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Field trials reveal trade-offs between grain size and grain number in wheat ectopically expressing a barley sucrose transporter

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ABSTRACT

Context: Optimizing assimilate partitioning to wheat grains is important to improve grain size and filling. Manipulation of gene expression of assimilate transporters is promising to improve uptake capacity and partitioning. The barley sucrose transporter HvSUT1 has been ectopically expressed in winter wheat controlled by the barley Hordein B1 promoter (HOSUT lines). In greenhouse experiments, HOSUT lines produced lager grains and higher grain yields than the untransformed control cultivar 'Certo'.
Objective: This study aimed to evaluate and verify the potential of three HOSUT lines regarding grain yield and quality under field conditions.
Methods: A three-year field trial in Switzerland using up to three N fertilization levels was performed. In addition to the three HOSUT lines and 'Certo', three Swiss reference lines were tested. Yield parameters, grain composition, phenology and disease scores were recorded.
Results: Grain weight was increased for HOSUT lines by 3–8 % compared to wildtype 'Certo', whereas grain number per spike was reduced by 4–12 %. Thus overall, grain yield per area remained largely unchanged. However, higher levels of iron and zinc found in HOSUT grains could be health-promoting. No negative unintended effects on chemical composition, disease susceptibility, and plant phenology were detected, with the exception of complete anther retention in one of the three HOSUT lines.
Conclusions: While stimulation of sucrose uptake and/or partitioning in HOSUT lines resulted in higher grain size, an increase of total grain yield may only be achieved if trade-offs between grain size and grain number are overcome.
Implications: The study contributes to the understanding of sucrose transporter modifications in wheat. It demonstrates the importance of field-testing crop plants that exhibit promising traits under controlled environments. More research is needed to identify the bottlenecks that appear under field conditions to unlock the potential of the HOSUT strategy for agronomic application.

1. Introduction

Wheat (Triticum spp.) is a major crop that accounts for one third of global grain production and almost half of the cereal nutrition (FAO, 2019). Although breeding has been progressing continuously, levels of yield increase remain below the estimated amounts required for the

growing human population in a climate-changing world (Ray et al., 2013). If breeding strategies succeed in optimizing physiological traits with minimal trade-offs, the lower estimate of the projected future wheat demand in 2050 (van Dijk et al., 2021) can be met without expanding the current agricultural cropping area (Guarin et al., 2022). In the past, wheat yield potential has been improved by increasing the

harvest index (weight of grains per total plant weight) and much less by higher total biomass gain (Reynolds et al., 2009). However, the current increase in wheat yield decelerates and the harvest index approaches a theoretical limit (Boehm et al., 2023). Novel strategies are therefore required to boost wheat yield potential further. Optimising photosynthetic performance and assimilate partitioning to grains, as suggested by "The International Wheat Yield Consortium" (Reynolds et al., 2011), are important approaches to improve grain size and grain filling (Paul et al., 2020; Fernie et al., 2020).

Grain yield covers different components that interact in a complex and multiplicative manner (Kuchel et al., 2007; Cuthbert et al., 2008; Sukumaran et al., 2015; Schulthess et al., 2017; Sabir et al., 2023; Slafer et al., 2023). The two main parameters grain number and grain size determine yield. Constant progress has been made by classical breeding in optimising wheat yield by increasing grain number. In contrast, grain size has been rather stable. There is often a negative correlation between these parameters (Sadras, 2007; Philipp et al., 2018; Wang et al., 2018; Brinton and Uauy, 2019). While it is obvious to assume that strong competition for assimilates among the growing grains leads to a trade-off between average grain weight and grain number, source-sink studies in wheat do not support this explanation (Slafer et al., 2022). If grain size differences within a spike and between main and secondary tillers are included as factors, other reasons for the negative correlation seem more likely. Grain number in wheat is limited by the number of florets that set grains and increasing the survival of distal florets will increase the grain number per spike (Sakuma and Schnurbusch, 2020). Since those additional grains are smaller than those most proximal to the rachis, however, the average grain size will decrease. Similarly, if an increased tiller number is used to increase grain number per area, the higher number of secondary tiller spikes that tend to carry a higher proportion of smaller grains will also decrease average grain size (Slafer et al., 2022). In addition, breeders selected for uniform grain size since this is demanded for industrial processing (Slafer et al., 2023).

Despite the large body of data showing a negative relationship between grain size and average grain weight, empirical evidence from classical breeding (Bustos et al., 2013) and genetic engineering (Calderini et al., 2021; Guo et al., 2022) exists that, in some wheat lines, high grain number and average grain weight were combined successfully.

Seed filling in wheat is metabolically regulated and largely dependent on the supply of assimilates, amino acids and sucrose (Weber et al., 2005; Ma et al., 2023). Increased assimilate partitioning to the developing spike and grain historically had the greatest impact on improving yield potential in wheat (Calderini et al., 1995; Aisawi et al., 2015). Wheat grain filling is assumed to be mainly sink-limited during most of the grain filling periods when grains grow under a saturated source supply (Sofield et al., 1977; Borrás et al., 2004; Serrago et al., 2013). It is therefore hypothesised that increasing the sink strength of the grains could improve yield (Paul et al., 2020). To achieve this, the sucrose transporter gene *HvSUT1* (Weschke et al., 2000) from barley (*Hordeum vulgare*) has been ectopically expressed in the winter wheat cultivar 'Certo', controlled by the barley *Hordein B1* promoter and the created wheat lines were referred to as "HOSUT lines". Sucrose uptake capacity and storage protein synthesis were stimulated in HOSUT lines (Weichert et al., 2010). Repeated experiments in phyto-chambers and greenhouses revealed yield advantages (Saalbach et al., 2014; Weichert et al., 2017, 2010), mainly by higher grain weight, indicating increased individual grain sink strength and improved sucrose transport and/or partitioning to the grains. However, this yield improvement has not yet been tested in field trials.

This study aimed to evaluate and verify the potential of three independent HOSUT lines with respect to grain yield and quality in a three-year field trial in Switzerland. Changes in sucrose influx into grains could affect nitrogen (N) metabolism by co-regulating leaf N metabolism and amino acid partitioning (Lu et al., 2020). Indeed, previous phyto-chamber trials with a HOSUT line showed that it produced higher seed yield when N fertilizer was increased from 200 to 300 kg N ha^{-1}, while wildtype 'Certo' did not profit from the extra fertilizer (Weichert et al., 2017). Therefore, the effect of different N fertilisation regimes was tested. Finally, we aimed to detect potential unintended effects of the chosen metabolomics engineering approach on chemical grain composition, plant phenology, and disease susceptibility.

2. Material and methods

2.1. Transgenic wheat lines and comparator wheat cultivars

Three genetically engineered, homozygous winter wheat lines, HOSUT12/44, HOSUT20/6, and HOSUT24/31, and the corresponding isogenic non-transformed cultivar 'Certo' were grown in a three-year field trial. The HOSUT lines, henceforth abbreviated as HOSUT12, HOSUT20, and HOSUT24, originated from separate *Agrobacterium* transformation events and express the barley sucrose transporter *HvSUT1* under control of the barley *Hordein B1* promoter. During HOSUT line development, plants containing only the *HvSUT1* transgene hemizygously but no copy of the selectable antibiotic resistance marker gene (which was used only during the transformation process), were selected and further propagated (Saalbach et al., 2014). Thus, the three HOUST lines used in this study were marker-gene free. Flow cytometry combined with PCR analysis as well as a cytological approach demonstrated that the transgene integrated into chromosome 7 A in HOSUT12, chromosome 5D in HOSUT20, and chromosome 4 A in HOSUT24 (Cápal et al., 2016; Takenaka et al., 2019). In the field season 2018, developing grains were collected 26 days after flowering and analysed by quantitative RT-PCR, confirming that the transgene *HvSUT1* was expressed in the three HOSUT lines (Yang et al., 2019). It is safe to assume that the plants grown in 2017 and 2019 also expressed the transgene, since the 2017 plants are the parental generation of the 2018 material, and the plants grown in 2019 are from the same seed batches used for the 2018 trial. Seeds were multiplied on the Protected Site (see below) in 2017.

