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Heat-Stable Phytases in Transgenic Wheat (*Triticum aestivum* L.): Deposition Pattern, Thermostability, and Phytate Hydrolysis

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The present paper addresses the question of thermotolerance of *in planta* synthesized heterologous enzymes using phytase as a model. Two individual transgenic wheat materials expressing an *Aspergillus fumigatus* phytase with a low denaturation temperature (62.5 °C) but a high refolding capacity, and a rationally designed consensus phytase engineered to a high denaturation temperature (89.3 °C), were evaluated. High levels of endosperm specific expression were ensured by the wheat high molecular weight glutenin 1DX5 promoter. Immunodetection at the light and electron microscopical level shows unequivocally that the heterologous phytase is deposited in the vacuole, albeit that the transformation constructs were designed for secretion to the apoplast. Evaluation of heat stability properties and kinetic properties unraveled that, under these deposition conditions, heat stability based on high unfolding temperature is superior to high refolding capacity and represents a realistic strategy for improving phosphate and mineral bioavailability in cereal-based feed and food.

KEYWORDS: Phytase; phytate; heat stability; micronutrients; phosphate

INTRODUCTION

Microbially produced enzymes are used today as additives for a range of industrial applications including feed and food production. Good enzyme thermotolerance is often required as many enzyme-facilitated processing steps are performed at high temperatures for reasons of economy and efficiency. Large-scale research and development efforts are therefore directed toward either the identification of thermotolerant enzymes or the design of formulations where carriers can protect the enzymes from heat-induced denaturation (*1*, *2*).

In recent years, plant-based production systems for enzymes and other proteins have received increasing attention. Several studies have shown that a range of enzymes can be produced to high levels in plants transformed for expression of microbial enzyme-encoding genes (*3*−*6*). The enzymes can be produced, via the use of specific promoters, in different organs and at different developmental stages. Furthermore, targeting to subcellular compartments or the apoplast via the use of appropriate signal sequences has become feasible.

However, the question of thermotolerance of *in planta* produced heterologous enzymes has rarely been addressed. Targeting to different cellular compartments and the apoplast may very well result in different levels of thermotolerance due to differences in post-translational modification. Likewise, it is conceivable that targeting to different compartments will present the enzyme to environments that either facilitate or reduce thermotolerance. Among the few cases where heterologous heat-stable enzymes have been produced *in planta*, Horvath et al. (*6*) found that a recombinant thermotolerant *Bacillus* (1,3-1,4)-β-glucanase synthesized either in the endosperm of developing barley (*Hordeum vulgare* L.) grains or the aleurone of germinating barley grains had retained the thermotolerance properties. Hence, substantial enzyme activity remained after heating of the grain to 65 °C for 2 h, a temperature which otherwise inactivates the endogenous barley (1,3-1,4)-β-glucanase. In contrast, when Lucca et al. (*7*) expressed an *Aspergillus fumigatus* phytase in rice (*Oryza sativa*), they found that only 8% of the initial phytase activity was retained after boiling transgenic grains for 20 min. When the commercial preparation of the fungal enzyme was boiled for the same time period, the activity amounted to 59% of the original activity. These differences in thermotolerance probably reflect that the *A. fumigatus* phytase is not a genuine heat-stable enzyme. In its purified form, it is denatured with a T_m of 62.5 °C but can refold into a fully active conformation after cooling (*8*). This indicates that somehow the *in planta* expression may interfere with refolding of the enzyme or provide an environment that is not favorable to refolding.

In the context of thermotolerance, phytase enzymes are particularly relevant. In the development of microbial phytases as feed additives, improving seed phytate P digestibility and

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hence decreasing the environmental phosphate load (*4*, *9*), there is substantial interest in heat stability as the enzymes must be able to withstand inactivation during the feed pelleting process (at 80−90 °C) in order to eliminate *Salmonella* infections. There is also substantial evidence that application of phytase to pig feed promotes zinc uptake (*10*−*14*). Phytate is generally considered to be the single most important antinutritional compound for the bioavailability of a range of essential minerals and also *Ins*P_5 and possibly *Ins*P_4 and *Ins*P_3 are known to have a negative effect on iron and zinc absorption (*15*). The antinutritional properties result from the ability of phytate and the lower *myo*-inositol phosphates to form complexes with Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, and $Fe^{2+/3+}$ (in decreasing order of stability) (*16*). Zinc as well as copper are often used as growth promoters in pig diets and supplied in excess to the physiological requirements to ensure a safety margin during production. The large-scale excretion of copper and zinc into the environment has raised concerns regarding the potential accumulation to toxic levels of the two minerals in the top soil as well as in streams, rivers, and lakes (*17*) (see (*18*) for review).

Phytate and phytases are also central topics of interest in the context of human nutrition. Current evidence suggests that phytate can efficiently impede the absorption of zinc and iron in the human digestive tract and such mineral deficiencies are estimated to afflict 2−3 billion people, primarily in the developing world (*19*−*23*). Accordingly, supplementation of phytase to human diets or increased phytase potential in major staple foods might be powerful tools for alleviating iron and zinc deficiencies in humans. Several studies have shown that *Aspergillus*-derived phytases can be produced in large quantities in a range of plants including cereals (*7*, *24*−*29*) with clear-cut positive effects on *Ins*P_6 degradation and phosphate bioavailability in animal-feeding trials (*25*, *30*, *31*). Potential effects on increased zinc bioavailability have not been reported but in vitro studies have shown that heterologous phytase expression promotes iron uptake in Caco 2 cells (*32*). It is thus conceivable that genetic engineering of staples for increased phytase expression could have potential for improving iron and zinc bioavailability.

However, as staples are processed by boiling or baking, high thermotolerance of the phytase enzyme will be of central importance. One extreme is rice that is cooked by prolonged boiling while, e.g., processing of wheat may include leavening which in turn allows for phytate degradation before baking (*33*, *34*). Unfortunately, most phytases possess only a limited thermotolerance and start to lose activity around 60 °C (*35*). The T_m of the most commonly used *Aspergillus niger phyA* phytase is 63.3 °C and the denaturation is associated with an irreversible conformational change with loss of 70−80% of the activity.

