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Associations between endogenous spike cytokinins and grain-number traits in spring wheat genotypes

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ABSTRACT

Genetic variation in grain number has been positively associated with levels of cytokinins in inflorescences in cereals, although studies quantifying endogenous levels in the field are currently lacking. The present study, using a spring wheat association mapping panel (HiBAP II) of 150 lines, quantified associations between spike hormone levels and grain number and associated traits. The HiBAP II panel was grown in the field in NW Mexico under irrigated conditions for one year and a subset of ten genotypes in the glasshouse under well-watered conditions for three years. The spike levels of four cytokinins (*trans*-zeatin riboside, *trans*-zeatin, isopentenyladenosine, and isopentenyladenine) were measured by using ultra-high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. In the glasshouse experiments, spike hormone levels were measured at booting and anthesis, and in the field experiment at anthesis. In the glasshouse experiments, cytokinin levels were also measured in the basal, central, and apical spikelets separately in addition to at the whole spike level. The spike cytokinin levels did not differ significantly between the basal, central and apical sections of the spike. or show a spike position × genotype interaction. In the glasshouse experiments, significant genetic variation was detected for the expression of the four cytokinins in spikes at booting. At booting, spike *trans*-zeatin concentration ranged amongst genotypes from 4.5 to 16.0 ng g^{-1} FW and was positively correlated with grain number per main shoot ($r = 0.77$, $P < 0.05$). In the field at anthesis, the spike levels of each of *trans*-zeatin, *trans*-zeatin riboside and isopentenyl adenosine were positively correlated with grains per m^2 ($r = 0.17$–0.19, $P < 0.05$). Our results indicated that selection for high spike cytokinin levels in wheat germplasm offers scope to raise grain number and yield potential in wheat.

1. Introduction

Wheat is one of the three major cereal crops and produces ~ 766 million tonnes per year (FAOSTAT, 2021) supplying 20% of the calories and protein for the human population (Shiferaw et al., 2013). Grain yield was increased during the Green Revolution in the 1960s and 1970s through the introgression of *Rht* semi-dwarfing genes, favouring assimilate partitioning to the spike during stem elongation and increasing grains per m^2 (GN) and harvest index (Hedden, 2003). Despite this major advancement in grain number, the yield potential of modern cultivars is still limited by grain sink strength during grain filling under favourable conditions (Rivera-Amado et al., 2020). Therefore, with a growing population and a requirement to double wheat production by 2050 for food security, breeders need to enhance grain number by identifying new traits underlying its expression (Bruinsma, 2009; Slafer et al., 2023).

Previous investigations in wheat have shown that genetic variation in fruiting efficiency (ratio of grain number to spike dry matter at

Abbreviations: CK, cytokinin; 2iP, isopentenyladenine; *t*-Z, *trans*-zeatin; iPA, isopentenyladenosine; *t*-ZR, *trans*-zeatin riboside; ABA, abscisic acid; IAA, indole-3-acetic acid; GN, grain number; A+7, *anthesis +7 days*; FE, fruiting efficiency.

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anthesis, FE) is strongly associated with GN (Garcia et al., 2014; Gonzalez et al., 2011), and could therefore be used as a target to increase GN in wheat breeding (Slafer et al., 2023). Fruiting efficiency (i.e., the efficiency for using dry matter allocated to the juvenile spike before anthesis to set a certain number of grains) is a measure of the outcome of processes related to floret development and degradation pre-anthesis, as well as grain abortion post-anthesis (Slafer et al., 2023). Fruiting efficiency is a heritable trait that exhibits transgressive segregation (Martino et al., 2015) and responds to selection (Alonso et al., 2018; Pedro et al., 2012). However, to deploy FE in breeding, an improved understanding of its physiological basis is required to identify the underlying mechanisms determining genetic variation. One promising avenue to improve fruiting efficiency is optimising the levels of spike cytokinins (CKs) influencing floret fertility and grain number.

CKs stimulate cell division and regulate shoot meristem size, spikelet primordia number and lateral meristem activity and there is evidence that genetic variation in grain number in cereals is influenced by levels of CK in the inflorescence (Ashikari et al., 2005; Jablonski et al., 2020). CK plant hormones have been strongly implicated in many aspects affecting grain yield, particularly grain number and size (Jameson and Song, 2016; Yamburenko et al., 2017). Through their involvement in cell division, CKs could influence floret development by promoting mitosis (Schaller et al., 2014). A gradient of CKs has been reported through the spike in barley, with the lowest levels in the basal spikelets and the highest in the apical spikelets (Youssef et al., 2017). However, currently little is known about the distribution of CKs within the spike in wheat and whether there may be an association with the fertility of individual spikelets in the spike (Slafer et al., 2023).

The active forms of CKs are nucleobases (free bases) such as isopentenyladenine (2iP) and *trans*-Zeatin (*t*-Z), while the inactive forms, which serve as a reservoir for CK storage, are nucleosides (five-carbon sugar added to nucleobase) such as isopentenyladenosine (iPA) and *trans*-zeatin riboside (*t*-ZR). CK levels are regulated by the balance between the activity of biosynthesis enzymes (e.g., isopentenyl transferase, IPT) and degradation enzymes (e.g., cytokinin oxidase/ dehydrogenase, CKX). The grain sink strength of the spike meristem could therefore be enhanced by altering cytokinin homeostasis through the upregulation or the downregulation of these enzymes, respectively, to coordinate growth and floret fertility (Li et al., 2019). While there has been speculation on a trade-off between fruiting efficiency and spike dry weight at anthesis (Lazaro and Abbate, 2012), manipulating spike hormones may offer breeders one avenue for simultaneously raising both.

When synthetic CK 6-benzylaminopurine (6-BAP) was added to Chinese winter wheat grown in the field 25 days after stem elongation initiation, floret abortion rates were reduced by up to 77% compared to the control sprayed with water (Zheng et al., 2016). A gene encoding for CK oxidase/dehydrogenase (*OsCKX2*) has been shown to correspond to the grain number QTL *Gn1a* on the short arm of chromosome 1 in rice (Ashikari et al., 2005). When the expression of *OsCKX2* was reduced, CK accumulated in the inflorescence meristems and grain number increased. The expression levels of two *TaCKX2* genes were positively correlated with grains per spike among 12 Chinese wheat varieties (Zhang et al., 2011). Transformation of cv. NB1 to lower expression of *TaCKX2.4* led to an increase of 12.6% in grains per spike in line JW39–3A compared to cv. NB1 in wheat (Li et al., 2018a). These studies suggest that manipulation of the genes to reduce CKX levels can increase grain number in wheat. This may occur both locally through spike CK levels or long-distance signalling messaging through the xylem and phloem, controlled by both endogenous and environmental signals (Kudo et al., 2010). CKs are also known to regulate plant development through crosstalk with other plant hormones. For example, CKs have been reported to regulate the expression and accumulation of the auxin efflux proteins PINFORMED (*PIN*) family, which resulted in increased auxin efflux affecting shoot branching in Arabidopsis (Waldie and Leyser, 2018).

