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Differences in Phytase Activity and Phytic Acid Content between Cultivated and Tibetan Annual Wild Barleys

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The Qinghai-Tibetan Plateau in China is considered to be one of the original centers of cultivated barley. At present, little is known about the phytase activity (Phy) or phytic acid content (PA) in grains of Tibetan annual wild barley. Phy and PA were determined in grains of 135 wild and 72 cultivated barleys. Phy ranged from 171.3 to 1299.2 U kg^{-1} and from 219.9 to 998.2 U kg^{-1} for wild and cultivated barleys, respectively. PA and protein contents were much higher in wild barley than in cultivated barley. Tibetan annual wild barley showed a larger genetic diversity in phytase activity and phytic acid and protein contents and is of value for barley breeding. There is no significant correlation between phytase activity and phytic acid or protein content in barley grains, indicating that endogenous phytase activity had little effect on the accumulation of phytic acid.

KEYWORDS: Barley (*Hordeum vulgare* L.); mineral nutrient; phytase; phytic acid; wild barley

INTRODUCTION

Phytic acid, *myo*-inositol 1,2,3,4,5,6-hexakisphosphate (InsP6), has long been known as the principal storage form of phosphorus (P) and inositol in cereal grains, which is an effective polyanionic chelating agent. It is deposited in grains as phytate salts of mineral cations such as K, Mg, Ca, Fe, Zn, Cu, and Mn (*1*−*3*). Phytate deposition plays an important role in storage and homeostasis of both P and some other mineral nutrients during grain development and maturation. However, phytic acid has been termed an "anti-nutrient" due to its direct or indirect ability in binding minerals. Thus, phytic acid altered the solubility, functionality, digestibility, and absorption of mineral nutrients, which significantly restrict the bioavailability of mineral nutrients in a meal (*4*, *5*). Phytic acid content in cereals is greatly affected by both genetic and environmental factors (*4*) and is also involved in stress responses, membrane biogenesis, and intracellular signaling (*6*).

Barley grains contain appreciable amounts of minerals, but their availability is low due to the formation of insoluble complexes with phytate (*7*), which is one of the main inhibitors for iron and zinc absorption in humans (*8*). The intake of large amounts of foods rich in phytate may cause several mineral deficiency symptoms (*9*).

Phytase (*myo*-inositol hexaphosphate hydrolase) hydrolyzes phytic acid to *myo*-inositol and inorganic phosphate. Hence, enhancement of endogenous phytase activity could improve the bioavailability of iron and zinc in cereals (*10*). Phytases have been studied intensively in the past few years because of the great interest in using such enzymes for reducing phytate in animal feed or food for human consumption and for reducing the total load of phosphorus released into the environment (*11*, *12*). Many efforts have been made to improve phytase activity or reduce phytate concentration in edible tissues. One successful way, approved with the development of transgenic technology, is overexpression of the phytase gene in some cereals (*13*−*15*). Besides generation of high-phytase plants, one of the transgenic strategies is producing crops with low phytate contents.

It is also reported that phytase activity increases markedly during germination of most seeds including cereals, accompanied by a significant decrease in phytate content and an increase in the phosphate content (*16*, *17*). Two phytate-degrading enzymes have been purified and identified from 4-day-old barley seedlings (*18*). One phytase (P2) was identified as a constitutive enzyme, whereas the other one (P1) was induced during germination; the activity of P1 was increased by almost 35-fold.

Reliable characterization of a wide range of traits is an essential step toward a fuller utilization of the wild genetic resources in barley improvement (*19*). Wild barley, the progenitor of cultivated barley, has often been considered to be an important source for obtaining genes of special interest in barley breeding programs (*20*), including many agronomic traits such as abiotic and biotic stress tolerances, grain protein quality and quantity, and micronutrient concentrations (Zn, Fe, and Mn) (*21*). The Qinghai-Tibet Plateau, called the "ridge of the world" and well-known for its harsh environment, is one of the original centers of cultivated barley, which is rich in genetic diversity. It has been shown that there is a wide biochemical, morphological, and physiological diversity in Tibetan annual wild barleys (*22*). However, there is no report of the phytase activity or phytic acid content in grains of Tibetan annual wild barley.

The objectives of the present investigation were to screen the barley genotypes or accessions for phytase activity and phytic acid content from Tibetan annual wild barley and compare the genetic differences between wild and cultivated barleys.