In the current study, we have generated transgenic lines of wheat that express a rationally designed phytase, termed Consensus Phytase (ConPhy). This engineered phytase has a T_m of 89.3 °C and is encoded by a synthetic gene that combines sequence motifs which are important for determining high heat stability and activity (*36*). For construction of transgenic wheat lines we used a 1DX5 promoter from wheat for driving high-level transcription in the endosperm and introduced a barley α-amylase signal sequence for targeting to the apoplast. These and another set of lines that were transformed with the same construct but housing the *A. fumigatus phyA* gene were subjected to three different studies. First, we have elucidated the deposition of the heterologous phytase in the subcellular compartments of the host cell; second, the heat-stability properties of the deposited/expressed enzyme was evaluated; and finally the kinetic properties of the *in planta* accumulated heat-stable phytases were investigated. The results obtained are discussed in the context of using *in planta* produced heterologous thermotolerant phytase for alleviating mineral deficiencies in humans and livestock.

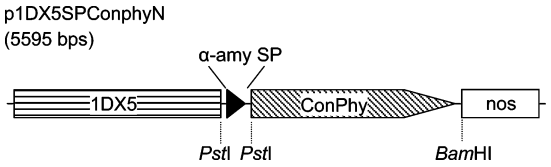

Figure 1. Vector for expression of consensus phytase in wheat endosperms. 1DX5, the wheat HMW-GS 1DX5 promoter; α-amy SP, the barley α-amylase signal peptide (*39*); ConPhy, the consensus phytase 10-thermo-[3]-Q50T-K91A (*36*); *nos*, the terminator sequence of the *Agrobacterium tumefaciens* nopaline synthase gene (*47*).

MATERIALS AND METHODS

Expression Vectors. A 1349 bp fragment of the consensus phytase 10-thermo-[3]-Q50T-K91A was PCR amplified using the upper strand primer 5′-*GGGGATC***CTGCAG**AACTCTCACTCTTGTGACACTGTTGACGG-3′ corresponding to bases 1−29 of the ConPhy gene and the lower strand primer 5′-*CGC***GGATCCGAGCTC**AGCGAAACATTCTTCCCAGTTACCAC-3′ corresponding to bases 1298−1321 of the gene. The upper strand primer generated a terminal PstI site (bold) and the lower strand primer terminal BamHI and SacI sites (bold). Sequences were included to ensure efficient terminal restriction endonuclease digestion of the PCR product (italics). The PCR product was ligated into the pCR 2.1-TOPO vector (Invitrogen) and the resulting plasmid pConPhy was sequenced on an ABI Prism 310 Genetic Analyzer (Applied BioSystems) using standard M13 forward and reverse primers. Following digestion with PstI and BamHI, the ConPhy gene was ligated into the PstI and BamHI sites of plasmid p1DX5SPPhy-Fum-modN (*37*), generating plasmid p1DX5ConPhyN. The 80 bp barley α-amylase signal peptide was isolated as a PstI−PstI fragment from plasmid PUSPPhyN (*28*) and ligated into the PstI site of p1DX5ConPhyN, yielding p1DX5SPConPhyN (**Figure 1**).

Generation and Identification of Transgenic Wheat Plants. The plasmid p1DX5SPConPhyN was introduced into immature embryos of wheat (*Triticum aestivum* L.) cv. Bobwhite using the DuPont PDS 1000 helium biolistic system, as described previously (*38*). Selection and regeneration of plants were performed as described by Brinch-Pedersen et al. (*28*). Plants transformed with p1DX5SPConPhyN were identified by PCR using the upper strand primer (Oligo #1) 5′-GATGGCGAACAAACATTTGTCCCTCTCCCTC-3′ corresponding to bases 1−31 of the barley α-amylase signal peptide (*39*) and the lower strand primer (Oligo #2) 5′ACCTGGGTCAGCCAACTTAGCA-3′ corresponding to bases 537−558 of the consensus phytase gene (*36*). The amplified fragment was 638 bp in length. The generation of the lines transformed with the *A. fumigatus phyA* gene has been described previously (*37*).

RT-PCR. Total RNA was isolated from leaf and endosperm tissues using the FatRNA kit-Green (Bio 101). cDNA was synthesized using oligo #2 and PCR was performed using oligo #1 in combination with oligo #2.

Western Blot. The techniques used were as previously described (*28*). Antibody against the consensus phytase-10-thermo-[3]-Q50T-K91A and goat-antirabbit alkaline phosphatase conjugate were used in 1:2000 and 1:5000 dilutions, respectively.

Microscopy and Immunolabeling. Thin slices of endosperm were cut from both wild type and ConPhy-L-04-01-04 (T_2 homozygous) developing seeds (20 dap) and immediately immersed in a fixative solution (4% (w/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde in phosphate buffer (0.1 M, pH 7.4)) overnight at 4 °C. After several washing steps with phosphate buffer (0.1 M, pH 7.4), samples were dehydrated through an ethanol series and then infiltrated in LR White resin and polymerized. For light microscopy, semithin sections (1 μm

in thickness) were mounted on glass slides and for electron microscopy sections showing silver interference colors were mounted on gold grids. The sections were preincubated in 5% (w/v) bovine serum albumine (BSA fraction V) in phosphate buffer (0.1 M, pH 7.4) and subsequently in polyclonal rabbit anti-phytase antibody. Thereafter, the sections were treated with a goat secondary antibody diluted in phosphate buffer (0.1 M, pH 7.4) and labeled with Alexa Flour 594 for fluorescence microscopy and 10-nm gold particles for electron microscopy. For electron microscopy, the sections were finally stained with 2% (w/v) aqueous uranyl acetate and observed using a Philips EM-400 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Phytase Activity and Heat Treatment of Wheat Flour. Seeds were milled and 0.1 g of flour was suspended in 1 mL of 220 mM sodium-acetate buffer (pH 5.5) preheated to 60, 87, or 100 °C and subsequently incubated for 0, 1, 5, or 20 min. The temperatures were selected to challenge phytase stability with temperatures below and above their T_m of 62.5 °C (*A. fumigatus*) and 89.3 °C (ConPhy), respectively. After heat treatment, the suspension was cooled to 37 °C and subsequently vortexed for 1 h at RT. After centrifugation at 3000*g* for 10 min, the supernatant was collected and assayed for after heat treatment residual (AHTR) phytase activity using the procedure previously described (*40*).

