c) further confirmed the assignment of the compound as d/l-Ins(1,3,4,5)P₄. Signal broadening in the ¹H-NMR spectrum (Figure 3b) was most probably due to a trace of paramagnetic ions present in the sample.

Inositol phosphate accumulation during grain development

Accumulation of inositol phosphates during grain development was analysed as a first step to characterize the metabolic
Inositol phosphates from barley low-phytate mutants

Figure 3 Compound purification and NMR spectroscopy

Following anion-exchange chromatography on a 70 ml Q-Sepharose FF column, inositol tetrakisphosphates were identified by TLC analysis (a). Peak fractions (numbers 48 and 49) were additionally analysed by MDD-HPLC to confirm the isomeric identity of the compounds (results not shown). Fraction 48 was lyophilized and resuspended in 2H2O in order to record 600 MHz 1H-NMR one-dimensional reference (b) and two-dimensional clean TOCSY (c) spectra at 25 °C (pH 7.01). The intensive cross peaks in TOCSY originate from primary three-bond proton–proton couplings. The one-dimensional peaks are labelled according to the structure of Ins(1,3,4,5)P4 shown in (d).

Figure 4 Phytate and D/d-Ins(1,3,4,5)P4 analysed during grain development

MDD-HPLC analysis of inositol phosphates was performed on TCA extracts obtained from developing grains. Representative chromatographic profiles of Pallas-P01 (a–d) as well as PLPIA mutant grain extracts (e–h) are shown. Time points of harvesting are indicated in days (d) after flowering. Peak i, d/l-Ins(1,4,5,6)P4/Ins(1,2,3,4,6)P5; peak j, d/l-Ins(1,2,3,4,5)P5, peak k, Ins(1,2,3,4)P3; peak l, d/l-Ins(1,2,4,5,6)P5; peak m, phytate; peak n, d/l-Ins(1,3,4,5)P4.

4a–4d). Accumulation of phytate (peak i) was accompanied by accumulation of D/l-Ins(1,4,5,6)P4/Ins(1,2,3,4,6)P5 (peak f), D/l-Ins(1,2,3,4,5)P5 (peak g) and D/l-Ins(1,2,4,5,6)P5 (peak h).

In grains from A-type mutants, a minute amount of phytate could be detected 7 days after flowering (Figure 4e) whereas D/l-Ins(1,3,4,5)P4 was first detected after another 14 days (Figure 4f). In the remaining development period the tetrakisphosphate accumulated together with phytate (Figures 4g and 4h). In comparison with the parent line, phytate synthesis was much slower and reached just about 25% of the phytate level present in mature Pallas-P01 grains.

Analysis of enzymic phytate degradation patterns

Phytate degradation by endogenous phytase activity in vitro was studied in order to investigate whether mutationally enhanced and/or modified phytase activities could be the reason for the altered inositol phosphate profiles observed in A-type grain mutants (Figure 5). Incubation for 12.5 min created a representative spectrum of degradation products in Pallas-P01 which comprised D/l-Ins(1,2,3,4,5)P5 (peak g), D/l-Ins(1,2,5,6)P5 (peak e), Ins(1,2,3,4)P3 (peak d) and various inositol bis- and tris-phosphates (peaks b and c).

In the mutant PLPIA, enzymic phytate degradation generated the same products as observed in the parent variety (peaks b–e).
Pallas-P01) is dominated by phytate, comprising around 90%.

Ins(1,3,4,5)

and numbers (chemical hydrolysate) is analogous to Figure 1. Indication of peaks by letters (phytate degradation products) by MDD-HPLC and compound assignment was carried out in comparison with the HPLC profile of a chemical phytate hydrolysate. A very similar spectrum of inositol phosphates was reported in both barley aleurone-layer phytase under germination-like conditions. The fact that similar inositol phosphates were detected in both parent line and mutant extracts (Figures 1 and 5). Although quantitative analysis of Ins(1,3,4,5)P$_4$ levels in parent and mutant lines is not feasible at the moment, the mere detection of Ins(1,3,4,5,6)P$_6$ represents an important caveat for the hypothesis of a matured Ins(1,3,4,5)P$_4$ 6-kinase gene.