Three conventional winter wheat cultivars commonly grown in Switzerland (i.e., 'CH Nara', 'Sailor' and 'Hanswin'), designated here as "Swiss lines", were included in the experiments for comparison. Seeds were obtained from Delley Samen und Pflanzen AG (Delley, Switzerland). 'CH Nara' provides grains with high protein content but low yield, 'Sailor' produces high yield with low protein content, and 'Hanswin' has medium yield and protein content (Courvoisier et al., 2016).

2.2. Field trials with transgenic wheat lines

Consent to release the transgenic lines was obtained from the Swiss Federal Office for the Environment under the Release Ordinance 2008 and the Gene Technology Act 2003 in compliance with the EU Directive 2001/18/EC (application B16001). Field trials were performed on the Protected Site at Agroscope in Zürich, Switzerland (Romeis et al., 2013). Within the area, wheat was rotated in a four-year cycle, and a cover crop mixture of annual clover species and *Phacelia* was pre-crop to the wheat trials. Before sowing, wheat seeds were treated with 2 ml Celest Trio per kg (Syngenta Agro AG, Stein, Switzerland; 25 g/l fludioxonil, 25 g/l difenoconazole, 10 g/l tebuconazoleand) and 1.5 ml/kg Smaragd (Bayer (Schweiz) AG, Zollikofen, Switzerland; 258.3 g/l clothianidin). Seed amounts were adjusted according to germination rates (98–100 % in all seed batches used) to attain a seed rate of 350 viable seeds per m^2. The trials were sown using a 7-row plot drill with 0.18 m row spacing. Experimental units were plots of 1.5 m x 8 m planted with one of the wheat lines. Since the edges of the plots showed variable plant density, plots were trimmed to 7.5 m (in 2017) or 7.4 m (2018 and 2019). The trial was surrounded by a 3 m wide border crop of Triticale (cultivar 'Larossa'). Grains were harvested plot-wise using a plot combine harvester. To analyse yield-related traits on spikes, mature spikes were cut manually a few days before grain harvest.

Phosphate (P), potassium (K) and magnesium (Mg) fertilizer was applied according to guidelines for crops in Switzerland (Richner and Sinaj, 2017) in all field seasons to all plots, while nitrogen fertilizer was added at different levels (see subsections 2.2.1 to 2.2.3). At the beginning of each field season, the available soil mineral nitrogen content (N_{min}) was assessed in 30 cm layers down to 90 cm soil depth, i.e., 0 – 30 cm, 30 – 60 cm and 60 – 90 cm (Agroscope, 2020), and total N_{min} was subtracted from the nitrogen dose to be applied. In each season, nitrogen fertilizer was split in three doses and applied at tillering, at the beginning of stem elongation and at flag leaf stage.

Herbicide was applied each spring (29 March 2017, 9 April 2018, 1 April 2019) using 0.8 kg/ha Artist (Bayer (Schweiz) AG; 24 % flufenacet and 17.5 % metribuzin) mixed with 0.2 kg/ha Chekker (Bayer; 12.5 % amidosulfuron and 1.25 % iodosulfuron-methyl-sodium). Cereal leaf beetles were controlled by spray application of 0.1 l/ha Audienz (Omya (Schweiz) AG, Oftringen, Switzerland; 480 g/l spinosad) on 18 May 2017 as well as on 14 and 22 May 2018 or 0.2 l/ha Talstar (Stähler Suisse SA, Zofingen, Switzerland; 80 g/l bifenthrin) on 17 May 2019. The fungicide Pronto Plus (Bayer, 13.6 % tebuconazole, 25.5 % spiroxamine) was applied 17 May 2019 against *Septoria* spp. at 1.5 l/ha and Proline (Bayer, 25 % prothioconazole) was sprayed 3 June 2019 against *Fusarium* Head Blight (FHB) and *Septoria* spp. Each year, bird protection nets were installed after sowing until the first leaf appeared (mesh size 25 mm×25 mm; in 2017 a fleece of 17 g m^{-1} was used instead) and from milk stage until harvest (mesh size 20 mm×20 mm in 2017 and 13 mm×13 mm in 2018 and 2019). This measure was required by the regulatory authority to prevent potential seed dispersal by birds.

2.2.1. Field season 2017

The wheat lines were sown on 2 November 2016 and harvested on 22 July 2017. Full flowering of Certo and the HOSUT lines was on 3 June 2017. Plots were arranged in a randomised block design with eight repetitions (Supplementary Fig. S1). Mineral N content in soil (February 2017) was 60 kg N ha^{-1}. Fertilisation was 50 kg N ha^{-1} (27 February 2017), 45 kg N ha^{-1} (31 March 2017) and 15 kg N ha^{-1} (12 May 2017), in total, 170 kg N ha^{-1}. This corresponds to the fertilisation level N1 in the subsequent field seasons.

2.2.2. Field season 2018

Sowing was performed on 16 October 2017, and harvesting on 16 July 2018. Full flowering of Certo and the HOSUT lines was on 26 or 27 May 2018. Mineral N content in soil (February 2018) was 55 kg N ha^{-1}. Three fertilisation levels were applied, N0, without fertilisation; N1, 45 kg N ha^{-1} (27 March 2018), 35 kg N ha^{-1} (16 April 2018), and 35 kg N ha^{-1} (7 May 2018), in total, 170 kg N ha^{-1}; N2, 75 kg N ha^{-1} (27 March 2018), 60 kg N ha^{-1} (16 April 2018), and 60 kg N ha^{-1} (7 May 2018), in total, 250 kg N ha^{-1}. The three Swiss lines were only cultivated under N1 fertilisation.

The wheat lines were grown in a split plot design with seven complete replicates (Fig. S1). Replicates were blocked, and within each replicate, the three N fertilizer treatments were blocked and randomly assigned (main plot or whole plot factor). Within each fertilizer block, wheat lines (experimental plots) were randomized (subplot or split-plot factor). All experimental plots were separated by a 1 m wide path on the short side (1.5 m), avoiding N fertilizer migration or washing off to neighbouring plots. On the long side (7.4 m) of the plot, however, the path was only 0.3 m wide. Therefore, a buffer plot (1.5 m x 7.4 m) with Triticale (cultivar 'Larossa') was sown between blocks of different N-levels. Each half of the buffer plot was fertilized at the same N-level as its adjacent experimental plot.

2.2.3. Field season 2019

Plants were sown on 18 October 2018 and harvested on 26 July 2019. Full flowering of Certo and the HOSUT lines was between 7 and 9 June 2019. Mineral N content in soil (February 2019) was 45 kg N ha^{-1}. Two fertilisation levels were applied, N1 (170 kg N ha^{-1}) and N2

(250 kg N ha^{-1}). Fertilizer was applied on 20 March 2019 (N1 45 kg N ha^{-1}, N2 75 kg N ha^{-1}), 9 April 2019 (N1 40 kg N ha^{-1}, N2 65 kg N ha^{-1}) and 16 May 2019 (N1 40 kg N ha^{-1}, N2 65 kg N ha^{-1}). The three Swiss lines were only cultivated under N1 fertilisation.

The wheat lines were grown in a split-plot design with eight complete replicates (Fig. S1). Analogous to the release 2018, replicates were blocked, N fertilizer treatments were blocked and separated by a triticale buffer plot, and lines were randomized within each block.

2.3. Analysis of yield parameters and grain composition

To determine spike density, all spikes within a 0.5 m-strip (2017), two 1 m-strips (2018) or three 1 m-strips (2019) of a single row per plot were counted. Only the 3 central rows of a plot were considered for counting, and the first and the last metre of a row were avoided. Spike density was calculated for each plot based on the mean spike number per metre in a row and a row width of 0.18 m (area of 0.18 m^2).