Analysis of Inositol Phosphate Hydrolysis before and after Heat Treatment. Analysis of the inositol phosphate (*Ins*P) degradation profiles was performed on seeds from wild type, from transgenic plant line PhyFum-mod-L08 (transformed with a codon optimized *A. fumigatus phyA* phytase gene) and from line ConPhy-L04-01-04 (transformed with the consensus phytase-10-thermo-[3]-Q50T-K91A). The analysis included *Ins*P composition in seeds before any heat treatment or incubation, after 1 h incubation at 37 °C, and after 20 min at 100 °C followed by 1 h of incubation at 37 °C. To analyze the *Ins*P composition in seeds before heat treatment and incubation ($t = 0$), seeds were milled and 0.5 g of the flour was mixed into 10 mL of 0.5 M HCl. The suspension was incubated under constant shaking (500 rpm) for 2 h at room temperature. Following incubation, the suspension was frozen in liquid N_2, thawed, and shaken for another 1 h at room temperature. After centrifugation (6238*g*, 10 min, 4 °C), the supernatant was recovered and 500 μL was filtered through a Microcon YM-30 filter using the procedure described by the manufacturer (Millipore). To analyze the IP composition after 1 h of incubation, the flour was mixed into 220 mM sodium-acetate buffer (pH 5.5) and incubated for 1 h at 37 °C. Following incubation, the suspension was centrifuged (6238*g*, 10 min, 4 °C) and 500 μL of the supernatant was filtered through a Microcon YM-30 filter. To analyze the IP composition after heat treatment and incubation, the suspension was heated to 100 °C for 20 min. After heat treatment, the samples were cooled to 37 °C and were subsequently incubated for 1 h at 37 °C followed by centrifugation and filtration as already described. The filtered samples were transferred to vials for HPIC and analyzed as described previously (*41*).

RESULTS

Transgenic Materials. Two lines expressing high levels of the consensus [ConPhy-L04-01-04 (T_2 homozygous)] and the *A. fumigatus* [PhyFum-mod-L08 (T_3, heterozygous)] (*37*) phytases respectively were analyzed for phytase activity, heat stability, and inositol phosphate hydrolysis in the seeds. Both lines were phenotypically normal like the other transgenic lines generated with the two constructs. RT-PCR analyses revealed that the ConPhy gene was expressed in leaves and seeds of the transformed wheat plants (data not shown). Western blotting demonstrated the presence of a single immuno-reacting band in extracts of water-soluble seed protein but not in leaf extracts. We have obtained similar results for the *A. fumigatus* phytase (*16*), showing that while the 1DX5 promoter also drives expression of the heterologous phytase genes in leaves, the amount of phytase protein generated was below the detection limit. The absence of an immuno-reacting band in wild type as well as in lines still segregating for the trait (**Figure 2**) further

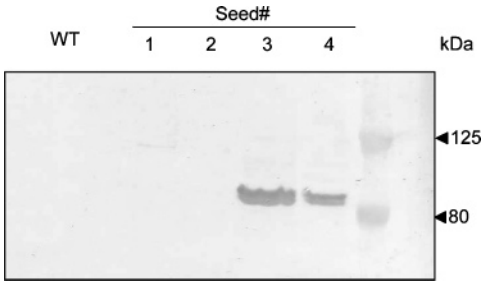

Figure 2. Western immuno blot of water-soluble protein from seeds of wild type (WT) and segregating T_2 transgenic ConPhy-L04-01-02 plants approximately 45 days after pollination. Seeds #1 and #2 lack the phytase due to segregation while seeds #3 and #4 express the phytase.

supported the conclusion that the Western analysis detected the consensus phytase. The molecular mass of the immuno-detected protein is about 90 kDa. The mature enzyme has a theoretical M_r of ~51 kDa but as the enzyme contains seven potential N-linked glycosylation sites and has been targeted for secretion, the difference between the apparent and theoretical M_r can be attributed to glycosylation. Similar glycosylation-dependent differences between the theoretical and the *in planta* observed M_r have been detected for the *A. niger phyA* encoded phytase when expressed in several plants such as wheat, soybean, and tobacco (*24*, *28*, *29*, *42*, *43*).

Localization of the Heterologous Phytase. Wheat endosperm cells store proteins in protein bodies within protein storage vacuoles (**Figure 3A**). The immunolocalization of phytase performed in wild type and ConPhy-L04-01-04 seeds revealed a very prominent labeling of the protein bodies, while there were no indications for the presence of larger amounts of ConPhy in other cell compartments or the apoplast (**Figure 3B**). No signal was detected in the wild type seeds (**Figure 3C**). Electron microscopy provides a more detailed image of the distribution of the recombinant phytase within the protein bodies. Wheat storage proteins form aggregates already in the endoplasmic reticulum that bud off and once free in the cytoplasm they are sequestered by protein storage vacuoles. Abundant gold probes are found in protein bodies within rough endoplasmic reticulum-like cisternae (**Figure 4A**), as well as in cytoplasmatic protein bodies at the periphery of a protein storage vacuole (**Figure 4B**). Major protein bodies were also heavily labeled with the exception of the triticin inclusion bodies (**Figure 4C**). This indicates an intracellular route for the heterologous phytase that comprises translation at ribosomes of the rough endoplasmic reticulum, co-translational transfer into the ER, and aggregation with other storage proteins in vesicles of the endoplasmic reticulum where after the vesicles are pinched off and transported to the large storage protein vacuoles (**Figure 4C**).