The fine-tuning of CK levels in wheat spikes represents a novel approach for engineering spikes with enhanced grain number. To date, no previous study has quantified the variation in spike CKs in modern wheat genotypes at different developmental stages and in different spikelet positions and quantified associations with fruiting efficiency, grain number and grain yield. Therefore, field and glasshouse studies were conducted over three years to quantify genetic variation in spike CK levels in 150 spring wheat genotypes of the HiBAP II association panel to investigate how levels of CK in the spike influence fruiting efficiency, grain number and other yield-related traits.

2. Materials and methods

2.1. Glasshouse experiments: Plant materials and experimental design

Three experiments were conducted, one in each of 2017, 2018 and 2019, under fully irrigated conditions in the glasshouse at Nottingham University, Sutton Bonington Campus, UK. A subset of ten genotypes from the CIMMYT High Biomass Association Panel (HiBAP II) was used (Table 1), which contains spring wheat genotypes with high expression of biomass and yield potential (Molero et al., 2019). The subset comprised nine elite cultivars or advanced lines and one synthetic-derived advanced line. The subset was selected based on the contrasting expression of fruiting efficiency using previous CIMMYT field data (Sierra-Gonzalez, 2020). Plants were grown in 2 L pots (one per pot) using John Innes No. 2 soil medium. The experimental design was a complete randomised block with four replicates in 2017 and 2019 and five replicates in 2018. Four different sets of plants were grown as technical replicates per replicate to allow destructive sampling for spike hormone levels at late booting (GS49) and anthesis (GS65) and plant growth analysis at GS65 and physiological maturity (GS89). All non-destructive measurements, such as plant height, were taken on the set of plants designated for plant growth analysis at physiological maturity.

The plants were sown on 2 August 2017, 13 June 2018 and 3 June 2019. Herbicides, fungicides, and pesticides were applied as required to minimise the effects of weeds, diseases, and pests (Table S1). Irrigation was supplied throughout the cycle every day using a complete nutrient solution applied through drip irrigation. Supplementary lighting was provided in 2017 from 30th October onwards to maintain a 16-hour day length. A Tinytag Ultra 2 data logger (Gemini Data Loggers, Chichester, UK) was placed in the glasshouse from sowing to maturity to measure temperature every 15 min (Fig. S1).

2.2. Field experiment: Plant materials and experimental design

In 2018–19, 150 genotypes of the HiBAP II panel were grown in a field experiment under fully irrigated yield potential conditions at the Norman E. Borlaug experimental station near Ciudad Obregón, Sonora, Mexico. The plants were sown on 27th November and the emergence

Table 1
Spring wheat HiBAP II genotypes in the glasshouse experiments FE_A main shoot: fruiting efficiency calculated using spike dry matter at anthesis. SD: synthetic derivative. High and low FE values based on field data from Sierra-Gonzalez (2020).

Genotype	Name	Origin	FE_A
1	CHEWINK #1	Elite	Low
2	PASTOR//HXL7573/2 *BAU/3/WBLL1	Elite	Low
3	DPW 621–50	Elite	High
4	SOKOLL	SD	Low
5	F2SR2–69//YANGLING SHAANXI/PASTOR	Elite	High
6	BORLAUG100 F2014	Elite	High
7	FRET2 * 2/BRAMBLING//BECARD/3/WBLL1 * 2/ BRAMBLING	Elite	High
8	KIDEA	Elite	Low
9	JANZ	Elite	High
10	BCN/RIALTO//ROLF07	Elite	Low

date was 3 December. The soil is a coarse sandy clay, mixed montmorillonitic typic calciorthid, low in organic matter and with a slightly alkaline pH of 7.7, as described in Sayre et al. (1997). The plants were grown in 1.6 × 5.0 m plots with three replicates in a raised-bed planting system (two beds and two rows per bed), randomised in an α-lattice design. Each bed had a 24 cm gap between rows and there was a 56 cm gap between beds. Herbicides, fungicides, and pesticides were applied to minimise the effects of weeds, diseases, and pests (Table S2). Irrigation was supplied throughout the cycle every 3–4 weeks using a gravity-based system where the channels between beds were flooded to keep the plants free from water stress. N fertiliser was applied as urea before sowing (50 kg N ha^{-1}), and then an application of P as triple superphosphate at sowing (50 kg P ha^{-1}). A second and third N application (200 and 150 kg N ha^{-1}, respectively) was applied as urea with the second and third irrigations. No plant growth regulators were applied. The seed rate was 10.25 g m^{-2}, with plant establishment of 132–214 plants per m^2.

2.2.1. Glasshouse experiments: Plant growth analysis

Samples for growth analysis were collected at anthesis (GS65) and physiological maturity (GS89) by cutting the plant at the soil level. The development stage was assessed on the main shoot (MS) of each plant twice a week according to the Zadoks decimal code (Zadoks et al., 1974). The plant height of the MS was measured from the soil surface to the tip of the spike, excluding awns, shortly before physiological maturity.

At anthesis, plants were separated into the MS, other fertile shoots (those with a spike) and infertile shoots. Each fertile shoot was then separated into the spike, leaf lamina and stem (true stem and leaf sheath attached, MS separately from other fertile shoots). Each plant component was then dried for 48 h at 70 °C and the weight of each component was recorded.

The number of fertile florets per spike was assessed on the MS spike according to the Waddington scale (Waddington et al., 1983) at anthesis, where any floret at a developmental score of W9.5 (styles and stigmatic branches spreading outward, stigmatic hairs well developed) or above was considered a fertile floret; this was done for each spikelet down one side of the ear.

