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“Wild barley serves as a source for biofortification of barley grains”

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ABSTRACT

The continuing growth of the human population creates an inevitable necessity for higher crop yields, which are mandatory for the supply with adequate amounts of food. However, increasing grain yield may lead to a reduction of grain quality, such as a decline in protein and mineral nutrient concentrations causing the so-called hidden hunger. To assess the interdependence between quantity and quality and to evaluate the biofortification potential of wild barley, we conducted field studies, examining the interplay between plant development, yield, and nutrient concentrations, using HEB-YIELD, a subset of the wild barley nested association mapping population HEB-25. A huge variation of nutrient concentration in grains was obtained, since we identified lines with a more than 50% higher grain protein, iron, and zinc concentration in comparison to the recurrent parent ‘Barke’. We observed a negative relationship between grain yield and nutritional value in barley, indicated by predominantly negative correlations between yield and nutrient concentrations. Analyzing the genetic control of nutrient concentration in mature grains indicated that numerous genomic regions determine the final nutritional value of grains and wild alleles were frequently associated with higher nutrient concentrations. The targeted introgression of wild barley alleles may enable biofortification in future barley breeding.

1. Introduction

Worldwide population growth results in increasing demands for the supply with sufficient amounts of food, as well as superior food quality [1–3]. Cereals, including barley (*Hordeum vulgare* ssp. *vulgare*) as the fourth-most important crop on a global scale [4,5], provide around 50% of the required calories worldwide [3,6]. Their contribution can even account for up to 70% of calories in least developed countries, primarily in Africa and Asia [6], where barley still has a pronounced role as staple food [4,7]. Moreover, cereals function not only as source for carbohydrates, but also for proteins, fiber, and nutrients [8–10], especially in countries where the consumption of animal-based products is unaffordable [3,11]. In addition, over 40% of the world production of barley, maize and wheat is used in livestock feed with the barley

proportion in the order of 67% [5,12,13].

The main breeding goal of the ‘Green Revolution’ was to improve grain yields, which had tremendous success [14,15]. However, the higher yields have one substantial drawback as they lead to a reduction in protein and mineral nutrient contents of grains, reducing their quality and nutritional value [16–18]. Roughly one billion people suffer from low intake of proteins and mineral nutrients, especially iron, zinc, and calcium [19–21]. Furthermore, an adequate supply with nutrients is also necessary for the plant itself to achieve high yields [22]. Therefore, the re-biofortification of our elite crop material represents a worthwhile approach to achieve a balanced diet for humans and livestock [20,23].

Barley represents an appropriate model for cereal research due to its relatively simple diploid genetics [24]. This suits barley as model

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species for members of the *Triticeae* tribe (e.g. soft wheat, durum wheat, and rye), since those species are closely related, allowing to transfer knowledge gained in barley to other *Triticeae* species [24]. Moreover, barley shows high tolerance against abiotic stresses [25–27]. As the already negative impacts of climate change will become more severe in the future, especially in large parts of Africa, the Arabian Peninsula, Southeast Asia and Central South America [28,29], the higher abiotic stress tolerance of barley might be an option to extend its production and provide a secure source for human food.

As a result of domestication and repetitive rounds of selection, many modern crops, including barley, suffer from genetic erosion, which is a loss of genetic variation [30–32]. The introgression of new genetic variation from wild progenitors, like wild barley (*Hordeum vulgare* ssp. *spontaneum*) from the Fertile Crescent and Tibet [33,34], is one option to replenish the gene pools of modern elite crops [31,35]. In this regard a successful example is the *Gpc-B1* locus in bread wheat, which was introgressed from wild emmer (*Triticum turgidum* ssp. *dicoccoides*) into wheat through chromosomal substitution [36,37]. The locus has positive impacts on the concentration of Zn, Fe, Mn and proteins in mature grains without a distinct negative impact on yield [38,39]. Several studies indicated that wild barley harbors huge phenotypic variation for a multitude of agronomic traits [40–45]. However, the usefulness of wild barley as source for biofortification has only rarely been examined.

Therefore, we conducted a study to capture the available variation of macronutrient and micronutrient concentrations in wild barley grains using the wild barley population HEB-YIELD, a selected subset of the nested association mapping (NAM) population HEB-25 [46]. For this purpose, HEB-YIELD was grown during two years in Dundee (United Kingdom) and Halle (Germany) with standard fertilizer application, as well as under nitrogen deficiency to examine the impact of nitrogen supply on mineral nutrient concentrations. In addition, we investigated the interplay between plant development, yield, and mineral concentrations by scoring key agronomical traits throughout the growing season.

2. Material and methods

2.1. Plant material

HEB-YIELD, a subset of the wild barley nested association mapping (NAM) population Halle Exotic Barley-25 (HEB-25) [46], was evaluated in yield trials. HEB-25 originated from crossing 25 diverse wild barley accessions (*Hordeum vulgare* ssp. *spontaneum* and *H. v.* ssp. *agriocrithon*) with the German elite spring barley cultivar Barke (*Hordeum vulgare* ssp. *vulgare*, released in 1996 by breeder Breun). HEB-25 comprises 1420 BC₁S₃-derived lines (backcrossed with Barke) grouped into 25 families (for more details see Maurer et al. [46]).

The HEB-YIELD subset consists of 48 HEB-25 lines that were selected from HEB-25 to ensure good threshability and the absence of brittleness to enable accurate yield estimation in field trials. In addition, the final HEB-YIELD lines were selected to independently segregate for homozygous elite versus homozygous wild barley alleles at the four major flowering time loci, which exhibited major plant developmental effects in HEB-25: *Ppd-H1*, *Sdw1*, *Vrn-H1* and *Vrn-H3* [40,44,46].

2.2. Genotypic data

The complete HEB-25 population was genotyped in generation BC₁S₃ using the barley Infinium iSelect 9k SNP chip (see Maurer et al. [46]). The diagnostic markers *i*_{BK}₁₆, *i*₁₂₃₀₉₂₄, *i*₁₁₁₀₇₀₅ and *i*₁₂₁₀₂₁₈, co-segregating with the four flowering time genes *Ppd-H1*, *Sdw1*, *Vrn-H1* and *Vrn-H3*, respectively, were used for selection of segregating HEB-YIELD lines that were homozygous for alternative alleles at the four loci (Table S1).