**Metabolic scenarios leading to accumulation of d/L-Ins(1,3,4,5)P$_4$ in A-type mutants**

To our knowledge, this is the first time that the occurrence of d/L-Ins(1,3,4,5)P$_4$ has been reported in a higher land plant, and the question concerning its metabolic role is an intriguing one. Detection of substantial amounts of this compound in A-type grains (≈ 300 µg/g of flour) prompted us to look for its presence in roots, stem and leaves. Phytate levels in these tissues were about two orders of magnitude lower than in seeds and d/L-Ins(1,3,4,5)P$_4$ could not be detected (results not shown). The absence of this compound in vegetative tissues suggests that accumulation of d/L-Ins(1,3,4,5)P$_4$ is the result of a mutation affecting exclusively grain-specific inositol phosphate metabolism [36]. When trying to associate d/L-Ins(1,3,4,5)P$_4$ with a particular inositol phosphate pathway it has to be considered whether this pathway is related to phytate degradation, inositol phosphate signalling or phytate synthesis. Investigation of phytate degradation patterns in vitro (Figure 5) indicated that phytase activities from A-type and Pallas-P01 grains degraded phytate in a similar manner, which argues against d/L-Ins(1,3,4,5)P$_4$ being the product of a mutationally altered phytase. InsP signalling, as another metabolic network that could potentially utilize Ins(1,3,4,5)P$_4$, is not as well understood in plants as it is in animal cells [37]. A Ca$^{2+}$-releasing effect of Ins(1,4,5)P$_3$ could be demonstrated in plant microsomes [38] but a plant Ins(1,4,5)P$_3$ 3-kinase, homologous with the ones found in animals, has not yet been identified [39].

Whereas d/L-Ins(1,3,4,5)P$_4$ cannot be readily associated with phytate degradation, a role for this compound in phytate synthesis seems more plausible. Evidence indicating such a role is provided by data showing accumulation of phytate and d/L-Ins(1,3,4,5)P$_4$ during grain development (Figure 4). Association of d/L-Ins(1,3,4,5)P$_4$ with phytate synthesis in barley suggests that the late steps of soya bean phytate synthesis described in [9] are also taking place in barley (Scheme 1). Assuming conservation of phytate synthesis pathways in higher land plants, it seems very likely that accumulation of d/L-Ins(1,3,4,5)P$_4$ in grains from barley A-type mutants is due to a defective Ins(1,3,4,5)P$_4$ 6-kinase. Consequently, our observation of enhanced d/L-Ins(1,3,4,5)P$_4$ levels in A-type grains (Figure 2) could indicate a feedback inhibition effect of d/L-Ins(1,3,4,5)P$_4$ on Ins(1,3,4)P$_3$ 5/6-kinase. Evidence for such a feedback inhibition mechanism acting on the soya bean Ins(1,3,4)P$_3$ 5/6-kinase was reported in [9].

However, if the Ins(1,3,4,5)P$_4$ 6-kinase gene is indeed hit by a mutation in A-type mutants and if this gene really plays such an important role in land-plant phytate synthesis, how can one then explain the observation that PLPA mutants nevertheless synthesize about 25% of the phytate present in the parent variety, Pallas-P01? One explanation could be that the mutation does not lead to a complete shut-down of enzyme activity but gives rise to a partially functional kinase with a slow turnover rate. Evidence supporting this hypothesis comes from the occasional appearance of a small peak or shoulder eluting after peak h, which indicates the presence of Ins(1,3,4,5,6)P$_6$ in both parent line and mutant extracts (Figures 1 and 5).