Phytase Activity and Heat Stability. The phytase activities in flour from seeds of wild type, PhyFum-mod-L08, and ConPhy-L04-01-04 wheat were measured to 803, 3655, and 4777 FTU/kg, respectively, at 37 °C (**Figure 5**). The flour was then incubated in preheated sodium-acetate buffer at 60, 87, or 100 °C for 1, 5, or 20 min where after the flour−buffer slurry was cooled to 37 °C and assayed for after heat treatment residual (AHTR) phytase activity.

One minute incubation at 60 °C did not negatively affect the phytase activity in wild type or transgenic lines and only a minor decrease was observed after 5 min incubation in the transgenics (**Figure 5A**). After 20 min at 60 °C, the activities were reduced substantially to 1960 FTU/kg in ConPhy-L04-01-04 and 1715 FTU/kg in Phyfum-mod-L08 corresponding to 41 and 47% residual activities, respectively. Surprisingly enough, the en-

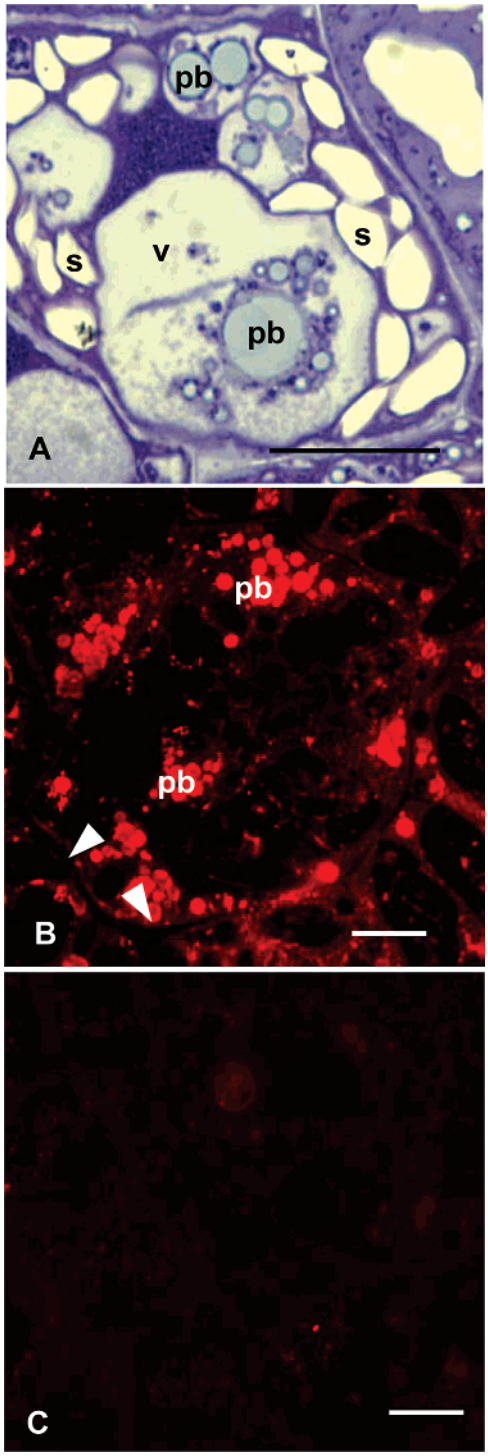

Figure 3. Light microscopy analysis of endosperm structure and consensus phytase localization. **A**, toluidine blue stained semithin section of wild type endosperm cells. Protein bodies (pb) are seen within the vacuoles (v). Starch granules are labeled with an S. The dark blue spheres on the periphery of the protein bodies correspond to triticin-containing inclusion bodies. **B**, immunofluorescence detection of the recombinant phytase in 1 μm thick sections of endosperm of the ConPhy-L04-01-04 line. The protein bodies are clearly labeled while there is no fluorescence from any other compartment of the cell or the apoplast (arrowheads). **C**, immuno fluorescence analysis of 1 μm thick section of wild type endosperm cells. There is virtually no labeling of any of the components of the wild-type endosperm cell. Scale: bar corresponds to 20 μm.

dogenous wheat phytase activity in wild type flour was not reduced throughout the 20 min test interval.

In the 87 °C experiment we found a slight phytase activity increase in the wild type samples during the first minute of incubation (**Figure 5B**). However, after 5 and 20 min, the residual phytase activities were reduced strongly to around 125 FTU/kg, corresponding to ~16% of the initial activity. ConPhy-L04-01-04 showed unaltered activity after 1 min, but after 5 and 20 min, the activities were reduced to 983 and 922 FTU/kg, corresponding to 21 and 19% of the initial activities. Heating of PhyFum-mod-L08 to 87 °C for 1 min caused a minor reduction from 3655 to 3297 FTU/kg whereas 5 and 20 min of heat treatment reduced the activities markedly to 380 and 319 FTU/kg, corresponding to 10 and 9% of the initial activities of PhyFum-mod-L08.

Boiling reduced the phytase activity considerably in all samples (**Figure 5C**). In wild type, a minor increase was observed after 1 min but after 5 and 20 min of boiling the activity was reduced to 4% and 1% of the initial activity. In PhyFum-mod-L08, 1, 5, and 20 min at 100 °C resulted in residual activities of 48%, 11%, and 8%, respectively. ConPhy-L04-01-04 was the least affected by the heat treatments. One minute of boiling reduced the activity from 4777 to 3925 FTU/kg, corresponding to an 82% residual activity. After 5 and 20 min at 100 °C the activity had decreased to 588 and 563 FTU/kg, corresponding to ~12% of the initial activity in both cases.