2.3. Field trials

The HEB-YIELD population was grown at two locations during two years (2015 and 2016), resulting in four environments. The locations were Dundee (United Kingdom; 56°28'53.71"N 3°6'35.17"W) and Halle (Germany; 51°29'46.05"N 11°59'29.58"E). A detailed description for each location is given in Table S2a. The full set of the 48 HEB-YIELD lines was sown at both locations (Table S2b). In addition to HEB-YIELD the recurrent parent 'Barke' and local cultivars were used as checks: 'Odyssey' (released by Limagrain, 2011) and 'Tyne' (RAGT, 1986) in Dundee and 'Marthe' (Nordsaat, 2005), 'Quench' (Syngenta, 2006), and 'Scarlett' (Breun, 1995) in Halle.

At both locations the plants were cultivated under regular fertilization (= control condition) and under nitrogen deficiency (= stress condition; Table S2c). In contrast to the control condition, lines in the stress treatment received no additional mineral N fertilizer in Dundee and Halle. The difference between both treatments regarding N were among 60 and 70 kg/N per hectare in both years by considering the results of the N_{min} analysis, which was performed in early spring prior to sowing to determine the availability of N for the HEB-YIELD lines. In Dundee additionally P, K and S were applied to the control blocks following local practice (Table S2c).

A randomized complete block design with four replicates was chosen as test design for the trials (Figure S1). The trials were conducted following local practices regarding tillage and pest management. Additional information on plant cultivation is provided in Table S2d.

2.4. Phenotypic data

In this study 17 traits were investigated and grouped into developmental (e.g. flowering time), yield-related (e.g. grain yield), and grain nutrient traits, including grain raw protein concentration (GPC) and grain concentration of carbon (C), phosphorus (P), potassium (K), sulfur (S), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu) and sodium (Na). A list of these traits is given in Table S3, including their method of measurement and in which location and year the traits were scored.

2.5. Determination of macronutrients and micronutrients in grains

After air-drying the harvested grains for two weeks, 6–8 g of grains of each plot were ground and homogenized using the mixer mill MM 400 (Retsch GmbH; Haan, Germany).

The dry matter concentration (DM) of each sample was determined after drying the barley flour for 3 h in a drying cabinet at 105 °C (method 3.1 modified [47]).

The elements C, N, and S were measured with a CNS analyzer (vario EL cube; Elementar Analysensysteme, Langensfeld, Germany), which is based on combustion analysis [48]. In this analysis 30 mg of flour per sample were combusted at 1500 °C in oxygen atmosphere for 160 s following the standard protocol of the vario EL cube. During combustion the gaseous products CO₂, NO₂, and SO₂ arose. Subsequently, after transformation and separation, their quantity was measured by a thermal conductivity detector. Grain raw protein concentration (GPC) was calculated by multiplying N by 6.25, according to the general assumption that barley proteins contain on average 16% of nitrogen [49].

For determination of macronutrients (P, K, Ca, Mg), micronutrients (Fe, Mn, Zn, Cu) and Na, inductively coupled plasma - optical emission spectrometry (ICP-OES) was used (Varian 715-ES ICP-OES; Varian, Palo Alto, California, USA). For this, 2 g of flour per sample were combusted in a muffle furnace at 550 °C for 14 h. The resulting ash was digested in three steps by adding two times 10 ml of hydrochloric acid (HCl, 6.0 M) and finally 10 ml of nitric acid (HNO₃, 1.8 M). After addition of HCl the solution was boiled down on a laboratory sand-bath. HNO₃ was evaporated to two thirds of the initial volume. The remaining solution was

transferred into a volumetric flask, filled up to 100 ml with bi-distilled water, filtrated, and analyzed by ICP-OES (methods 10 & 11 modified [47]).

2.6. Statistical analyses

All statistical analyses were carried out with SAS 9.4 (SAS Institute Inc., Cary, NC, USA [50]). Variance components (defined as random) were estimated with PROC VARCOMP and broad sense heritabilities (h^2) for each trait within locations and treatments were calculated across years following the formula:

$$h^2_{(control\ or\ stress)} = \frac{V_g}{V_g + \frac{V_{gy}}{Y} + \frac{V_r}{YR}} \quad (1)$$

where

V_g	=	genotypic variance	Y	=	number of years
V_{gy}	=	genotype by year interaction variance	R	=	number of replications
V_r	=	error variance			

In addition, the heritability was calculated within locations but across both treatments:

$$h^2_{(across)} = \frac{V_g}{V_g + \frac{V_{gy}}{Y} + \frac{V_{gt}}{T} + \frac{V_{gyt}}{YT} + \frac{V_r}{YTR}} \quad (2)$$

where

V_{gt}	=	genotype by treatment interaction variance	T	=	number of treatments
V_{gyt}	=	genotype by year by treatment interaction variance			

The repeatability (rep) of each trait was computed for each location and year following the formulas:

$$rep_{(control\ or\ stress)} = \frac{V_g}{V_g + \frac{V_r}{R}} \quad (3)$$

$$rep_{(across)} = \frac{V_g}{V_g + \frac{V_{gt}}{T} + \frac{V_r}{TR}} \quad (4)$$

The analysis of variance (ANOVA) across locations was calculated with PROC MIXED to test for the presence of genotype, location and year effects. For this purpose, the main effects (genotype, location and year), as well as their corresponding interaction effects were treated as fixed effects in the following model:

$$y_{ijk} = \mu + \mathbf{g}_i + \mathbf{l}_j + \mathbf{y}_k + (\mathbf{gl})_{ij} + (\mathbf{gy})_{ik} + (\mathbf{ly})_{jk} + (\mathbf{gly})_{ijk} + e_{ijk} \quad (5)$$

where

y_{ijk}	=	observed phenotype of the i th genotype in the j th location and the k th year
μ	=	intercept
\mathbf{g}_i	=	effect of the i th genotype
\mathbf{l}_j	=	effect of the j th location
\mathbf{y}_k	=	effect of the k th year
$(\mathbf{gl})_{ij}$	=	interaction effect between the i th genotype and the j th location
$(\mathbf{gy})_{ik}$	=	interaction effect between the i th genotype and the k th year
$(\mathbf{ly})_{jk}$	=	interaction effect between the j th location and the k th year
$(\mathbf{gly})_{ijk}$	=	interaction effect between the i th genotype, the j th location and the k th year
e_{ijk}	=	residual/error of y_{ijk}

Fixed effects are written in bold.

