

Research Paper

Analysis of grain elements and identification of best genotypes for Fe and P in Afghan wheat landraces

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This study was carried out with the aim of developing the methodology to determine elemental composition in wheat and identify the best germplasm for further research. Orphan and genetically diverse Afghan wheat landraces were chosen and EDXRF was used to measure the content of some of the elements to establish elemental composition in grains of 266 landraces using 10 reference lines. Four elements, K, Mg, P, and Fe, were measured by standardizing sample preparation. The results of hierarchical cluster analysis using elemental composition data sets indicated that the Fe content has an opposite pattern to the other elements, especially that of K. By systematic analysis the best wheat germplasms for P content and Fe content were identified. In order to compare the sensitivity of EDXRF, the ICP method was also used and the similar results obtained confirmed the EDXRF methodology. The sampling method for measurement using EDXRF was optimized resulting in high-throughput profiling of elemental composition in wheat grains at low cost. Using this method, we have characterized the Afghan wheat landraces and isolated the best genotypes that have high-elemental content and have the potential to be used in crop improvement.

Key Words: wheat landraces, elements, high-throughput method, best genotypes.

Introduction

Elements are required not only for plant growth, but also as minerals for human nutrition and health; at least 50 are vital for the wellbeing of humans (Tolonen 1990).

Hence it is important to analyze the bioavailability of elements in plants, especially in food crops that are considered important staples. Cereals are one of these crops and in

particular wheat accounts for 20% of the world population's calorie intake. Mg, Fe, and Zn are mainly present in the aleurone layer of bread wheat grains, and whole wheat products are an important source of the daily requirements for these minerals and trace elements in humans (Piergiorganni *et al.* 1997). Potassium ions (K⁺) are the major cationic osmoticum in plants, detailed reviews of K⁺ homeostasis can be found elsewhere (Pettigrew 2008). Phytate, which is abundant in cereal grains, reduces the bioavailability of micronutrients, particularly Zn and Fe, to humans and monogastric animals (Fan *et al.* 2008). The chemical composition of wheat grain was well reviewed by Šramková *et al.* (2009). However, this crop contains suboptimal quantities

Communicated by Luigi Guarino

Received March 8, 2016. Accepted July 7, 2016.

First Published Online in J-STAGE on August 23, 2016.

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of micronutrients, especially iron (Fe) and zinc (Zn), and most of this content is removed by milling. To measure the bioavailability of elements, or the ionome, the quantitative and simultaneous measurement of the element composition of living organisms requires the selection of specialized instrumentation and sample preparation protocols based on various selection criteria. So far several analytical procedures have been used to determine the concentrations of elements in plants (Salt *et al.* 2008). Atomic spectroscopy like FAAS (Flame Atomic Absorption Spectrometry) and ICP-MS (Inductively Coupled Plasma Mass Spectrometry) are the usual technologies for this purpose. However, in most methods plant samples are destroyed by elemental acids prior to measurement, which may cause contamination and affect the results. In addition, preparation of the samples is different depending on the element being measured, which is time-consuming and expensive. X-ray fluorescence analysis (XRF) has been widely used for element analysis in geochemistry, manufacturing and forensic science (West *et al.* 2012). Energy-dispersive XRF (EDXRF) is the most commonly used bench-top method as an alternative technology where the simultaneous measurement of multi-elements in many samples can be done in a short time span. In wheat the application of EDXRF started recently but has been limited to Zn and Fe content analysis (Paltridge *et al.* 2012, Velu *et al.* 2011, 2012). In another example, Rüdiger *et al.* (2009) used EDXRF to determine elements in 20 plants that are commonly used medicinally in Nigeria. They reported 14 elements including both macro- and microelements in the samples. Various analytical techniques, EDXRF, FAAS, and Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES), were applied to investigate the concentration of elements in herbal drug samples from plants originating from the Lamiaceae family (Ražić *et al.* 2005). The content of multiple elements in the shoots and roots of 3 plants growing in 3 zones, which had different geochemical and mineralogical characteristics in the Cartagena-La Unión range, was determined by EDXRF (Gonzalez-Fernandez *et al.* 2011). Interestingly, the concentration of elements in these plants was dependent on the tissue and growing conditions.