At physiological maturity, for all fertile shoots, the spike was separated from the straw (stem and leaves), and the weight of the straw and spike was recorded separately after drying for 48 h at 70 °C (MS separately from other fertile shoots). The number of spikes was counted, and the spikes were then hand-threshed before the grains and chaff were weighed after drying for 48 h at 70 °C (MS separately). All the grains were then counted (MS separately). These data were then used to calculate harvest index (HI, the proportion of above-ground dry matter (DM) in the grain), above-ground DM at physiological maturity ($AGDM_{PM}$) and fruiting efficiency (FE, calculated two ways as either the ratio of grain number to spike DM at anthesis, FE_A, or as the ratio of grain number to chaff DM at maturity, FE_{chaff}).

2.2.2. Field experiment: Growth analysis

Samples were collected seven days after anthesis (GS65 +7) and physiological maturity (GS89) (Zadoks et al., 1974). At GS65 + 7, shoots were cut at ground level in a quadrat of 0.5 × 1.6 m. A 50-shoot sub-sample was taken on a fresh weight basis and the sub-sample dried at 70 °C for 48 h and weighed. From the remainder of the sampled material, 12 fertile shoots (those with a spike) were randomly selected, and each shoot was separated into the spike, leaf lamina and stem (true stem and leaf sheath attached) and each component was weighed after drying for 48 h at 70 °C.

At physiological maturity, 50 fertile shoots per plot were randomly selected and cut at ground level. The spike was separated from the straw (stem and leaves), and the weights of the spike and straw were recorded separately after drying for 48 h at 70 °C. Spikes were then threshed using a Wintersteiger LD350 Laboratory Thresher (Wintersetiger, Ried, Austria) and grains were weighed after drying for 48 h at 70 °C, and 200 grains were counted and weighed to determine thousand grain weight (TGW). Each plot was machine-harvested in an area ranging from 3.2 to 5.6 m^2 after borders were removed to avoid any border effect, and values were adjusted to the moisture percentage measured in each plot. These data were then used to calculate harvest index (HI, proportion of above-ground DM in the grain), above-ground DM ($AGDM_{PM}$, GY/HI), grain number per m^2 (GN, GY/TGW), and fruiting efficiency (FE_{A+7}, ratio of grain number to spike DM at anthesis + 7 days, or FE_{chaff}, ratio of grain number to chaff DM at maturity).

2.2.3. Plant hormone analysis: Glasshouse experiments

In each year, the central spikelets of one MS spike were sampled and placed in liquid nitrogen for plant hormone analysis (four CKs: 2iP, *t*-Z, iPA, *t*-ZR and ABA and IAA) at GS49 and GS65 for each genotype in each replicate. Ten central spikelets were sampled at booting and six at anthesis. Additionally, in 2019, for two genotypes in each replicate (selected to show contrasting fruiting efficiency (Chewink #1 low and Janz high) based on results in 2017 and 2018) at GS49 the flag leaf and peduncle of one MS were sampled per genotype in each replicate (the flag leaf and peduncle sampled were taken from the same main shoot from which the spike was sampled at GS49); and at GS65 six of the basal and apical spikelets (dividing total spikelet number by three) of one MS per genotype in each replicate were sampled as well as the central spikelets, flag leaf and peduncle (all from the same MS).

In the laboratory, 100 mg of frozen plant material per sample was subsampled, and the material was stored at − 80 °C. The samples were then analysed for plant hormone profiling using ultra-high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UHPLC/ESI-MS/MS) as described by Müller and Munné-Bosch (2011). The frozen plant material was ground in liquid nitrogen and the plant hormones were extracted with 200 µl extraction solvent using ultrasonication, before centrifugation (10,000 rpm for 15 min at 4 °C) and collection of the supernatant. The pellet was re-extracted twice with 100 µl of extraction solvent and the supernatants from each extraction were combined and filtered before analysis with UHPLC/ESI-MS/MS. Internal standards were used for estimating recovery rates for quantification.

2.2.4. Plant hormone analysis: Field experiment

Each plot was sampled at anthesis (GS65) in two replicates. Due to the time-consuming nature of the spike sampling for hormone analysis and available resources, it was only feasible to carry out the spike hormone analysis in two of the three replicates. Two spikes per plot, one from each inner row of the two beds, were sampled and frozen in liquid nitrogen immediately in the field. Then in the laboratory, keeping the two spikes frozen on dry ice, approximately three central spikelets from each spike were sampled and combined (resulting in a 6-spikelet sample) to make a 100 mg fresh weight sample of frozen material, which was stored at − 80 °C. The spike hormone analysis was carried out on this material using UHPLC/ESI-MS/MS as described for the glasshouse experiments (Müller and Munné-Bosch, 2011).

2.3. Statistical analysis

In the glasshouse experiments, genotype means in individual years were estimated from the Analysis of Variance (ANOVA) using Genstat 19th edition (VSN International, 2021), where the genotypes were considered as fixed effects and replicates as random effects. Genotype means across years were estimated from the cross-year ANOVA, where the genotypes were considered as fixed effects and replicates and years as random effects using Genstat 19th edition. In the field experiment, genotype means and standard deviations were calculated in R v 4.3.1 using the package Psych (Revelle, 2023). Statistical significance is considered throughout at the 5% level ($P < 0.05$). We have also commented on trends for effects which were between the 5–10% significance level. A covariate for days from sowing to anthesis was included in

the ANOVA when it had a significant effect, to take account of any environmental change with development. For the two genotypes where different plant components (flag leaf, peduncle, basal spikelets, central spikelets and apical spikelets) were measured for plant hormone levels, a separate ANOVA was carried out where the genotypes were considered as fixed effects and plant component position and replicates as random effects. Broad-sense heritability (H^2) was calculated as described in Eq. 1, where $\sigma^2 g$ and $\sigma^2 e$ are the genotypic and environmental variance, respectively, and $\sigma^2 ge$ is the genotype × environment interaction. The number of environments and replicates are represented by *e* and *r,* respectively.

$$H^2 = \frac{\sigma^2 g}{\sigma^2 g + \frac{\sigma^2 ge}{e} + \frac{\sigma^2 e}{re}} \quad (1)$$

Genstat 19th edition was used for calculating linear regressions and the R v 4.3.1 package Hmisc (Harell, 2022) for calculating Pearson's correlation coefficients using the genotype means from the ANOVA.