Best linear unbiased estimators (BLUEs) were estimated using the PROC MIXED procedure. The BLUEs for each HEB-YIELD line were computed across years for each location and treatment level (gt) separately, as well as across treatments (t). Genotype and treatment were modelled as fixed effects and year as a random effect:

$$y_{ikmn} = \mu + \mathbf{g}_i + y_k + \mathbf{t}_m + (\mathbf{gy})_{ik} + (\mathbf{gt})_{im} + (y\mathbf{t})_{km} + b[y\mathbf{t}]_{nkm} + e_{ikmn} \quad (6)$$

where

y_{ikmn}	=	observed phenotype of the i th genotype in the k th year and the n th block in the m th treatment
μ	=	intercept
\mathbf{g}_i	=	effect of the i th genotype
y_k	=	effect of the k th year
\mathbf{t}_m	=	effect of the m th treatment
$(\mathbf{gy})_{ik}$	=	interaction effect between the i th genotype and the k th year
$(\mathbf{gt})_{im}$	=	interaction effect between the i th genotype and the m th treatment
$(y\mathbf{t})_{km}$	=	interaction effect between the k th year and the m th treatment
$b[y\mathbf{t}]_{nkm}$	=	effect of the n th block nested in the k th year and the m th treatment
e_{ikmn}	=	residual/error of y_{ikmn}

Fixed effects are written in bold.

Pearson correlation coefficients (r) between trait BLUEs were calculated via PROC CORR. Furthermore, to test for significant treatment effects a t -test (PROC TTEST) and an ANOVA within locations were performed (PROC MIXED). The ANOVA model included the main effects (genotype, treatment and year) and their corresponding interaction effects as fixed effects (comparable to model 5). A further t -test was computed to test for differences between the locations.

Performance of the HEB-YIELD lines was compared to the recurrent parent 'Barke' by conducting a Dunnett test [51] with PROC MIXED. The resulting P -values were adjusted following Bonferroni-Holm [52]. To enable a comparison between the traits the relative performance (RP) was calculated as:

$$RP [\%] = \frac{(BLUE (HEB\ line) - BLUE ('Barke'))}{BLUE ('Barke')} * 100 \quad (7)$$

All figures were created using R (3.5.0 [53]) with the package ggplot2 (2.2.1 [54]), except the Circos plots [55].

2.7. Single marker regression

A simple linear model was fit to regress a trait's value on the quantitative SNP marker score obtained from the IBD genotype matrix of Maurer et al. [56]. For this purpose PROC GLM was used to fit the model:

$$y = \mu + Marker + e \quad (8)$$

where

y	=	observed phenotype
μ	=	intercept
$Marker$	=	effect of SNP marker
e	=	residual/error

Subsequently, single marker P -values resulting from an F -test (full model versus reduced model without marker effect) were plotted for each trait in a Circos plot and candidate genes were indicated.

3. Results and discussion

3.1. Phenotypic data

We examined the interplay between plant development, yield, and

nutrient concentrations in the wild barley introgression population HEB-YIELD, a diverse subset selected from the NAM population HEB-25 [46], by scoring 17 traits at two test sites in Dundee (United Kingdom) and Halle (Germany) (Table S2a). The examined traits can be grouped into developmental, yield-related, and nutrient traits, including seven macronutrients (C, N, P, K, S, Ca, Mg) and four micronutrients (Fe, Mn, Zn, Cu), as well as Na (Table S3). All traits were determined under a standard nitrogen fertilizer application regime, following local practice (= control condition) and without nitrogen fertilizer (= stress condition; Table S2c), resulting in 1593 analyzed plots (Table S4a).

The majority of traits exhibited a wide range of variation within each location and treatment, indicated by high coefficients of variation (CV; Figures S2 & S3; Table S5). The extremely low CVs for C with less than 0.5% were striking and confirm that the carbon concentration in plants is very constant with values between 45 and 50% [57]. For the remaining elements, the CVs ranged from around 5% for Mg up to more than 20% for Na, whereby macronutrients showed in general lower CVs than micronutrients.

The ANOVA results indicated that all investigated factors (genotype, year, and location) had significant effects on all traits, except for Cu and ears per square meter (EAR) where location was not significant (Table S6a).

For most nutrients (GPC, P, K, S, Ca, Mg, Fe, Mn, Zn, Cu) we observed heritabilities > 0.54 (on average 0.82; Table 1). The above-mentioned lack of variation for C resulted in low heritabilities at both locations (on average 0.15). Moreover, in Dundee Na had low heritabilities with values < 0.25. The developmental and yield-related traits on average exhibited a heritability of 0.78, whereof the trait EAR was lowest with an average of 0.36.

BLUES across treatments were calculated to obtain a single value per genotype that allowed an easier comparison with already published results (Table S4b). The observed nutrient concentrations fit very well to those presented in the literature (Figure S4; Table S7), except for Na where the studies of Mengesha [58] and Jeroch et al. [59] reported more than fourfold higher values for barley grains. Elemental concentrations varied substantially between genotypes. We identified genotypes which had more than 50% higher concentrations of Na, Fe, or Zn than the recurrent parent Barke (Figure S5). Notably, line HEB_09_163 had 64% and 50% higher GPC than Barke in Dundee and

Halle, respectively. Overall, we identified lines with a maximum GPC of 13.5% and 15.9% in Dundee and Halle, respectively (Table S5). Wild barley might therefore offer a good source for the improvement of GPC, as already indicated by a survey with Tibetan wild barley [60].

