Title

Localization of iron, zinc, and protein in seeds of spelt (Triticum aestivum ssp. spelta) genotypes with low and high protein concentration

Authors

Ozturk, Levent
Altintas, Gamze
Erdem, Halil
et al.

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Introduction

Inadequate nutrition with Fe and Zn can cause serious health problems such as anemia, impaired immune system and poor cognitive function. It is estimated that Fe and Zn deficiency exists in about half of the world population (Welch and Graham 2004). The prevalence and extent of Fe and Zn deficiency among populations is known to be associated with nutritional habits and dependence to cereal based staple foods. Among many efforts to prevent micronutrient malnutrition, enrichment of cereal grains with micronutrients through breeding and micronutrient fertilization is widely accepted as a feasible and sustainable solution (Cakmak, 2008). The existence of a large and useful genetic variation is of great importance for a successful breeding program aiming at improving cereal grains with micronutrients. The genetic variation in micronutrient concentrations of modern cultivated wheat is, however, fairly limited. Compared to modern wheat, the primitive spelt wheat (*Triticum aestivum* ssp. *spelta*) possess a higher genetic variation in protein and micronutrients (Moudry and Dvoracek, 1999; Campbell, 1997), suggesting that it is a potential source for breeding biofortified (micronutrient-dense) modern cultivars.

A close relationship between high grain protein content and micronutrients, particularly Fe and Zn, has been reported in number of studies conducted with modern and wild wheats (Peterson et al., 1986; Cakmak et al, 2004, Ozturk et. al., 2006; Kutman et al, 2009). Increasing evidence suggest that enhancement of grain protein content of wheat would also greatly contribute to biofortification with micronutrients (Distelfeld et al 2007; Morgounov et al., 2007; Kutman et al., 2009). The present study was conducted to (i) demonstrate the localization of Fe, Zn, and protein and (ii) compare the qualitative data produced by specific staining methods with the analytical data produced by instrumental analysis of spelt genotypes having either low or high seed protein concentration.

Materials and Methods

Seed Material and Analysis of Fe, Zn and protein

Seeds of spelt genotypes were selected from a collection of 750 spelt genotypes obtained from USDA-ARS gene bank. Following analysis of Fe, Zn and protein the data was ranked for protein concentration and seed weight. Ten spelt genotypes with similar seed weight were selected for staining experiments of which five had low (i.e. ~12 %) and five had high (i.e. ~25 %) seed protein concentration. Seed Fe and Zn concentrations were analyzed by ICP-OES following acid digestion by a microwave digestion system. Grain protein concentration was calculated by multiplying the grain N concentration by a factor of 5.83 (Merrill and Watt, 1973). Grain N concentration (% dry wt.) was measured by an automated N analyzer.

Staining for Fe, Zn and Protein Localization

Seeds were excised longitudinally along the crease by a scalpel and placed in 20 ml of the following staining solutions for determination of Fe, Zn and protein localization. Iron staining solution (Perls' Prussian blue) was prepared by mixing 10 ml of 2 % potassium hexacyanoferrate (w/v) with 10 ml of 2% HCl (w/v). Zinc staining solution was consisted of 500 mg L^{-1} 1,5-diphenyl thiocarbazone (DTZ) dissolved in analysis-grade pure methanol (Ozturk et al., 2006). Protein staining solution was prepared by diluting the standard Bradford reagent (Bradford, 1976)

by analysis-grade pure ethanol at a ratio of 2:1 (v/v). Staining of seeds for Fe and Zn localization was performed at room temperature for 30 min, whereas for protein seeds were stained at 70^{o}C for 15 min. The stained seed surfaces were examined and photographed by using a reflectance light microscope with a high-resolution digital camera.

Results

The selected high-grain-protein (HGP) spelt genotypes had about two times higher seed protein concentration than the low-grain-protein (LGP) genotypes, while the HGP or LGP genotypes were very similar in their protein concentrations (Table 1). Average Zn and Fe concentrations were 30 and 36 for LGP and 57 and 71 mg kg^{-1} for HGP genotypes (Table 1). The HGP genotypes had about 2-fold Zn and Fe than the LGP. Average seed weights were similar for both LGP and HGP genotypes, suggesting that differences in seed nutrient concentration would not be associated to seed biomass for the selected genotypes. Consequently, there was no correlation between seed weight and Fe, Zn or protein concentration of the genotypes tested. With out any exception, HGP genotypes had higher seed Fe and Zn concentration (Table 1). As a result, there were significant correlations between seed Fe, Zn and protein concentrations of the spelt genotypes.