3. Results

3.1. Glasshouse experiments

In 2017 the experiment was sown later than the other two years (02/08/17 vs. 13/06/18 and 03/06/19), resulting in lower temperatures during grain filling and a longer growing season. Photoperiod was supplemented in 2017 to ensure 16-hour light days. Anthesis was reached on average 13–15 days later in 2017 than in 2018 and 2019 (66 days emergence to anthesis (DTA) vs. 51 and 53 DTA, respectively, Table 2, Table S3). The mean daily temperatures in the experiments are shown in Fig. S1.

3.1.1. Plant yield, yield components and biomass

Averaging over years, there was genetic variation in above-ground dry matter (AGDM) per plant at anthesis (5.45–9.24 g, $P < 0.001$, Table 2; Table S3) and physiological maturity (11.05–22.01 g, $P < 0.001$, Table 3). There was also a year × genotype interaction for AGDM per plant at both stages ($P < 0.05$) At physiological maturity, fruiting efficiency based on MS spike DM at anthesis (FE_A) ranged among genotypes from 78.7 to 115.8 grains g^{-1} ($P = 0.015$); and based on chaff DM (FE_{chaff}) from 73.3 to 116.9 grains g^{-1} ($P = 0.004$) (Table 3). Grains per MS (GN_{MS}) ranged from 29 to 48 ($P = 0.004$) and grains per plant (GN_P) from 136 to 275 ($P < 0.001$). At maturity, high heritability (> 0.50) was observed for all traits except for HI for the MS (0.46). The year × genotype interaction was not statistically significant for fruiting efficiency for the MS (based on either spike DM at anthesis or chaff DM) or grains per MS. However, there was a significant year × genotype interaction for grain yield (per MS and per plant; $P < 0.01$ and $P < 0.001$, respectively) and for grains per plant ($P < 0.001$). The interaction for grain yield per MS was related to an interaction for TGW for the MS ($P < 0.05$) and the interactions for grain yield per plant and grains per plant to an interaction for spikes per plant ($P = 0.01$).

FE_{chaff} was positively associated among genotypes with grains per MS (R^2 =0.56, $P = 0.013$, Fig. 1b), but FE_A did not associate with grains per MS (R^2 =0.12, ns, Fig. 1a). Grains per plant and grain yield per plant were positively associated (R^2 =0.73, $P = 0.002$, Fig. 1d). The line 5 (F2SR2–69//YANGLING SHAANXI/PASTOR) had a relatively high weighting on these associations (Fig. 1b and d); however, the associations were still statistically significant omitting line 5 from the regressions (data not shown).

3.1.2. Genetic variation in spike hormone levels and association with grain sink traits

Averaging across years, the genetic ranges for the spike hormone analyte levels at late booting (GS49) and anthesis (GS65) are shown in Fig. 2. Cross-year analysis showed genotype had a significant effect on levels of all hormones at GS49 except 2iP and ABA (Table 4). However, at GS65, genotype was only significant for 2iP, ABA and IAA. For the four CKs, there was genetic variation at GS65 for 2iP from 0.6 to 1.1 ng g^{-1} ($P < 0.001$), and at GS49 for *t*-Z from 4.5 to 12.8 ng g^{-1} ($P < 0.001$), iPA from 0.8 to 1.5 ng g^{-1} ($P < 0.05$) and *t*-ZR from 1.4 to 4.8 ng g^{-1} ($P < 0.001$). The genotype × year interaction was significant for all plant hormones at GS49, except ABA, and for all at GS65 except iPA and *t*-ZR (Table 4). Regarding the developmental stage, all hormones apart from iPA and 2iP showed a significant change in analyte level from late booting to anthesis (Fig. 2). For example, *t*-Z, *t*-ZR and IAA increased from late booting to anthesis, whereas ABA showed a significant decrease between these two stages ($P < 0.05$).

The genetic variation in analyte levels for the individual years is shown in the boxplots in Fig. S2 and Table S4**.** For most spike hormones, the pattern of the change between GS49 and GS65 was similar in each of the three years. The genotype effect in individual years was not always significant, particularly in 2019, where only ABA showed genotype differences and there was a trend for genotype differences for 2iP ($P = 0.08$) at GS65 (Table S5). Regarding the assessments at the two growth stages, the genotype × growth stage interaction was not statistically significant for any of the six plant hormones (Table S6).

The correlations among genotypes between spike hormone levels and grain yield, HI and fruiting efficiency are shown in Table 5. At late booting, spike *t*-Z level was positively correlated with grains per MS ($r = 0.77$, $P < 0.01$), and negatively with MS TGW ($r = -0.66$, $P < 0.05$). There was also a trend for a positive correlation between *t*-Z level and FE_{chaff} ($r = 0.60$, $P < 0.10$). In addition, at late booting, there was a trend for a positive correlation between spike 2iP level and fertile florets per spike ($r = 0.62$, $P < 0.10$; Table 5) and between *t*-ZR and MS HI ($r = 0.61$, $P < 0.10$). For spike hormones at anthesis, there was a trend for a positive correlation between *t*-ZR level and FE_{chaff} ($r = 0.56$, $P < 0.10$). iPA showed a trend for a positive correlation with grains per MS ($r = 0.59$, $P < 0.10$) and IAA a trend for a negative correlation was seen with MS HI ($r = -0.60$, $P < 0.10$).

3.1.3. Variation in hormone levels within spikes and between plant organs

In 2019, for two genotypes, the flag leaf and peduncle were assessed for plant hormone levels at both GS49 and GS65; and at GS65 each of the basal, central and apical spikelets for the spike was assessed. At GS49, there were significant differences amongst the plant components in

Table 2
Phenotypic ranges, heritability, least significant differences (LSD, P = 0.05) and significance (p-values) for 10 spring wheat HiBAP II genotypes in the glasshouse for traits at anthesis. Cross-year means of 2017, 2018 and 2019.

		(Min-Max)	Heritability	LSD (5%)	Genotype significance	Gen x Year significance
Main Shoot	DTA	51–60	0.34	2.45	< 0.001	< 0.001
	Plant Height cm	60.11–80.29	0.44	6.54	< 0.001	0.388
	$AGDM_A$ g	1.20–1.91	0.26	0.21	< 0.001	0.131
	Fertile florets per spike	32.74–45.38	0.54	6.17	< 0.001	0.026
	Spike DM g	0.31–0.43	0.55	0.06	0.001	0.097
Plant	$AGDM_A$ g[†]	5.45–9.24	0.64	1.21	< 0.001	0.043

DTA: days emergence to anthesis, $AGDM_A$: above-ground dry matter at anthesis, [†] DTA added as covariate if $P < 0.05$.

