

Symposium: Plant Breeding: A New Tool for Fighting Micronutrient Malnutrition

Transgenic Approaches in Commonly Consumed Cereals to Improve Iron and Zinc Content and Bioavailability¹

Preben B. Holm,² Klaus N. Kristiansen and Henrik B. Pedersen

The Danish Institute of Agricultural Sciences, Department of Plant Biology, Research Centre Flakkebjerg, DK-4200 Slagelse, Denmark

ABSTRACT Modern genetic and molecular technologies provide a number of tools that can be utilized for the development of staple foods with a higher iron and zinc content and improved bioavailability of these minerals. This article summarizes current strategies aimed at increasing the iron-sequestering capacity of the endosperm and improving mineral bioavailability via in planta synthesis of microbial phytases. A case study is presented for wheat, and future strategies are discussed addressing the importance of phytase thermostability. *J. Nutr.* 132: 514S-516S, 2002.

KEY WORDS: • iron • zinc • phytate • phytase • bioavailability • transgenic

The implementation of the tools of molecular genetics and molecular biology may prove essential for breeding cultivars that are more nutrient-dense and that have improved bioavailability of nutrients. Today, modern breeding has unprecedented access to a large knowledge base with respect to plant genes, including the sequence of the *Arabidopsis* and soon the rice genome. This will allow a better understanding of the basic mechanisms involved in the synthesis and accumulation of vitamins and minerals in plant tissues. Because of the metabolic unity of organisms through evolution, the knowledge obtained from one organism can be readily applied to other organisms. Moreover, a large variety of techniques are available for generation of mutants, mapping of traits by molecular markers, positional cloning, gene manipulations, and genetic transformation, all of which enhance the power and speed of plant breeding.

A number of questions relating to iron and zinc uptake, transport, mobilization and deposition in plants, thus, may be addressed using modern molecular and genetic techniques (1,2). The outcome of this research might be plants with a higher iron and zinc content or improved mineral bioavailability that can be used as valuable experimental material in feeding experiments or eventually for developing a high iron/zinc cultivar. In conventional breeding programs, identification of markers specific for genes involved in micronutrient uptake, mobilization and deposition allows for rapid and precise marker-assisted breeding programs whereby high iron/zinc

traits can be introgressed into elite cultivars from other cultivars or related species. It is, thus, apparent from the results of the Consultative Group on International Agricultural Research Micronutrient Project (this volume) that there is substantial genotype-determined differences among different cultivars in their ability to accumulate iron and zinc in grain that can be explored in plant breeding.

A number of studies have addressed the possibilities for improving iron and zinc uptake in roots and transport and deposition in the vegetative parts of the plants [see (3) for a review]. However, in wheat and rice, the most widely eaten food for the poor in developing countries, only a small fraction (wheat, 20% and rice, 5%) (1,3,4) of the iron is transported from the senescing leaves to the grain. In contrast >70% of the zinc is mobilized (3). Second, in cereals the two minerals are almost exclusively stored in the husk, the aleurone and the embryo and large proportions, therefore, are lost during milling and polishing (5). This implies that the full potential of the genotype-determined increments in iron and zinc content is not realized for improving human nutrition.

It is, thus, apparent that more research is required to elucidate the mechanisms determining mineral mobilization from leaves to grain. In this context, the mechanisms underlying iron and zinc transport and deposition in the different tissues of grain are of particular importance. A second aspect that needs to be addressed is the effect of phytic acid, a compound generally assumed to be the major antinutritional factor for iron and zinc uptake in the human digestive tract (6,7). A reduction in the amounts of phytic acid, therefore, is regarded as an important strategy for improving iron and zinc bioavailability (8).

Iron deposition

In general, iron in plants is stored in vesicles of ferritin, consisting of 24 polypeptide subunits that can sequester some

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² To whom correspondence should be addressed. E-mail: prebenb.holm@agrsci.dk

4000 iron atoms in a nonreactive form as hydrous ferric oxide-phosphate. The ferritin structure can also concentrate some 600 molecules of phosphate and is accordingly also a phosphate storage molecule (9). Ferritin, thus, serves the dual purpose of providing iron for the synthesis of chlorophyll and iron proteins such as ferredoxin and cytochromes and of preventing damage from free radicals produced by iron-dioxygen interactions. Ferritin is mainly present in nongreen plastids, such as proplastids, etioplasts, chromoplasts and amyloplasts (10).

In recent transformation experiments, endosperm-specific expression of a soybean (11) or *Phaseolus vulgaris* (12) ferritin gene in rice resulted in an up to threefold increase or doubling, respectively, of the iron content of the seed. This implies that the low-iron concentration in the seed may not result from low-iron availability for transport, but rather from a lack of sequestering capacity in the seed. However, when the soybean ferritin gene expression was driven by a so-called constitutive promoter to increase ferritin synthesis throughout the plant, there was only an increase in the iron content of the vegetative parts but not in the seed (13).

Phytic acid

In cereal grains, most of the phosphate is bound in phytic acid (phytate, myo-inositol 1,2,3,4,5,6-hexakisphosphate) (14) that primarily is deposited together with protein and minerals in aleurone storage vacuoles (15). In maize, the embryo and the scutellum are the primary depositories for phytate. During germination, inorganic phosphorus is released via the action of the hydrolytic enzyme phytase (myo-inositol hexakisphosphate phosphohydrolase). In the dry seeds and in the digestive tract of nonruminant animals including humans, there is little or no phytase activity (16,17). The undigested phytic acid excreted in the manure contributes significantly to the environmental phosphate load, an increasing ecological concern. There are also economic and resource perspectives. Digestible inorganic phosphate compounds must be added to the animal feed to ensure a proper nutritional composition and the high-quality phosphate mineral resources are being rapidly depleted. Finally, as described above, phytic acid is considered the single most important antinutritional factor for the availability of minerals, such as iron, calcium and zinc (18). Cheryan (19) concluded that phytic acid readily forms complexes with Zn^{2+} , Ni^{2+} , Co^{2+} , Mn^{2+} , Ca^{2+} and Fe^{2+} in decreasing order of stability.