Each year, 20–25 representative mature spikes from main tillers were sampled from each plot a few days before harvest. The outer rows and half a meter from either end of the plot were avoided. The spikelet number per spike was counted for 5 (2017, 2018) or 20 (2019) spikes, the grain number per spike for 5 (2017), 25 (2018), or 20 spikes (2019), and the grain yield per spike (weight of all grains per spike) was recorded for 20 (2017, 2019) or 25 spikes (2018). To determine yield per plot, the seeds harvested by plot combine harvester were cleaned from husks and chaff with a laboratory thresher or a windsifter (Kurt Pelz, Bonn, Germany) and weighted. A random subsample of these mature dry grains (ca. 40–60 g in 2017 and 2018, ca. 10 g in 2019) was taken and the length, width, and area of the grains was determined using the digital seed analyser MARVIN (Version 5.0, GTA Sensorik GmbH, Neubrandenburg, Germany). After measuring the grain size, the same batch of seeds was weighted to determine the thousand grain weight (TGW).

For the analysis of nutrients, 10 g of the mature grains were milled in a bead mill (MM400, Retsch GmbH, Haan, Germany) for 90 s at 30 Hz and the flour was dried for 24 h at 70°C. The starch and sucrose contents were determined using a coupled enzyme assay as described by Weigelt et al. (2009). Total carbon and nitrogen were determined with a Vario EL Elemental analyser (Elementar Analysensysteme GmbH, Langenselbold, Germany). For the analyses of macro- and microelements, material from the ground and dried mature grains was weighed into PTFE tubes and digested in HNO_3 under pressure using a microwave digester (UltraCLAVE IV; MLS Mikrowellen-Labor-Systeme GmbH, Leutkirch, Germany). Macro- and microelements were measured by inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 6500, Thermo Fisher Scientific, Germany) combined with the CETAC ASX-PRESS™ PLUS rapid sample introduction system, and a CETAC auto-sampler (CETAC Technologies, Omaha, NE, USA). Element standards were prepared from certified reference materials from CPI international (Eroglu et al., 2017).

2.4. Monitoring of plant phenology and disease resistance

At least once per week, all plots were visually assessed for plants with unexpected phenotypes, like delayed development, brighter leaf colour, or aberrant leave shape. In addition, ear emergence (heading) and flowering date were recorded. Degree days were calculated by adding average daily air temperatures (°C) from one day after sowing to the date of heading or flowering, while days with temperatures below zero were added as zero. Plant height was determined at the milk stage by manually measuring from the soil surface to the top of the spike using a grade rod with 0.01 m resolution. Measurements were taken approximately in the middle of the front, middle and rear third of each plot, always estimating the average height of the plants surrounding the grade rod, and the mean value of the 3 recordings per plot was taken for data analysis.

During the main season, plants were checked weekly for above-ground disease symptoms of powdery mildew (caused by *Blumeria graminis*), leaf rust (*Puccinia triticina*), FHB (*Fusarium* spp.), and *Septoria* leaf blotch (*Septoria* spp.). If there were significant infection levels in at least one plot, disease scores of all plots were taken by estimating the average disease severity of all plants of a plot. The frequency of subsequent scorings was adapted to the progress of each disease in each season. Powdery mildew infection and leaf blotch were scored from 0 (no infection) to 9 (whole plant infected) based on the scale of Kmecl et al. (1995). This scale considers the percentage of symptomatic leaf and head area of the entire plant with special focus on the flag leaf and head. *Mycosphaerella graminicola* (syn. *Septoria tritici*) and *Phaeosphaeria nodorum* (syn*. Septoria nodorum* and *Stagonospora nodorum*) were not distinguished from each other (and from other potentially occurring *Septoria* species) and a single score was recorded for leaf blotch disease symptoms (causal agents were abbreviated as *Septoria* spp). Leaf rust was scored on the flag leaf by estimating the percentage of infected leaf surface (Schnurbusch et al., 2004). For FHB, disease incidence was expressed as the total number (2017) or the percentage (2019) of symptomatic wheat heads (i.e., fully or partially bleached heads) per plot. For powdery mildew and leaf rust data analysis, the area under the disease progress curve (AUDPC) was calculated (Shaner and Finney, 1977).

In 2018, there was a strong infection with leaf rust, *Septoria* spp. and *Fusarium* spp. and during the course of the season, it was impossible to distinguish the damage caused by each disease separately. Therefore, a general score was taken by estimating the percentage of damaged area of the flag leaf.

2.5. Statistical analysis

Statistical analyses were conducted in R (version 4.2.2). All analyses were based on N = 7 (2018) or N = 8 (2017, 2019) replicated plots. For technical replicates (samples) within plots, the mean per plot was calculated and used for analyses. Each year was analysed separately. Linear models (LM) and linear mixed effects models (LMER) were used for all parameters (Supplementary Tables S1-S5). For 2017, a two-way ANOVA with the factors wheat line and replicate was conducted (parameter ~ wheat line + replicate). For 2018 and 2019, LMER models for the split plot design were fitted with the fixed factors wheat line, fertilization level (Flevel), wheat line × Flevel interaction, and replicate, and the random factor replicate × main plot (parameter ~ wheat line + Flevel + wheat line:Flevel + replicate + (1|replicate:mainplot)). Main plot refers to the arrangement of the blocked fertilization levels in the design (see Fig. S1). In 2018, the Flevels were N0, N1, and N2, while in 2019 only N1 and N2 were available. Significant results for the factor wheat line were further analysed using Dunnett posthoc tests, where each HOSUT line was compared with 'Certo'. Significances in Flevels in 2018 were compared using Tukey posthoc tests. Because a number of significant wheat line × Flevel interactions were observed for compositional parameters (Table S2), all compositional parameters were analysed separately for the different Flevels (parameter ~ wheat line + replicate) (Table S3).

In addition to analyses per year, we also analysed the data for all years together, but only for the standard fertilization (N1), because data for this level were available for all three years. These analyses used essentially the same models and posthoc tests as described for the data analysis of 2018 and 2019, but the factor Flevel was replaced by the factor year (parameter ~ wheat line + year + wheat line:year + replicate + (1|replicate:mainplot)). Disease scores were analysed by Kruskal-Wallis ANOVAs followed by Dunn posthoc tests. Data for Swiss reference lines and weather data were not analysed statistically, but results are shown in Supplementary Fig. S2-S5.

3. Results

3.1. Yield parameters

Six yield-related traits were analysed in all three years: grain yield per area, thousand grain weight (TGW), grain number per spike, grain yield per spike, number of spikelets per spike, and spike density. While only standard fertilization (N1) was used in 2017, the experiment in 2018 included plots without N fertilization (N0) and plots with elevated fertilization (N2). In previous greenhouse and phyto-chamber trials with HOSUT lines, standard N fertilization was 200 kg N ha^{-1}, while the recommended quantity for winter wheat in Switzerland is only 140 kg N ha^{-1}. As a compromise, we decided to use 170 kg N ha^{-1} as N1, and 250 kg N ha^{-1} as N2. The N level of 170 kg N ha^{-1} is also used as high-input treatment in Swiss winter wheat variety trials (Herrera et al., 2020).

In the field experiment in 2017, the grain yield per hectare was not significantly different among the wheat lines (Fig. 1A, Table S1, S6). Grain yield of HOSUT12 and HOSUT24 did not significantly differ from 'Certo' in 2018, while yield of HOSUT20 was 3 % (N2, N1) and 14 % (N0) lower than that of 'Certo' (Fig. 1B). In 2019, grain yield was 1 % (N2) or 6 % (N1) higher in HOSUT12 and 4 % (N2) or 8 % (N1) higher in HOSUT24 than in 'Certo', and these four differences were statistically significant. In contrast, no significant effect was observed in HOSUT20 (Fig. 1C, Table S1, S6). Over all three years, there was no significant effect on yield per area among the wheat lines with standard fertilization (Table S5). Yield was lowest in 2018 and highest in 2017 (Table S5, S7). In 2018, the fertilized plots showed ca. 40–60 % higher yields per area than the unfertilized plots (N0), but no differences in yield between standard (N1) and elevated fertilization (N2) were evident. Similarly, no difference between N1 and N2 was observed in 2019 (Fig. 1B, C, Table S1).

