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## Characterizing Barley Seed Macro- and Micro-nutrients under Multiple Environmental Conditions

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Crop seeds are the main staples in human diet, especially in undeveloped countries. In any case, the diet needs to be rich not only in macro-nutrients like carbohydrates and protein, but also in micro-nutrients. Nevertheless, both the macro- and micro-nutrients presented in seeds largely vary in consequence of field and environment conditions. In this research, 60 lines of a barley RILs population segregating for the SSR marker *Hvm74*, which is genetically linked to the *GPC* (grain protein content) locus (*HvNAM-1*), were studied in 4 environments (two growing years and two field managements) by carrying out a comprehensive profile of seed macro- (starch, total nitrogen and total soluble protein) and micro-nutrients (phytate, phenolics, flavonoids, P, Zn and Fe). Under field conditions, all the components were largely affected by the environment, but TN (total nitrogen) exhibited high genotype contribution, while micro-nutrients displayed higher genotype  $\times$  environments interactions (GEI) than macro-nutrients. In order to approach the effects of carbon-nitrogen (C–N) balance on other seed components, two C/N ratios were calculated: C/TN (CNR1) and C/TSP (CNR2). CNR2 exhibited stronger negative correlations with all micro-nutrients. Hence, the significant GEI and its negative relationships with CNR2 highlighted the different characters of micro-nutrients in barley seeds.

**Keywords:** seed component, macro-nutrient, micro-nutrient, genotype  $\times$  environments interactions, carbon/nitrogen ratio, *Hvm74*

### Introduction

Carbon (C) and nitrogen (N) are the major elements stored in crop seeds which highly related to the final crop yield (Triboi and Triboi-Blondel 2002). Robust evidences from field trials suggested remarkable negative correlations between GPC and total yield (Blanco et al. 2012). Genetically, *GPC* locus (*HvNAM-1*), a homologous gene of *GPC-*

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*B1(TtNAM-B1)* from wild emmer wheat (*Triticum dicoccoides*) (Uauy et al. 2006), was detected in barley that controls leaf senescence and resulted in N remobilization (Distelfeld et al. 2008), thereby also enhanced barley GPC (Parrott et al. 2011). However, the direct relationship between C compositions and *GPC* locus has not yet been reported in barley, even though C compounds are the major components in barley seeds.

There are plenty of seeds components with levels lower than 1% of total grain, which are regarded as ‘micro-nutrients’, but played crucial roles for human health. In spite of the high heritability obtained in greenhouse conditions (Mamo et al. 2014), this group of traits exhibited low heritability (Joshi et al. 2010) in fields since the prevailing effects of environments (Marwede et al. 2004). The genotype  $\times$  environment interactions (GEI) are the major sources for phenotypic variation in micro-nutrients and have been investigated in wheat (Ficco et al. 2009). Few studies were devoted to the relationships between macro- and micro-nutrients (Gomez-Becerra et al. 2010). Specifically, the general relationships between micro-nutrients and C–N balance are still poorly understood in seeds. Phosphorus (P) does not belong to micro-elements in plants because of its large percentage in dry matter. However, the total P in dry seeds is lower than 1% of total grain and could therefore be classified as micro-nutrient in seed researches. In addition to its functions for human diet, seed P is considered to be of benefit for seedling establishment and finally crop yields (White and Veneklaas 2012).

Considering the plenty of environmental experiments on seed components, large cultivar collections are commonly taken as materials to observe the complicate GEI by several mathematic models (Joshi et al. 2010), but the diverse genetic background might enlarge their GEI. Moreover, these studies are often lacking of a comprehensive profiling for seed components (Hristov et al. 2010). In barley seeds, the GEI of seed components are frequently combined with studies of malting quality for further investigation (Holopainen et al. 2015). Notwithstanding it is a C-accumulating seed crop, in barley the C–N balance is also rarely observed under multiple environments. Therefore, in our study, 60 lines of bi-parental RILs population with simple genetic background were investigated for GEI on both macro- and micro-nutrients with comprehensive profiling of seed components among 4 different environments, the further effects of C–N balance on micro-nutrients were also characterized in this study.