MATERIALS AND METHODS

Plant Materials and Sample Preparation. One hundred and thirty-five Tibetan annual wild barley accessions, including 90 two-rowed accessions

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(*Hordeum vulgare* L. ssp. *spontaneum*) and 45 six-rowed accessions (*H. vulgare* L. ssp. *agriocrithum*), and 72 cultivated barley genotypes (*H. vulgare* L. ssp. *vulgare*), including 54 two-rowed and 18 six-rowed genotypes, were used in this study. Tibetan annual wild barleys, collected from the Qinghai−Tibet Plateau of China, were kindly provided by Professor Dongfa Sun from Huazhong Agricultural University of China. All genotypes or accessions were planted in early November 2008, in adjacent plots in a farm field, and each genotype or accession consisted of 2 m length rows with three replications (Huajiachi campus, Zhejiang University, China). Field management was the same as applied locally. At maturity, the plants were harvested and stored in a refrigerator at 4 °C, and grains were mixed and milled to pass through a 0.5 mm screen for analysis.

Phytase Activity Assay. Phytase activity was analyzed according to a method reported previously (*11*). Phytic acid sodium salt hydrate (Sigma P0109 from rice) was used as substrate. The phytase activity was expressed as units, which is defined as the amount of inorganic phosphorus liberated from sodium phytate solution at a rate of 1 μmol min^{-1} at pH 5.5 and 37 °C.

Phytic Acid Content Measurement. Phytic acid content was measured according to the methods given in references *4* and *23* with some modifications. The barley sample (0.3 g) was placed into a 50 mL centrifuge tube, and 10 mL of 0.2 M HCl was added; following vibration in a shaker overnight, the contents were centrifuged at 10000*g* for 10 min. Two milliliters of 0.2% $FeCl_3$ was added to 2.5 mL of supernatant, and the mixture was boiled in a bath for 30 min and then centrifuged at 10000*g* for 15 min after cooling. The supernatant was discarded, and the tube was washed twice with 5 mL of deionized water. Three milliliters of 1.5 M NaOH was added into the residue, which was then vortexed for 2 min and centrifuged at 10000*g* for 10 min. The supernatant was discarded, and 3 mL of 0.5 M HCl was added to dissolve the residue. Finally, deionized water was added to the solution up to the volume of 10 mL. Fe content was determined by atomic absorption spectrophotometer (Shimadzu AA6300, Japan), and phytic acid content was calculated by multiplying Fe content by a factor of 4.2.

Total Protein Content and Mineral Element Concentration Measurement. Total protein content in barley grains was determined by near-infrared reflectance spectroscopy (NIRA, Matrix-1, Bruker Co., Germany) using a previously established calibration curve (*24*). Mineral element concentration in barley grains, such as Fe, Cu, Zn, Mg, and Ca, was determined according to the method given in ref *25* with some modifications. The barley sample (1.0 g) was placed into a 50 mL centrifuge tube, and 20 mL of 1 M HCl was added; the mixture was vibrated in a shaker for 24 h and finally filtered. Mineral elements were determined by atomic absorption spectrophotometer (Shimadzu AA6300).

Statistical Analysis. Each measurement was carried out in three replications. Analysis of variance and correlation were performed using general linear models (PROC GLM) of SAS V8.0 (SAS Institute, Cary, NC).

Table 1. Phytase Activity and Phytic Acid and Total Protein Contents in Grains of Cultivated and Tibetan Wild Barleys

	Phy (U kg^{-1})		PA (mg g^{-1})		protein (mg g^{-1})	
	WB[a]	CB[b]	WB	CB	WB	CB
min	171.3	219.9	2.1	3.4	84.1	70.2
max	1299.2	998.2	15.4	9.2	174.9	133.3
mean	492.4	566.4	7.1	5.5	116.0	96.8
CV[c] (%)	33.9	26.5	41.4	24.4	14.4	15.7

[a] WB, wild barley, n = 135. [b] CB, cultivated barley, n = 72. [c] CV, coefficient of variation.