3.2. Effects of nitrogen fertilization

Nitrogen undisputedly represents the key nutrient for crops, since this element limits yield in nearly every agricultural cropping system, and there is an increasing demand for it as long as the world population grows. The identification of variation and finally the improvement of the nitrogen use efficiency could be one possible solution to keep the required demand within limits [61,62]. This attempt would help to reduce the costs and energy consumption during the production of inorganic N fertilizer, as well as to secure ecosystems from environmental damage through the application of excessive amounts of N fertilizer [63].

Therefore, we conducted nitrogen deficiency field trials at both locations to assess the effects of the N supply of barley plants on yield and on nutrient concentrations in the grains. The outcome of this experiment indicated that nutrient concentrations in the grains were only to a minor degree influenced by the N supply level of plants, and the treatment effects were noticeably lower than those on grain yield (Fig. 1; Table S5). The only exception from this statement are Zn and Na, which showed a substantial increase in concentration under stress condition (without N fertilizer) in Dundee. Interestingly, the treatment effect for Zn is opposite in Halle in comparison to Dundee and we have no coherent explanation for the different behavior of the N treatment for Zn so far. In any case, the high heritability (> 0.8) points to reliable data. The effects found for grain yield with -17.9 dt/ha and -6.3 dt/ha by comparing control versus stress in Dundee and Halle, respectively, circumstantiate that the treatment was effective and that N is crucial for achieving high yields. The strong N effects on yield are, however, only partly reflected in GPC, especially in Dundee, although N is a main constituent of proteins [63]. In a recently published study Guttieri et al. [64] also observed that N fertilization appears to have only a minor impact on different nutrient concentrations.

In the present study the responses of genotypes to the N treatment were similar, indicated by non-significant genotype-by-treatment

Table 1
Descriptive statistics summary for BLUES.

Trait ^a	Unit ^b	Dundee						Halle					
		Control			Stress			Control			Stress		
		Mean	CV [%]	<i>h</i> ²	Mean	CV [%]	<i>h</i> ²	Mean	CV [%]	<i>h</i> ²	Mean	CV [%]	<i>h</i> ²
HEA	days	84.3	6.4	0.91	85.9	7.0	0.91	66.1	8.1	0.95	67.2	8.2	0.95
EAR	number/m ²	603.9	15.2	0.47 ^c	468.4	11.5	0.33 ^c	571.2	8.4	0.17	470.9	10.5	0.46
GNE	number	19.4	15.9	0.94 ^c	18.5	13.7	0.89 ^c	19.1	14.5	0.85	19.5	12.5	0.85
TGW	g	47.8	7.3	0.82	46.8	6.9	0.79	50.6	8.0	0.92	50.9	8.3	0.92
YLD	dt/ha	55.3	16.7	0.93	37.4	14.8	0.80	40.1	16.8	0.78	33.7	16.2	0.84
C	% DM	45.9	0.4	0.28	45.7	0.4	0.06	46.5	0.4	0.03	46.5	0.3	0.24
GPC	% DM	9.9	12.3	0.91	9.8	10.2	0.85	13.0	9.1	0.80	11.9	10.0	0.89
P	g/kg DM	3.6	7.6	0.91	3.7	7.0	0.88	3.5	7.7	0.83	3.7	7.3	0.87
K	g/kg DM	4.3	7.3	0.87	4.5	5.9	0.84	4.2	9.3	0.85	4.5	8.7	0.89
S	g/kg DM	1.2	7.8	0.65	1.2	6.4	0.76	1.4	7.2	0.83	1.3	7.2	0.81
Ca	g/kg DM	0.3	10.6	0.79	0.3	10.6	0.81	0.4	11.5	0.86	0.4	12.0	0.91
Mg	g/kg DM	1.2	6.3	0.85	1.2	5.8	0.81	1.2	6.1	0.92	1.2	6.3	0.93
Fe	mg/kg DM	29.9	17.5	0.91	29.7	15.2	0.85	35.2	12.5	0.80	31.9	13.5	0.92
Mn	mg/kg DM	8.0	11.4	0.73	7.7	10.0	0.72	11.1	12.2	0.87	10.6	13.2	0.83
Zn	mg/kg DM	19.8	16.2	0.86	23.1	16.0	0.85	26.7	13.3	0.89	25.3	14.4	0.80
Cu	mg/kg DM	5.0	10.5	0.62	5.2	9.2	0.54	5.0	9.5	0.82	5.0	8.0	0.61
Na	mg/kg DM	31.4	20.7	0.25	40.5	17.9	0.23	72.6	29.6	0.80	74.5	27.4	0.78

a) HEA (Flowering time), EAR (Number of ears), GNE (Grain number per ear), TGW (Thousand grain weight), YLD (Grain yield), C (Carbon), GPC (Grain protein concentration), P (Phosphorus), K (Potassium), S (Sulfur), Ca (Calcium), Mg (Magnesium), Fe (Iron), Mn (Manganese), Zn (Zinc), Cu (Copper) & Na (Sodium).

b) DM (dry matter).

c) Repeatability rather than heritability was calculated as only one year of measurements was available.