## Materials and Methods

### *RILs and field experiments*

Sixty lines of 146 barley RILs population were chosen as material basing on its segregation on SSR marker *Hvm74* on chromosome 6H which was demonstrated to be tightly linked to the *GPC* locus (Distelfeld et al. 2008). The RILs population was obtained by crossing cultivar “Karl”(6-row) and “Lewis”(2-row) which also proved to be diverse at GPC trait (Parrott et al. 2011). All the 60 barley lines belong to six-row type. Field experiments carried out at two Yunnan-Guizhou plateau sites including Guiyang (Site 1, 26°34'N, 106°27'E, 1100 m a.s.l) and Anshun (Site 2, 26°15'N, 105°55'E, 1395 m a.s.l),

Guizhou province of P. R. China. Two types of environment were applied to profile the seed components including different growing years and field managements. Two growing years were completed at Site 1 during years of 2010–2011 (Y2011) and 2011–2012 (Y2012) without fertilization. Whereas, two field managements were conducted at Site 2 during years of 2012–2013 but distinct by two fertilizer conditions, one set was fertilized by 30 kg/ha N:P:K 15:15:15 (Y2013F), comparing to the other condition without any fertilizations (Y2013NF). All barley seeds were sowed in furrows (headrows) over all field conditions with 1.5 m width and 50 cm intervals. The fertilizations were applied into furrows directly during seed sowing. Three replicates were made by randomized block design in each field, respectively. The soil samples of all field sites were collected at the depth of 0–30 cm and analyzed with parameters of pH, organic matter content and element concentrations. Seeds sample were cleaned and grinded into wholemeal flour by FastPrep-24 Automated System (MP Biomedicals, USA), then filtered by 0.45 mm sieve and dried in oven at 40 °C for a week before the analysis.

#### *Analysis of seed components*

Starch (Sta) was conducted by Megazyme Kit (K-TSTA 07/11) and the procedures were modified from the AACC76.13/AOAC996.11. TN was determined by Kjeldahl method using Tecator Kjeltac 8400 (FOSS, Sweden). The parameter of GPC was multiplied by coefficient of 5.68. Total soluble protein (TSP) was analyzed by UV-Spectrophotometer (Beckman, USA) based on the BCA protocol.

Total flavonoid (Fla), phenolics (Phe), phytate (Phy) and inorganic phosphorus ( $P_i$ ) content were determined by stepwise extractions on one sample. Samples were extracted by 50% methanol following 1 h ultrasonic treatment, 30 min water bath at 65 °C and 30 min shaking at room temperature by PCMT Thermo-Shaker (Grant-bio, UK). After 10 min centrifuge, samples were placed at 4 °C with minimum 48 h for sugar sedimentation. The supernatants were measured for Fla and Phe content, respectively. The residues were vacuum-dried by Concentrator Plus (Eppendorf, Germany) for 8 h, 0.5M HCl was added to extract residues and the supernatants were measured for Phy and  $P_i$  content independently. Fla content was determined by the method of Jia et al. (1999). The Folin-phenol method (Ainsworth and Gillespie 2007) was applied to determine the Phe content. The supernatants after second extraction were analyzed for Phy based on Wade reagent (Latta and Eskin 1980). The  $P_i$  content was measured by the methods of Ficco et al. (2009).

For the mineral determination of Zn and Fe, samples were digested with  $HNO_3$  (65%) by MARS5 microwave digestion system (CEM Analytical, USA). The diluted solutions after digestion were analyzed for Zn and Fe concentrations by atomic absorption spectrometry (AA6000, Techcomp, China). Single kernel weight (SKW) and hundreds kernel weight (HKW) were measured independently considering the seeds variation in the six-row type of barley, repeating 10 and 4 times, respectively.

### Statistical analysis

Two-way ANOVA was carried out by STATISTICA7 (StatSoft, USA) considering genotypes (G) as a fixed factor and environment (E) as a random factor, wherein the data were transformed by  $\text{Log}_{10}$ . Nested ANOVA was conducted with genotypes nested in haplotype (*Hvm74*). Tukey HSD was applied by JMP 6.0. Principal component analysis (PCA) were conducted by software PAST and the graphs were drew by Sigmaplot 12.0 with data normalization of dividing average values. Pearson correlation matrix was calculated by R 2.1.1 with same data normalization as PCA, then were visualized into networks using the open source bioinformatics software platform Cytoscape 2.8.1.