Germplasm is key for any crop improvement. Due to sub-optimal availability of elements in the wheat varieties currently grown, it is vital to look for natural genetic variation present in the wheat gene pool. Landraces are one source of germplasm where there is a high possibility of finding plants with variations in elemental concentration as they may have undergone unique adaptation to enable them to grow under low-input conditions. Hence, the Afghan wheat landraces collected by Dr. Hitoshi Kihara and his team between 1955 and 1970, and kept at the Kihara Institute for Biological Research were used for this study (hereafter referred to as KAWLR – Kihara Afghan Wheat Landraces) (Manickavelu *et al.* 2014). The objective of this work was to identify wheat core-set germplasm from the landraces using EDXRF technology that maximizes the content

of desirable elements for human health, and plant growth and development, as a new genetic resource for improving the elemental composition of wheat.

Materials and Methods

Plant material

In total, 446 accessions of KAWLR along with 10 reference lines were grown in field conditions with standard crop management (Manickavelu *et al.* 2014). For element analysis, 266 landraces along with all reference lines, which yielded good grain shape, were chosen (**Supplemental Table 1**; <http://shigen.nig.ac.jp/wheat/komugi/>). While selecting samples, border plants were excluded and the grains of the remaining plants were pooled from each plot. Twelve seed grains were selected randomly from the pooled grains for elemental analysis. To avoid any contamination of soil, threshing and grain selection were carried out by hand. After selecting the best genotypes, the same grain grown in different locations (Agricultural Research Stations, Darulaman and Badambagh, Afghanistan, and CIMMYT Agricultural Research Station, Haymana, Turkey) was taken for re-analysis and confirmation.

Measurement of element content using EDXRF

The wheat grains were completely dried by incubating at 65°C overnight. Four grains were wrapped in powder paper and crushed using a hammer. The crushed grains were put between 2 tungsten beads of 6.0 mm diameter (Tungsten Beads TC-604, Bio Medical Science) in a tube (Master Tube Hard MT020-01H, Bio Medical Science, Japan) and homogenized using a shaker (Shaker Master Auto, Bio Medical Science) for 4 minutes. The powder derived from each tube was placed in a plastic ring of 8 mm external diameter, 6 mm internal diameter, and 3 mm height without any chemical treatment and pressed at 20–32 MPa to make a tablet of 10 mm external diameter, 8 mm internal diameter, and 1 mm height. Three tablets per sample were made to measure the element levels. EDXRF (EDX-700-HS50, Shimazu, Japan) was used with a voltage of 0 to 20 KeV and data was collected after 250 seconds. The results were analyzed using the in-built software (DXP-700, Shimazu, Japan) of EDXRF. In order to express the elemental concentrations as % or ppm depending on the content of each element, we made standard curves for K (potassium), Mg (magnesium), Fe (iron), Ca (calcium), and P (phosphorus) by using standard reference materials (SRMs), which were obtained from the National Institute of Standards and Technology. SRMs were apple, peach, tomato, and spinach leaves (SRM1515, 1547, 1573a, and 1570a, respectively).

Measurement of element content in wheat grains using ICP-AES

To further compare the values of EDXRF, the ICP-AES analytical method was used on selected genotypes and reference lines. Approximately 0.3 g of grain (ca. 10) were

washed in 0.1 M HCl for 3–5 seconds and dried to a constant weight in a hot oven (>50°C). This was then digested in nitric acid in a glass tube at 200°C on a hot stove, and H₂O₂ (20 µL) was added 2–3 times. When all the grain had been digested, the remaining white residue was further resolved with 10% nitric acid (v/v). K, Mn (manganese), Fe, Cu (copper), Zn (zinc), Na (sodium), P, and S (sulfur) were measured in the 3 replicates by inductively coupled plasma atomic emission spectroscopy (ICP-AES; Ciros CCD, Rigaku, Japan).

Data analysis

One-way analysis of variance (ANOVA) was carried out to test the significance of among-landrace variation. For each element, an average of 3 replications was obtained for each landrace, and the averages of all landraces were standardized to have a mean of 0 and a standard deviation of 1 for further data analysis. Hierarchical cluster analysis using Ward’s method based on Euclidian distance was performed to understand the relationships between elements and between landraces. A heat map representing the quantity of elements was generated based on the results of hierarchical cluster analysis. Least significant difference (LSD) was calculated using Fisher’s LSD. We used R for all these analyses.