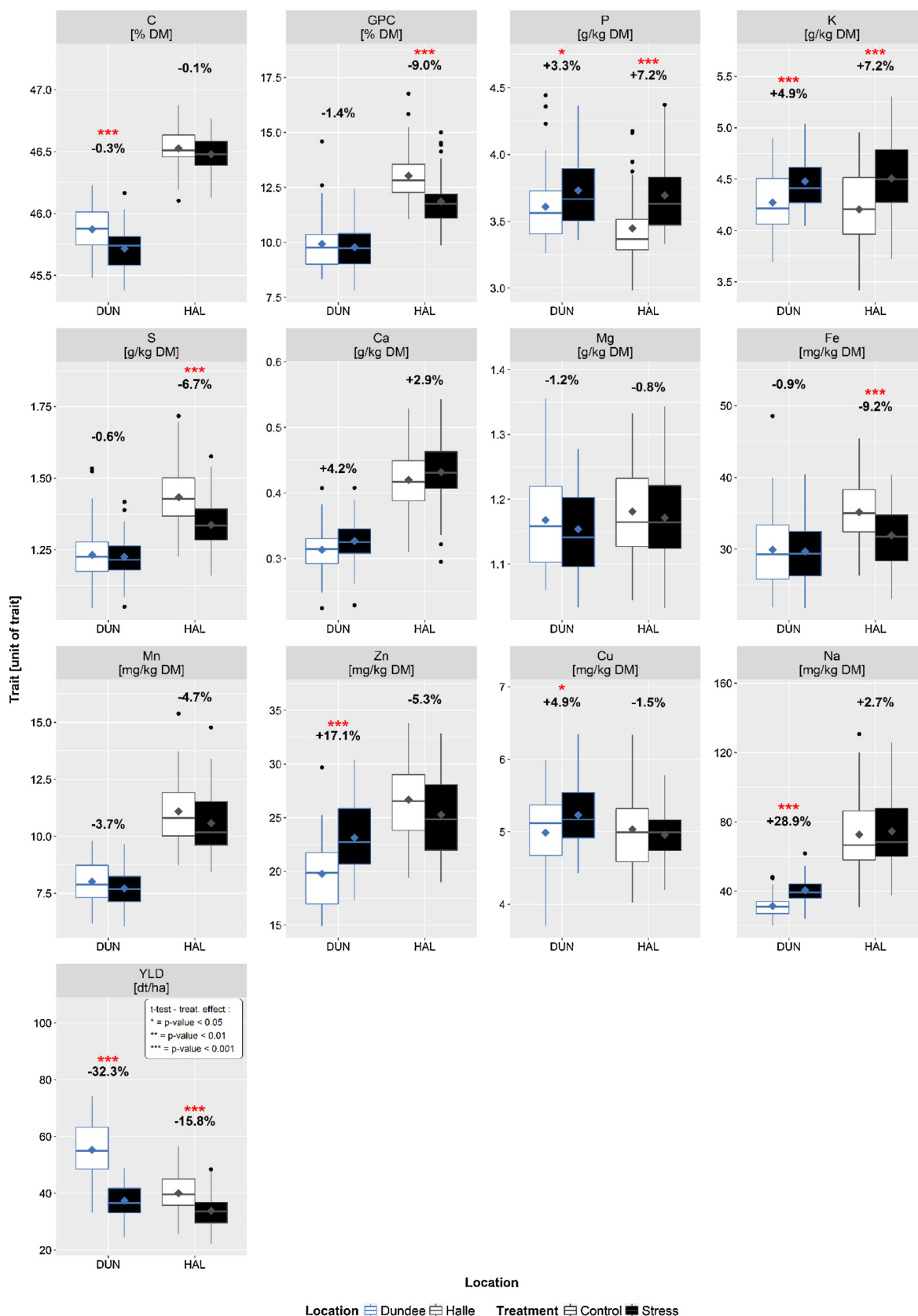


Fig. 1. Trait variation and verification of treatment effects for the studied traits. The trait names and units of the traits are indicated in the grey rectangles above each subplot. Trait abbreviations are listed in Supplementary Table 3. The color of the boxes represents the location, which is also indicated on the x-axis: blue for Dundee (DUN) and grey for Halle (HAL). The y-axis reflects the value of the traits in its specific unit. Non-filled boxes refer to the control condition and filled boxes to the stress condition. Statistically significant treatment effects were obtained via t-test and are indicated by red asterisks above the boxes with $P < 0.05 = *$, $P < 0.01 = **$ or $P < 0.001 = ***$. Additionally, the difference between the means of the two treatments is given in relation to the mean of the control condition in percentage above each box. The figure is based on BLUEs across years. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

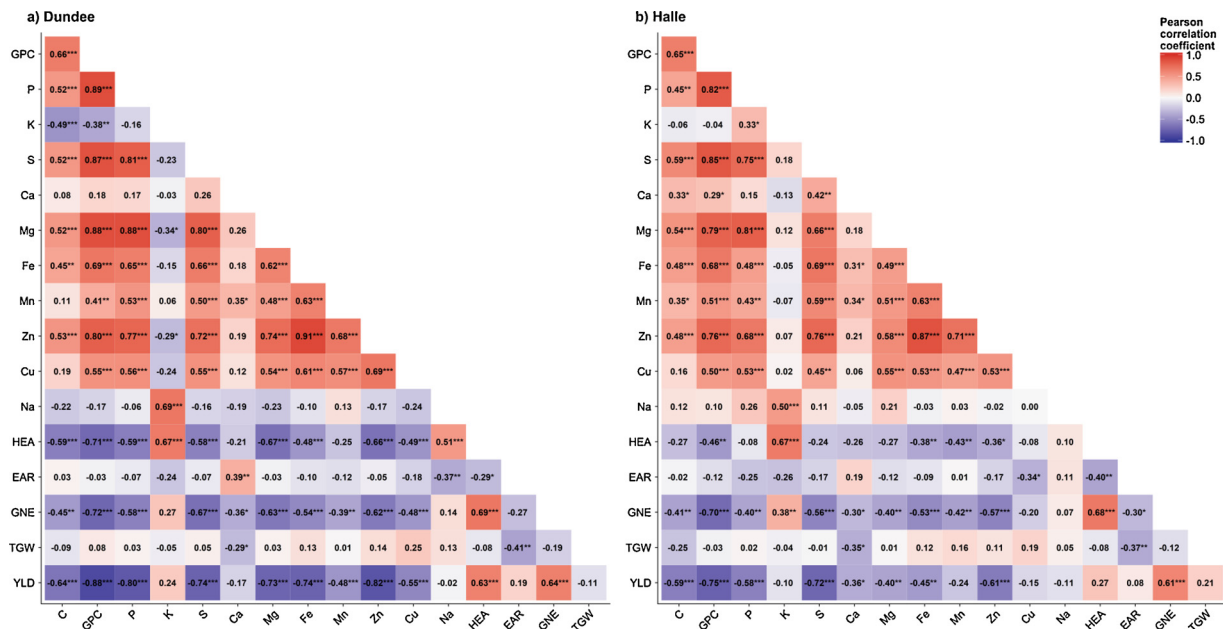


Fig. 2. Pearson correlation heat maps for the studied traits in Dundee (a) and Halle (b). The correlations are colored based on their direction (blue: negative; red: positive) and strength (bright color: weak correlation; dark color: strong correlation). Significance of the correlations is given by asterisks with $P < 0.05 = *$, $P < 0.01 = **$ or $P < 0.001 = ***$. The trait abbreviations are C (Carbon), GPC (Grain protein concentration), P (Phosphorus), K (Potassium), S (Sulfur), Ca (Calcium), Mg (Magnesium), Fe (Iron), Mn (Manganese), Zn (Zinc), Cu (Copper), Na (Sodium), HEA (Flowering), EAR (Number of ears), GNE (Grain number per ear), TGW (Thousand grain weight) and YLD (Grain yield). The figure is based on BLUES across years and treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

