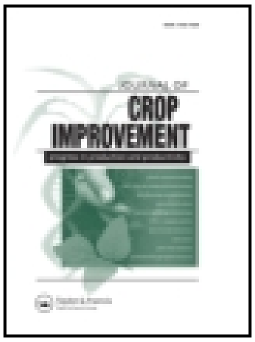

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Searching a spring wheat mutation resource for correlations between yield, grain size, and quality parameters

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ABSTRACT

*To broaden genetic variation, an irradiated wheat (*Triticum aestivum L.) M_5 population was generated in the background of spring wheat cv. Almaken. This resource was used to measure components of productivity, including grain number and grain weight (GW) per main spike, GW per plant (GWP), 1000-grain weight (TGW), grain size and grain shape, and some quality parameters. Some mutant lines, mostly in the 200-Gy-dosed germplasm, had 2–4 times higher grain iron and zinc concentrations and 7–11% higher protein content relative to the parent line. Some irradiated lines had significantly larger TGW, and grain area (GA), length, and width than the parent, cv. Almaken. The largest GA and grain length (GL) were 30–40% greater than those of the parent. Correlations for Zn concentration versus GA = 0.191, $p < 0.01$, grain protein content (GPC) versus GA = 0.128, $p < 0.05$, GPC versus GL = 0.113, $p < 0.05$, and GPC versus grain width = 0.191, $p<0.001$ were observed in 200 Gy-dosed mutants. In 100 Gy-dosed mutants, correlations for Fe concentration versus GWP = 0.302, $p < 0.001$ and Fe concentration versus TGW = 0.153, $p < 0.01$ were found. The mutant lines showed the capacity to biofortify wheat grain without negatively impacting on crop productivity and this population offers promising donors for improving grain parameters such as GA, length, and width and quality. The data presented showed how the genetic variation generated through radiation could be used to test the linkage between various important grain parameters.

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Introduction

Wheat (*Triticum aestivum* L.) is a major cereal crop for both human and animal nutrition, providing 28% of the world's edible dry matter and up to

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60% of the daily calorie intake in developing countries (FAOSTAT 2008; http://faostat.fao.org; Godfray et al. 2010). It is also a major source of essential minerals for human nutrition (Balyan et al. 2013; Borrill et al. 2014; Lafiandra, Riccardi, and Shewry 2014; Pearce et al. 2014; Shewry et al. 2011). Iron and zinc deficiency affect more than half of the world's population (Borrill et al. 2014; Campos-Bowers and Wittenmyer 2007). Wheat is an essential component of most human diets, and for this reason, there is a need for genetic enhancement of the nutrient value of this crop to provide a cost-effective way of diminishing global micronutrient malnutrition (Welch and Graham 2004). Biofortification through plant breeding rather than food supplementation has multiple advantages (Borrill et al. 2014; Velu et al. 2013). Across years, wheat breeding has reduced genetic diversity by replacing traditional cultivars with modern higher yielding varieties (FAO Document Repository 2013). Plant breeding efforts have pushed for higher agronomic yield and this has resulted in decreased nutritional quality; for example, many years ago, it was shown that yield is negatively correlated with protein content (Bhatia and Rabson 1976). During the past 50 years in the United Kingdom, the use of high yielding, semi-dwarf varieties has been accompanied by decreased grain zinc, iron, copper, and magnesium contents (Fan et al. 2008).

The nutritional value of a crop is dependent on the quantity and composition of the protein, and grain protein content (GPC) is an important nutritional quality trait with great impact on end products (Balyan et al. 2013). The GPC, as a quantitative trait (high protein for bread making and low protein for animal feed and other uses), is routinely screened in wheat breeding programs. The current range of GPC variation in commercial cultivars is limited because breeding for improved GPC is difficult. Moreover, there is a negative correlation between GPC and grain yield (Bhatia and Rabson 1976; Brevis and Dubcovsky 2010). Improvement in GPC in modern wheat cultivars, without yield reduction, requires developing genotypes with higher N-use efficiency, which involves improved N-uptake and/or N-remobilization (Brevis and Dubcovsky 2010). Elevated temperature and elevated [CO_2] under future climatic conditions are predicted to decrease yield and increase grain protein deficiency (Ingvordsen et al. 2016; Kang and Banga 2013).

The nutritional value of wheat depends on yield quality traits, but grain, physical parameters may also be important. Wheat yield is related to grain weight (GW), which in turn is related to grain size and shape (Huang et al. 2015). Grain size and shape are important traits in breeding programs; they are regarded as phenotypically stable yield components, influencing end-use qualities, such as milling and flour production (Gegas et al. 2010). Grain size is characterized by length, width, and area (Gegas et al. 2010; Okamoto et al. 2012) and seed morphology may be important for flour production. Therefore, increasing genetic diversity may provide better traits to be used for the genetic improvement of wheat with respect to food production and end-use qualities (Okamoto et al. 2012).

Successful breeding for yield-associated traits and grain quality traits requires genetic variation, which has to be distinguishable from environmental effects. Advances in desired traits must be achieved without negative effects on other important traits, such as yield and resistance to biotic and abiotic stresses. It is widely considered that the genetic diversity of major crops has decreased primarily as a consequence of breeding; including the repeated use of local germplasm and the adoption of breeding schemes that do not favor genetic recombination (Akfirat and Uncuoglu 2013; Reif et al. 2005). Mutagenesis, a powerful tool for wheat improvement, has been used for yield improvement, but this technique has not been as widely applied for improving nutritional quality of the grain, including protein, iron, and zinc concentrations. During the past 80 years, new mutant varieties were produced for both seed and vegetatively propagated crops (Maluszynski et al. 2009; Parry et al. 2009; Shu and Lagoda 2007; Tomlekova, Kozgar, and Wani 2014). The FAO/IAEA Mutant Variety Database in 2014 reported 3220 mutant plant varieties of 214 plant species worldwide (http://mvgs.iaea.org/). Radiation and chemical mutagens can be used to introduce new genetic variability, from which mutants with desired traits can be selected (Shu and Lagoda 2007). The use of induced novel genetic variation is particularly valuable in major food crops that have restricted genetic variability (Parry et al. 2009).

Gamma irradiation has been shown to generate a mix of small (1–16 bp) and large (up to 130 kbp) deletion mutations in the genomes of Arabidopsis and rice (Cecchini et al. 1998; Morita et al. 2009). In the present study, gamma irradiation doses of 100 and 200 Gy were used to expand the genetic diversity of spring wheat lines and then grow selected high-yielding lines through to the M_5 generation. We investigated yield components, such as grain number per main spike (GNS), grain weight per main spike (GWS), grain weight per plant (GWP), 1000-grain weight (TGW), phenotypic structure of grain size and shape variation, grain protein content (GPC), grain iron concentration (GIC), and grain zinc concentration (GZnC). Correlation analysis was used to test the relationships between grain physical parameters and quality components, such as protein and metal content, in this irradiated population. We argue that this mutant germplasm is a genetic resource showing the potential to breed for the improved grain nutritional quality and increased yield.