Table 3
Phenotypic ranges, heritability, least significant differences (LSD, P = 0.05) and significance (p-values) for 10 spring wheat HiBAP II genotypes in the glasshouse for traits at physiological maturity. Cross-year means of 2017, 2018 and 2019.

		(Min-Max)	Heritability	LSD (5%)	Genotype significance	Gen x Year significance
Main Shoot	$AGDM_{PM}$ g	2.15–3.42	0.51	0.46	< 0.001	0.023
	Grain yield g	0.91–1.51	0.81	0.28	0.005	0.005
	TGW g	25.03–43.72	0.74	5.36	< 0.001	0.049
	HI	0.40–0.47	0.46	0.05	0.05	0.048
	FE_A grns g^{-1}	78.7–115.8	0.72	24.18	0.015	0.081
	FE_{chaff} grns g^{-1}	73.3–116.9	0.76	20.64	0.004	0.328
	GN_{MS}	29.20–48.00	0.52	9.11	0.004	0.429
Plant	No. fertile shoots	6–9	0.72	1.26	< 0.001	0.010
	$AGDM_{PM}$ g	11.05–22.01	0.77	2.80	< 0.001	0.005
	Grain yield g	4.75–8.29	0.83	1.48	< 0.001	0.001
	TGW g	25.96–34.94	0.71	5.45	0.044	0.091
	HI	0.32–0.46	0.64	0.04	< 0.001	0.018
	FE_{chaff} grns g^{-1}	73.0–112.0	0.92	14.77	< 0.001	0.464
	GN_P.	136.3–275.3	0.84	39.60	< 0.001	0.002

$AGDM_{PM}$: above-ground dry matter (DM) at physiological maturity, TGW: thousand-grain weight, HI: harvest index, FE_A: fruiting efficiency calculated using spike DM at anthesis, FE_{chaff}: fruiting efficiency calculated using chaff DM at physiological maturity, GN: grain number

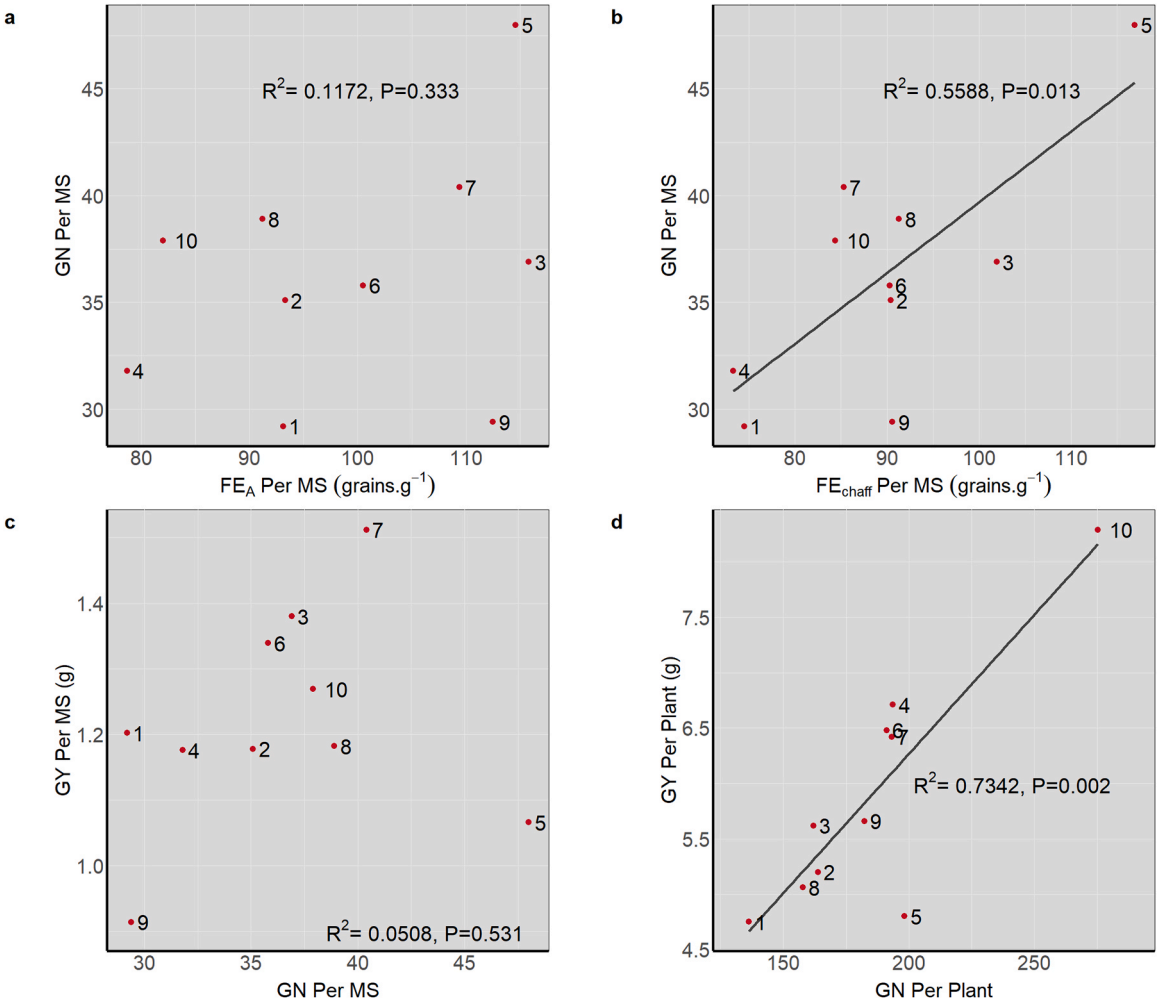

Fig. 1. Linear regressions of a) grain number (GN) per main shoot (MS) on fruiting efficiency based on spike DM at anthesis (FE_A), b) GN per MS on fruiting efficiency based on chaff DM at maturity (FE_{chaff}), y = 0.33x+ 6.53, c) grain yield (GY) per MS on grain number (GN) per MS and d) GY per plant on GN per plant, y = 0.02x+ 1.24. Values represent means for 10 HiBAP II genotypes in the glasshouse across 2017, 2018 and 2019, numbered points indicate genotypes (see Table 1).

hormone levels for 2iP and ABA (Fig. 3). The levels of 2iP and ABA at GS49 were higher in the spike (1.6 and 46.8 ng g^{-1}, respectively) and flag leaf (1.5 and 44.2 ng g^{-1}, respectively) than in the peduncle (0.4 and 21.1 ng g^{-1}, respectively, P < 0.05). At GS65, the plant component effect was significant for iPA, zeatin, zeatin riboside and ABA (Fig. 4).