interactions for the majority of nutrients (Table S6b). This is also supported by the high positive Pearson's correlation coefficients between control and stress condition for the nutrient traits with an average of 0.84 in Dundee and 0.88 in Halle (Table S8a). Whilst the control plots at Dundee also received supplementary P, K and S, the concentrations of these elements were either less or no different in this treatment when compared to the stress treatment. The same trend was observed at Halle, suggesting that the addition of these elements at Dundee had not biased the results (Table 1). Based on the minor effects of the N deficiency treatment on nutrient concentrations, we decided to merge both datasets for further analyses to increase the statistical power.

3.3. Correlations between plant development, yield, and nutrient traits

To get a first glimpse on the interplay between plant development, yield, and nutrient concentrations, we calculated the correlations between all scored traits across treatments (Fig. 2; Table S8b), as well as within treatments (Figures S6 & S7; Table S8b). Independent of the location, there were two striking findings: first, the high negative correlations between the majority of nutrients (except for K, Ca, and Na) with flowering time (HEA), grain number per ear (GNE), and grain yield (YLD), and second, predominantly positive correlations between most nutrients except K and Na.

The first observation suggests that the mineral concentrations are negatively affected by a 'dilution effect', which is in agreement with a number of previous studies [64–66]. An improvement in yield can be achieved by increasing the grain number per area (= EAR and GNE) or the grain size (= thousand grain weight; TGW) [67,68]. In both cases, the nutrients are distributed into more or larger grains whereas the absolute amount of accumulated nutrients seems to stagnate, causing the well-known 'protein or nitrogen dilution effect' [16,69,70]. In the wild barley introgression population HEB-YIELD, lines that exhibit late HEA and/or high GNE are characterized by superior yields (for more details see Wiegmann et al. [45]), which explains why the nutrient concentrations are also negatively correlated with HEA and GNE. Overall, the negative correlations are more strongly pronounced in

Dundee than in Halle, presumably as a result of the higher yield level by 9 dt/ha. Based on the localization of the majority of mineral nutrients in the aleurone layer of a grain, we speculated that high TGW could have a negative impact on nutrient concentrations because larger grains have a reduced surface-to-volume and aleurone to endosperm ratio, resulting in a higher proportion of starch [71]. However, our results indicate no pronounced effects of TGW on nutrient concentrations in general, since the only significant negative correlation of TGW was observed with Ca (-0.29/-0.35; Dundee/Halle), which is in agreement with studies from McDonald et al. [72] and Zhao et al. [66].

The positive correlations between the majority of nutrients indicate that these elements might share common features in uptake, distribution, or storage. Uptake and transport processes are governed by a multiplicity of proteins, which are involved in the steps from mobilization and uptake from the rhizosphere until the final translocation into the seeds. This includes xylem and phloem loading and unloading, tissue distribution, as well as trafficking and sequestration within the cell [73–76]. Examples for the co-handling of mineral nutrients are the transport of both Fe and Mn by transporters of the MTP and NRAMP families [77,78], the transport of Mn and Ca by BICAT proteins [79,80] or the concerted uptake of Fe and Zn through unspecific divalent metal cation transporters [81,82], which is supported by our data as these nutrients showed the highest positive correlations in both locations (0.91/0.87). Recently, a study in barley investigated the role of HvIRT1, a member of the ZIP family of transporters, which is largely responsible for Mn uptake, translocation and accumulation in the mature grain [83]. Moreover, HvIRT1 also transports Zn because of a broad specificity [83]. This interaction is apparent in our data, since concentrations of Mn and Zn showed highly significant positive correlations (0.68/0.71). A further example for the interdependency of nutrient concentrations is the positive relationship between N/GPC and P, possibly due to an increased root growth through N, which improves the P uptake from the rhizosphere [84]. Several independent studies reported the existence of such patterns of correlations between specific nutrients [64,66,85].

In addition, the correlations between K and Na are noteworthy,

since they are mostly contrary to the remaining nutrients. Interestingly, K and Na show only slightly negative or even positive correlations with YLD. The stability of K concentrations in cereal grains under various conditions has been reported before. For example, in a long-term experiment, low K supply rates caused a drop in grain yield of barley and in K concentration in straw [86]. However, K concentrations in grains were invariant and, similar to the findings in the present study, not negatively correlated with yield. This indicates that plants specifically regulate K import during grain filling by unknown mechanisms [87]. K and Na exhibited similar correlations, probably because they partially share the same transport mechanisms and because Na can partly substitute for K in cellular functions [88,89]. Nevertheless, at present it is unclear why K and Na behave different from the majority of other nutrients.

3.4. Improved genotypes for barley breeding

During the last decades wild material has been frequently used for the introgression of genes and alleles encoding for favorable attributes into elite germplasm [31,90]. However, this was mostly successful for the improvement of resistance against pathogens and tolerance to abiotic stresses, rather than the improvement of quality and yield [91]. There have been only a few diversity studies which examined the genetic potential for crop biofortification [64,66,85,92]. The present study is one of the first to evaluate cereal genetic resources to improve grain nutrient concentrations.

The huge phenotypic variation of the “Halle Exotic Barley” wild introgression population has already been exemplified for plant development [40,44], resistance to fungal pathogens [43,93], tolerance to abiotic stresses [41,94] and yield [45,95]. The HEB lines offer the possibility to estimate potentially positive wild allele effects in an adapted background, as they are embedded into the elite barley cultivar Barke (for more details see Maurer et al. [46]). Using such a background enables the direct use of these lines as crossing parents in elite barley breeding programs.