Materials and methods

Plant material

Seeds of the spring bread wheat awn-less variety "Almaken" (*T. aestivum* L.) were irradiated with 100 and 200 Gy doses from a ^{60}Co source at the Kazakh Nuclear Centre. Seeds were planted after irradiation to raise M_1 plants (Kenzhebayeva et al. 2014). The M_1 generation was grown in the experimental field of the Kazakh Institute of Agricultural and Farming near Almaty (43°15′N, 76°54′E, elevation 550 m above mean sea level). Single spikes were harvested from each plant for the M_2 generation, and selection of the best lines based on yield of individual plants continued to M_5 generation. In the M_3 and M_4 generations, some seeds were grown in a randomized block design for a Fusarium head blight resistance screen (Kenzhebayeva et al. 2014). Seed was gathered from the main spike; although tiller number and size varied, each plant produced only a single main spike. Seeds from the best yielding mutant lines were selected individually in each generation. The selection criteria for these lines were GWS and GWP, which were applied in the M_3 and M_4 generations (2011 and 2012) and based on the values for the parent cv. Almaken grown under the same trial conditions. For example, in 2011, the parent line had a mean GWS of 0.95 ± 0.4 g and a mean GWP of 1.7 ± 0.2 g. In 2012, for the M_4 generation, the threshold criteria chosen for selection were GWS > 1.2 g and GWP > 2 g for mutant lines. The initial number of lines in the M_1 generation was 300 each for the 100- and 200-Gy radiation doses. In the M_3 generation, 61 lines (20%) were selected from the 100-Gy radiation dose population and 48 lines (16%) were selected from the 200-Gy dose population. The same numbers of lines for each radiation dose were selected for the M_4 generation.

After harvesting the M_5 plants, 15 lines from the original 100 Gy radiation dose were selected. These lines were numbered as follows: 75(2), 76(2), 76(3), 79(1), 79(5), 81(1), 82(2), 82(4), 82(5), 84(2), 84(4), 89(5), 89(8), 91(1), and 91(2). Another 15 lines were selected from the 200-Gy radiation dose and these were numbered: 94(2), 94(4), 95(2), 95(3), 95(5), 95(7), 95(8), 98(1), 98 (2), 98(4), 98(6), 101(1), 101(3), 101(5), and 101(6). These two populations, selected from the two different levels of radiation, were then used for further analysis. Grain samples from each mutant line, together with the parent Almaken, were planted in a field trial for further evaluation. Each line was grown in three-replicate three-row plots, 2 m long, 1.20 m wide, with 20 cm between rows and 30 seeds planted per row. The trial was managed according to locally recommended agronomic practices. Phosphate was applied as superphosphate (19%) and ammonium phosphate (46%) at 250 kg/ha plowed in during the previous autumn. Nitrogen application as ammonium nitrate (46%) was made at 100 kg/ha in the spring. The soil was a brown medium-loess type (pH = 7.5); the topsoil (0–20 cm) humus content was 2–2.6% and the total nitrogen content was 0.189–0.2%, with mobile phosphorus varying from 16.3 to 17.8 mg/kg and exchangeable potassium from 451 to 498 mg/kg for dry soil. Ten randomly selected spikes or plants from each line were taken for analysis (five samples per row). To record yield-associated traits, the following plant parameters were measured: GWP, grain number per main spike (GNS), and GWS. The TGW was calculated as the mean weight of three sets of 100 grains per line multiplied by 10. Dry GW was measured up to two decimal places using a standard laboratory balance.

Grain morphology analysis

Morphology measurements were made by using the WinRHIZO image analysis system (version 1.38 2007, Reagent Instruments Inc., Canada) for grain length (GL) and grain area (GA) on 50–60 grains per line. The GL/GW ratio was also calculated.

Determination of GPC

GPC was determined with near-infra red reflectance spectroscopy on whole grains (ZX50 Portable Grain Analyzer, USA) using proprietary calibration software provided (Zeltex Hagerstown, MA, USA). Three repetitions were done using 25 grains per line.

Assessment of grain iron and zinc concentrations

Grain samples (100 and 200 Gy M_5 mutant lines and cv. Almaken) were washed with sodium dodecyl sulfate (0.1%), rinsed in deionized water, dried to a constant weight at 65–70°C, and then ground with a mixer mill (Retsch MM400 GmbH). A 0.2-g sample was digested with a mixture of nitric acid (65% HNO_3, analytical grade) and hydrogen peroxide (30% H_2O_2) (5:1, v/v) using digestion automat K-438 and scrubber K-415 triple scrub systems (BUCHI Corporation, New Castle, USA). Sample digestion was done using the following temperature regime: heating to 70°C for 40 min, 90°C for 45 min, 130°C for 3 hr, 150°C for 1 hr, cooling and then waiting until 25°C was reached. The sample was diluted to 20 mL with twice-distilled water from a duran D50 glass system (GFL 2102, Germany). Iron and zinc concentrations were measured using flame atomic absorption spectroscopy (Model NovAA350, AnalytikJena, Jena, Germany). Measurements of mineral nutrients were checked against the certified reference values from the State standard samples LLC "HromLab", Zn 7837-2000, Fe 7835-2000 diluted by 0.3% HNO_3. Three extract and analysis repetitions were performed.

Data analysis

All data were analyzed using R 3.0.2 (R Core Development Team 2013). The simultaneous tests of general linear hypotheses, Dunnett contrasts, were used for multiple comparisons of the means. Summary data are reported as mean values ± standard deviation. Data with box plots and Bonferroni tests, correlation coefficients between productivity components and grain quality parameters, and *p* values were calculated using the GenStat software (10th edition). Linear or Pearson correlation coefficients were used to test for a linear relationship between grain parameters. When testing significance, testing r^2 values are more meaningful as they describe the amount of co-variation explained by an *r* value and these are shown as % values. A *p*-value ≤0.05 was considered statistically significant.

Results

We have plotted the pooled grain chemical analysis data to show the range of values generated by the irradiation treatments. The GPC for the pooled data showed considerable variation, from 12.9 to 14.9%, with a mean of 14.0 ± 0.4% (n = 106) (Figure 1a). Nine genotypes (30.0%), mainly in the 200-Gy-dosed treatment, had 7.3–11.3% higher GPC than the parent line. Evaluation of wheat mutant germplasms for GIC showed considerable genetic variation among the M_5 lines (Figure 1b). Significant differences between treatments were found for the GIC, with the values ranging from 16.8 to 111.3 mg/kg, with a mean of 59.8 ± 23.3 mg/kg (n = 90). Seventeen M_5 lines (56.7%) had significantly enhanced GIC relative to the parent line, exceeding it by 1.9–3.6 times. The highest values of GIC were found in 200 Gy mutant germplasm. Frequency distributions of GZnC are presented in Figure 1a. The total range of GZnC values in lines was from 28.8 to 95.6 mg/kg, with a mean of 53.6 ± 22.9 mg/kg (n = 90). Fourteen mutant genotypes (46.7%), which were from the 200-Gy treatment, had 1.5- to 2.8 times higher GZnC than the parent line. A comparison of mutant lines for grain Fe and Zn concentrations revealed that 11 of the main lines (36.7%) generated from the 200-Gy dose which showed significantly enhanced GIC and GZnC (Figure 1d). This range of values defines the genetic variability that exists in the parent and gamma-irradiated M_5 lines under one set of environmental conditions.