## Results

### Profiling of barley seed components from multiple environments

The brief soil conditions and average temperature information from three filed sites have been described in Table S1\*, which were highlighted by variations of  $T_{\text{april}}$ , soil pH, Mg and Mn. In PCA of all seed traits (Fig. 1), 4 environments are significantly separated by first two components which explain 60.1% of total variation. Seed components in Y2013F

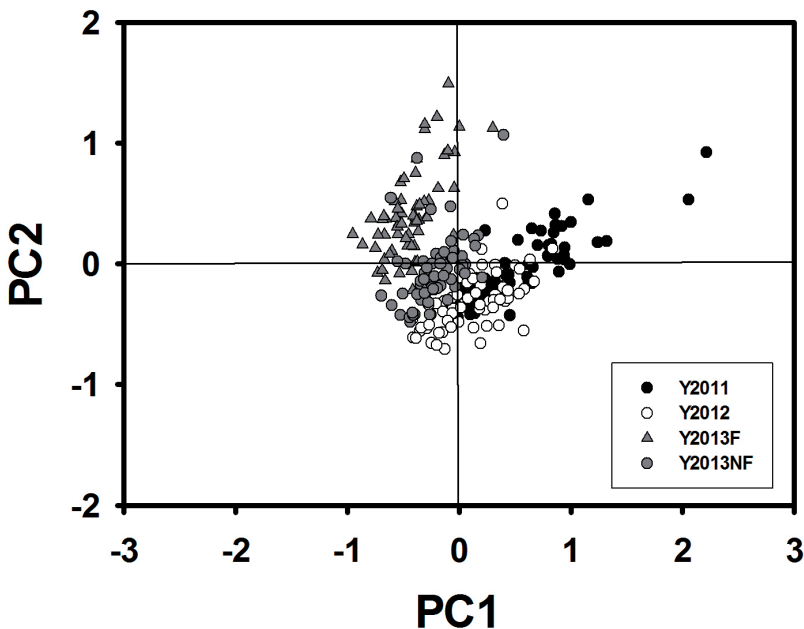


Figure 1. Principal component analysis of barley seed components, HKW and SKW in 4 environments. The first two components explain 36.1% and 24.0% of variations, respectively

\*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

are clearly shifted from other environments suggesting the strong influences from the field managements. Meanwhile, the large variations between Y2011 and Y2012 also proved the significant effects from different growing years which varied in both soil and weather conditions (Table S1), wherein the unfavorable temperature during seed developments and its relatively lower micro-nutrients in soil condition were found Y2011. Nine seed components of 60 RILs and 2 parents from 4 environments are exhibited in Fig. S1, as well as HKW and SKW. Briefly, Sta and TN were accumulated among 4 environments in a contrary way; whereas this pattern was not clearly followed by the micro-nutrients.

#### *Effects C–N balance on barley seed components and correlations between macro/micro-nutrients*

Two C/N ratios were calculated in Fig. S1 using Sta as C, GPC (C/N ratio 1, CNR1) or TSP (C/N ratio 2, CNR2) as N, concomitant with average ratio of 6:1. There are significances in CNR1 among all environments but no significant difference between Y2013F and Y2013NF in CNR2. This result proved that field managements could alter CNR1 but not CNR2, and indicated TSP is more stable in barley grain than TN. Correlation-based networks were applied on all environments dataset and the interactions between groups of macro- and micro-nutrients were illustrated in Fig. 2A. Strong correlations from Phy and Phe to TSP were positively linked with the reversed patterns to Sta. The core networks of CNR were highlighted in Fig. 2B with uncorrelated traits of SKW and HKW. Interestingly, CNR2 was negatively correlated with all micro-nutrients but CNR1 only showed two links with  $P_i$  and Fe. The CNR2 improved the correlations with micronutrients comparing to the individual C and N in Fig. 2A.