Results

Measurement of elemental composition in wheat

We employed a destructive method for sample preparation to determine the complete profile of the elements in whole grain of KAWLR. The optimization of the sampling method was established by considering all factors and a tablet-like sample was prepared for each germplasm on which the measurements were to be taken. Out of the many elements, 3 major, K, P, and Mg, and 1 minor, Fe, were detected in our material harvested from a field in Maioka, Japan (Supplemental Table 1, Table 1). One of the macroelements, Ca, was not detected, indicating either that there was poor availability in the wheat grain or that it could not be measured by EDXRF. In contrast, the bulk amount of

some of the macroelements reflects their importance in wheat; for example, the level of K is more than both P and Mg together. This result corresponds with previous reports using wheat grains (Ryan *et al.* 2004). Additionally, the abundance of K was more consistent than that of the other macroelements (P and Mg), which is apparent from the lower standard deviation (Table 1, Fig. 1). These results coincide with those of Ryan *et al.* (2004), in that minor variations in the abundance of N and K occurred in relation to that of P in grains harvested from wheat grown under various fertilizer regimes. This may indicate there is strict regulation of K levels in wheat grains. Of the microelements, only 1, Fe, was measured but it is crucial for human health. The range between the maximum and minimum content of this element in the landraces is huge compared with the reference lines. This shows the diversity of the landraces and the potential they offer for use in crop improvement to aid biofortification.

In order to see the advantage of our methodology over previous studies we compared our data to the Fe data of the International Maize and Wheat Improvement Center (CIMMYT) that was obtained with non-crushed grains using EDXRF (data not shown). We found that the values of all our samples are higher mainly due to the modified methodology we used. Although the results may be affected by

Table 1. Elemental availability in Kihara Afghan Wheat Landraces (KAWLR)

	Content in KAWLR				Content in reference lines			
	K (%)	P (%)	Mg (%)	Fe (ppm)	K (%)	P (%)	Mg (%)	Fe (ppm)
Ave	1.567	0.531	0.169	84.710	1.547	0.471	0.152	85.773
SD	0.054	0.081	0.047	12.502	0.029	0.046	0.027	9.347
Max	1.791	0.914	0.388	122.200	1.539	0.578	0.214	97.282
Min	1.430	0.339	0.075	55.142	1.504	0.397	0.120	70.726

The results are presented as the average (Ave), standard deviation (SD), and maximum (Max) and minimum (Min) values for each elemental content in grains of 266 landraces and 10 reference lines grown in a field in Maioka, Japan.

Each elemental concentration is expressed as % or ppm depending on the element.

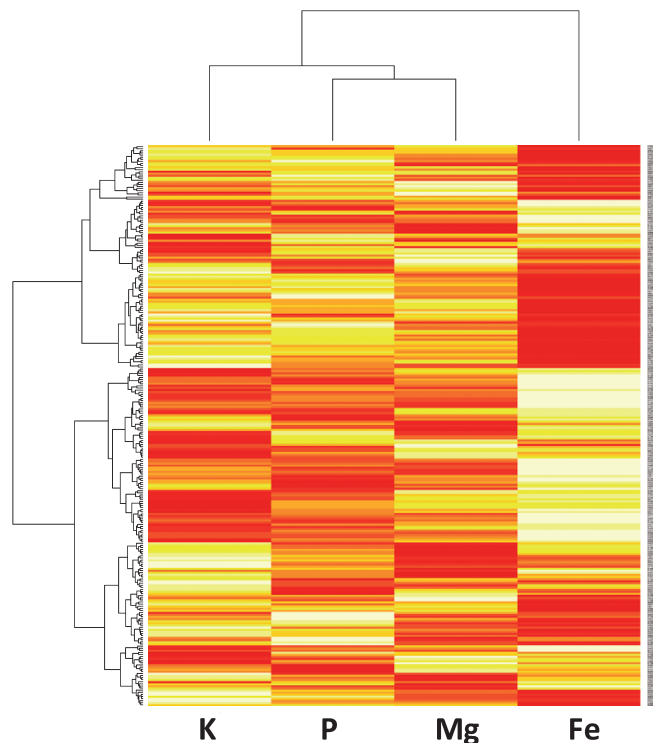


Fig. 1. Correlation clustering of elements in Kihara Afghan Wheat Landraces (KAWLR). The correlation between elements and genotypes is shown. The brighter the color the higher the accumulation of the elements, Iron (Fe), Phosphorus (P), Magnesium (Mg), and Potassium (K), in grains harvested from each KAWLR. White and red indicate higher and lower accumulation, respectively.

location, i.e. soil nutrients, the data clearly show a significant difference. Hence it is apparent that using a destructive method of sample preparation is advantageous in order to see the complete profile of elements present in wheat grains.