For the parent and M_5 mutant lines in this study, the ranges of grain morphometric parameters, such as GA, grain width (GW), GL, and GL and GW ratio (GL: GW ratio), are tabulated (Table 1). The GA values ranged from 18.0 to 24.8 mm^2, with a mean of 21.4 ± 1.9 mm^2 (n = 90). The GA of 22 genotypes (73.3%) from the M_5 generation was 11–42% higher than that of the parent line. GL varied from 6.8 to 8.4 mm, with a mean of 7.8 ± 0.4 mm in M_5 lines (n = 90). The parent line had the lowest GL of 6.4 mm (n = 3), whereas the majority of M_5 lines (93.3%) showed 12–31% longer grains than the parent line (Table 1). Eight M_5 lines (26.7%)

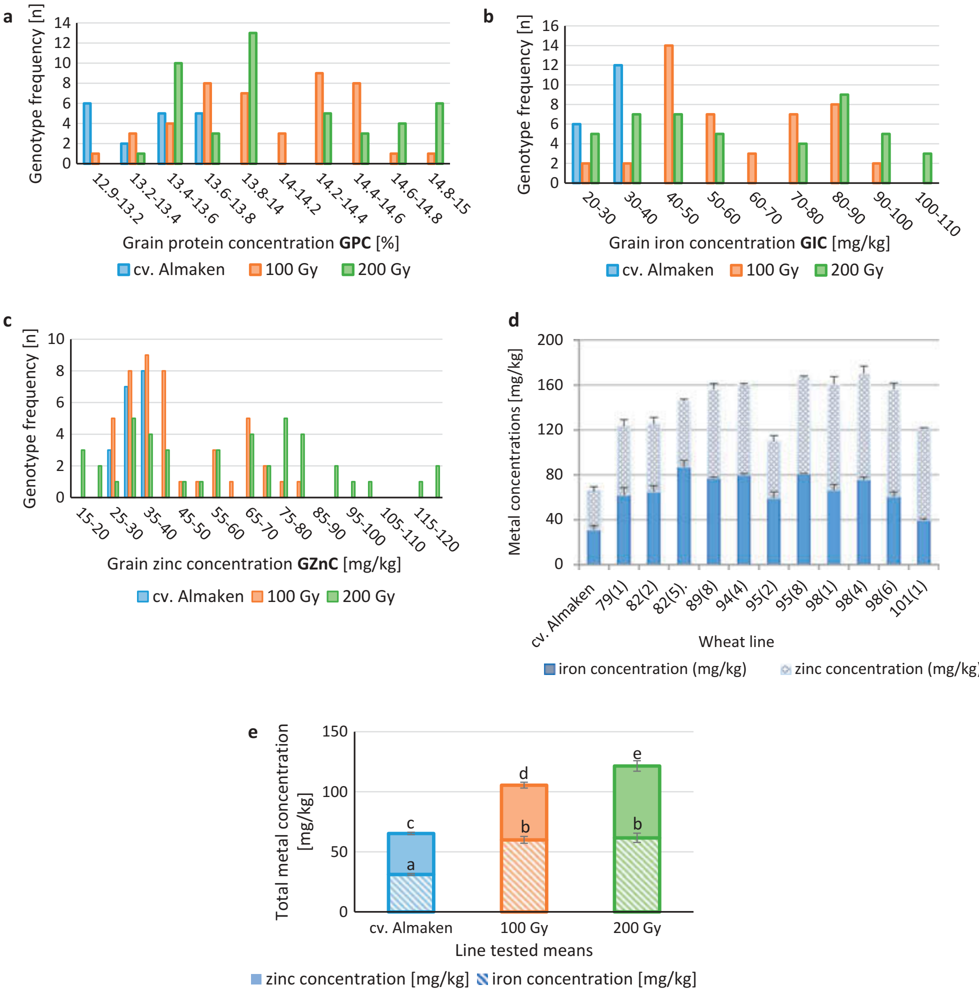

Figure 1. Frequency distribution for pooled grain parameters to show the range of values. Data include: (a) GPC, (b) GIC, (c) GZnC, (d) lines with significantly enhanced GIC and GZnC, (e) shows the mean values for GIC and GZnC in 100 and 200 Gy-dosed M_5 wheat mutant lines and parent (cv. Almaken). (Errors are SEM, n = 18–45, significance based on $p < 0.05$, One-way ANOVA multi-comparison Tukey test Genstat 18th edition).

showed the longest GL (>8.0 mm). The GW values ranged from 3.6 to 4.8 mm, with a mean of 4.3 ± 0.17 mm (n = 90) for the combined irradiated lines. The mean GW data for each radiation dose were 4.0 ± 0.2 in 100 Gy lines (n = 45) and 4.6 ± 0.2 in the 200 Gy lines (n = 45), whereas the parent line mean was 3.4 ± 0.2 (n = 3) (Figure 1a). With the exception of line 84(2), most of the lines (96.7%) had significantly higher GW (11.5–39.3%) than the parent line. Eleven genotypes (36.7%) had mean GW values of >4.5 mm and also showed higher GL and GA means when compared with the parent line $p < 0.0001$) (Table 1).

Table 1. Comparing grain size parameters of advanced M_5 mutant lines of spring wheat developed using 100 and 200 Gy and the parent cv. Almaken.