The levels of *t*-Z and *t*-Zr were higher in each of the spike sections compared to the peduncle or flag leaf. The levels of iPA in the peduncle were higher than in the flag leaf or spike, while ABA showed the

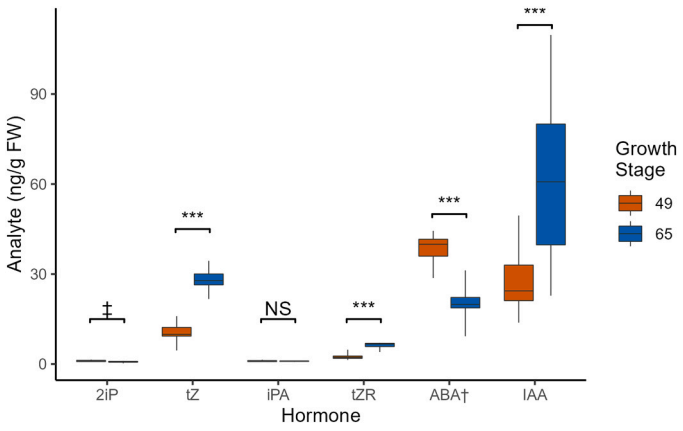

Fig. 2. Boxplots showing genetic ranges for 10 HiBAP II genotypes in spike hormonal analyte levels at late booting (GS49) and anthesis (GS65) in glasshouse experiments. Values represent means across 2017, 2018 and 2019. The line within the box represents the mean, bottom and top of boxes represent upper and lower quartiles and lines below and above box minimum and maximum values, respectively. Significance values indicate if growth stage is significant *** $P < 0.001$, ‡$P < 0.1$, NS: not significant. †: DTA added as a covariate if $P < 0.05$, 2iP: isopentenyladenine, t-Z: zeatin, iPA: isopentenyladenosine, t-ZR: zeatin riboside, ABA: abscisic acid, IAA: indole-3-acetic acid (auxin).

Table 4
Significance (p-values) of hormonal analyte levels for 10 HiBAP II genotypes in the glasshouse experiments from cross-year ANOVA 2017, 2018 and 2019 and least significant difference (LSD, $P = 0.05$) for genotype.

Hormone		LSD	Year P value	Genotype P value	Gen x Year P value
GS49	2iP	0.57	< 0.001	0.100	0.027
	t-Z	2.75	0.003	< 0.001	< 0.001
	iPA	0.44	< 0.001	0.038	0.005
	t-ZR	1.08	0.007	< 0.001	< 0.001
	ABA	10.21	< 0.001	0.098	0.259
	IAA	12.39	0.021	< 0.001	< 0.001
GS65	2iP	0.35	0.001	< 0.001	< 0.001
	t-Z	11.92	0.008	0.556	0.034
	iPA	0.73	< 0.001	0.997	0.796
	t-ZR	2.37	< 0.001	0.263	0.081
	ABA†	6.76	< 0.001	< 0.001	0.051
	IAA	31.46	0.005	< 0.001	0.04

†DTA added as a covariate if $P < 0.05$
2iP: isopentenyladenine t-Z: zeatin, iPA: isopentenyladenosine, t-ZR: zeatin riboside, ABA: abscisic acid, IAA: indole-3-acetic acid (auxin)

opposite effect. Regarding the basal, central and apical spikelets, although there was an apparent tendency for each of the CKs to increase slightly from the basal to apical spikelet positions, there were no statistically significant differences among the spike positions or significant effects for the spike position × genotype interaction (Fig. 4, Table S7).

3.2. Field experiment

For grain yield and grain number traits, heritability was consistently high at > 0.5 (Table 6). Grain yield ranged among the 150 HiBAP II lines from 494 to 743 g m^{-2} and grains m^{-2} from 11,974 to 17,991 ($P < 0.001$). Fruiting efficiency calculated using spike DM at anthesis + 7 days ranged from 33.8 to 86.1 grains g^{-1} ($P < 0.001$) and calculated using the chaff ranged from 51.4 to 98.0 grains g^{-1} ($P < 0.001$) (Table 6). FE_{A+7} and FE_{chaff} were each positively correlated among genotypes with grains m^{-2} ($r = 0.55$ and $r = 0.61$, respectively, $P < 0.001$; Fig. 5). Grains m^{-2} was also positively correlated with grain yield ($r = 0.44$, $P < 0.05$; Fig. 5).

In the field, genetic variation was significant for all hormones measured on the 150 genotypes at anthesis except 2iP and IAA (Fig. 6). The analyte level was highest for *t*-Z and *t*-ZR (Fig. 6), with the other hormones having overall lower analyte levels and narrower ranges. (Fig. 6). Although the correlations were relatively weak, among the 150 genotypes the CKs *t*-Z ($r = 0.17$, $P < 0.05$), iPA ($r = 0.20$, $P < 0.05$) and *t*-ZR ($r = 0.29$, $P < 0.001$) were each positively correlated with FE_{chaff} (Fig. 5). In addition, 2iP ($r = 0.37$, $P < 0.001$) and *t*-ZR ($r = 0.18$, $P < 0.05$) were positively correlated with FE calculated using spike DM at anthesis + 7 days (A+7) (Fig. 5), and there was a trend for a positive correlation between iPA ($r = 0.14$, $P < 0.10$) and FE_{A+7}. All four CKs also correlated positively with grains m^{-2} ($r = 0.17$–0.19, $P < 0.05$, Fig. 5). However, none of the four CKs were associated with spikes per m^2. Neither were any correlations observed between either ABA or IAA and grains m^{-2}, FE_{A+7} or FE_{chaff} (Fig. 5).

3.3. Correlation between traits from the glasshouse to the field

With regard to the correlation between genetic variation in the glasshouse and the field for the subset of 10 genotypes, GY ($r = 0.77$, $P = 0.01$), HI ($r = 0.68$, $P = 0.03$), above-ground DM at physiological maturity ($r = 0.64$, $P = 0.05$) and above-ground DM at anthesis ($r = 0.66$, $P = 0.04$) all had a strong positive correlation (Table S8). This was not the case for other yield traits such as GN or TGW ($r = 0.23$, 0.31, ns, Table S8). The spike *t*-Zr level at GS65 also showed a positive correlation between the two environments ($r = 0.74$, $P = 0.01$, Table S8).