In both locations we could identify HEB-YIELD lines, which significantly outperformed the recurrent parent, cv Barke, regarding the concentration of nearly every investigated nutrient (Fig. 3; Table S9). In particular, for GPC, Ca, Na, Fe, and Zn we could identify a number of promising lines with more than 50% higher elemental concentrations. Some of these lines showed higher concentrations than the recurrent parent Barke for several traits simultaneously, for instance HEB_08_096, HEB_09_163, HEB_11_025, HEB_14_045, HEB_15_082, HEB_15_094, HEB_18_225, HEB_19_076 and HEB_25_020. The increased concentrations in these lines were stable across both investigated environments, although in general nutrient concentrations are influenced by pronounced genotype-by-environment interaction effects (Table S6a) [96,97]. These distinct interactions render it difficult to select superior lines in a breeding program based on a single or few environments [98,99]. Additionally, we found lines that showed increased concentrations of single elements, like for Na (e.g. HEB_07_063 in Dundee and HEB_08_202 in Halle) and Ca (e.g. HEB_10_184 in Dundee and HEB_01_132 in Halle), whereby the latter lines had roughly the same yield as Barke.

Nevertheless, it must be mentioned that the majority of lines had a considerably reduced grain yield. This is best exemplified by HEB_09_163, which exhibited significantly higher concentrations of GPC, P, S, Ca, Mg, Fe, and Zn in both locations, but also a more than 50% lower yield level. As discussed in 3.3, this negative relationship between nutrient concentration and yield is well-described [64–66], making it difficult to select for higher nutrient concentrations without reducing yield. Moreover, a reduction in yield is hardly acceptable, as we need to raise yields in the next decades to supply the growing world population with a sufficient amount of food [2,3].

3.5. Nutrient yield

We further explored the relationship between nutrient concentrations and yield by calculating the nutrient yield (= product of plot grain yield and its respective nutrient concentration; in agreement with Khan et al. [100]).

Nearly all HEB-YIELD lines showed significantly lower nutrient yields than Barke, particularly in Halle where we found strong reductions in nutrient yield for the elements K (up to 55%), Ca (51%), Fe (47%), P (44%) and Zn (44%) (Figure S8; Table S9a). In contrast, in Dundee we could detect lines that had significantly higher nutrient yields for Ca (+32%), Fe (+27%) and Zn (+18%) (Figure S9; Table S9a). However, the general trend is unambiguous that most HEB-YIELD lines had inferior nutrient yields to Barke. There are only a few studies available that investigated the relationship between nutrient concentrations and nutrient yields, but there is an in-depth knowledge present about the relationship between protein concentration and protein yield [16,101–103]. One common observation is that an improvement in protein yield is mainly achieved by raising grain yield rather than protein concentration. This is in agreement with the achievements of the last decades of breeding and selection for higher grain yields, which resulted in lower grain protein concentrations, but improved protein yields [17,102]. Based on our data we suggest that this is also valid for other nutrient yields, since those lines having significantly lower grain yields than Barke are characterized by marginal nutrient yields, whereas the local check cultivars mostly exhibited superior nutrient yields in both locations (for instance GPC: Figure S10, and Zn: Figure S11).

Our findings clearly support the existence of high variations of nutrient concentration in HEB-YIELD and a pronounced negative relationship between yield and the majority of the investigated nutrient concentrations with the exception of K. One breeding approach would be to cross the best-performing HEB-YIELD lines regarding nutrient concentration (e.g. HEB_09_163 for GPC) with a high-yielding elite line and derive a random inbred population that could be used to determine if the two characters can be separated genetically and, if so, identify not only suitable recombinants but also genetic markers that could be used in future selection programs. As reported by Bogard et al. [104] different strategies have already been applied to reduce the negative correlation, including the introgression of genes from related species, even though all of these strategies failed so far.

However, potentially methods based on genetic engineering may be more successful, if they are transferable to field conditions. Two recently published studies report on quite considerable success by over-expressing the Fe and Mn transporter TaVIT2 [105] and the Zn transporter HvMTP1 [106] under control of an endosperm-specific promoter in wheat and barley. In both cases grain nutrient concentrations were improved without negative impacts on growth and yield in greenhouse trials [105,106].

3.6. Associated genomic regions

By dissecting the genetic architecture of the investigated nutrient traits through genetic mapping, quantitative trait loci (QTL) that control the trait variation can be identified [107,108]. Therefore, we applied a single marker regression analysis aiming to identify QTLs that improve nutrient concentration in HEB-YIELD without negative impacts on yield, as recommended by Bogard et al. [109]. However, it must be noted that the relatively small population size of HEB-YIELD (48 lines) might lead to biased results, indicated by a lower QTL detection rate, more false positives, and an overestimation of effect sizes [110–112]. Nevertheless, the obtained results can give a first glimpse on the genetic control of our studied traits. We plan to verify detected QTLs by a follow-up study with heterogeneous inbred families (HIFs [113,114]) segregating for the two alternative alleles present at a promising QTL.