**DISCUSSION**

Evidence for basal phytase activity during maturity and development

The MDD-HPLC data presented in this paper show that the inositol phosphate composition in mature barley grains (cv. Pallas-P01) is dominated by phytate, comprising around 90% of all inositol phosphates present. Using a chemical phytate hydrolysate as a reference, the remaining 10% were assigned as d/L-Ins(1,2,3,4)P$_4$, d/L-Ins(1,2,5,6)P$_4$, d/L-Ins(1,2,3,4,5)P$_5$, d/L-Ins(1,4,5,6)P$_6$/Ins(1,2,3,4,6)P$_5$ and d/L-Ins(1,2,4,5,6)P$_6$ (Figure 1). A very similar spectrum of inositol phosphates was reported by Brearley and Hanke [16], who investigated the products of barley aleurone-layer phytase under germination-like conditions. The fact that similar inositol phosphates were detected in both mature and germinating (imbibing) barley grains indicates that barley phytase is not only active during germination but also during maturity, although on a basal level. The proportionality between phytate peak size and areas of peaks f-h observed during grain development (Figure 4) suggested further that re-phosphorylation of phytate degradation products establishes a steady-state equilibrium between phytate synthesis and basal phytase activity. Evidence for such futile InsP$_i$/InsP$_j$ cycles has been reported from the phytate metabolism of the slime mould Dictyostelium [12].
Alternatively, continuation of phytate synthesis in A-type mutants could be explained by one or more salvage pathways departing from Ins(1,3,4)P$_4$ (Scheme 1). The existence of such alternative pathways in barley seems likely when investigations of inositol phosphate kinases from other land plants are considered. In addition, the results of inositol kinase activity in Arabidopsis, a homologous enzyme activity was observed, which yielded Ins(1,3,4,5)P$_5$ as the major and Ins(1,3,4,6)P$_5$ as the minor phosphorylation product [8] and 2:6/L-Ins(1,3,4,5)P$_5$ 6-kinase in barley. In this pathway, Ins(1,3,4,5)P$_4$ was found to be the major phosphorylation product of Ins(1,3,4,5)P$_5$ 5/6-kinase. In Arabidopsis a homologous enzyme activity was observed, which yielded Ins(1,3,4,5)P$_5$ as the major and Ins(1,3,4,6)P$_5$ as the minor phosphorylation product [8] and 2:6/L-Ins(1,3,4,5)P$_5$ 6-kinase in barley. In this pathway, Ins(1,3,4,5)P$_4$ was found to be the major phosphorylation product of Ins(1,3,4,5)P$_5$ 5/6-kinase. In Arabidopsis, a homologous enzyme activity was observed, which yielded Ins(1,3,4,5)P$_5$ as the major and Ins(1,3,4,6)P$_5$ as the minor phosphorylation product. In barley A-type mutants, salvage pathways via Ins(1,3,4,5)P$_4$ and/or Ins(1,2,3,4)P$_4$ (broken arrows) may be responsible for residual phytate synthesis in the barley. In this pathway, Ins(1,3,4,5)P$_4$ 6-kinase is mutationally blocked or slowed down. Alternative scenarios which also could lead to 2:6/L-Ins(1,3,4,5)P$_5$ accumulation are discussed in the text.

Scheme 1 Hypothetical pathway of phytic acid synthesis in land plants

Accumulation of 2:6/L-Ins(1,3,4,5)P$_5$ in A-type low-phytate mutants suggests that Ins(1,3,4)P$_4$ 5/6-kinase is involved in barley phytate synthesis. In preceding studies, purification and characterization of inositol phosphate kinases from soya bean seeds led to the proposition of a phytate synthase pathway via Ins(1,3,4)P$_4$, Ins(1,3,4,5)P$_5$ and Ins(1,3,4,5,6)P$_5$ [9,10]. In this pathway, Ins(1,3,4,5)P$_4$ was found to be the major phosphorylation product of Ins(1,3,4,5)P$_5$ 5/6-kinase. In Arabidopsis, a homologous enzyme activity was observed, which yielded Ins(1,3,4,5)P$_5$ as the major and Ins(1,3,4,6)P$_5$ as the minor phosphorylation product [8] and 2:6/L-Ins(1,3,4,5)P$_5$ 6-kinase in barley. In this pathway, Ins(1,3,4,5)P$_4$ was found to be the major phosphorylation product of Ins(1,3,4,5)P$_5$ 5/6-kinase. In Arabidopsis, a homologous enzyme activity was observed, which yielded Ins(1,3,4,5)P$_5$ as the major and Ins(1,3,4,6)P$_5$ as the minor phosphorylation product. In barley A-type mutants, salvage pathways via Ins(1,3,4,5)P$_4$ and/or Ins(1,2,3,4)P$_4$ (broken arrows) may be responsible for residual phytate synthesis in the barley. In this pathway, Ins(1,3,4,5)P$_4$ 6-kinase is mutationally blocked or slowed down. Alternative scenarios which also could lead to 2:6/L-Ins(1,3,4,5)P$_5$ accumulation are discussed in the text.