ANOVA and hierarchical clustering analysis of elements

As a result of one-way analysis of variance (ANOVA) among landraces, the variation was highly significant ($P < 0.001$) for all 4 elements (Supplemental Table 2). Hierarchical cluster analysis indicates that the quantity of Fe is more likely to have an opposite pattern to the quantity of the other elements, especially K (Fig. 1). P and Mg shared similar patterns to the other elements. In the KAWLR, 2 major and a further 4 sub-clusters of landraces were derived from the availability of elements.

Identification of potential landraces

The data generated has helped to select a core-set of landraces with a higher elemental content that can be used for further studies. As a first selection, we isolated a total of 42, 29, 31, and 36 landraces, which showed more than 0.6% P, 1.6% K, 0.22% Mg, and 100 ppm Fe content, respectively. Some of these genotypes and their elemental content were compared with reference lines and are shown in Fig. 2. Although many candidates that could be used as genetic resources were identified, elemental levels in grains are highly environmentally dependent, and are particularly sensitive to the concentration of these elements in the soil (Srinivasa *et al.* 2014). Therefore, the core-set lines were grown in 3 different locations, in Badambagh and Darulaman, both in Afghanistan, and in Turkey. The results from these trials were compared to those previously obtained. This resulted

in the isolation of the 2 best landraces for high P content (756 and 782) and the 7 best for Fe content (504, 528, 665, 700, 718, and 761), all were from the KAWLR, because these lines had better accumulation than the reference lines in all the locations in which they were grown (Tables 2, 3). The results also indicate that they can be effectively utilized for further studies. In order to further validate the best candidates based on the EDXRF method, 2 landraces for P and 4 landraces for Fe were re-measured using the ICP-AES method (Fig. 3). The results confirmed the higher bioavailability of elements in selected landraces. It also shows the reliability of EDXRF compared to the ICP-AES method, and although the latter is more sensitive it is more expensive per sample.

From previous studies it has been unclear whether there is a relationship between grain weight and elemental composition (Morgounov *et al.* 2007, Velu *et al.* 2012). We measured grain weight of the whole set and found a negative

Table 2. Stability of elements showing endogenous levels of P in the grains

	Badambagh	Darulaman	Turkey	Average	LSD
586	0.552	0.484	0.411	0.482	0.030
664	0.524	0.483	0.420	0.475	0.144
665	0.454	0.527	0.425	0.468	0.131
666	0.328	0.467	0.419	0.404	0.055
756	0.624	–	0.463	0.543	0.091
782	0.509	0.492	0.513	0.508	0.129
857	0.435	0.533	0.464	0.477	0.059

Grains harvested from landraces (3-digit numbers) grown at 3 different locations (Afghanistan – Badambagh and Darulaman; Turkey) were compared and the best genotypes are shown.

LSD indicates least significant difference.