Genotypes/ Mutant	Grain area (GA) [mm^2]	Grain length (GL) [mm]	Grain width (GW) [mm]	Grain length and grain width ratio (GL:GW ratio)
cv. Almaken	17.45 ± 0.22	6.44 ± 0.23	3.41 ± 0.24	1.89 ± 0.96
75(2)	18.67 ± 0.12	7.29 ± 0.17***	3.91 ± 0.13*	1.86 ± 1.31*
76(2)	18.47 ± 0.13	7.55 ± 0.27***	3.93 ± 0.08**	1.92 ± 3.38**
76(3)	18.35 ± 0.12	6.84 ± 0.56	4.18 ± 0.16***	1.64 ± 2.88***
79(1)	18.44 ± 0.09	8.44 ± 0.31***	4.25 ± 0.24***	1.99 ± 1.29***
79(5)	18.99 ± 0.13	8.31 ± 0.16***	4.11 ± 0.22***	2.02 ± 0.73***
81(1)	19.42 ± 0.17***	8.32 ± 0.19***	3.97 ± 0.12**	2.1 ± 1.58**
82(2)	17.97 ± 0.15.	7.03 ± 0.05	3.85 ± 0.18*	1.83 ± 0.33*
82(4)	18.92 ± 0.08	7.61 ± 0.23***	3.83 ± 0.13	1.99 ± 1.77
82(5)	18.35 ± 0.29	8.29 ± 0.11***	4.29 ± 0.18***	1.93 ± 0.61***
84(2)	19.79 ± 0.33***	7.19 ± 0.13**	3.62 ± 0.21	1.99 ± 0.62
84(4)	24.17 ± 0.68***	7.7 ± 0.17***	3.93 ± 0.13**	1.96 ± 1.42**
89(5)	22.13 ± 0.38***	7.82 ± 0.14***	4.05 ± 0.09***	1.93 ± 1.56***
89(8)	23.59 ± 0.23***	7.84 ± 0.17***	4.1 ± 0.13***	1.91 ± 1.42***
91(1)	24.68 ± 0.21***	7.59 ± 0.23***	4.07 ± 0.07***	1.86 ± 3.29***
91(2)	22.06 ± 0.06***	7.76 ± 0.11***	4.08 ± 0.18***	1.9 ± 0.61***
94(2)	22.65 ± 0.22***	7.67 ± 0.15***	4.33 ± 0.12***	1.77 ± 1.25***
94(4)	21.2 ± 0.21***	7.64 ± 0.2***	4.36 ± 0.24***	1.75 ± 0.83***
95(2)	24.33 ± 0.3***	7.79 ± 0.15***	4.73 ± 0.13***	1.65 ± 1.15***
95(3)	21.59 ± 0.23***	7.66 ± 0.18***	4.69 ± 0.24***	1.63 ± 0.75***
95(5)	20.69 ± 0.25***	7.59 ± 0.23***	4.54 ± 0.27***	1.67 ± 0.85***
95(7)	21.93 ± 0.11***	8.05 ± 0.26***	4.63 ± 0.21***	1.74 ± 1.24***
95(8)	22.97 ± 0.1***	7.6 ± 0.23***	4.69 ± 0.18***	1.62 ± 1.28***
98(1)	24.84 ± 0.16***	8.21 ± 0.79***	4.73 ± 0.18***	1.74 ± 1.67***
98(2)	23.77 ± 0.1***	7.93 ± 0.11***	4.66 ± 0.22***	1.7 ± 0.52***
98(4)	23.04 ± 0.19***	7.82 ± 0.15***	4.54 ± 0.13***	1.72 ± 1.15***
98(6)	23.92 ± 0.12***	7.82 ± 0.08***	4.61 ± 0.20***	1.7 ± 0.35***
101(1)	21.46 ± 0.16***	7.72 ± 0.14***	4.65 ± 0.20***	1.66 ± 0.70***
101(3)	20.65 ± 0.21***	8.31 ± 0.11***	4.36 ± 0.14***	1.91 ± 0.79***
101(5)	21.12 ± 0.12***	8.39 ± 0.16***	4.28 ± 0.14***	1.96 ± 1.14***
101(6)	22.81 ± 0.12***	7.97 ± 0.09***	4.75 ± 0.15***	1.68 ± 0.60***

*, **, and ***denote significance at 0.05, 0.01, and 0.001 probability levels, respectively. The lines are significantly different from parent line. Grain area, grain length and grain width, grain length and grain width ratio are means of three replicates.

GA: Grain area; GL: grain length; GW: grain width; GL:GW: grain length and grain width ratio.

Scatter plots of data for grain parameters were compared with quality parameters, such as protein and metal contents; some example plots are shown in Figure 2a–c. These plots illustrate the spread of values and a fitted correlation line was used to test if there was any evidence for a relationship between the parameters. These data showed that for some parameters, both the radiation dose levels (100 or 200 Gy) gave very similar results, which were different from those for the parent line (Figure 2a, GNS vs. GW per spike). By contrast, the other parameters showed two different patterns that depended on the irradiation dose (Figure 2c, GPC vs. GZnC). Comparing the distribution of points in Figure 2b for each of the patterns showed how gamma irradiation had produced a much higher level of variation in the grain metal contents when compared with the parent line. Statistical testing

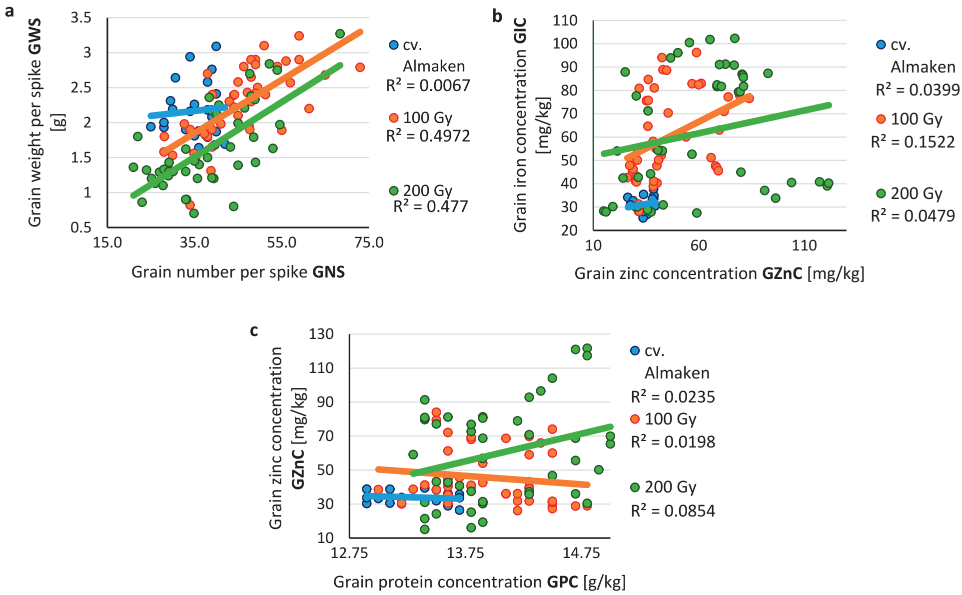

Figure 2. Scatter plots to show the spread of grain data comparing the parental line with the M_5 generation after two levels of gamma-irradiation dose and yield selection. (a) Grain number per spike (GNS) plotted against grain weight per spike (GWS). (b) Grain iron concentration (GIC) plotted against grain zinc concentration (GZnC). (c) Grain protein content (GPC) plotted against grain zinc concentration (GZnC).

was used to compare the relationships between pairs of grain morphological and chemical parameters to show when significant correlations could be identified. The results of the significance testing for the relationships are shown as box plots (Figures 3 and 4). Statistically significant correlations ($p < 0.05$) for grain morphological parameters are shown in Figure 3 and for protein content and metal concentrations in Figure 4. Statistical testing indicated that the GW had significantly increased in the irradiated lines relative to the parent line; effect of the 200-Gy dose was significantly larger than that of the lower dose (Figure 3a). Similarly, the grain length (GL, Figure 3b), area (GA, Figure 3c), and TGW (Figure 3d) parameters were also significantly larger in the samples obtained from the irradiated plant populations when compared to the parent line. For these parameters, no significant difference could be obtained between grain samples given high and low doses of irradiation. For the other grain parameters (GWP, GWS, GNS), there were no significant differences between the parent and the lines obtained from the two irradiation treatments. Grain chemical analysis of protein, iron, and zinc concentrations showed significantly higher mean values for some lines for both 100 and 200 Gy treatments when compared with the parent (Figure 4a–c). However, comparing all three types of chemical analysis showed that there were no significant differences between the 100- and 200-Gy-irradiated lines.