Table 5
Pearson's correlation coefficients between traits measured at anthesis and physiological maturity and spike hormone analyte levels at booting (GS49) and anthesis (GS65) for 10 spring wheat HiBAP II genotypes. Values based on means over 3 years (2017, 2018 and 2019) in glasshouse experiments, †: DTA (days to anthesis) added as a covariate if $P < 0.05$, correlation significance: ‡$P < 0.10$, * $P < 0.05$, ** $P < 0.01$.

Hormone		$AGDM_A$ MS	Spike DM MS	No. Fertile Florets/Spike	$AGDM_{PM}$ MS	GN MS	GY MS	HI MS	FE_A MS	FE_{chaff} MS	TGW MS
GS49	2iP	0.53	0.42	0.62‡	0.46	0.36	0.44	-0.05	-0.19	-0.02	0.09
	t-Z	0.03	0.07	-0.22	-0.03	0.77 **	0.03	0.15	0.39	0.60‡	-0.66 *
	iPA	-0.07	-0.05	0.26	0.15	-0.07	0.2	0.2	0.01	-0.04	0.39
	t-ZR	-0.24	-0.13	0.19	0.17	-0.17	0.37	0.61‡	0.22	0.06	0.4
	ABA	0.2	-0.42	-0.02	0.13	0.15	0.04	-0.26	0.26	0.13	0.08
	IAA	-0.23	0.03	0.37	0.04	-0.2	0.21	0.53	0.04	-0.18	0.23
GS65	2iP	-0.31	0.18	-0.32	-0.57‡	0.31	-0.5	0.09	-0.22	0.43	-0.56‡
	t-Z	-0.04	0.11	0.09	0.01	-0.15	0.1	0.36	-0.22	-0.14	0.29
	iPA	0.26	0.39	0.23	0.09	0.59‡	0.1	0	-0.22	0.41	-0.23
	t-ZR	-0.32	-0.26	-0.12	-0.16	0.24	-0.11	0.05	0.39	0.56‡	-0.37
	ABA†	0.28	-0.29	-0.3	0.03	0.28	-0.14	-0.52	0.17	0.21	-0.2
	IAA	-0.03	-0.23	-0.29	-0.09	0.08	-0.27	-0.60‡	0.11	0.13	-0.04

2iP: isopentenyladenine t-Z: zeatin, iPA: isopentenyladenosine, t-ZR: zeatin riboside, ABA: abscisic acid, IAA: indole-3-acetic acid (auxin), MS: main shoot, $AGDM_A$: above-ground dry matter (DM) at anthesis, $AGDM_{PM}$: above ground DM at physiological maturity, GN: grain number, GY: grain yield (g), HI: harvest index, FE_A: fruiting efficiency calculated using spike DM at anthesis, FE_{chaff}: fruiting efficiency calculated using chaff DM at maturity, TGW: thousand-grain weight (g)

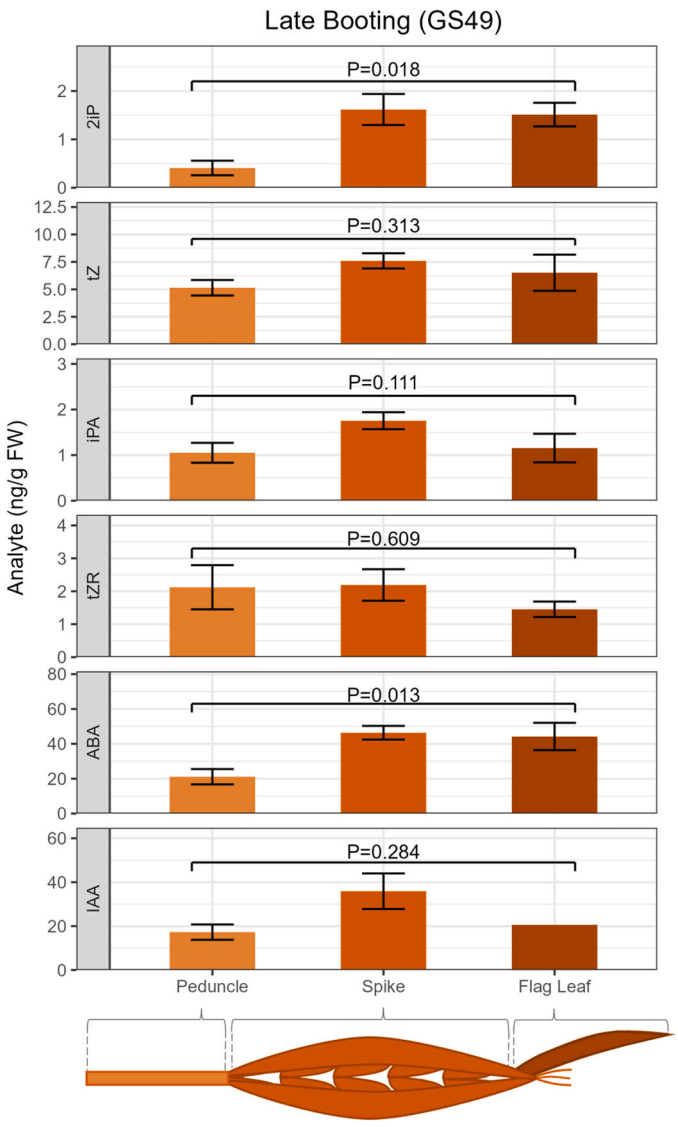

Fig. 3. Hormone analyte levels in the peduncle, flag leaf and spike at late booting (GS49). Values represent the means of two HiBAP II genotypes (genotypes 1 and 9, Table 1) in 2019 in the glasshouse experiments. Error bars represent standard error, 2iP: isopentenyladenine t-Z: zeatin, iPA: isopentenyladenosine, t-ZR: zeatin riboside, ABA: abscisic acid, IAA: indole-3-acetic acid (auxin), P values showing position significance calculated from 2-way ANOVA.