Yield per area is determined by the number of seeds harvested per area and their average weight. Therefore, we looked deeper into these yield components. In 2017, TGW was significantly increased by 5–6 % in all HOSUT lines compared with 'Certo' (Fig. 1D, Table S1, S6). In 2018, higher TGW was evident in HOSUT20 and HOSUT24 (Fig. 1E), and in 2019, grains of HOSUT20 were heavier than those of 'Certo' (Fig. 1F). When 2017–2019 were summarized, TGW in HOSUT lines (N1) was 3–8 % higher than in 'Certo' (Table S5, S6). In 2017, TGW was lowest and in 2019 highest (Table S5, S7). Elevated fertilization consistently resulted in lighter grains with statistical differences between each fertilization level (Fig. 1E, F, Table S1).

Grain morphology was measured to learn whether differences in grain weight resulted from growth in length, width, or both. Results for grain area were comparable to results for TGW in all years (Table S1). Similarly, both grain length and grain width were affected by genotype, although less significant than for TGW and grain area. Over all years, grain area, grain length, and grain width in N1 were significantly increased by 1–4 % in all HOSUT lines except grain length in HOSUT12 (Table S5, S6). Fertilization decreased mainly grain width and to a lesser extend also grain length (Table S1).

In addition to yield per area, TGW and grain morphology, also yield parameters per spike were examined. While the number of grains per spike was not statistically different among wheat lines in 2017, significantly fewer grains were produced in HOSUT20 in 2018 and 2019 compared with 'Certo' (Fig. 2A-C, Table S1). Grain numbers per spike for HOSUT12 and HOSUT24 were not statistically different from 'Certo' in 2018 and 2019. Over all years, the number of grains per spike in HOSUT lines in N1 was 4–12 % lower than in 'Certo', and this difference was statistically significant for HOSUT24 and close to significance (0.05 < p < 0.1) for the other HOSUT lines (Table S5, S6). The highest number of grains per spike was observed in 2017, the lowest in 2019 (Table S5, S7). N fertilization increased the number of grains per spike (N0 vs. N1 and N2), but no differences between standard and elevated fertilization (N1 vs N2) were observed in 2018 and 2019 (Table S1).
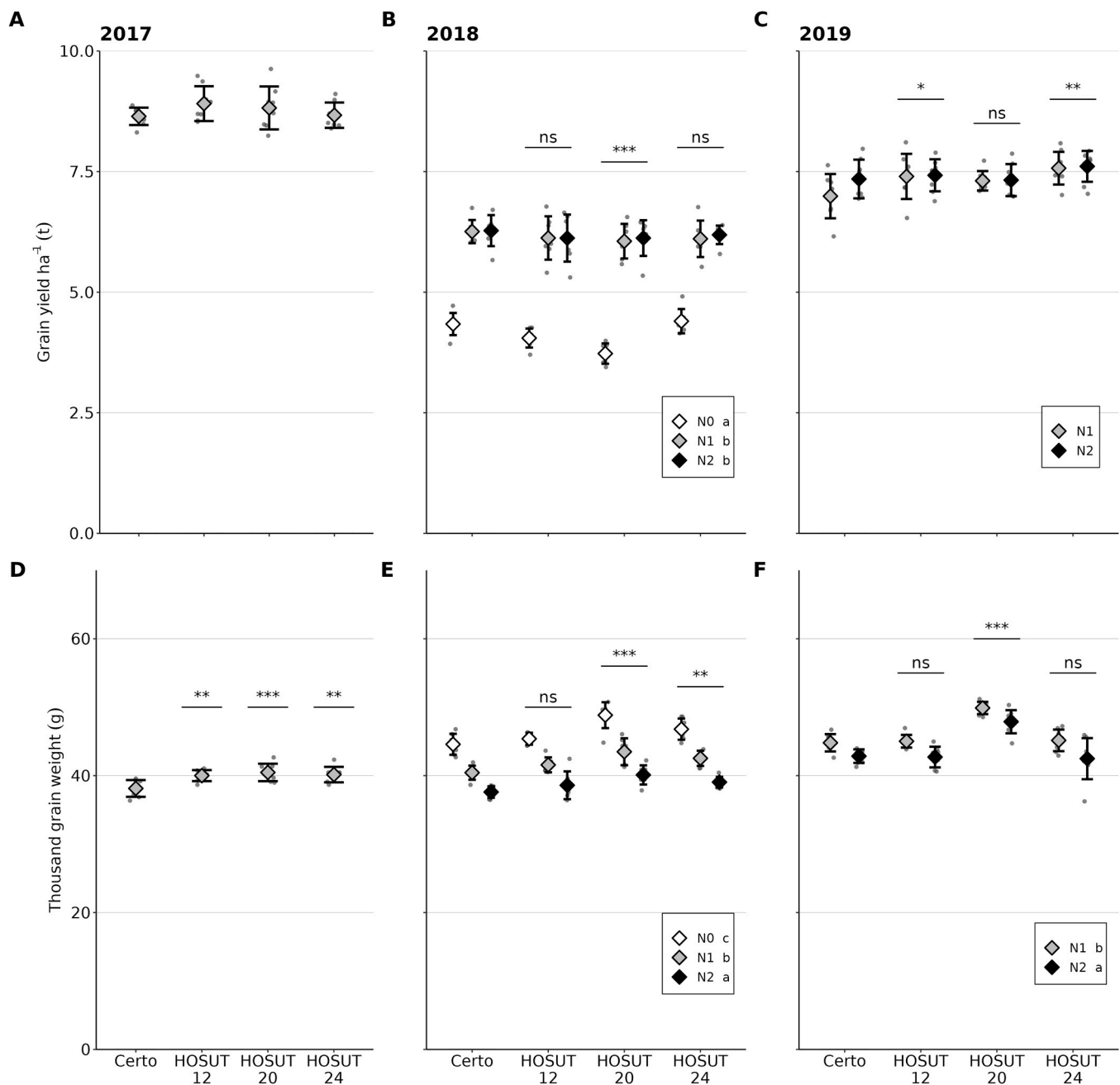
Fig. 1. General yield parameters: grain yield and thousand grain weight (TGW). Means are indicated as diamonds and fertilization levels by fill colour (white = N0, grey = N1, black = N2). Standard deviations are shown with error bars. If linear models revealed a significance for wheat line, Dunnetts tests were conducted where each HOSUT line was compared to 'Certo' (results shown in the plots, ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). If present, significances in fertilization levels are shown in the legend (different letters indicate significant differences).

The grain yield per spike (total weight of grains per spike) did not differ among wheat lines in any year (Fig. 2D-F, Table S1) and also when analysed over all years (Table S5). If the years are compared, fewer grains per spike were counted in 2019 than in the other years (Table S7). In 2018, standard fertilization (N1) resulted in higher grain yield compared with no fertilization (N0) and elevated fertilization (N2), but no difference between N1 and N2 was evident in 2019 (Table S1).

The number of spikelets per spike was similar among wheat lines in 2017 and 2018 (Fig. 2G,H, Table S1). In 2019, however, slightly less spikelets were produced per spike in all three HOSUT lines compared with 'Certo' (Fig. 2I). The number of spikelets per spike also was not significantly different among wheat lines when means were averaged over all three years in the N1 fertilization treatment (Table S5). In 2018,

spikes contained the lowest number of spikelets and in 2017 the highest (Table S7). Fertilization did not affect the number of spikelets in 2018, but more spikelets were counted with elevated fertilization in 2019 (Fig. 2 H,I, Table S1).