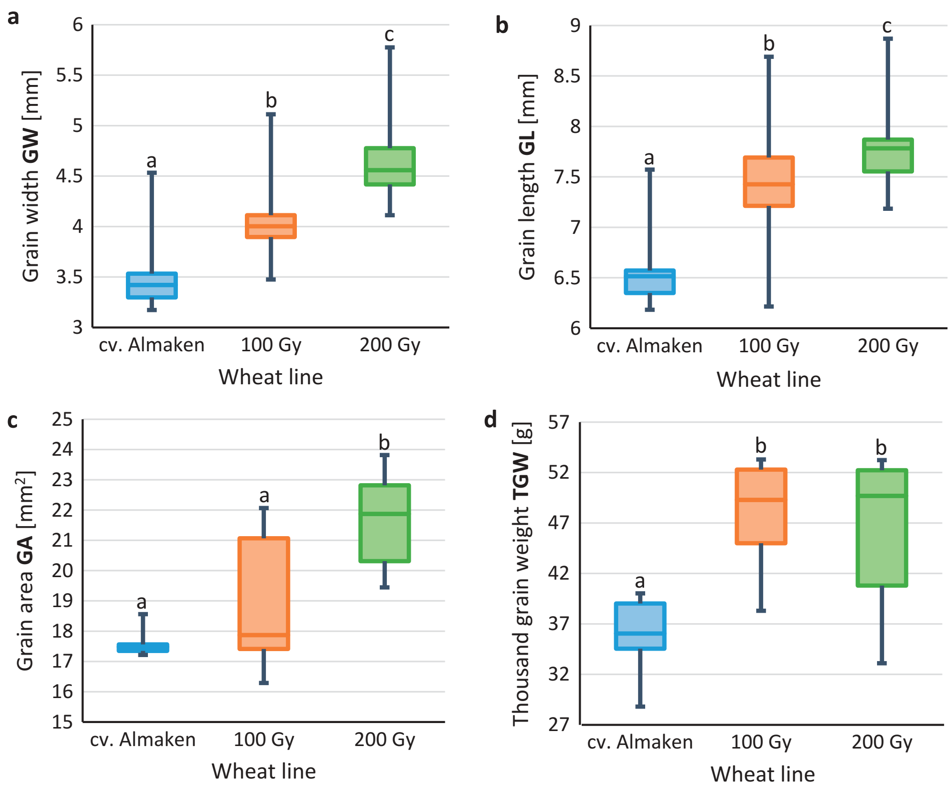

Figure 3. Box plots showing the statistical testing for the relationships between grain morphological parameters for the parent, 100 (low) and 200 (high) Gy irradiated wheat. Parameter pairs are tested comparing the parental line with the M_5 generation after two levels of gamma-irradiation dose (100 and 200 Gy) and yield selection. (a) Grain width GW [mm], (b) grain length GL [mm], (c) grain area GA [mm^2], (d) thousand grain weight TGW [g].

Detailed analyses of the data showed that the radiation doses of 100 and 200 Gy had generated mutations with respect to many parameters when compared with the parental line, with the greatest variation being for GWP and TGW (Table 2, Figure 3d). For the pooled data sets, the GWP ranged from 1.8 to 9.8 g, with a mean of 4.0 ± 1.9 g (n = 112). Ten genotypes (33.3%), mostly from the 100-Gy treatment, had significantly more GWP than other mutant lines and parent (Table 2). The variation for GNS was significant in eight M_5 lines (26.7%), which was chiefly found in 100 Gy-treated lines, with a range from 26.0 to 56.1 grain numbers per main spike (n = 112). There were no significant differences for GWS between M_5 lines and parent cv. Almaken. The TGW varied from 34.9 to 57.5 g, with a mean of 48.0 ± 5.8 g (n = 106). Twenty lines representing 66.7% of the total were characterized by significantly higher TGW than the parent line. Three M_5 lines [81(1), 84(2), and 101(5)] (10.0%) had significantly higher GNS, GWP, and TGW than the parent line. Four mutant lines resulting from the 100-Gy

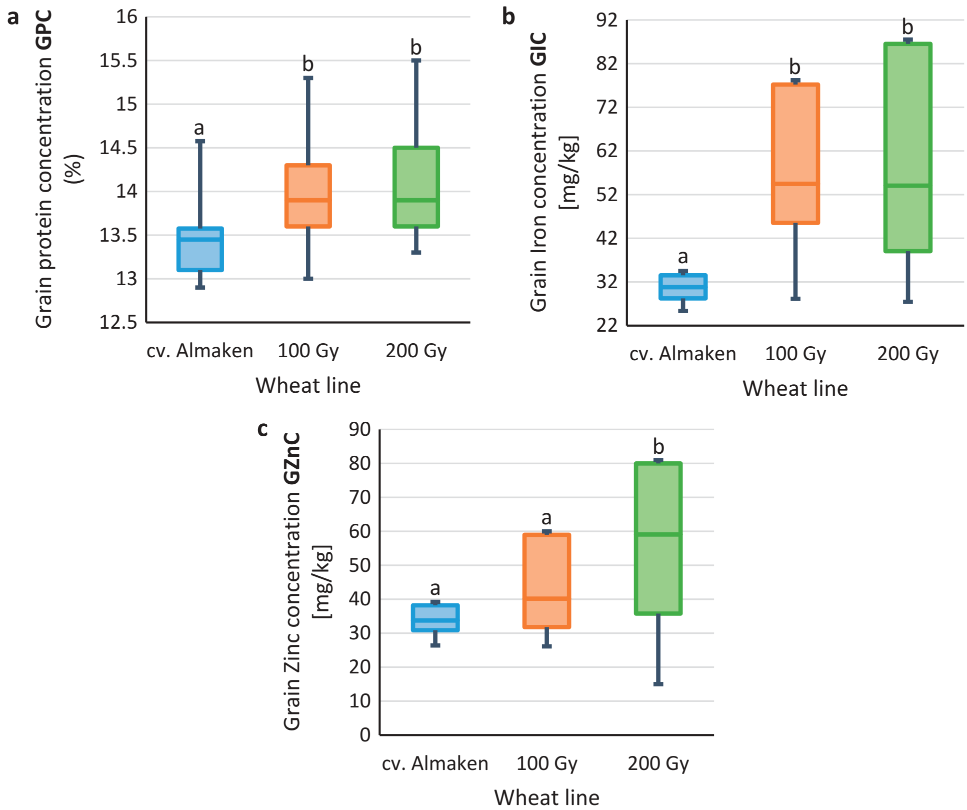

Figure 4. Box plots showing the statistical testing for the relationships between grain analysis parameters for the parent, 100 (low) and 200 (high) Gy irradiated wheat. Parameters are tested compared to the parental line for the M_5 generation after two levels of gamma-irradiation dose (100 and 200 Gy) and yield selection. (a) Grain protein content GPC [%], (b) grain iron concentration GIC (mg/kg), (c) grain zinc concentration GZnC (mg/kg).