#### *ANOVA for GEI and haplotype Hvm74*

Environments had significant effects ( $p < 0.001$ ) on all traits and specifically large contributions to Sta, TN and Zn, but these effects were minor to Phy and  $P_i$ , as well as HKW and SKW (Table S2). In antioxidants, Phe is less affected than Fla by multiple environments. Also, TSP is less influenced by environments than TN. However, genotypes contributed different significances to seed components. There are no significances observed between genotypes in traits of Sta, TSP, phytate, Phe and  $P_i$ , but significances with  $p < 0.001$  in traits of TN, Fe, HWK and SKW, and significances with  $p < 0.05$  in traits of Fla and Zn. These results suggested that macro-nutrients of C (starch), soluble N (total soluble protein), and P (phytate and  $P_i$ ) of barley grains were not inherited in this RILs population, whereas, micro-nutrients of Fla, Zn and Fe exhibited high hereditary under multiple environments. GEI showed no significances in Sta and TSP, these results were same as genotype contributions. High levels of GEI were observed in all micro-nutrients except Zn and Fe. Two types of C/N ratios displayed similar results as TN and TSP, respectively.

The segregation of *Hvm74* divided 60 lines into 34 and 26 lines with “Lewis” and “Karl” alleles, respectively. In the nested ANOVA (Table S3), genotype was nested in the

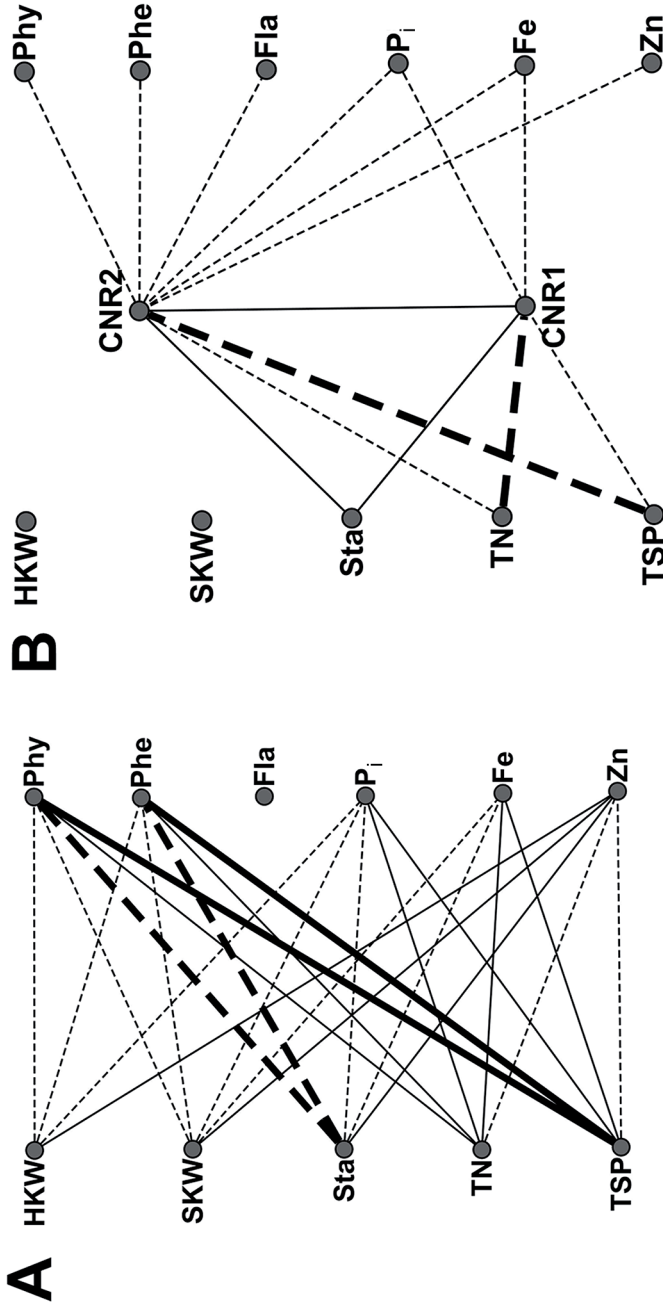


Figure 2. Correlation-based network analysis of barley seed components in 4 environments. The networks used the data from 4 environments. Figure A represents the correlations between macro- and micro-nutrients. Figure B only refers to the correlations between CNR and seed components. The solid and dotted lines refer to the positive and negative correlations, respectively. Edges with increased width indicated the coefficients up from “ $r^2 < 0.5$ ” to “ $r^2 > 0.5$ ”. Sta = starch, TN = total nitrogen, TSP = total soluble protein, Phy = phytate, Phe = total phenolics, Fla = total flavonoids, P<sub>i</sub> = inorganic phosphorus, Zn = zinc, Fe = iron, HKW = hundreds kernel weight, SKW = single-kernel weight, CNR1 = starch/total nitrogen ratio, CNR2 = starch/total soluble protein