RESULTS

Phytase Activity in Cultivated and Tibetan Wild Barleys. The genotypic variation in phytase activity (Phy) of cultivated and Tibetan wild barleys is shown in **Table 1**. Phy for 135 wild barley accessions (WB) ranged from 171.3 to 1299.2 U kg^{-1}, with a mean of 492.4 U kg^{-1}, whereas Phy for 72 cultivated barley genotypes (CB) ranged from 219.9 to 998.2 U kg^{-1}, with a mean of 566.4 U kg^{-1}. The ratios of the maximum Phy to the minimum Phy were 7.58 and 4.54 for WB and CB, respectively, indicating that there is a greater difference among WB than among CB. A wild accession, coded XZ92, had Phy of about 1300 U kg^{-1} (with 4.81 and 150.7 mg g^{-1} phytic acid and protein contents, respectively), which is valuable for barley breeders in developing cultivars with high phytase activity.

Phytic Acid and Protein Contents in Cultivated and Tibetan Wild Barleys. It can be seen from **Table 1** that PA content of WB ranged from 2.1 to 15.4 mg g^{-1}, with a mean of 7.1 mg g^{-1}, and CB ranged from 3.4 to 9.2 mg g^{-1}, with a mean of 5.5 mg g^{-1}. The average PA content of WB was much higher than that of CB. In addition, WB had a larger coefficient of variation (CV) than CB. Protein content ranged from 84.1 to 174.9 mg g^{-1} and from 70.2 to 133.3 mg g^{-1}, with a mean of 116.0 and 96.8 mg g^{-1} for WB and CB, respectively.

Phytase Activity and Phytic Acid and Total Protein Contents in Six-Rowed and Two-Rowed Tibetan Wild Barleys. The genotypic variation in Phy and PA and protein contents of six-rowed and two-rowed wild barleys is shown in **Table 2**. The Phy of six-rowed wild barley (SWB) ranged from 190.5 to 822.1 U kg^{-1}, with a mean of 466.7 U kg^{-1}, and that of two-rowed wild barley (TWB) ranged from 171.3 to 1299.2 U kg^{-1}, with a mean of 505.3 U kg^{-1}. PA content of SWB ranged from 2.1 to 14.5 mg g^{-1}, with a mean of 7.0 mg g^{-1}, and that of TWB ranged from 2.4 to 15.4 mg g^{-1}, with a mean of 7.1 mg g^{-1}. Protein content ranged from 84.1 to 130.2 mg g^{-1} and from 94.1 to 174.9 mg g^{-1}, with means of 106.4 and 120.7 mg g^{-1}, for SWB and TWB, respectively. On the whole, there was no major difference in phytase activity and phytic acid content in grains between SWB and TWB, except for protein content.

Mineral Element Concentrations in Six-Rowed and Two-Rowed Tibetan Wild Barleys. Like phytase activity in barley grains, the concentrations of iron (Fe), copper (Cu), zinc (Zn), magnesium (Mg), and calcium (Ca) in grains had no marked difference between six-rowed and two-rowed wild barleys (**Table 2**). The ranges of SWB for Fe, Cu, Zn, Mg, and Ca concentrations were

Table 2. Phytase Activity, Phytic Acid and Total Protein Contents, and Mineral Nutrient Concentrations in Grains of Six-Rowed and Two-Rowed Tibetan Wild Barleys

type		Phy (U kg^{-1})	PA (mg g^{-1})	protein (mg g^{-1})	Fe (mg kg^{-1})	Cu (mg kg^{-1})	Zn (mg kg^{-1})	Mg (g kg^{-1})	Ca (g kg^{-1})
six-rowed	min	190.5	2.1	84.1	53.4	6.3	47.7	1.16	0.21
	max	822.1	14.5	130.2	108.8	9.2	85.1	1.67	0.53
	mean	466.7	7.0	106.4	73.9	7.6	60.6	1.37	0.37
	CV[a] (%)	30.9	43.0	8.6	17.4	9.5	12.9	6.4	22.0
two-rowed	min	171.3	2.4	94.1	57.3	6.2	50.6	1.34	0.16
	max	1299.2	15.4	174.9	141.5	10.9	119.9	1.84	0.56
	mean	505.3	7.1	120.7	85.1	8.3	69.1	1.48	0.34
	CV (%)	35.0	40.8	14.6	19.3	11.9	17.3	6.7	23.9

[a] CV, coefficient of variation.