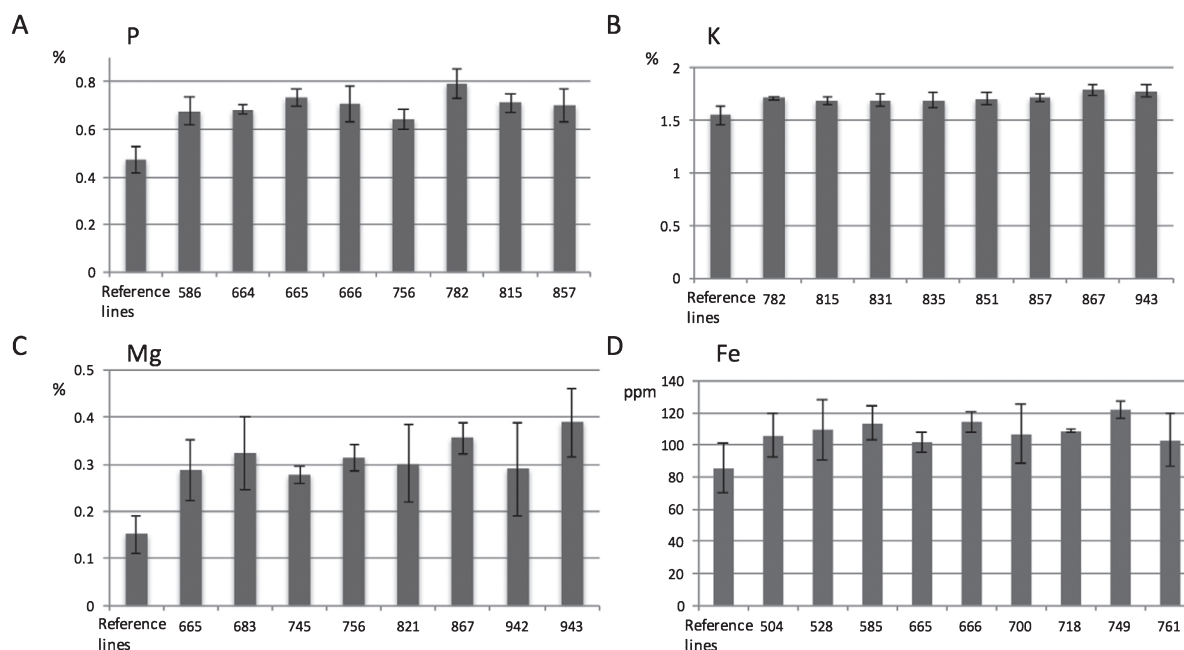


Fig. 2. Core-set selection of Kihara Afghan Wheat Landraces (KAWLR) with respect to elements (P, K, Mg, and Fe). The landraces, indicated by the 3-digit numbers, were selected and plotted with the reference lines. Error bars indicate standard deviation.

Table 3. Stability of elements showing endogenous levels of Fe in the grains

	Badambagh	Darulaman	Turkey	Average	LSD
504	106.12	96.589	134.397	112.368	33.112
528	99.511	95.562	130.238	108.437	42.430
585	107.52	125.357	150.571	127.816	30.162
665	—	121.875	179.066	150.471	38.811
666	106.715	102.951	97.139	102.268	25.569
700	96.195	104.386	151.628	117.403	29.436
718	87.330	108.788	149.558	115.225	43.412
749	87.433	89.507	—	88.470	13.024
761	99.718	100.826	131.565	110.703	14.293

Grains harvested from landraces (3-digit numbers) grown at 3 different locations (Afghanistan – Badambagh and Darulaman; Turkey) were compared and the best genotypes are shown.

LSD indicates least significant difference.

correlation between grain weight and the amounts of the macroelements K, P, and Mg. In contrast, we did not find a significant correlation between grain weight and the microelement Fe (**Supplemental Table 3**). It has been found that there is no correlation between the weight of a thousand grains and the content of Zn, a microelement, in adapted wheat lines but there is a negative correlation in unadapted wheat (Zhao *et al.* 2012). Taking this report into account, it is understandable that we found no correlation between grain weight and the microelements as the material that we used is well adapted to local conditions. Since there was a possibility of a negative correlation between grain weight and elemental composition, the grain weights of selected landraces grown in Barambah in Afghanistan were confirmed (**Table 4**). As a result, landraces 782 and 528 were chosen as the best resources as they both have high elemental composition along with good grain weight, and will be ideal candidates for use in breeding materials to improve adapted wheat varieties in Afghanistan.

Table 4. Comparison of grain weight and elemental composition

Landraces	Average (mg) ± SD	Phenotype (content)
756	29.4 ± 7.4	P (0.624%)
782	42.4 ± 5.1	P (0.509%)
504	38.3 ± 7.0	Fe (106.118 ppm)
528	41.9 ± 7.4	Fe (99.511 ppm)
585	36.9 ± 7.0	Fe (107.519 ppm)
665	25.6 ± 6.4	Fe (121.875 ppm)
700	37.0 ± 5.7	Fe (96.195 ppm)
718	23.8 ± 6.3	Fe (87.330 ppm)
761	21.9 ± 8.0	Fe (99.718 ppm)

The landraces which showed high accumulation of P and Fe were selected and grown in a field in Badambagh, Afghanistan.

The grain weights of these landraces were measured (n≥10). The element that each landrace accumulated most and its amount are presented as the phenotype (content).

The results are presented as the average and standard deviation (SD).

Each P and Fe concentration is expressed as % or ppm depending on the content.