dose showed significantly higher GWP and TGW when compared to the parent line. In contrast to the parent line, the mutant lines showed significantly stronger positive correlation relationships between GNS and GWS (r^2 = 0.497, p < 0.001 for 100 Gy and r^2 = 0.477, p < 0.001 for 200 Gy), and between GWS and GWP (r^2 = 0.217, p < 0.01 for 100 Gy and r^2 = 0.274, p < 0.001 for 200 Gy) (Table 3).

The association between grain nutrients and yield is important. Interestingly, the parent line GPC showed a significant, positive correlation with GNS (r^2 = 0.261, p < 0.05) (Table 3). For grain chemical analysis, significant correlations were not observed (Table 3).

In general, the GIC correlations were small and only the GIC of the 100-Gy treatment lines exhibited a significant, positive correlation with the plant productivity components, TGW (r^2 = 0.153, p < 0.01), and GWP (r^2 = 0.302, p < 0.001). In the 100-Gy-treated mutant lines, we observed that there was no significant correlation between GIC and GA (r^2 = 0.097) and GW (r^2 = 0.038);

Table 2. Comparing yield-associated traits of advanced M_5 mutant lines of spring wheat developed using 100 and 200 Gy and the parent cv. Almaken.

Genotypes/ Mutant	Grain number per main spike	Grain weight per main spike [g]	Grain weight per plant [g]	Thousand-grain weight [g]
cv. Almaken	35.20 ± 4.60	2.17 ± 0.40	2.69 ± 0.57	36.48 ± 3.16
75(2)	29.00 ± 1.00	1.48 ± 0.42	2.16 ± 0.39	41.90 ± 2.01
76(2)	49.9 ± 7.94*	2.39 ± 0.45	5.26 ± 1.51***	44.00 ± 1.85
76(3)	54.00 ± 9.07**	2.74 ± 0.33	4.86 ± 0.84***	39.77 ± 1.29
79(1)	48.9 ± 4.53	2.47 ± 0.32	5.49 ± 0.95***	45.83 ± 1.96
79(5)	35.88 ± 5.30	1.23 ± 0.46	3.24 ± 1.21	50.37 ± 2.93***
81(1)	49.50 ± 6.32*	2.47 ± 0.32	8.81 ± 1.66***	51.30 ± 1.75***
82(2)	48.5 ± 7.14*	2.3 ± 0.58	7.3 ± 1.92***	45.63 ± 0.74
82(4)	53.00 ± 2.16**	2.81 ± 0.22	3.63 ± 1.78	46.27 ± 2.64
82(5)	49.1 ± 8.69	2.58 ± 0.57	9.8 ± 1.82***	51.53 ± 2.88***
84(2)	55.00 ± 5.01**	2.88 ± 0.34	4.13 ± 0.54*	52.27 ± 1.42***
84(4)	40.00 ± 3.06	2.00 ± 0.56	2.85 ± 0.83	53.03 ± 2.06***
89(5)	47.75 ± 5.39	2.10 ± 0.47	5.02 ± 0.82***	57.53 ± 2.79***
89(8)	43.40 ± 4.42	2.46 ± 0.47	3.87 ± 0.82	53.73 ± 1.63***
91(1)	34.14 ± 9.24	1.65 ± 0.56	2.16 ± 0.87	51.37 ± 2.20***
91(2)	38.47 ± 2.18	1.99 ± 0.50	2.90 ± 0.75	47.60 ± 2.67**
94(2)	26.00 ± 2.29	1.08 ± 0.85	2.86 ± 1.24	48.10 ± 2.55**
94(4)	36.25 ± 3.09	1.17 ± 0.54	3.47 ± 1.21	38.40 ± 1.67
95(2)	37.33 ± 3.22	1.19 ± 0.44	2.80 ± 1.53	39.20 ± 0.92
95(3)	27.00 ± 2.16	1.55 ± 0.08	2.32 ± 1.05	37.73 ± 2.91
95(5)	28.00 ± 3.52	1.17 ± 0.30	1.82 ± 0.60	44.70 ± 1.93
95(7)	30.50 ± 5.80	1.21 ± 0.27	2.67 ± 1.56	34.87 ± 1.80**
95(8)	34.39 ± 9.01	1.68 ± 0.52	2.30 ± 0.88	54.20 ± 2.14***
98(1)	29.75 ± 1.53	1.07 ± 0.16	3.56 ± 1.67	51.94 ± 0.60***
98(2)	44.20 ± 6.46	1.46 ± 0.59	3.44 ± 0.96	53.18 ± 2.64***
98(4)	41.53 ± 9.41	2.08 ± 0.60	3.18 ± 0.83	53.53 ± 0.32***
98(6)	34.39 ± 9.01	1.68 ± 0.52	2.30 ± 0.88	48.73 ± 2.96***
101(1)	44.25 ± 7.03	2.29 ± 0.38	4.95 ± 0.44***	53.50 ± 2.03***
101(3)	52.20 ± 7.32**	2.85 ± 0.39	3.98 ± 0.52	48.97 ± 2.70***
101(5)	56.10 ± 7.57***	2.94 ± 0.46	4.33 ± 2.76**	52.80 ± 1.92***
101(6)	49.00 ± 7.31	2.37 ± 0.42	3.95 ± 1.45	49.27 ± 1.60**

*, **, and ***denote significance at 0.05, 0.01, and 0.001 probability levels, respectively. The lines are significantly different from parent line.

Grain number and weight per main spike, grain weight per plant are a means of three replicates. Each replication was analyzed as the average value from the ten randomly selected spikes/plants.

but the relationship of GIC with GZnC was significantly positive ($r^2 = 0.152$, $p < 0.01$). In the 200-Gy treatment, no significant correlations could be found between the GIC, GZnC, and GPC parameters (see Figure 2; Table S3). GPC was positively correlated with GW ($r^2 = 0.191$, $p < 0.01$) and GA ($r^2 = 0.128$, $p < 0.05$) at the 200-Gy dose but not at 100-Gy dose (Table 3).