Table 3. Correlation Coefficients among Phytase Activity, Phytic Acid and Protein Contents, and Mineral Nutrient Concentrations in Barley Grains[a]

	Phy	PA	protein	Fe	Cu	Zn	Mg	Ca
Phy	1							
PA	−0.0839	1						
protein	−0.1037	0.2232**	1					
Fe	−0.1263	0.0928	0.4353**	1				
Cu	−0.0763	0.1869**	0.5363**	0.3471**	1			
Zn	−0.1940**	0.0926	0.6339**	0.5784**	0.4066**	1		
Mg	−0.2533**	0.1711*	0.7336**	0.3360**	0.4405**	0.5248**	1	
Ca	−0.1941**	0.1557*	0.2182**	−0.0422	0.1226	−0.0100	0.2186**	1

[a] * and ** indicate significance at 0.05 and 0.01 probability levels, respectively; n =207.

53.4−108.8 mg kg^{-1}, 6.3−9.2 mg kg^{-1}, 47.7−85.1 mg kg^{-1}, 1.16−1.67 g kg^{-1}, and 0.21−0.53 g kg^{-1}, with means of 73.9 mg kg^{-1}, 7.6 mg kg^{-1}, 60.6 mg kg^{-1}, 1.37 g kg^{-1}, and 0.37 g kg^{-1}, respectively. For TWB, the ranges and means of all mineral concentrations were basically similar to those in SWB, but the maximum values of Fe and Zn concentrations were much higher in TWB than in SWB.

Relationships among Phytase Activity, Phytic Acid and Protein Contents, and Mineral Element Concentrations. Analysis of the correlations among Phy, PA and protein contents, and mineral element concentrations of 207 cultivated and Tibetan wild barleys showed that Phy was significantly and negatively correlated with Zn, Mg, and Ca concentrations in barley grains (**Table 3**). The correlation between Phy and PA or protein content was not significant, indicating that endogenous phytase activity in barley grains has little effect on the accumulation of phytic acid. It may be assumed that it is possible to develop barley cultivars with high phytase activity and low phytic acid content, simultaneously.

DISCUSSION

Phytic acid acts as the primary phosphorus reserve, accounting for up to 85% of the total P in cereals and legumes, which is utilized during seed germination and supports seedling growth by supplying biosynthetic needs of the growing tissues (*3*). Genetic variation in mineral concentration has been studied in cereal crops (*26*). In this study, genotypic variation in PA content was found in both cultivated and Tibetan wild barleys (**Table 1**). Grain Fe, Zn, Ca, Mg, and Cu concentrations in maize, rice, and barley are cultivar dependent (*26*). The current study showed that there was a large genotypic variation in each mineral concentration for WB. Cu, Mg, and Ca concentrations in WB were much higher than those in CB, with means of 8.1 mg kg^{-1}, 1.45 g kg^{-1}, and 0.35 g kg^{-1} for WB and 7.1 mg kg^{-1}, 1.33 g kg^{-1}, and 0.27 g kg^{-1} for CB, respectively. However, there was no significant difference in Fe and Zn concentrations between WB and CB (data not shown). In addition, there was a significant and positive correlation between PA content and Cu, Mg, and Ca concentrations in barley grains, but no significant correlation was found between PA content and Fe or Zn concentration. Weak correlation between phytate and Fe, Zn, Ca, or Mg concentration was found in other crops (*26*). Moreover, significantly positive correlations among grain Fe, Cu, and Zn concentrations were detected in this study (**Table 3**), indicating the possibility of simultaneously increasing concentrations of Fe, Cu, and Zn in barley breeding.

Phytic acid is mainly stored in protein bodies of seeds as spherical inclusions, called globoids, and chelates minerals to form a protein matrix (*27*, *28*). In this study, PA content was significantly and positively correlated with protein content, supporting our previous finding (*4*). Moreover, protein content was significantly and positively correlated with all examined mineral concentrations (Fe, Cu, Zn, Mg, and Ca) (**Table 3**), suggesting that breeding for elevated levels of protein content is likely to increase these mineral concentrations in barley grains.

This is the first study on phytase activity and phytic acid content in the grains of Tibetan annual wild barley. Compared with cultivated barley, the wild barley showed a wider variation in phytase activity. We identified some wild barley accessions with high phytase activity. There is no significant correlation between phytase activity and phytic acid or protein content in barley grains. It could be supposed that endogenous phytase activity in barley grains had little effect on the accumulation of phytic acid.

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