In addition to grain size and the number of grains per spike, the spike density directly affects total yield. There were no significant differences between the HOSUT-lines and 'Certo' for the number of spikes per m^2 in any year (Table S1). However, over all years with the N1 fertilization level, 7–12 % more spikes were counted in the HOSUT plots compared with 'Certo', and this difference was statistically significant for HOSUT20 and HOSUT24 (Table S5, S6). The lowest spike density was recorded in 2018, the highest in 2017 (Table S7). Standard N fertilizer application increased spike density compared to non-fertilized plots, and
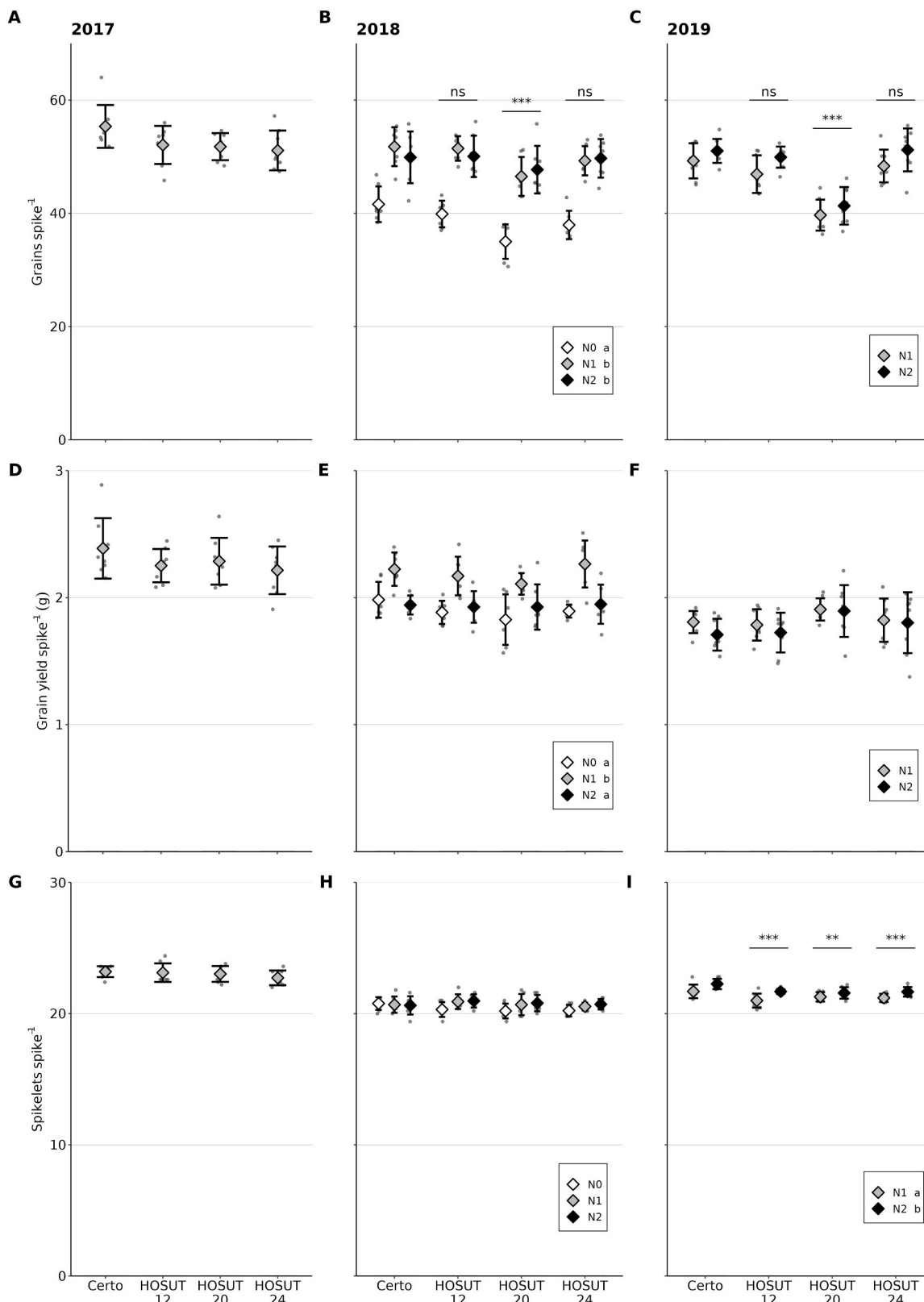
Fig. 2. Yield parameters per spike: number of grains, grain yield, and spikelets. Means are indicated as diamonds, fertilization levels by fill colour (white = N0, grey = N1, black = N2). Standard deviations are shown with error bars. If linear models revealed a significance for wheat line, Dunnett tests were conducted where each HOSUT line was compared with 'Certo' (results shown in the plots, ns = not significant, * p < 0.05, ** p < 0.01, *** p < 0.001). If present, significances in fertilization levels are indicated in the legend (different letters indicate significant differences).

additional fertilizer (N2) enhanced this effect (Table S1).

For the three Swiss lines that were grown with standard fertilization (N1), grain yield per hectare, TGW, and spike density were determined (Fig. S2). Although not analysed statistically, 'CH Nara' produced the lowest yield per plot, 'Sailor' the highest, and 'Certo' and the HOSUT lines in between. Similarly, TGW was lowest for 'CH Nara' and highest for 'Sailor' with 'Certo' and the HOSUT lines in between in 2017 and 2018. In 2019, 'Hanswin' had the highest TGW among the Swiss lines, but 'Certo' and the HOSUT lines exceeded the TGW of all three Swiss lines. Spike density for 'Certo' and the HOSUT lines was similar or higher than that of 'CH Nara' and 'Hanswin', while the lowest density was observed with 'Sailor' (Fig. S2).

The different yield parameters influenced each other (Fig. S6A). The number of spikelets and grains per spike as well as the yield per hectare were correlated, *i.e.*, clustered in the principal components analysis (PCA). This group was opposite (negatively correlated) to a cluster of the grain shape parameters width and surface (grain area) as well as TGW. This suggests that the increased grain size was at the cost of the total number of grains, and is negatively correlated to total yield (yield per area).

Genotype × year_Flevel biplots show that years generally cluster closer together than N fertilizer levels, indicating that weather conditions had a bigger impact than N fertilization (Fig. S7). Not surprisingly, 'Certo' was separate from the HOSUT lines in terms of TGW, and to a lesser extent to yield per area, while there was no clustering pattern for number of grains, spikelets and yield per spike, as well as for the number of spikes per area. Interestingly, HOSUT20 was closer to 'Certo' than the other HOSUT lines for all parameters.

3.2. Grain composition

Effects of genotype on the composition of mature grains were analysed for each fertilization level and year separately (Table S3). In heat maps (Fig. 3), values of HOSUT lines are displayed as relative to 'Certo'. Levels of iron, zinc, sulphur, and magnesium were higher (green shading) in most cases compared with 'Certo', in some instances significantly (red values in Fig. 3). In contrast, HOSUT lines contained lower levels of sucrose, potassium and calcium (purple shading). No differences in any year and fertilization level were revealed for carbon, starch, phosphorous, and manganese. In general, differences in nutrient composition were less pronounced in HOSUT24, while highest differences were present in HOSUT20 grains collected in 2018, i.e., iron 16 % higher in N0, zinc 12 % higher in N0 and N1, sulphur 13 % higher in N0, sucrose 10 % lower in N1, potassium 5 % lower in N1, and calcium 13 % lower in N2. There was no consistent trend regarding the influence of fertilization level on the difference in nutrient levels among wheat lines. Over all years, HOSUT lines showed 2–5 % less sucrose (significant for HOSUT20 and HOSUT24), 3–5 % less potassium (significant for HOSUT12, trend for HOSUT20), 3–7 % less calcium (HOSUT20), and 4–8 % more zinc (HOSUT20 and HOSUT24) than 'Certo' (Table S5, S6). In addition, HOSUT12 grains contained 4 % more iron than 'Certo'.

The year of cultivation affected all measured nutrients significantly (Table S7). Highest differences were observed for iron, which was 43 % higher in 2017 than in 2019. Sucrose was 40 % higher in 2018 than in 2017 and manganese showed 27 % higher levels in 2018 than in 2019. Other nutrients varied between 2 % and 19 % among the years.