Inositol Phosphate Hydrolysis before and after Heat Treatment. To further characterize the phytase activities in flour samples obtained from the three materials, we analyzed the *myo*-inositol phosphate isomeric profiles by HPIC. The profiles from mature seed flour that had not been heated or incubated were identical for the three different materials and revealed one major peak, representing *Ins*P_6 as well as trace levels of *Ins*P_5 isomers, represented by DL-*Ins*(1,2,4,5,6)P_5 and DL-*Ins*(1,2,3,4,5)P_5 (**Figure 6**). Interestingly, the *Ins*P_6 peaks in ConPhy-L04-01-04 and Phyfum-mod-L08 were slightly bigger than those in wild type, a difference that we ascribe to the fact that the studied transgenic seeds were smaller than the wild-type seeds. Hence, after weighing the samples, the embryo and aleurone tissues holding almost all seed *Ins*P_6 contributed more in the transgenic materials than in the wild type. Incubation of the flour from the ConPhy line for 1 h at 37 °C resulted in complete hydrolysis of all *Ins*P_6 and all lower inositol phosphates (not shown).

The *myo*-inositol phosphate profiles after boiling of flour for 20 min and incubation of the flour was unravelled in wild type and ConPhy-L04-01-04, representing the maximum AHTR-phytase activity (563 FTU/kg) (**Figure 5**). For wild type flour only minor changes were observed in comparison to the profile obtained from analysis of untreated flour. This finding was in agreement with the rapid inactivation and virtual elimination of the endogenous phytase activities. The *Ins*P_6 level was reduced by 17% relative to the initial *Ins*P_6 level and there were only minor amounts of *Ins*P_5 isomers, primarily DL-*Ins*(1,2,3,4,5)-P_5, which constituted 50% of all *Ins*P_5's (**Table 1**). Detection of these specific pentakisphosphate isomers is consistent with endogenous wheat phytase belonging to the 6-phytases. As shown in a previous study, incubation of wild type flour at 37 °C for 20 min causes a *Ins*P_6 reduction by approximately 45% and a substantial accumulation of lower inositol phosphates (*29*).

The *myo*-inositol profile observed after boiling the flour of ConPhy-L04-01-04 reflected a significant AHTR-phytase activity (**Figure 7**). The *Ins*P_6 level was reduced by 42% (**Table 1**) and several isomers of lower inositol phosphates from *Ins*P_2 to *Ins*P_5 accumulated in the profile. In agreement with the heterologous phytase being a 3-phytase, the major *Ins*P_5 isomer was DL-*Ins*(1,2,4,5,6)P_5, representing 48% of the total *Ins*P_5