Discussion

The ionic potential of the orphan wheat germplasm of Afghanistan, KAWLR, was determined by EDXRF using a modified sampling method. For sample preparation, a destructive method was established with the aim of analyzing the elemental composition in the whole grain. In general, EDXRF is considered a high-throughput method of element analysis and can be used without any pre-treatment (Ražić *et al.* 2005). It is known that bran is rich in B vitamins and elements, and that the germ also contains reasonably high levels of elements (4.5%) (Šramková *et al.* 2009). The Fe data obtained with non-crushed grains actually showed that in order to know the complete profile of the elements in a grain, it is necessary to use a destructive method. The optimizations in sampling for measurement using EDXRF produced

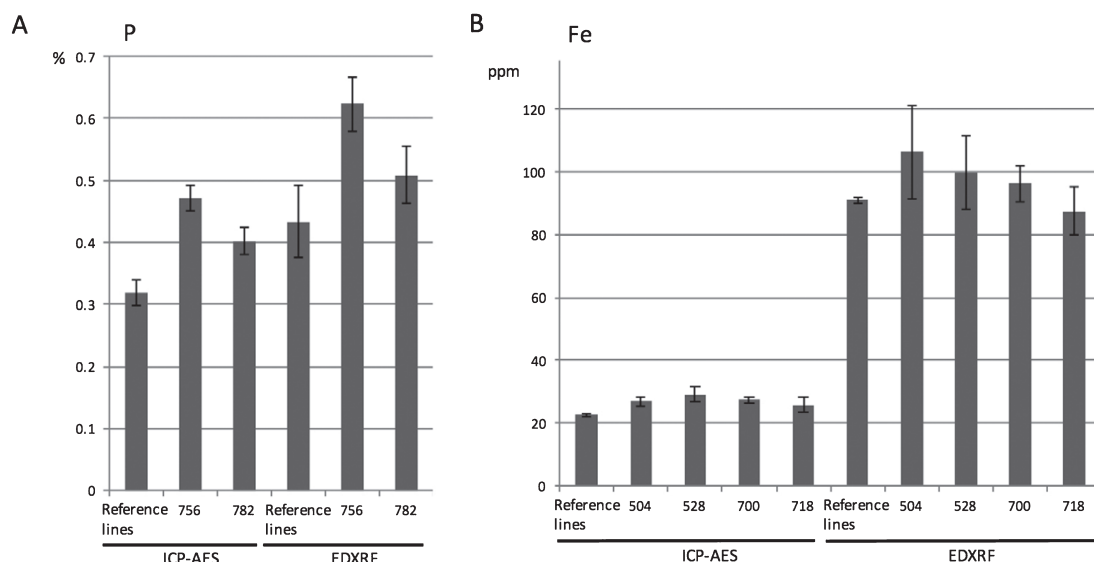


Fig. 3. Comparison of elemental analysis. The selected landraces for P and Fe and the reference lines were analyzed with ICP-AES.

a high-throughput evaluation of the elemental composition in wheat grains at low cost.

Although the range between the maximum and minimum content of all detectable elements in whole grains was huge compared with reference lines, the range of P and Fe content is especially remarkable (**Table 1**). The differences in concentration between the soil solution and the plant cells are more than 2,000-fold and the concentration of inorganic phosphate (Pi) in the soil solution (1 μM) is lower than the Km for plant uptake (Bielecki 1973, Schachtman *et al.* 1998). Moreover, bound P, which constitutes insoluble complexes with cations, such as aluminum and iron under acid conditions, is unavailable for plant uptake (Runge-Metzger 1995, Vance *et al.* 2003, von Uexkull and Mutert 1995). P is, therefore, one of the most limiting elements of the 17 essential elements for plant growth and its availability is limiting crop yield on 30–40% of arable land in the world (Sanchez and Uehara 1980). Hence, although P is not one of the most important elements to target in the human diet, it is vital for the plant to be able to take up and keep as much P in its cells as possible to ensure a good yield (Hazell 1985). Fe content was effectively targeted by its incorporation into the HarvestPlus strategy for Fe and Zn improvement (Velu *et al.* 2014). Micronutrient malnutrition ('hidden hunger') presently affects over 40% of the world's population and is increasing, especially in many developing countries. This type of malnutrition causes increased mortality and morbidity rates, reduction in labor productivity, quality of life and so on. Presently, iron and iodine deficiencies are seen as the most urgent issues within nutrition communities and health-care officials (Welch and Graham 2004).