Fertilization had a high impact on nutrient levels in wheat grains (Table S2, S8, Fig. S8, S9). Independent from the wheat line, increasing fertilization (N0 to N1 and N1 to N2) resulted in increased levels of nitrogen (12–29 %), iron (11–21 %), sulphur (8–17 %), manganese
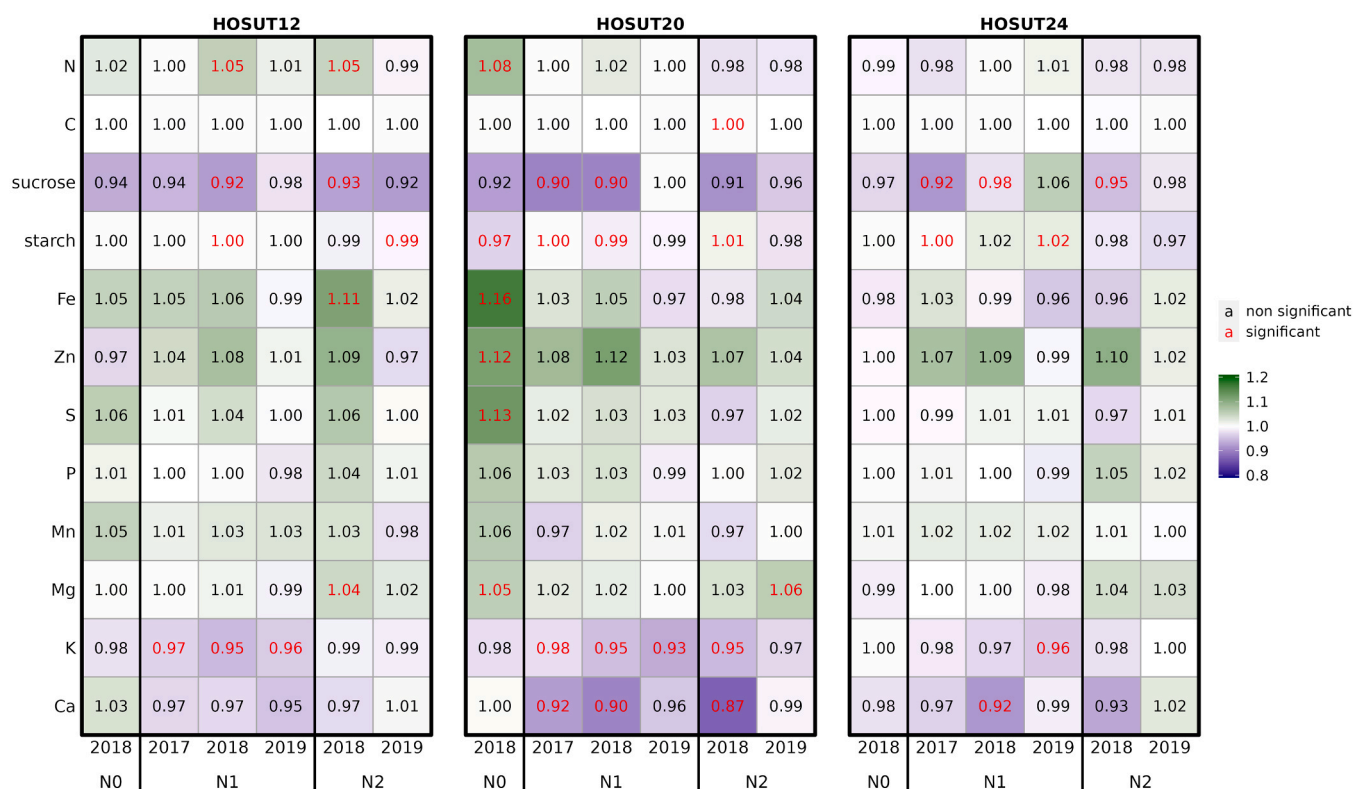

Fig. 3. Outcome of statistical analyses for compositional data. Each fertilization level and year were analysed separately with linear models (LM): parameter ~ wheat line + replicate. For significant results, Dunnett tests were performed. For graphical display (heatmap), the means of each HOSUT line were divided by the mean of 'Certo' to obtain relative values that are easily comparable among the different parameters (numbers in the tiles). Values below 1 (purple tile colour) indicate lower values, values above 1 (green) higher values for the HOSUT lines compared with 'Certo'. Red numbers show significant differences between HOSUT and 'Certo'. N0, N1, and N2 indicate the different fertilization levels.

(10–12 %), and calcium (15–19 %). Furthermore, fertilization from N0 to N1 increased potassium (4 %) and decreased zinc (10 %). In 2019, higher fertilization from N1 to N2 decreased potassium (2 %) and magnesium (3 %), while no such effect was evident in 2018. For most nutrients, effects from N0 to N1 were stronger than from N1 to N2.

In a PCA, the different compositional parameters were widely spread over the two main dimensions (x- and y-axes). Nitrogen and sulphur clustered (correlated) on one side, while sucrose, potassium and zinc clustered on the opposite side (negatively correlated) (Fig. S6B).

3.3. Plant phenology

During regular field visits, no obvious differences in development stage between the HOSUT lines and 'Certo' were observed. Records of the degree days of ear emergence (heading) and the degree days of flowering confirmed that there was no consistent developmental difference between the HOSUT lines and 'Certo' in any year, except in 2018, when heading of HOSUT20 was approximately 1 day later and flowering of HOSUT24 one day earlier than for the other lines (Table S4). Similarly, no effects among the wheat lines on heading and flowering were evident when all years were analysed together (Table S5). In contrast, year had an influence on heading and flowering (Table S7). Lowest degree days for both parameters were noted in 2017 and highest in 2019. Fertilization had no influence on heading and flowering times (Table S4). One markable observation was that the flowers of HOSUT20 completely lacked anther extrusion during flowering time (strict cleistogamy) in all three field seasons. Therefore, flowering time could not be recorded accurately for this wheat line. In general, heading and flowering time was very similar for all wheat lines including the Swiss lines (Fig. S3).

No other obvious morphological differences were noticed. Measurements of the plant height revealed no consistent difference between the HOSUT lines and 'Certo' in the three field seasons and under different fertilisation treatments (Table S4), as well as when all years were analysed together (Table S5). HOSUT20 plants were 1–2 cm shorter in 2017 and HOSUT24 plants longer in 2018 compared with 'Certo'. In 2017 and 2018 plants were shorter than in 2019 (Table S5). Increased fertilization resulted in higher plants in 2018, while no differences between standard and elevated fertilization were evident in 2019 (Table S4). When looking at the Swiss lines, 'CH Nara' plants were shortest, 'Sailor' plants highest, and 'Certo' and the HOSUT lines in between (Fig. S3).

3.4. Disease resistance

In all field seasons, natural infection with powdery mildew, leaf rust, *Septoria* and FHB occurred (Fig. S4). In some cases, disease levels were extremely low, and 'Certo' as well as the HOSUT lines showed only traces of infection symptoms. In the field season 2017, there was a moderate infection with powdery mildew while no infection was noticed in the other years. Leaf rust infection was low in 2017 and 2019 and high in 2018. *Septoria* symptoms were pronounced in 2018, and to a much lesser extent in 2017 and 2019. FHB infection was low in 2017 and more frequent in 2019. In 2018, FHB could not be reliably scored because of early ripening and presence of other disease symptoms on the heads. In all disease assessments, there were no obvious differences between the HOSUT lines and 'Certo', with two exceptions: HOSUT20 was significantly more resistant to FHB than 'Certo' in both assessment years (2017 and 2019) and HOSUT12 was more resistant to leaf rust than 'Certo' in 2018. N fertilization generally increased disease severity and the Swiss lines were in most cases more resistant to diseases than 'Certo' and the HOSUT lines (Fig. S4).

4. Discussion

One way of increasing grain yield in wheat is to increase the potential

of sucrose uptake and/or partitioning to the grains. This study reports the field-testing of three independent transgenic HOSUT lines, expressing the barley sucrose transporter *HvSUT1* under the control of the barley *Hordein B1* promoter (Saalbach et al., 2014; Weichert et al., 2017, 2010). In phyto-chambers and greenhouses, these lines were promising as they showed increased yield potential. Here, the lines were cultivated in a three-year field trial in Switzerland under different nitrogen (N) fertilisation regimes. Grain yield per plot, TGW, grain morphology, spike density, and grains per spike and spikelet were analysed together with grain composition. The aim was to study whether the genetically improved sucrose uptake capacity into grains can be translated into higher grain yield under field conditions. The results, however, revealed that neither of the three HOSUT lines showed significant and consistent improvement of grain yield per hectare compared with the wildtype 'Certo', irrespective of N fertilization level. While TGW was clearly increased in the HOSUT lines compared with 'Certo', grain number and spikelet number per spike were decreased.