haplotype *Hvm74*. The traits of Sta, TN, Fe and CNR1 displayed significances in haplotype contributions with  $p < 0.001$ . However, genotype-nested-*Hvm74* exhibited significant contributions to TN, Fe and CNR1 but not to Sta. Moreover, the contributions of genotype-nested-*Hvm74* in these three traits are much higher than the contribution from haplotype, which indicated that *Hvm74* is still not the major factor for those traits. In addition, haplotype *Hvm74* also exhibited significances on traits of Phy and Fla but with minor contributions.

## Discussion

### *Macro- and micro-nutrients of barley seeds give different GEI*

Seed components are classified according to their levels, which are associated with different GEI under multiple environments. The GEI of GPC and Sta under multi-environments trials have been discussed by plenty of studies, with general opinions that both GPC and Sta are genetically controlled (Bao et al. 2004; Souza et al. 2004) but also largely influenced by environmental factors (Zhang et al. 2001). Their GEI could be as large as the contribution of the genotype (Jeuffroy et al. 2014). In our study, Sta not only showed low GEI, but also low genotype contributions, mainly due to the non-significance of the difference in Sta content between bi-parents. In contrast, TN exhibited 21% variation due to genotype, which is twice higher than the corresponding value of GEI. Thus, the GEI of TN is relatively lower than the percentage of variability explained by environment and genotype, thereby supporting that genetic materials with simpler genetic background might reduce the GEI of corresponding traits. However, TSP did not show the same results as TN for both genotype and GEI contributions. Apparently, TSP is not strictly related to GPC under multiple environments. This suggests that other nitrogen-containing components, like insoluble proteins and inorganic nitrogen, show a large GEI.

In our results, all the micro-nutrients were largely affected by environments, wherein Zn (47%) exhibited highest environmental contributions in ANOVA results (Table S2) and might be influenced by the varied soil Zn contents among three field sites. However, different soil Fe contents were also detected but concomitant with relatively lower contributions (27%) from environments in ANOVA, which might be offset by the positive effects from *GPC* locus with supports from nested ANOVA (Table S3). Moreover, 4 traits of Phy (51%), Phe (43%), Fla (33%) and  $P_i$  (43%) displayed higher GEI than Zn and Fe, which emphasized the distinguished characters of antioxidants and P portions from micro-elements. High GEI of antioxidants in other crop seeds has been observed with 35% of Phe and ~30% of other antioxidants in durum wheat (Brankovic et al. 2015). Similarly, 34.7% and 44.3% GEI have been found for Phy in bread wheat and durum wheat, respectively (Brankovic et al. 2015), but only 7% in rice seeds (Liu et al. 2005). In Brankovic's study,  $P_i$  showed relatively lower GEI in both bread wheat (15.5%) and durum wheat (27.0%), which is different from our barley seeds. High GEI of both Phy and  $P_i$  in barley were probably due to the seed hulls, as it was proved that hulled barley is able to maintain more stable P content, especially the Phy (Liu et al. 2007). Large GEI for Zn (51%) and

Fe (54%) were also observed in durum wheat (Ficco et al. 2009), but slightly lower GEI for Zn (31%) had been observed in spelt wheat (Gomez-Becerra et al. 2010). In milled rice grain, Zn and Fe exhibited 18% and 46% GEI, respectively (Pandian et al. 2011). Few studies reported insignificant and small GEI for both Zn and Fe in seeds (Joshi et al. 2010). The different GEI of antioxidants and P portions from micro-elements might be due to their distinguished transfer mechanism during seed development. Antioxidants are mainly synthesized during seed maturation, which could be largely affected by environmental conditions during seed desiccation (Caldwell et al. 2005). The P content in seeds also depends on the P utilization efficiency (PUE), which is determined by the available P in soil (Ozturk et al. 2005). Even the soil total P was not varied from three site, but the different soil pH also could largely change the soil available P and might increase the GEI of seed P portions. The *GPC* locus might bring potential effects on micro-elements in barley, and its positive effect on Fe has been proved in our study by the nest-ANOVA (Table S3). The remobilization of N compounds might improve translocation of Zn and Fe into seeds. Therefore, even the soil micro-nutrients were shifted from three sites, the relatively lower GEI was still observed for Zn and Fe in our multiple environments study suggesting the strong genetic influences from *GPC* locus.