Discussion

Wheat genetic improvement requires the identification of key traits in high-performing cultivars to deploy in breeding programs. Breeding has targeted increased yield but not the nutritional value of stable food crops (Borrill et al. 2014; Chatzav et al. 2010; Graham et al. 1999; Cakmak et al. 2010; Ingvordsen

Table 3. *R*-squared correlation coefficients with *p* values between yield-associated traits (TWG, GNS, GWS, and GWP), grain protein content and micro-elements concentrations, and grain morphometric parameters (GA, GL, and GW) in parent (cv. Almaken) and spring wheat M_5 mutant lines.

	GWS [g]	GWP [g]	TGW [g]	GPC [%]	GIC [mg/kg]	GZnC [mg/kg]	GL [mm]	GW [mm]	GA [mm²]
cv. Almaken									
GNS	0.007	0.016	0.007	0.261*	0.145	0.015	0.485	0.775	0.620
GWS [g]		0.000	0.180	0.045	0.056	0.043	0.994	0.949	0.996*
GWP [g]			0.000	0.002	0.060	0.016	0.983	0.820	0.930
TGW [g]				0.036	0.053	0.091	0.074	0.303	0.161
GPC [%]					0.006	0.000	0.987	0.833	0.939
GIC [mg/kg]						0.040	0.020	0.187	0.075
GZnC [mg/kg]							0.833	0.559	0.721
GL [mm]								0.909	0.981
GW [mm]									0.971
100 Gy M_5 mutant lines									
GNS	0.497***	0.177**	0.002	0.009	0.023	0.018	0.085	0.011	0.123*
GWS [g]		0.217**	0.001	0.007	0.003	0.000	0.038	0.045	0.081
GWP [g]			0.004	0.003	0.302***	0.036	0.096*	0.033	0.324***
TGW [g]				0.079	0.153**	0.009	0.227**	0.004	0.279***
GPC [%]					0.001	0.020	0.105	0.000	0.029
GIC [mg/kg]						0.152**	0.001	0.038	0.097
GZnC [mg/kg]							0.006	0.016	0.035
GL [mm]								0.001	0.335*
GW [mm]									0.001
200 Gy M_5 mutant lines									
GNS	0.477***	0.387***	0.153**	0.017	0.024	0.009	0.039	0.009	0.062
GWS [g]		0.274	0.196	0.020	0.001	0.037	0.103	0.034	0.071
GWP [g]			0.153	0.000	0.000	0.002	0.001	0.029	0.041
TGW [g]				0.034	0.034	0.002	0.007	0.000	0.000
GPC [%]				0.085	0.085	0.085	0.113*	0.191**	0.128*
GIC [mg/kg]						0.048	0.024	0.047	0.051
GZnC [mg/kg]							0.069	0.058	0.191**
GL [mm]								0.253***	0.374***
GW [mm]									0.194**

*, ** and ***denote significance at <0.05, <0.01, and <0.001 probability levels, respectively.

GWS: Grain weight per spike; GWP: grain weight per plant; TGW: thousand grain weight; GPC: grain protein content; GIC: grain iron concentration; GZnC: grain zinc concentration; GL: grain length; GW: grain width; GA: grain area; GNS: grain number per spike.

et al. 2016; Kang and Banga 2013; Pearce et al. 2014). Wheat is a staple food in many countries of the world and therefore, biofortifying the crop can help improve human health. However, breeding programs concentrated on increased yield have narrowed the genetic base of modern crop plants. To introduce more genetic diversity and introduce new traits, one breeding strategy has been to employ the wild relatives of crop plants (Chatzav et al. 2010). Another approach to this problem is to generate new sources of variation in the genetic background of modern varieties by introducing more major re-organizations within the genome. The genetic changes produced by irradiation are much larger than the subtle single-base changes introduced by chemical mutagens. Mutated lines generated by irradiation can be used as potential donors for genes/alleles beneficial for wheat-breeding programs to increase yield and improve grain quality. In the current study, genetically stable M_5 mutant lines of spring wheat were generated from parent seed given two doses of radiation (100 and 200 Gy). Comparing the M_5-mutated lines showed that considerable variation was generated for some traits by the irradiation treatment, for example, TGW, grain iron, and zinc concentrations (Figures 1b and 2, Table 2).

To consider the results in more detail, we first discuss the morphological parameters (Figures 2 and 3) and then examine the chemical data (Figures 1a, 2 and 4). Relative to the parent, the actual grain physical parameters, GW, GL, GA, and TGW (Figure 3a–d), were significantly increased in the irradiated lines, but only GW showed a dose-dependent pattern (Figure 3a). These are all grain-size parameters, suggesting that there is considerable scope to alter these parameters in wheat. By contrast, the other grain parameters measured (GWP, GWS, and GNS) were not significantly different from those of the parent. These parameters are expressed per plant or main spike and suggest that there may be a limitation imposed by the physical structure of the wheat plant; perhaps, the ability of the stem to support crop limits the maximum grain size. Together these data showed that although the grain size could be changed by mutation, the yield per plant or spike was not significantly changed when compared with the parent line. A significant reduction in phenotypic variation in grain shape in the modern germplasm pool has been previously shown when compared to ancestral wheat species, probably as a result of a relatively recent bottleneck of genetic variation (Gegas et al. 2010). The emerging genetic model of phenotypes indicates that during wheat domestication, a long, thin, primitive grain has transformed to a wider, shorter modern grain. In the present study, new mutant M_5 lines of spring wheat were quantified for grain-size variables (GA and GW) and grain-shape variables (GL, including GL:GW ratio). The means of mutant M_5 lines showed that GA and to a lesser degree GL were the most variable phenotypic traits. Variations in GW and the GL:GW ratio were moderate and less variable (Figure 5). There was significantly more variation

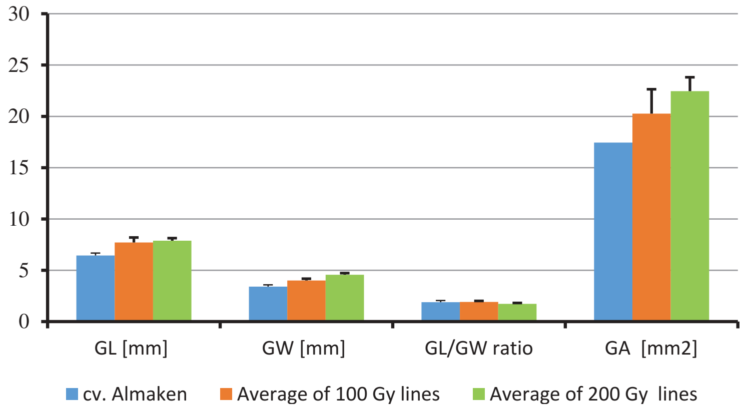

Figure 5. Phenotypic variation in grain size parameters (Grain length GL, Grain width GW, ratio of GW/GL, and Grain area GA in M_5 mutant lines generated by irradiation with 100 and 200 Gy doses and cv. Almaken (the parent line). Mean of values of GL, GW, GL/GW, and GA with standard error bars.

in GA (range 7.0 mm) and GL (range 1.4 mm) in mutant lines than GW (range 1.1 mm). The highest means of GL and GW in M_5 lines were 27.5–30.3% and 33.1–38.7% higher than the parent cv. Almaken (Table 1).). Moreover, most of the heavier and longer grain lines were found in the 200-Gy-dosed germplasm. Unlike the parent and 100-Gy-dosed lines, GA in 200-Gy mutant lines was closely related to GL (r^2 = 0.374, $p < 0.001$) and GW (r^2 = 0.194, $p < 0.01$), showing an expected relationship between GW and dimensions of grain (Table 3).