4.1. Larger grains but no yield gains were achieved under field conditions

The data from the current study confirm that the primary effect of the genetic modification in the HOSUT lines is the increased grain size. All parameters of grain morphology (area, length, and width) were increased, suggesting effects both early, when grain length is determined, and later in the grain development stages, when grain width is resulting from filling (Pielot et al., 2015). The effect on both grain length and width suggests a general stimulation of cell proliferation in the HOSUT grains. This is in accordance with previous results, which showed that HOSUT grains were larger and revealed higher cell numbers in the endosperm from 15 to 25 days after fertilisation (Saalbach et al., 2014; Weichert et al., 2017), indicating a possible assimilate effect on endosperm cell proliferation.

While grain size was generally increased in HOSUT lines, some important yield components were decreased in some cases, such as grains per spike and spikelets per spike. As a result, total grain yield was not altered significantly over the experimental period of three years. While grain numbers per spike were not different between HOSUT24 and 'Certo' in phyto-chamber experiments (Weichert et al., 2017), a tendency towards lower grain numbers per spike were revealed in several different HOSUT lines in field-like trials in small greenhouses in soil beds without regulation of temperature, humidity and lighting (Saalbach et al., 2014). This might indicate that HOSUT lines possibly perform better than 'Certo' only under optimal conditions of a closed system, while they fail to use their full potential under stressful field conditions.

A simple explanation for the difference between field and greenhouse results would be that smaller seeds of HOSUT lines got lost during the mechanical harvesting process. If the size difference of grains within a spike (Brinton and Uauy, 2019) was more pronounced in HOSUT spikes than in 'Certo', the fraction with smallest grains could have been segregated and discarded together with the husks by the combine harvester, leading to a decreased grain yield in HOSUT lines with larger average grain size. However, we prevented this technical artefact by lowering harvester ventilation speed to avoid any seed losses (and also to reduce volunteer plants in the following season - a requirement by the regulatory authorities). As a consequence, there were still a lot of husks and chaff in the harvested material and they had to be removed later with a laboratory thresher or a windsifter adjusted to retain small grains. In addition, data from hand-harvested spikes confirm that average grain weight was indeed increased: Grain yield per spike (total weight of grains per spike) did not differ among wheat lines in any year (Fig. 2D-F, Table S1) and also when analysed over all years (Table S5). In contrast, over all years, the number of grains per spike in HOSUT lines in N1 was 4–12 % lower than in 'Certo', and this difference was statistically significant for HOSUT24 and close to significance ($0.05 < p < 0.1$) for the other HOSUT lines (Table S5, S6), indicating a higher average grain

weight. Thus, data from hand-harvested single spikes and plot yield data are consistent.

While pathogen infection was not recorded in the previous trials (Saalbach et al., 2014; Weichert et al., 2017), different pathogens attacked 'Certo' and the HOSUT lines in the field, particularly in 2018. To detect possible differences in disease resistance, no fungicides were used in 2017 and 2018, and a reduced spraying was applied in 2019. There were, however, no differences between 'Certo' and the HOSUT lines, except for a reduced FHB infection in HOSUT20 (Fig. S4). We did not assume significant disease-driven effects on yield in 2017 and 2019 since disease levels were generally low and infection occurred late in the season (after flowering).

The use of bird protection nets represents another difference to the previous studies. They were applied during germination and emergence as well as after flowering until harvest (another biosafety requirement). Due to their relatively large mesh size, they are not expected to have influenced grain yield by microclimatic changes or shading. In addition, grain number per area and yield is largely determined in the period soon after the onset of stem elongation to around one week after anthesis (Carrera et al., 2024), thus there was a very limited temporal overlap.

The addition of the three Swiss lines to the experiment allows to discuss the performance of 'Certo' and its transformed lines in the context of local cultivars (Courvoisier et al., 2016). In our field trials, the Swiss lines yielded from 5 t ha^{-1} ('CH Nara' in 2018) to almost 9 t ha^{-1} ('Sailor' in 2017), while Certo and the HOSUT lines yielded on average 8.76 (2017), 6.14 (2018) and 7.32 t ha^{-1} (2019) under standard (N1) fertilization (Fig. S2, Table S7). In comparison, average yield from 2008 to 2018 was 7.7 t ha^{-1} in Lindau, a location of the Swiss trial network of winter wheat varieties and advanced breeding lines closest to our trial site where the same N fertilization level was applied as in our N1 treatment (Herrera et al., 2020). This suggests that in general, our yield trials delivered comparable results to standard yield trials used in variety testing in Switzerland.

As expected, 'CH-Nara' had the lowest yield as this line is known to have a high protein content but low yield (TOP quality, high gluten content ideal for bread-making). 'Hanswin' is known to have medium yield and protein content (class I, well suited for extenso cultivation), and this was also observed in our experiments. 'Sailor' is a feed wheat with high yield and low protein content (Class II quality). 'Certo' (and the HOSUT lines), is also grown as feed wheat. However, in the Swiss context, 'Certo' did not reach the yield level of 'Sailor', except for the field season 2017. This is not surprising as 'Sailor' is adapted to Swiss conditions and thus also on the recommended variety list in Switzerland, whereas 'Certo' is not recommended in Switzerland. Thus, we hypothesize that due to the lack of local adaptation of 'Certo' and the HOSUT lines, the latter may not have been able to overyield 'Certo' as they did under controlled conditions.

4.2. Larger grains in HOSUT lines are compensated by lower grain number

We can only speculate about the causes of the difference between phyto-chamber or greenhouse and field results. Apparently, increased grain size was counter-balanced by the number of grains per spike under field conditions. This result can be explained by the well-known trade-off between grain size and grain number, which is an intrinsic property of many crops and non-crop plants (Philipp et al., 2018; Brinton and Uauy, 2019; Mora-Ramirez et al., 2021) and is not easy to overcome by conventional breeding (Acreche and Slafer, 2006; Quintero et al., 2018). From an evolutionary point of view, the adjustment between these two traits guarantees yield stability (Sadras, 2007). Whether the HOSUT trait causes a true trade-off that unveils under field conditions or if the negative correlation can be overcome would need further investigation. The introduction of the HOSUT trait in other wheat genotypes and tests of the existing lines in further field environments or under varying climate chamber conditions could give a first answer.

4.3. Enhanced grain sink strength in HOSUT lines may need combination with other traits to increase yield

The comparison of traditional with improved cultivars as well as landraces with elite varieties showed that previous breeding improvements mainly derived from increased spike numbers and higher grain numbers per spike and spikelet, which resulted in higher grain numbers per hectare (Philipp et al., 2018). Theoretically, a higher number of grains could also be achieved by increasing spikelet numbers. However, the number of spikelets per spike in wheat is negatively correlated with most other traits, with the consequence that this trait has not been favoured during breeding (Philipp et al., 2018). Grain numbers were largely increased by allocating high levels of assimilates from stems to grains, as shown for the semi-dwarf wheat varieties (Brancourt-Hulmel et al., 2003). However, breeding did not yet generate much gain in TGW. Furthermore, spike architecture in regard to the distribution of grain yield and grain numbers along the spike has been surprisingly stable (Philipp et al., 2018). This indicates potential competition for assimilates in the spike, being affected by assimilate loading and unloading in the vasculature and short-distance transport within spike, rachis and spikelets. Such limitations could impact biomass distribution among individual tissues within spikes (Reynolds et al., 2009).