Alternatively, continuation of phytate synthesis in A-type mutants could be explained by one or more salvage pathways departing from Ins(1,3,4)P$_4$ (Scheme 1). The existence of such alternative pathways in barley seems likely when investigations of inositol phosphate kinases from other land plants are taken into consideration: Ins(1,3,4)P$_4$ 5/6-kinase from Arabidopsis produced both Ins(1,3,4,5)P$_5$ and Ins(1,3,4,6)P$_5$ under in vitro conditions [8], and the corresponding soya bean kinase yielded Ins(1,3,4,5)P$_5$ as the major and Ins(1,3,4,6)P$_5$ as minor product(s) [9].

In an altogether different but equally likely scenario, D/L-Ins(1,3,4,5)P$_4$ accumulation could be the consequence of a metabolic redirection of the InsP$_4$ anabolism. Here, a mutationally induced defect of Ins(1,4,5)P$_5$ 6-kinase [39] is assumed to cause reductions in D/L-Ins(1,3,4,5,6)P$_5$, a compound that co-elutes with Ins(1,2,3,4,6)P$_6$ (peak f in Figure 1). In fact, the area of peak f is clearly reduced in chromatograms from A-type mutants, but our efforts to separate D/L-Ins(1,3,4,5)P$_4$ from Ins(1,2,3,4,6)P$_6$ have so far been unsuccessful. Nevertheless, a defective Ins(1,4,5)P$_5$ 6-kinase might lead to activation of an Ins(1,4,5)P$_5$ 3-kinase reaction and accumulation of D/L-Ins(1,3,4,5)P$_4$, would then result from a lack of a further anabolic enzyme utilizing this product. An observation directing further attention to this scenario is that accumulation of phytate seems to precede the rise of D/L-Ins(1,3,4,5)P$_4$ in the early stages of grain development (Figure 4). In this context, accumulation of D/L-Ins(1,3,4,5)P$_4$ could represent a regulated type of compensation mechanism which becomes active in order to produce high concentrations of InsP$_4$ in a metabolic situation where phytate is lacking. A similar ‘buffer pathway’ model was suggested in a recent study by Raboy et al. [40] where decreases in phytate were observed together with increases in D/L-Ins(1,2,4,5,6)P$_5$ and D/L-Ins(1,4,5,6)P$_5$ in maize lpa2–1 mutants.

To further investigate the metabolic role of D/L-Ins(1,3,4,5)P$_4$ in barley we have initiated purification of inositol phosphate kinase activities from grains. Future work will also have to determine the true enantiomerism of D/L-Ins(1,3,4,5)P$_4$ isolated from A-type mutants. Since soya bean and Arabidopsis Ins(1,3,4)P$_5$ 5/6-kinases utilize D/L-Ins(1,3,4)P$_4$ as a substrate [8,9] it seems very likely that the tetrakisphosphate in PLPA mutants is a D-enantiomer as well. Nevertheless, this has to be proven by physical or chemical means.

MIPS as the candidate gene in B-type mutants?

In comparison with A-type lines (PLP1A, PLP2A and PLP3A) investigations of PLP1B revealed rather moderate changes. Phytate levels were somewhat reduced (60–75% of the Pallas-P01 level) and unusual inositol phosphate accumulations were not observed (Figure 1). These observations argue against a mutation affecting later steps of phytate synthesis, as apparently seems to be the case in A-type mutants. Phenotypically, PLP1B is similar to the lpa1 mutants in maize and Harrington barley as described by Raboy and co-workers [4,22]. In the case of the lpa1 mutation, the MIPS gene was proposed as a candidate gene. This hypothesis seemed very likely since the gene product of MIPS controls an early and therefore a regulating step of phytate synthesis. While mapping data from lpa1 maize confirmed the proposed hypothesis, no such evidence could be found in corresponding barley mutants (cv. Harrington) [24]. In this context, it can be expected that future mapping studies with the lpa1-like PLP1B mutant may lead to a better understanding of the problems which currently surround the MIPS candidate-gene hypothesis.

This work was supported in part by the Danish Cereal Network, framework 1 (1996–2001).

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Received 30 May 2000/13 November 2000; accepted 15 December 2000


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