Table 1: Concentration of protein, Fe, Zn and seed weight of selected low and high protein containing spelt genotypes.

Protein Status	Genotype	Protein	Fe	Zn	Seed wt.
		(%)	(mg kg^{-1})		(mg seed^{-1})
	Sp 211	13	40	26	40
	Sp 912	12	25	33	45
Low-grain-protein	Sp 207	12	35	26	37
	Sp 89	11	36	26	40
	Sp 21	10	45	37	39
Mean		12	36	30	40
	Sp 663	26	59	51	35
	Sp 244	25	79	66	41
High-grain-protein	Sp 926	25	84	54	37
	Sp 818	25	70	53	37
	Sp 804	24	61	60	41
Mean		25	71	57	38

Perls' Prussian blue staining for Fe resulted in staining of scutellum and aleurone in a greenish-blue color (Fig 1). In LGP genotypes with lower seed Fe, the intensity of overall staining was less than the HGP genotypes. In LGP genotypes, Fe staining was more apparent in the scutellum than the aleurone. However, in the case of HGP genotypes, Fe staining was particularly high in the aleurone. When compared to the LGP genotypes, Fe staining was more evident in the endosperm of the HGP genotypes. Incubating the excised seeds in dithizone for Zn localization resulted in staining of the whole embryo in red color, including the scutellum. The

intensity of the red color formation in the embryo was similar in LGP and HGP genotypes. Apart from the embryo, endosperm also stained in a pinkish-red color, particularly in the HGP genotypes. The only apparent difference in Zn staining was the darker staining of the endosperm in HGP genotypes. Staining of seeds for protein resulted in formation of typical blue color of Coomassie dye binding on the protein-rich seed parts. In all spelt genotypes tested, embryo and partly aleurone were the main targets of Coomassie dye binding. Similar to dithizone staining, the major difference in protein staining was the darker staining of the endosperm in the HGP genotypes.

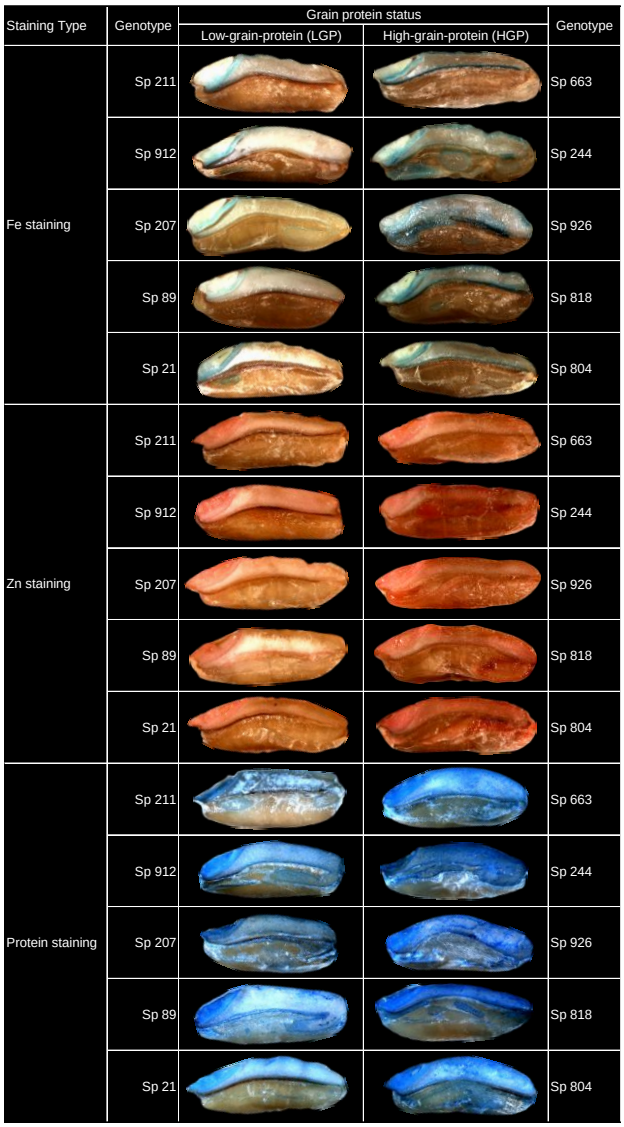

Fig 1: Localization of Fe, Zn and protein in spelt (*Triticum aestivum* ssp. *spelta*) genotypes containing low (i.e. ~12 %) or high (i.e. ~25 %) grain protein concentration.