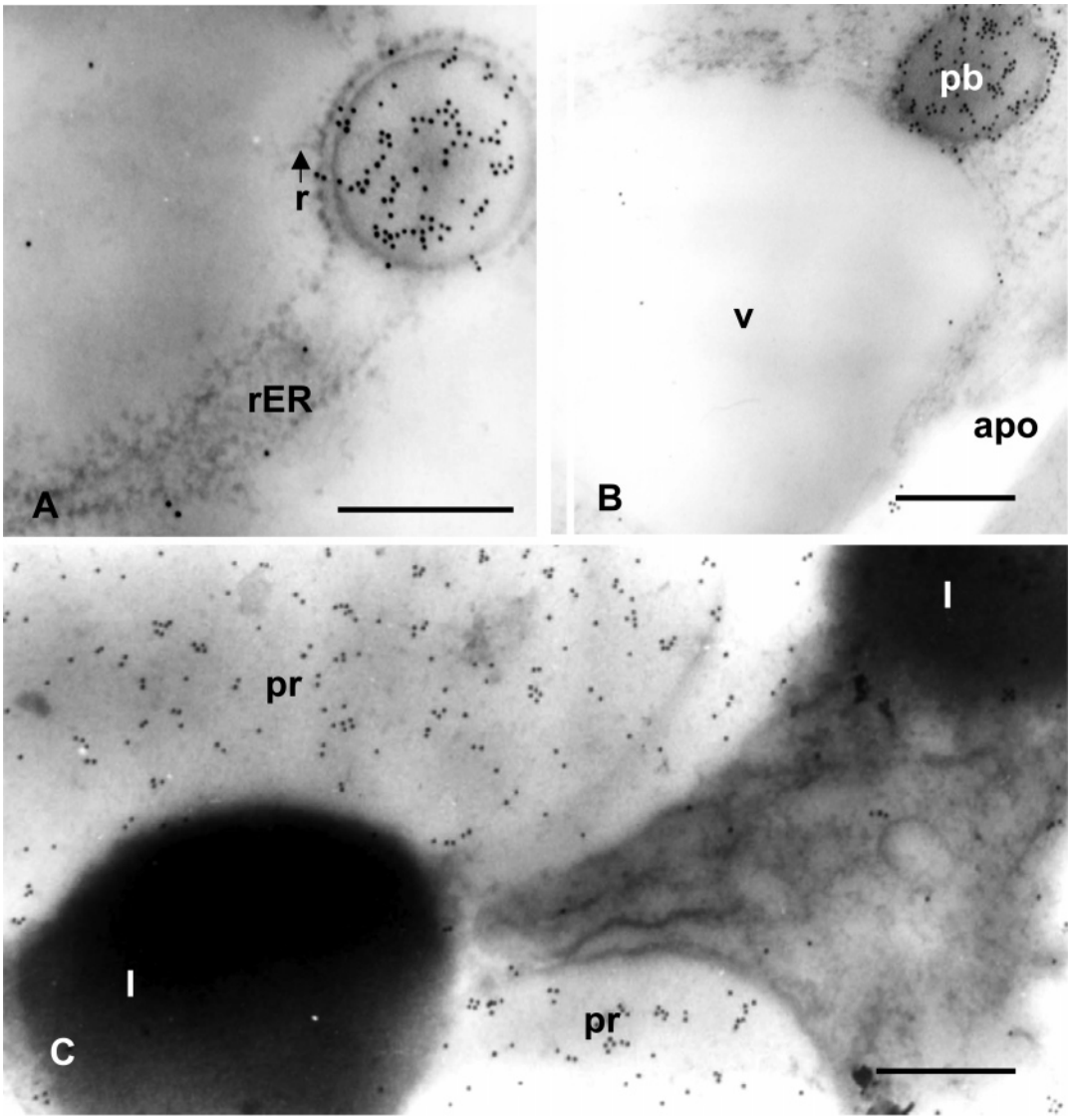

Figure 4. Immunoelectron microscopical analysis of the localization of the recombinant phytase. **A**, gold labeling of a spherical protein body within a cisterna of rough endoplasmic reticulum (rER). See the ribosomes (r) bound to the cytoplasmic face of the membrane. **B**, a labeled protein body (pb) associated with a vacuole (V). **C**, labeling of a major protein body. Notice the absence of labeling of the triticin inclusion bodies (I). Scale: bar corresponds to 0.5 μm.

isomers (**Table 1**). In wild type, only 22% of the total *Ins*P_5 appeared as DL-*Ins*(1,2,4,5,6)P_5 (**Table 1**).

DISCUSSION

In the current study we have evaluated two heat-stable phytases expressed in transgenic wheat with respect to deposition pattern, thermostability properties, and the ability of AHTR phytase activities to hydrolyze *Ins*P_6 and lower inositol phosphates. We used the wheat HMW 1DX5 GS promoter for driving expression of the two phytase genes and although the promoter is not completely specific for the endosperm we only found immuno-detectable phytase in this tissue. The activities of the heterologous phytases exceeded that of the endogenous phytases by a factor of 5−6 which is higher than previously reported for other phytase lines of wheat (*28*, *29*, *37*).

An α-amylase signal peptide was included in the expression cassette in order to ensure secretion of the protein to the apoplast via the default secretory pathway. However, our immunolocalization studies showed unequivocally that the heterologous phytase was deposited in the protein storage vacuole. We can thus support the hypothesis that the endosperm provides an environment for protein trafficking that appears to overrule the normal secretion mechanisms to the apoplast and instead favors deposition in protein storage vacolues ((*44*) and Stoger et al., unpublished results). Arcalis et al. (*44*) thus found that an *A. niger* phytase gene produced in wheat likewise was deposited in the storage vacuole, although secretion to the apoplast was expected due to the insertion of a murine immunoglobulin signal sequence. Further details of these mechanisms need to be explored. Arcalis et al. (*44*) also found a glycosylation pattern of the heterologous phytase in wheat that indicates a preferential passage from the endoplasmic reticulum to the Golgi. We have not performed a glycosylation analysis but it appears from the electron microscopy studies that there is also a pathway that comprises assembly of the phytase enzymes in protein bodies within the ER with subsequent translocation to the protein storage vacuole.

In the analysis of the thermotolerance of the two phytase enzymes we found that the *A. fumigatus* phytase retains 8% of its activity after boiling of the flour for 20 min while the consensus 10-thermo-[3]-Q50T-K91A phytase maintained 12% of its original activity. The 8% retained activity of the *A.*

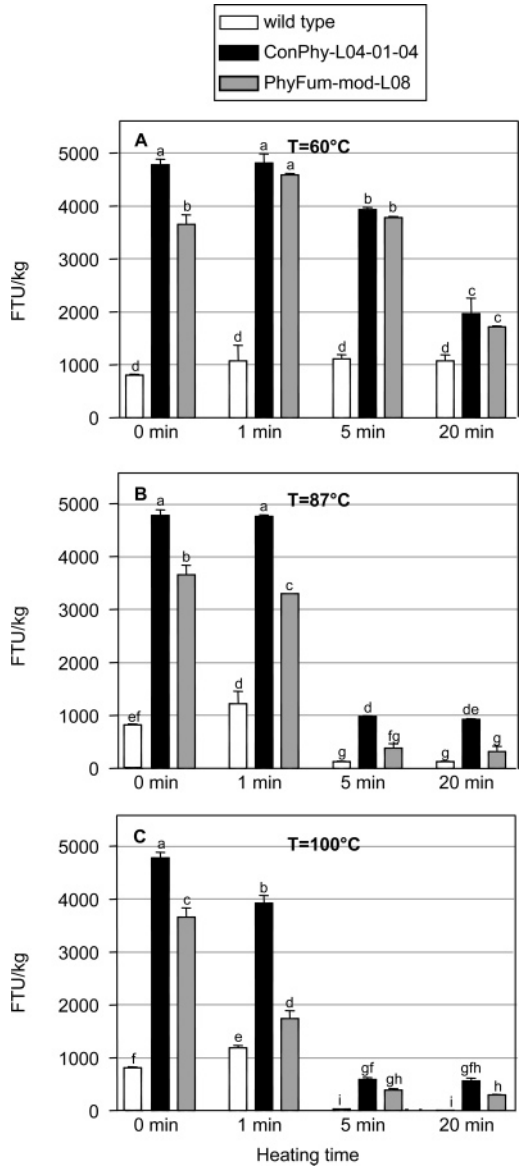

Figure 5. Analysis of phytase activity in seeds from wild type, ConPhy-L04-01-04 and PhyFum-mod-L08 plants. Flour from milled seeds was suspended in sodium-acetate buffer heated to 60, 87, or 100 °C and incubated for 0, 1, 5, or 20 min. After the heat treatment, the suspension was cooled and the water-soluble protein was extracted and assayed for phytase activity. One FTU is the amount of enzyme that liberates 1 μmol orthophosphate per minute under the test conditions. Bars on top of each histogram represent the standard error. Each treatment represents an average of at least two replicates. Columns labeled with the same letter do not have statistically different means, $P < 0.05$, t-test.