Next, we discuss the results of the grain chemical analyses for iron, zinc, and protein contents. The irradiated mutant lines exhibited wide variations in grain concentrations of metals, with significantly higher concentrations of grain Fe and Zn relative to the parent cv. Almaken. The highest values of GIC revealed in 200-Gy-mutant germplasm exceeded the parent by 3–4 times. Increase in grain Zn concentration was less; nevertheless, the highest values of GZnC in the mutant lines were about threefold greater than in the parent, demonstrating a huge potential for an enhancement of these micro mineral concentrations. As we found for GIC, the 200-Gy-mutated lines generally showed the higher GZnC.

Most of the 200-Gy-mutant lines showed significant simultaneously enhanced GIC and GZnC, as well (Figure 1b). This finding suggests that donors for high GIC and GZnC can be found among these mutant resources and also that the genetic backgrounds and the factors by which they are created play an important role. The effect of the radiation doses on the averages of GIC and GZnC in mutant lines showed greater genetic variation for the GZnC than another grain quality parameter (Figure 1e).

The large increases measured for Fe and Zn concentrations in some M_5 lines suggested that there was a great capacity for grain-micronutrient accumulation.

These grain–metal accumulations can occur without adversely affecting plant biochemical and physiological functions and they indicate the potential to induce mutations in genes involved in mineral homeostasis processes. The advantage of wild emmer over cultivated wheat for higher grain nutrient concentrations has been previously consistently demonstrated (Cakmak et al. 2004; Chatzav et al. 2010; Peleg et al. 2008). However, because of sexual incompatibility between the crop and its wild relative, it may require embryo rescue or use tissue culture to recover fertile embryos. To eliminate deleterious genes from the wild relative that are not related to the trait of interest, an extensive program of backcrossing is also required (Lafiandra, Riccardi, and Shewry 2014). The values reported for Fe concentration in hexaploid wheat, wild wheat, and landraces grown under field conditions have been reported as ranging from 28.8 to 56.5 (Graham et al. 1999), 19.0 to 88.4 (Oury et al. 2006), 22.9 to 67.6 (Liu et al. 2006), 37.8 to 44.1 (Tiwari et al. 2009), 27.0 to 43.0 (Velu et al. 2011), and 28.9 to 58.9 mg/kg (Choi et al. 2013). The reported concentration ranges for Zn are 25.2–53.3 (Graham et al. 1999), 16.4–39.5 (Oury et al. 2006), 16.2–32.4 (Liu et al. 2006), 41.9–48.4 (Tiwari et al. 2009), 15.0–51.0 (Velu et al. 2011), and 25.8–66.8 mg/kg (Choi et al. 2013). The highest metal concentrations obtained in the irradiated lines exceeded these upper limits in previously published data for both iron and zinc (see Figure 1b,c). Although environmental factors can influence grain metal concentrations, in this work, all the mutant lines and the parent were grown under the same field conditions, with no specific fertilizer supplementation of these metals.

The highest GPC measured in the irradiated mutants was 14.8% (Figure 1a), which was an 11.3% increase over the parent cv. Almaken. A significant positive correlation between GA and GWP and TWG ($p < 0.001$) was observed in only the 100-Gy-dosed mutant lines. As GL is a component of GA, this positive correlation is not surprising ($p < 0.001$) for both 100- and 200-Gy doses. In the case of 100- and 200-Gy-dosed M_5 lines, GPC also significantly positively correlated to GL, $r^2 = 0.105$, ($p < 0.05$) and $r^2 = 0.113$, ($p < 0.05$), respectively. Together, these results may suggest that physically larger grain, increased area, and length improve the capacity to accumulate a higher % protein.

The genetic diversity among different wheat genotypes is essential to identify potential donors with genes/alleles beneficial for wheat breeding programs and to develop novel cultivars with desirable attributes. Understanding the associations between micronutrients and grain yield, plant height, grain size, and end-use quality parameters would facilitate the selection of mineral-dense progenies by breeding with plants with desired phenological and other preferred traits (Velu et al. 2013). Correlations between grain concentrations of mineral nutrients may indicate the existence of one or more common genetic/physiological mechanisms underlying plant mineral homeostasis (Chatzav et al. 2010). A significant correlation was also detected between grain Fe and Zn concentrations at the lower irradiation dose (Figure2b; Table S3, $p < 0.01$), perhaps indicating that their accumulation may be controlled by the same loci. The close correlation between Fe and Zn concentrations may suggest possible common regulatory mechanisms to transport and store these micronutrients (Cakmak et al. 2000). However, this correlation is lost at the higher irradiation dose (Table 3). The regulation of transporter gene expression for the steps from leaves to grain during wheat monocarpic senescence is poorly understood (Pearce et al. 2014). A detailed understanding of these mechanisms will be required to engineer wheat varieties with improved nutritional quality through biofortification (Borrill et al. 2014). In domesticated wheat (Zhao et al. 2009), and synthetic hexaploids (Velu et al. 2013), there were positive correlations between Zn and Fe concentrations. This implies that the alleles for Zn and Fe grain deposition co-segregate or are pleiotropic, and therefore, Zn and Fe concentrations can be improved simultaneously. As the studies for bread wheat (Raboy et al. 1991), wild emmer wheat (Chatzav et al. 2010), and synthetic hexaploids (Velu et al. 2011) previously reported, we also found significantly positive correlations between GPC and Zn and Fe concentrations. However, in our data, these correlations were only found in the 100-Gy-dosed mutant lines for Zn (Table 3).

Creating high-yielding wheat varieties is a major goal for many breeding programs. Combining yield with higher Zn and Fe concentrations is a principal concern for breeders (Chatzav et al. 2010; Welch and Graham 2004). An inverse relationship between yield and grain-nutrient concentrations (especially GPC) is well-documented in cultivated wheat (Murphy, Reeves, and Jones 2008). In this work, The GPC, GIC, and GZnC showed positive correlations with grain morphometric parameters, such as GA, GL, and GW in the 100-Gy-dosed mutant lines, demonstrating a relationship between these parameters. These findings suggest that there is scope to improve wheat grain protein, iron, and zinc without compromising yield and this gamma-irradiated mutation resource may be a useful tool for improving grain quality.

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