Discussion

The results pointed out that seed Fe and Zn correlated well with seed protein concentration in spelt genotypes having a contrasting grain protein level. This finding is in line with those found in wild emmer wheat accessions (Cakmak et al., 2004; Peleg et al., 2008) and common wheat (Morgounov et al., 2007, Zhao et al., 2009). The changes in grain size or weight can result in "dilution" or "concentration" effects on grain protein and micronutrients. Since the genotypes used had very similar seed weight, role of any "concentration" or "dilution" effects on the reported Zn, Fe and protein concentrations can be excluded. The results presented here for spelt wheat and other reports (Ozturk et al., 2006; Kutman et al., 2009) suggest that seed proteins serve as a local sink for Fe and Zn in the wheat grain. Possibly, similar physiological or genetic factors are involved in grain accumulation of protein, Zn and Fe. Recent findings indicate the role of NAM genes in increasing grain Zn, Fe and protein concentrations by regulating senescence (Uauy et al., 2006; Distelfeld et al., 2007). Exploitation of genetic variation in grain protein constitutes a valuable breeding tool for enrichment of cultivated wheat with Fe and Zn. Selection or breeding of genotypes for high protein might be associated with a simultaneous selection for higher grain Zn and Fe.

Staining of spelt seeds by using specific dyes for Fe, Zn and protein revealed that Fe is preferentially localized in the scutellum and aleurone; however Zn and protein was localized in the whole germ (i.e. including scutellum, radicle and plumule) and the aleurone as well. Our qualitative findings for Fe and Zn localization are in well agreement with results of Mazzolini et al. (1985) where proton microprobe technique was utilized to analyze site specific variations of mineral nutrients in different seed parts. Mazzolini et al. (1985) reported analytical data for the accumulation of Fe and Zn in the embryonic parts such as the radicle, leaf primordium and scutellum and showed that Fe concentration was almost four times higher in the scutellum than the leaf primordium. Accordingly, in the current study, the Prussian blue staining of excised spelt seeds confirmed that the scutellum and aleurone are the main parts of Fe localization. In the literature, comprehensive information is available on the molecular and physiological aspects of metal uptake, translocation or re-mobilization in plants, and the major bottleneck in seed deposition of metals is thought to be the phloem unloading step (Grusak et al., 1999; Palmgren et al., 2008; White and Broadley, 2009). Very recently, a roadmap for zinc trafficking in the barley grain from phloem unloading until deposition based on gene expression profiles was proposed on a molecular scale (Tauris et al., 2009). The mechanisms affecting metal deposition into different components of seed and its dependency on N nutrition remains to be a challenging scientific research topic.

The majority of Fe was clearly shown to accumulate in the scutellum (Fig 1). One reason for this could be the retention of the phloem unloaded Fe by phytic acid present at very high quantities in the scutellum (Lin et al., 2005; Joyce et al., 2005). The Fe localization method applied in this study was not only helpful in locating the Fe-rich seed parts, but also visually distinguished high- and low-Fe containing spelt genotypes. Therefore, we suggest that Perls' Prussian blue staining can be used as a practical pre-screening method for selection of spelt genotypes with higher seed Fe concentration. The dithizone staining method used for Zn localization was less sensitive in visually distinguishing spelt seeds with up to two-fold difference in average Zn concentration (i.e. 30 vs. 57 mg kg^{-1} Zn). This result seems to be contrary to the results found with modern wheat cultivars (Ozturk et al., 2006). The reason for the absence of a sharp difference in red color formation among low- and high-Zn genotypes is

possibly due to insufficient sensitivity of the dye at the given Zn range (i.e. 30-57 mg kg^{-1}) (see Table 1.). The naturally occurring darker background color of spelt genotypes could also have a negative impact on the red color formation on the excised seed surface. However, it was clearly shown that embryo as a whole was the main site of Zn localization in spelt seeds, with only a little red color formation along the aleurone (Fig 1). The protein staining of seeds showed that embryo and aleurone were the most protein dense parts of the spelt seed and as the protein level increased, dye binding and thus blue color formation also increased virtually in all tissues of the seeds including the endosperm (Fig 1). The similarity in localization of Zn and protein particularly in embryo was noticeable, indicating their co-localization in embryo and in well agreement with the recent results obtained in durum wheat (Kutman et al., 2009). To our knowledge, this is the first report on protein staining of seed tissue in spelt wheat showing a substantial visual difference in relation to seed protein level. We conclude that the proposed method for protein staining will be helpful for pre-screening of large germplasms for high grain protein content. The method is practical and easily applicable requiring only basic lab ware and the well-known Bradford reagent (Bradford, 1976).

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