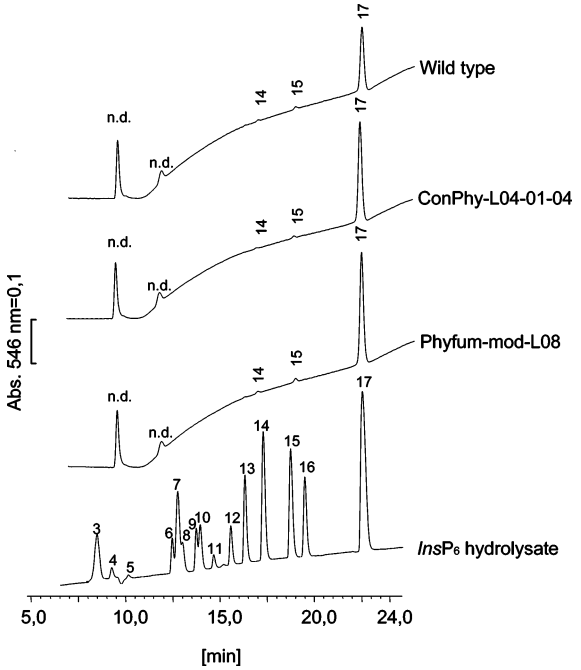

Figure 6. The inositol phosphate profile in flour from mature seeds of wild type, PhyFum-mod-L08, and ConPhy-L04-01-04 plants analyzed via HPIC. Parts of the elution profile of *Ins*P_6 hydrolysate are included for isomer identification. Peaks in the profiles from grains extracts are assigned by identifying corresponding peaks in the *Ins*P_6 hydrolysate trace. (3) *Ins*(1,3,5)P_3, D/L-*Ins*(1,3,4)P_3, D/L-*Ins*(1,4,5)P_3; (4) D/L-*Ins*(1,5,6)P_3; (5) *Ins*(4,5,6)P_3; (6) DL-*Ins*(1,2,3,5)P_4, DL-*Ins*(1,2,4,6)P_4; (7) DL-*Ins*(1,2,3,4)P_4; *Ins*(1,3,4,6)P_4; (8) DL-*Ins*(1,2,4,5)P_4; (9) DL-*Ins*(1,3,4,5)P_4; (10) DL-*Ins*(1,2,5,6)-P_4; (11) *Ins*(2,4,5,6)P_4; (12) DL-*Ins*(1,4,5,6)P_4; (13) *Ins*(1,2,3,4,6)P_5; (14) DL-*Ins*(1,2,3,4,5)P_5; (15) DL-*Ins*(1,2,4,5,6)P_5; (16) *Ins*(1,3,4,5,6)P_5; (17) *Ins*P_6.

Table 1. Percent Reduction of Seed *Ins*P_6 and Distribution between DL-*Ins*(1,2,4,5,6)P_5 and DL-*Ins*(1,2,3,4,5)P_5 after 20 min at 100 °C Followed by 1 h of Incubation at 37 °C

	InsP_6 % reduction	DL-*Ins*(1,2,4,5,6)P_5 % of total InsP_5	DL-*Ins*(1,2,3,4,5)P_5 % of total InsP_5
WT	17	22	50
ConPhy-L04-01-04	42	48	39

fumigatus enzyme after 20 min of boiling is in agreement with the data of Lucca et al. (*7*) for polished rice grains expressing the same enzyme. In contrast, the purified *A. fumigatus* phytase regained 59% of its activity after cooling. The intracellular deposition pattern of the phytase in the rice grain has not been reported but it appears that deposition of phytase in the rice as well as the wheat endosperm somehow impedes refolding of the enzyme into an active configuration. We further found that, at 60 °C, the endogenous wheat phytase is fully thermotolerant during the 20 min incubation period, a result in agreement with the fact that most plant phytases are inactivated at temperatures above 70 °C (*48*). After 1 min at 87 and 100 °C, the wild type phytase activity increased slightly but statistically significantly, an increase that we believe can be attributed to activity increases during the first phase of the incubation, although it cannot be ruled out that the high temperatures improved the extractability of the phytase. In contrast, the 60 °C incubation regime resulted in strong decreases in the activity of the heterologous phytases (to 47 and 41% of the initial activities of PhyFum-mod-L08 and ConPhy-L04-01-04 after 20 min). The abilities of the two different heterologous phytases to resist thermal inactivation were supported by the statistical data. Thus, it is clear that at 60 °C, which is below the T_m of both enzymes, ConPhy-L04-01-04 and PhyFum-mod-L08 activities were reduced to the same level of significance. However, at 87 °C where the T_m of only the *A. fumigatus* phytase were transgressed, the two types of phytases behave statistically different with the largest reductions seen in PhyFum-mod-L08, a reduction that after 5 min is statistically similar to the activity level seen in wild type. At the most extreme treatments, 100 °C for 1, 5, and 20 min, the statistical data reveal a clear significant difference between the two phytases after 1 min whereas after 5 and 20 min, the observed differences are not statistically significant.

To get an estimate of the potential of the endogenous and the heterologous phytase for mediating phytate degradation after