In this study, we try to identify the best genotypes or core-set that show consistent endogenous levels of elements. The trial identified the 2 and 7 best landraces showing high P and Fe content, respectively, in all the locations in which they were grown. The results are shown in **Tables 2, 3**. The best genotypes for P were collected from the North Western region of Afghanistan and landraces from the Central and Northern regions of Afghanistan showed higher Fe content. The soils of Afghanistan are alkaline and 50% have a pH between 8 and 8.5, and are generally rich in alkaline earth carbonates (Edouard 1972). Although phosphorous and micronutrients (Zn & Fe) are usually lacking in calcareous soil, the selected genotypes have the ability to take up these nutrients. The results show the highly heritable nature of the selected lines. They also disprove the previous conclusion that breeding for high Fe concentration may be an illusion due to the strong influence of the environment (Oury *et al.* 2006). In contrast to P and Fe, none of the landraces showed similar accumulation of K and Mg in the various locations (data not shown). This result suggests that the effect of the environment varies between elements. Other studies have reported that significant interactions between genotype and environment were seen for Zn and Fe grain concentrations (Joshi *et al.* 2010, Morgounov *et al.* 2007, Ortiz-Monasterio *et al.* 2007). It was also found in this study that the Fe con-

centration in grains which were harvested from plants grown in the Turkish field was higher than that from other fields, showing a strong interaction between Fe content in the grain and the environment (**Table 3**). However, high heritability of both Fe and Zn concentrations was observed (Velu *et al.* 2012), showing there is still a strong genetic component to the accumulation of these in the grain. Hence our study clearly shows the possibility of an environmental effect for some of the elements (K and Mg) and at the same time reports a high chance of heritability of Fe and P levels. Soil analysis was not taken into consideration, as the main aim of this study was to identify germplasm that has the capacity to take up nutrients irrespective of its location.

The comparative evaluation of EDXRF and ICP-AES indicates that the detection value for P and Fe was higher in EDXRF than ICP, although the trend was picked up by both methods, which confirms the analysis (**Fig. 3**). Particularly apparent with Fe, the identified landraces showed extreme accumulation in EDXRF in comparison with previously reported germplasm (Zhao *et al.* 2009). As our methodology showed high values even for the reference lines, which are supposed to be less than the HarvestPlus target of 35 ppm, there was also the possibility that EDXRF showed relatively high concentrations of Fe in comparison with other methods such as ICP-AES. The comparative evaluation of EDXRF and ICP-AES is remarkable, because, in this report, measurement using EDXRF indicates a higher concentration of Fe than that using ICP-AES (Djingova *et al.* 1998). In fact, ICP-AES analyses using some landraces showed that these landraces including reference lines have less than the target, although the relative quantities of Fe in the landraces and reference lines were invariant (**Fig. 3**).

This study reports the elemental profiles of wheat using EDXRF technology. This is the first time the KAWLR have been characterized and the best genotypes for high-elemental content isolated. Some of the selected materials have already been incorporated in international as well as in-house breeding activities. Currently, a huge initiative, HarvestPlus, by the Consultative Group on International Agricultural Research (CGIAR) targets Zn, Fe, and Vitamin A for biofortification (Bouis 2014). However, so far only Zn has been targeted in wheat for many reasons including the lack of germplasm. In our study we have found 9 landraces with the potential to be incorporated into the wheat gene pool. Of these, 2 landraces, 782 and 528, have a high P and Fe composition, respectively, with good grain weight and will be ideal candidates for use in crop improvement (**Table 4**). Notably, 782 may be particularly valuable as it has a high P composition and there is a negative correlation between grain weight and P composition (**Supplemental Table 3**). The comparison of grain sources from different locations proves the existence of high heritable elemental traits, as in the case of P and Fe, and low heritable traits such as for K and Mg. This will help to design genetic improvement accordingly. Also the results will help quantitative trait locus (QTL) mapping, which is at present in progress.

Acknowledgements

We thank Ms. Kyoko Yokomizo, Ms. Mieko Komochi, and Ms. Tomomi Sawada for measurement of elemental profiles using EDXRF, Dr. Naruoka for the initial care of materials, and Dr. Marta for providing selected seed material from Turkey. This work is the outcome of a SATRPES-Afghan project funded by JST (Japan Science and Technology Agency) and JICA (Japan International Co-operation Agency).

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