Inefficient assimilate delivery to spikes and grains occurs regardless of source capacity, indicating the importance of translocation arrangements (Seki et al., 2015). This may partially explain why the higher sucrose uptake and/or partitioning in the HOSUT lines affected only individual grain sink strengths, but did not translate into higher sink strength on the whole plant level improving grain yield per hectare. The vascular system of spikes and spikelets could have imposed resistance to assimilate movement, which is important for the 'sink activity' of the spikes and the yield (Bremner and Rawson, 1978). Disparity in number and dimensions of vascular bundles in different spike segments could be critical affecting ultimate size and grain number along the rachis (Asli and Houshmandfar, 2011). Genetic yield gain during breeding has not been accompanied by similar increases in the vascular size of the wheat spike and no clear association was found between genetic improvement and magnitude of vascular systems in peduncles (Lopez-Garrido et al., 2001). In rice, simultaneous increases in sink size and translocation capacity through the vasculature increased the number of vascular bundles and contributed to increased grain yield (Fujita et al., 2013; Terao et al., 2010). Thus, consistent improvement of grain yield may only be achieved by integrated approaches targeting several yield-component traits in parallel (Würschum et al., 2018), such as sucrose sink strength in the grains together with increased vascular capacity to enable the wheat plants to maintain similar or increased numbers of larger grains. However, knowledge on the vascular architecture during wheat spikelet development is currently limited (Wolde and Schnurbusch, 2019) and suitable target genes for future research still need to be identified.

4.4. Higher grain sink strength increased important grain micro-nutrients

Grain sink strength has been shown to be important for micronutrient delivery (Stomph et al., 2011). Moreover, differences in grain size can change the relationship between seed organs affecting grain composition and quality (Nuttall et al., 2017). Therefore, possible effects of genotype and fertilizer treatment on the composition of mature grains were analysed. HOSUT-grains contained 4 and 8 % more zinc compared with 'Certo' and slightly more iron (up to 4 % in HOSUT12), whereas levels of sucrose, potassium and calcium were lower. Previous experiments in phyto-chambers also revealed higher zinc and lower potassium levels in HOSUT lines compared with 'Certo' (Weichert et al., 2017) and experiments in field-like microplots showed more zinc and iron and lower sucrose levels in the same HOSUT lines that were used in the present study (Saalbach et al., 2014). The lower sucrose content may indicate a higher sink strength, e.g., caused by increased starch

biosynthesis. Vice versa, strongly increased sucrose levels have been measured in barley mutants with deficiencies in starch biosynthesis (Faix et al., 2012). Potassium levels can be involved in sucrose transport acting as a counter-ion during H^+-coupled co-transport of sucrose (Patrick and Offler, 2001). Higher contents of some of the most important micronutrients can be due to an increased grain sink strength, which has been shown to be significant for micro-nutrient delivery (Stomph et al., 2011). Thereby, feed-forward control of micro-element loading can be regulated by the sucrose influx and respond to the increased grain sink strength of HOSUT-grains. It is known that the transfer route of micro-nutrients into grains is similar to that of sucrose and that the main portion of iron and zinc is transported early in development, at 10–14 DAF (Pearson et al., 1998). If an improved sucrose uptake capacity also results in higher micro-nutrient contents, as partly observed in the present study with HOSUT grains, this could be exploited by breeding for health-promoting qualities, in particular regarding iron and zinc.

4.5. HOSUT lines had similar phenology and one line lacked anther extrusion

Metabolomic changes may potentially affect plant phenology or susceptibility to biotic stress, including pests and pathogens. In addition, unintended effects due to the transformation process and the insertion of foreign genes may occur when genetic engineering is applied. The HOSUT lines did not differ from the wildtype 'Certo' in respect to plant length, heading time and flowering time, indicating that plant phenology including growth remained unchanged in HOSUT lines and that *HvSUT1*-overexpression primarily affected grain development. However, spike density was higher than in 'Certo' in two of the three HOSUT lines when all years were analysed together, albeit not when years were analysed individually. In addition, we found no indication for increased disease susceptibility in the HOSUT lines. The improved FHB resistance of HOSUT20 can be explained by the complete retention of anthers, which has been shown to affect FHB susceptibility in conventional wheat lines (Kubo et al., 2013). Furthermore, previously published assessments in the same field trial showed no indication that the HOSUT lines suffer from increased aphid damage (Yang et al., 2019). In summary, there were no obvious unintended effects that would call into question the further development of the used metabolic engineering approach. In contrast, the strict cleistogamy phenotype observed in HOSUT20 may be interesting for the breeding of *Fusarium*-resistant wheat. In addition to this, HOSUT20 does not shed pollen due to the lack of anther extrusion, which prevents outcrossing. Therefore, this is an interesting trait to facilitate co-existence between GM and non-GM wheat fields. However, HOSUT20 had the lowest number of grains per spike, which might be a consequence of reduced fertilization due to a potential defect in anther development beyond the lack of extrusion. This effect was partly compensated by increased TGW so that the yield per spike remained unchanged compared with 'Certo'.

4.6. Nitrogen fertilization, year, and cultivar had stronger effects on yield parameters and nutrient composition than HOSUT transformation

For the HOSUT lines and 'Certo', N fertilisation increased yield, number of grains per spike, spike density, and plant height, but decreased TGW. Effects of fertilisation were mainly observed between the treatments without additional fertilisation (N0) and the standard fertilisation (N1) and less between standard and elevated fertilisation (N2). There was also no indication that higher N-fertilisation improved yield traits in the HOSUT-plants over 'Certo' (no interactions of fertilisation and wheat line). Increasing fertilizer doses generally increased nutrients in grains, e.g., nitrogen, iron, sulphur, manganese, and calcium, while it decreased starch. In addition, fertilization from N0 to N1 increased potassium, but decreased zinc. Overall, effects of fertilizer addition on yield parameters and grain composition were stronger than effects of the transformation, *i.e.*, HOSUT lines compared with 'Certo'.

Differences in yield parameters, nutrients, and phenology were significant between the three years of field experiments. 2017 resulted in the highest yield, which was accompanied by the highest grain length, numbers of grains per spike, yield per spike, spikelets per spike, and spike density. However, TGW, grain area, and grain width were lowest in 2017. In contrast, 2018 had the lowest yield and the lowest grain length, number of spikelets per spike, and spike density. In 2019, yield was intermediate, but TGW, grain area, and grain width had the highest values while grains per spike and yield per spike was lowest among all years. Overall, the largest differences among years were observed for yield (43 %) and spike density (43 %). Similarly, grain composition varied among years. Contents of iron, zinc, and calcium were highest in 2017 while nitrogen, sucrose, starch and potassium were lowest. In 2018, sucrose, starch, potassium, and manganese were highest while sulphur, phosphate and magnesium were lowest. In 2019, nitrogen, sulphur, phosphate, and magnesium were highest and iron, zinc, manganese, and calcium lowest. Also plant phenology varied among years with early heading and flowering and shortest plant length in 2018 and late heading and flowering and highest plants in 2019.

A major factor that influences plant growth and yield is the weather. Temperature and precipitation for the 3 field seasons is available in Fig. S5. Shortly after flowering is an important time point in the development of wheat, when grains start to develop. The filling of grains directly influences yield (Reynolds et al., 2022). In 2018, a warm and dry period from April to early May before flowering and a relatively dry period before harvest may have caused drought-stress in the plants and resulted in lower yields compared to the other years, where rainfall was more equally distributed over the growing season. In addition, after flowering in 2018, plants suffered from *Septoria* leaf blotch and other diseases, which may also have contributed to yield reduction. To avoid such disease symptoms in the following year, plants were treated with fungicide in 2019.

5. Conclusions

The increased potential of sucrose uptake and/or partitioning to the grains in transgenic HOSUT lines resulted in larger grains, but did not translate to increased yield per hectare, irrespective of N-fertilization level. Increased grain size was compensated by lower grain numbers per spike. While a trade-off between grain size and grain numbers is common in conventional breeding, the genetic breeding approach chosen for the HOSUT lines was not sufficient to overcome this trade-off under field conditions. Further improvements, such as increased vascular capacity in the spikes, may be goals for future breeding research. While no adverse unintended effects were evident in the current HOSUT lines, increased levels of zinc and partly also iron, and potential FHB resistance due to an anther retention phenotype, may be interesting traits for further breeding. Since strict cleistogamy prevents outcrossing, it is also an interesting trait to facilitate co-existence between GM and non-GM wheat fields.

CRediT authorship contribution statement

Susanne Brunner: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Formal analysis, Conceptualization. **Heiko Weichert:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Michael Meissle:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **Jörg Romeis:** Writing – review & editing, Methodology, Conceptualization. **Hans Weber:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

All data used for statistical analyses are available in the online supplemental material for this publication.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fcr.2024.109506.

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