In summary, the indigestibility of the primary phosphate reserves in seeds has major nutritional, environmental and economic consequences and the phytic acid problem has attracted substantial interest in animal feed research. Impairment of phytic acid biosynthesis by mutagenesis in diploid plant species such as maize, rice and barley seems to be a realistic strategy for reducing phytic acid in seeds and mutagenized maize cultivars are marketed. In these cultivars ~50% of the phosphate is present in a free form with a corresponding reduction in the amount of phosphate bound in phytic acid (20).

A third strategy for improving the mineral nutrition of seeds is the addition of microbial phytase to animal feed. Phytases are produced by a number of microorganisms and in most cases are secreted proteins (21). The phytases produced by *Aspergillus niger* var. *ficuum* are the most intensively studied. The *PhyA* gene encodes a phytase with pH optima of 2.5 and 5.0. Addition of *A. niger* phytase to feed has been shown to enhance the release of phosphate from phytate, to reduce the phosphate excretion, and to improve the bioavailability of

minerals, such as Mg, Zn, Cu and Fe, bound to phytic acid (22–24). Phytase addition to animal feed is currently widely implemented in The Netherlands and the United States.

In theory, mutagenesis of polyploid plant species, such as wheat, oilseed rape and soybean, is expected to be much more troublesome as more gene copies are present. For this reason, a transgenic approach has been taken in these crops. A large number of studies have shown that the *A. niger* phytase can be synthesized efficiently in transgenic plants, such as tobacco, canola, alfalfa, and soybean [see (25) for a review]. In planta synthesis of microbial phytase substitutes efficiently for the addition of exogenous phytase as illustrated in feeding trials with monogastric animals (26,27). Denbow et al. (27) showed that the addition of 1200 U phytase activity from transgenic soybeans caused a 50% reduction in the phosphorus excretion from broilers compared with a diet supplemented with an intermediate level (0.16%) of dietary nonphytate phosphorus. Moreover, excretion of phosphorus was reduced on average 11% compared with experiments in which a commercial microbial-derived phytase was added. The reduced excretion reflects an increased phosphorus digestibility of ~10%.

A case study in wheat

Below we describe our own work to engineer wheat for constitutive and endosperm-specific expression of an *A. niger* phytase (25). In summary, we designed constructs comprised of the strong constitutive promoter from the maize ubiquitin 1 gene and the *A. niger phyA* gene. To ensure targeting to the cell wall, we introduced a signal sequence from barley α -amylase upstream of the *phyA* gene (Ubi-Sp-phyA). The constructs were introduced into wheat immature embryos by particle bombardment and transgenic regenerable cell lines selected using the bar-Bialaphos selection technique.

Western immunoblotting with polyclonal antibodies raised against the *Aspergillus* phytase indicated that a phytase of the expected molecular weight had been synthesized and further that the protein as expected was glycosylated. The *A. niger* phytase contains 10 Asn-linked consensus sites (Asn-Xaa-Ser/Thr) and the mature fungal enzyme is known to be a secreted, glycosylated protein (28). At the early and mid-stages of grain filling, the heterologous phytase was primarily synthesized in one or more tissues of the pericarp, seed coat and aleurone, whereas the endosperm was the primary site for phytase synthesis toward the end of grain filling. Progeny analyses revealed that the transgenic trait was transferred to the next generation and that there was an up to fourfold higher phytase activity than measured in wild-type seeds.

Perspectives

The results obtained illustrate that wheat can serve as a host for the synthesis of a fungal phytase and we are currently optimizing the technology for increased levels of expression, endosperm-specific expression, and targeting to other cellular compartments such as storage vacuoles. However, one important aspect remains to be solved. *A. niger* phytases are inactivated at temperatures $>60^{\circ}C$. Accordingly, these phytases will not be useful in transgenic cereals for human consumption because the cooking or baking procedures typically will inactivate the enzyme rapidly. However, bread making involving a leavening process might allow for realization of the phytase potential.

The lack of heat stability of the *A. niger* phytase has led to an intensive search for more heat-stable variants. *Aspergillus fumigatus* secretes a phytase with a broad pH range that can

sustain boiling at 100°C for 20 min (29). In a recent study, rice was engineered for an endosperm-specific expression of this phytase (12). The enzyme was targeted to the cell wall and three lines were identified that synthesized functional phytase. In two of these the phytase activity was a factor twice above background, while in the third line the phytase activity had increased by a factor of 130 times. After boiling rice flour containing the isolated fungal enzyme for 20 min, the enzyme retained 59% of the phytase activity. However, when rice grains were cooked under the same conditions, only 8% of the phytase activity was retained.

These preliminary data suggest that for unknown reasons the in planta synthesized phytase is less heat-stable than when produced in fungi. The *A. fumigatus* enzyme is known to undergo denaturation and inactivation during heating but has in solution the capacity to refold into an active form when the temperature is reduced. Possibly, the cellular environment of the rice endosperm interferes with this process. Alternatively, this heat-tolerance differential may reflect a glycosylation pattern in the heterologous plant host that is different from that generated in the fungal host. In the future, it, thus, will be a challenge to identify other microbial phytases that do not denature in response to elevated temperatures, or via alternative targeting or modification techniques, to ensure a high in planta heat stability using *A. fumigatus* or other microbial phytases.

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