#### *C–N balance in barley seeds and its effects on other components*

C/N ratios were investigated in our study, and they showed improved correlations with micro-nutrients than the individual C and N nutrients; especially CNR2 exhibited negative correlations with all the micro-nutrients (Fig. 2B). In fact, C/N ratio has been used as a parameter in other seed research (He et al. 2005). CNR2 derives from the final levels of hydrolysable starch and soluble protein in dry seeds, which could provide part of information for both actual photosynthesis capacities and N uptake and remobilization levels. Based on the general opinions of N priority (Triboi and Triboi-Blondel 2002) in C/N ratios, most negative correlations of CNR2 with micro-nutrients might be contributed by TSP. Since antioxidants mainly belong to secondary metabolites and are synthesized at late seed developmental stage (Routaboul et al. 2006), it is also possible that they are highly related to the soluble protein levels due to the main functions of N compounds in seeds secondary metabolism (Scheible et al. 2004). The positive correlations between *GPC* and micro-elements (Zn and Fe) have been documented (Gomez-Becerra et al. 2010). The roles of Fe and Zn, which frequently are involved in protein synthesis and N metabolism, are also reported (Takahashi et al. 2009); for example, Zn-finger protein and nitrate fixation by Fe (Hell and Stephan 2003). However, no significantly negative correlations were found between seed P and *GPC* in rice seeds (Liu et al. 2005), suggesting the negative correlations between CNR2 and P compounds (Phy and P<sub>i</sub>) might be caused by C compounds. The C/N ratio also points out the seeds orientation or adaptation of C/N metabolisms under their specific environments. Following the concept of sink-source communication during seed development (Triboi and Triboi-Blondel 2002), lower C/N ratios could be raised by the increased seed-sink strength but mainly by the varied N remobilization induced by environments.



### *Effects of haplotype Hvm74 on seed components*

In the aim of understanding the effects of the *GPC* locus on seed components in barley seeds, haplotype *Hvm74* was studied and results proved that it not only had effects on Sta and TN, but it also exhibited a strong effect on Fe. The leaf senescence and N remobilization have been clearly demonstrated in *GPC* domain lines (Parrott et al. 2011). Even the Zn and Fe amounts had been reported to be associated with *GPC1* gene in wheat (Pearce et al. 2014), but the Fe enhancement by effect of the *GPC* locus had not been previously reported for barley seeds. The ions transform of Zn and Fe in phloem in is still not clearly understood, but the *GPC-B1* gene has been assumed to encode transporters or chelators for their mobilizations into seeds (Distelfeld et al. 2007). A recent investigation of Zn translocation in wheat seeds structures proved the high levels of mobilizations of Zn into seed rachis and crease at late seed developmental stage (Stomph et al. 2011), which might be associated with the N remobilization into seeds at same developmental stage. Ultimately, nested-ANOVA shows that though haplotype at the *GPC* locus has significant effects on several seed components, it still explains a minor source of variation with respect to the genotype, suggesting other genes are also involved in the accumulations of Sta, TN and Fe within this barley population.

In a word, we presented a comprehensive profile of barley seed components from 4 environments, with a remarkable high GEI in Phy, Phe, Fla and  $P_1$  but relatively low GEI in Zn and Fe. The segregations of *GPC* locus within the population and its strong effect on Fe suggested its significant influences on micro-elements. The dissections on C–N balance highlighted the CNR2 as a key parameter for the interactions between macro- and macro-nutrients in barley seeds.

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### Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at <http://www.akademai.com/content/120427/>

Electronic Supplementary *Table S1*. Average temperature, pH, organic matter content and element concentrations in the soil of three field locations in China

Electronic Supplementary *Table S2*. Two-way ANOVA of seed components from 4 environments

Electronic Supplementary *Table S3*. Nested ANOVA of seed components from 4 environments, genotype nested within haplotype *Hvm74*

Electronic Supplementary *Figure S1*. Comparative average of barley seed components, carbon/nitrogen ratio, HKW and SKW in 4 environments