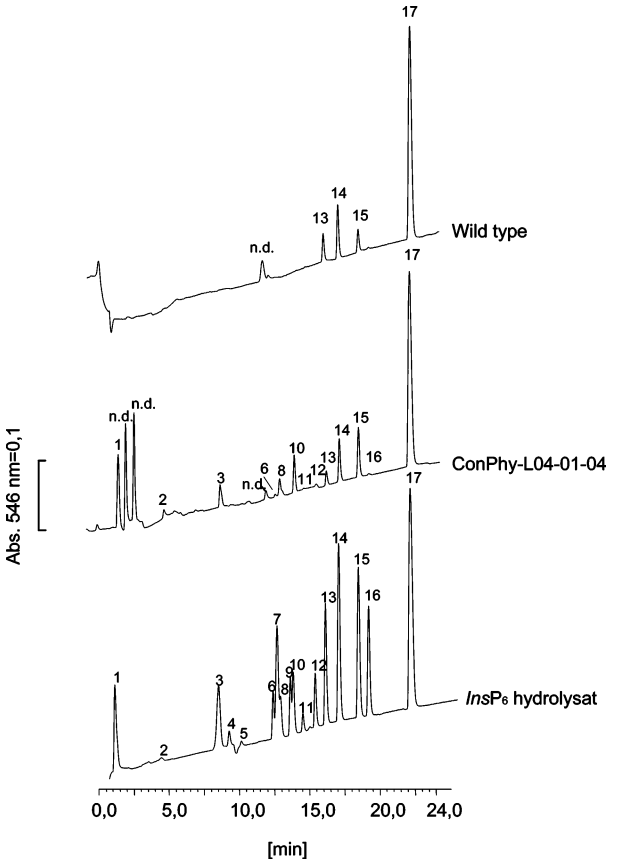

Figure 7. *Myo*-inositol phosphate profile of wild type and ConPhy-L04-01-04 after boiling the flour for 20 min and incubating the boiled samples at 37 °C for 1 h. The elution profile of *Ins*P_6 hydrolysate is included for isomer identification. Peaks in the profiles from grains extracts are assigned by identifying corresponding peaks in the *Ins*P_6 hydrolysate trace. (1) *Ins*P and P_i; (2) *Ins*P_2; (3) *Ins*(1,3,5)P_3, D/L-*Ins*(1,3,4)P_3, D/L-*Ins*(1,4,5)P_3; (4) D/L-*Ins*(1,5,6)P_3; (5) *Ins*(4,5,6)P_3; (6) DL-*Ins*(1,2,3,5)P_4, DL-*Ins*(1,2,4,6)P_4; (7) DL-*Ins*(1,2,3,4)P_4; *Ins*(1,3,4,6)P_4; (8) DL-*Ins*(1,2,4,5)P_4; (9) DL-*Ins*(1,3,4,5)-P_4; (10) DL-*Ins*(1,2,5,6)P_4; (11) *Ins*(2,4,5,6)P_4; (12) DL-*Ins*(1,4,5,6)P_4; (13) *Ins*(1,2,3,4,6)P_5; (14) DL-*Ins*(1,2,3,4,5)P_5; (15) DL-*Ins*(1,2,4,5,6)P_5; (16) *Ins*(1,3,4,5,6)P_5; (17) *Ins*P_6.

high-temperature treatments, we further measured the amount of residual phytate as well as the compositions of lower *myo*-inositol phosphates after incubation of flour boiled for 20 min. In earlier studies we have found that the endogenous phytase activities of wild type wheat with an activity on 735 FTU/kg can reduce seed *Ins*P_6 by 45% after 50 min incubation of flour at 37 °C (*29*). Furthermore, using the same incubation regime, transgenic wheat flour containing an *A. niger* phytase with an activity of 1467 FTU/ kg resulted in a reduction of the *Ins*P_6 level by 86% and complete dephosphorylation of all lower *myo*-inositol phosphates (*29*). The concerted action of the endogenous and the heterologous phytases could be monitored during the incubation, as the endogenous phytases are 6-phytases while the *Aspergillus* phytases initially removed the phosphate group at the 3-position. In the current study we found that the 563 FTU/kg residual activity, present after 20 min boiling of ConPhy-L04-01-04 flour, reduced the *Ins*P_6 level by 42%, an activity and reduction in the same range as in the nonboiled wild type and an indication that no, or only very little, *Ins*P_6 hydrolysis occurs during boiling of the flour. The reduction in *Ins*P_6 levels was accompanied by increases in *Ins*P_5, namely *Ins*(1,2,4,5,6)P_5 in the case of the transgenic line, which is in agreement with the heterologous phytases being 3-phytases.

The present article constitutes the first analysis where two heat-stable phytases, possessing a high unfolding temperature and a high refolding capacity, respectively, have been expressed in a cereal. For the first time we have evaluated in detail the accumulation of the heterologous phytase, compared the heat stability properties of the enzymes, and finally unravelled the capacity of the AHTR phytase activity to hydrolyze seed *Ins*P_6 to lower inositol phosphates. Additional experiments are required to describe the residual phytase activity and *Ins*P_6 degradation after feed pelleting and food processing. However, it is clear from these studies that heat-stable phytase can be accumulated in transgenic cereals and result in a substantial *Ins*P_6 hydrolysis even after extended boiling of milled seeds. Moreover, it is also clear that the storage protein vacuole does not appear to represent favorable protective environments for maintaining enzyme activity during prolonged incubation at high temperatures. Under these deposition conditions, heat stability based on a high unfolding temperature is superior to an enzyme where the AHTR phytase activity relies on high refolding of the denatured protein.

In summary, we find that transformation of staples for increased phytase activity may be a realistic strategy for alleviating micronutrient deficiencies in humans. Lines with substantially higher phytase activity can easily be generated. Recently, we have obtained barley lines with an *A. fumigatus* phytase activity of up to ~10.000 FTU/kg (Brinch-Pedersen, unpublished data). Present evidence from pig-feeding experiments suggests that, at low to moderate dietary zinc levels, supplementation with 500 FTU phytase activity per kg of feed leads to 20−50% increases in plasma zinc levels. This implies that even low residual phytase activities after food processing may have a significant effect on the zinc status. Previous studies on bread making have demonstrated a significant effect on reducing phytate levels during fermentation via addition of exogenous phytase to the dough (*33*, *34*). However, a number of issues have to be addressed and clarified before implementing a phytase transgene strategy for improving iron and zinc uptake in humans. Present evidence suggests that a certain level of inositol phosphates can be important due to suggested potential health benefits of inositol phosphates with functions such as antioxidants and anti-neoplastic, with the ability to reduce serum lipids and cholesterol levels and to prevent renal calculi via mineral binding, and with hypoglycemic effects of relevance in diabetes (*45*, *46*). It will thus be a major research objective to clarify if phytate can be reduced to a level that at the same time can maintain the potential health benefits as well as improve the iron and zinc bioavailability.

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