Based on our findings we detected a number of QTLs that

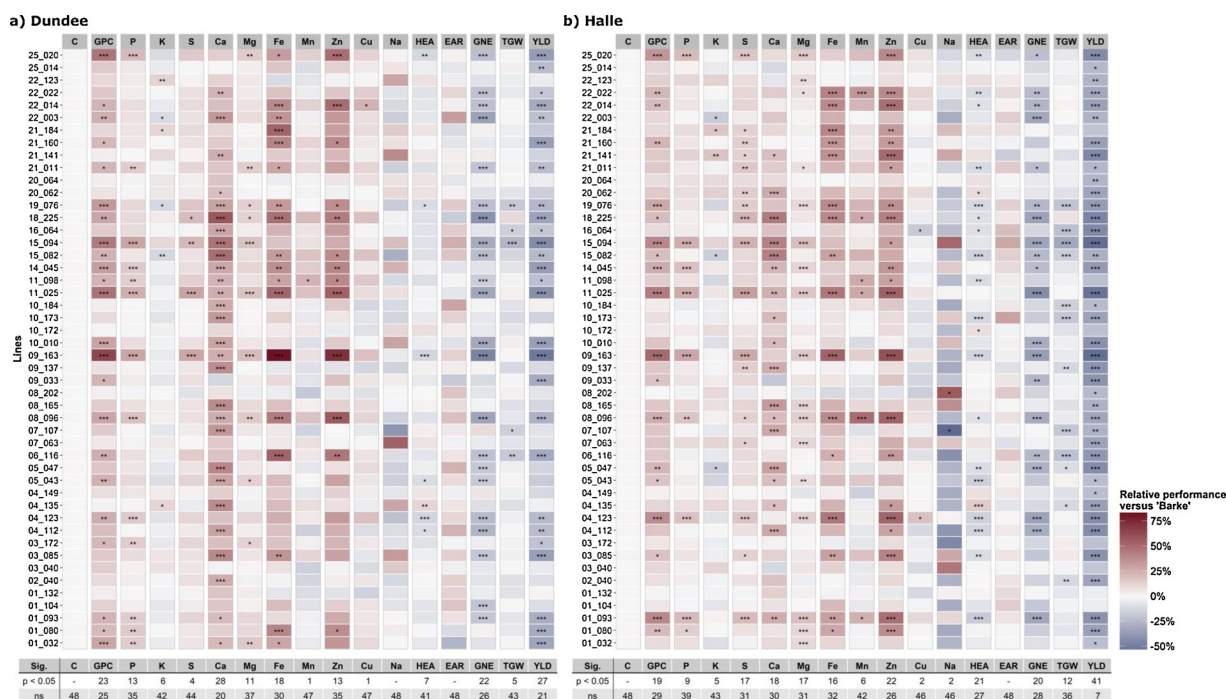


Fig. 3. Heatmap showing the relative performance of the 48 HEB-YIELD lines in comparison to the recurrent parent ‘Barke’ for the studied traits in Dundee (a) and Halle (b). The color of the tiles represents a positive (red) or negative (blue) deviation from Barke. In addition, the results of a Dunnett’s test with Barke as reference are indicated for each line inside the tile. Significant deviations are shown by asterisks with $P < 0.05 = *$, $P < 0.01 = **$ or $P < 0.001 = ***$. The p-values are Bonferroni-Holm corrected, and a summary table of the test is shown below the figure. The trait names are indicated in grey rectangles at the top, and their abbreviations are listed in Supplementary Table 3. The figure is based on BLUEs across years and treatments (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

simultaneously influenced the majority of investigated traits and, in several cases, corresponded to well-known candidate genes (Figs. 4 and 5; Tables S10 & S11). We identified these genomic regions simultaneously in both locations, indicating the robustness of the results. Below we will discuss a few of these regions, indicating the importance of plant development and yield, as well as nutrient uptake and translocation to determine the final grain nutrient concentration in HEB-YIELD.

3.6.1. Short arm of chromosome 2H

We detected significant effects on the traits HEA, K, Fe, Mn, and Zn in both locations, as well as on GPC, Ca, Mg, Cu, and Na in Dundee originating from the short arm of chromosome 2H (Tables S10 & S11). Except for HEA, K, and Na the wild barley allele increased the trait values. In most cases SNP markers, that are located directly within the *Ppd-H1* gene sequence, showed the lowest p-values. HEA effect sizes of around -8 days pinpoint to a possible role of *Ppd-H1*, confirming results already obtained in the whole HEB-25 population [40,44,46]. *Ppd-H1* is the main regulator of photoperiodism in barley and determines flowering time to a high extent [115], as well as exerting pleiotropic effects on a number of additional developmental and yield-related traits [40,44], however without a significant impact on yield in HEB-YIELD [45]. Most wild barley accessions possess the dominant responsive *Ppd-H1* allele, which accelerates development under long-day conditions [116,117]. From studies on *Arabidopsis* it is known that several nutrient transporters are regulated by the circadian clock and that the expression of *PRR7*, the *Arabidopsis thaliana* orthologue of *Ppd-H1*, is under clock control [118]. Consequently, the detected effects might be the result of the influence of *Ppd-H1* on nutrient transporter regulation, as well as on overall plant development.

3.6.2. Long arm of chromosome 3H

Sdw1 is the major semi-dwarf gene locus in barley, located on the

long arm of chromosome 3H. Exotic *Sdw1* alleles or genes in its proximity exhibited strong effects on our studied traits, especially on plant height [45] and YLD (Tables S10 & S11). In addition, the majority of nutrient traits showed positive effects arising from this region, which clearly supports the negative relationship between yield and nutrient concentration. The region on the long arm of chromosome 3H showed significant effects on GPC, P, Ca, Mg, and Zn in Dundee and Halle, whereupon the wild allele increased all traits except Ca, which was clearly reduced. Semi-dwarf alleles have been widely used in modern breeding programs and were one crucial component of the ‘Green Revolution’ boosting grain yields in the past [15,119,120]. Semi-dwarf barley cultivars are characterized by reduced plant height, late maturity, increased tiller numbers, and improved harvest index, altogether resulting in elevated grain yields [121,122]. This is in agreement to our observations that HEB-YIELD lines carrying the wild allele (= long straw allele) at *Sdw1* had an increased plant height and a distinctly reduced yield. The reduced yield might be one explanation why most nutrient concentrations showed positive effects coming from the wild allele of *Sdw1*, indicating the important relationship between yield and quality.

3.6.3. Short arm of chromosome 6H

We identified pronounced impacts of the short arm of chromosome 6H on nutrient concentrations, influencing the traits C, GPC, P, S, and Mg at both locations (Tables S10 & S11). In each case, the wild allele increased the nutrient concentration. The senescence-inducing gene *NAM-1*, located on the short arm of 6H [123], might be a probable candidate for this locus. This gene belongs to the family of NAC (NAM, ATAF-1,2, CUC) transcription factors, which influence a multitude of plant processes, such as development and senescence [124]. From studies in wheat and barley it is known that early senescence can improve nutrient concentrations, especially of GPC, Fe, and Zn, accompanied with negative impacts on yield [123,125,126]. These findings

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