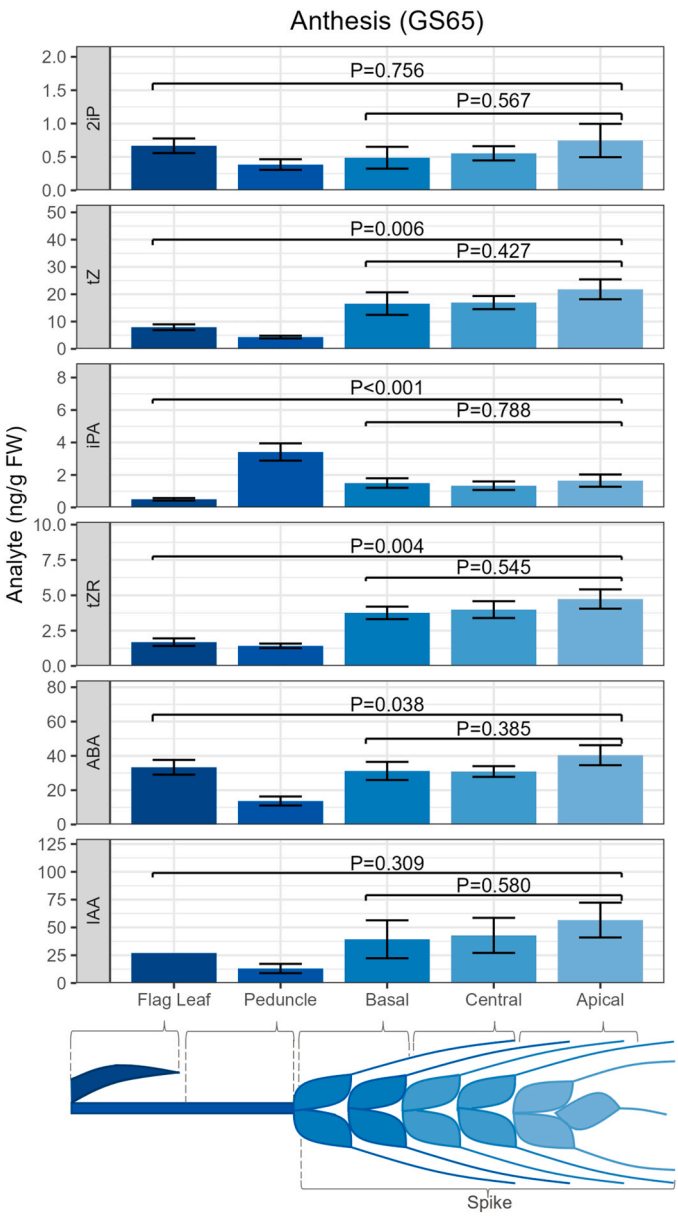

Fig. 4. Hormone analyte levels in the flag leaf, peduncle and spike sections (basal, central and apical) at anthesis (GS65). Values represent the means of two HiBAP II genotypes (genotypes 1 and 9, Table 1) in 2019 in the glasshouse experiments. Error bars represent standard error. 2iP: isopentenyladenine t-Z: zeatin, iPA: isopentenyladenosine, t-ZR: zeatin riboside, ABA: abscisic acid, IAA: indole-3-acetic acid (auxin), position significance calculated from 2-way ANOVA with the P-values shown for all plant organs and from ANOVA calculated with just spike organs.

4. Discussion

4.1. Evidence for genetic variation in cytokinin levels

Cytokinins promote cell division, growth and differentiation which potentially contribute to increasing grain number and FE (Jameson and Song, 2016). In the glasshouse experiments, either the genotype effect or the genotype × year interaction was statistically significant for each of the four CKs at both late booting and anthesis, except for IPA and *t*-ZR at anthesis. Genotype variation was also statistically significant for each of the spike CKs for the 150 genotypes in the field experiment at anthesis except for 2iP. The present results, therefore, demonstrated strong evidence for genetic variation in spike cytokinin levels in the modern bread wheat cultivars and advanced lines of the HiBAP II.

In the glasshouse, the levels of *t*-Z and *t*-ZR in spikes were always higher at anthesis than at late booting. A previous study reported a sharp increase in CKs (Z and ZR) immediately after anthesis, which coincides with the phase of grain endosperm cell division, in wheat cv. Kopara grown in the field (Jameson et al., 1982). Although comparisons across glasshouse and field environments must be made cautiously, a study by Liu et al. (2013) reported Z and ZR levels at anthesis in winter wheat (cv. Zhoumai 18) grown in the field were ~6 ng g^{-1} FW, which was similar to the level of *t*-ZR reported in our glasshouse experiments at 6.3 ng g^{-1} FW. However, the level of *t*-ZR in the field experiment at anthesis was higher at 104 ng g^{-1} FW, while *t*-Z levels were slightly lower at 60 ng g^{-1} FW.

For the two genotypes for which different plant components were assessed in the glasshouse experiments (Chewink #1 and Janz), at late booting 2iP levels were 4-fold greater in the spike than the peduncle, suggesting that higher levels of cytokinins in the spike at this stage may

Table 6
Phenotypic ranges, least significant differences (LSD, P = 0.05), heritability and significance (p-values) for 150 spring wheat HiBAP II genotypes for traits at physiological maturity in 2018–19 in the field experiment.

	(Min-Max)	Heritability	LSD (5%)	Genotype P value
$AGDM_{PM}$ (g m^{-2})	1115–1666†	0.743	98.91	< 0.001
Grain yield (g m^{-2})	494–743	0.807	50.01	< 0.001
TGW (g)	31.9–54.9	0.96	2.072	< 0.001
HI	0.40–0.51†	0.824	0.028	< 0.001
FE_{A+7} (grns g^{-1})	33.8–86.1†	0.539	16.99	< 0.001
FE_{Chaff}	51.4–98.0	0.823	10.29	< 0.001
GN. (grains m^{-2})	11974–17991	0.886	1242.2	< 0.001
SN (spikes m^{-2})	211–393	0.838	37.18	< 0.001

†DTA added as a covariate if P < 0.05, sig: significance, $AGDM_{PM}$: above-ground dry matter (DM) at physiological maturity, TGW: thousand-grain weight, HI: harvest index, FE_{A+7}: fruiting efficiency calculated using spike DM at anthesis + 7 days, FE_{Chaff} fruiting efficiency calculated using chaff DM at maturity, GN: grain number per m^2, SN: spike number per m^2

be required to promote floret survival and fertile florets per spike. Similarly, at anthesis, *t*-Z level was 4-fold higher in the spike than the peduncle, and *t*-ZR was 2.8-fold higher. This suggests that elevated levels of CKs are required in the spike at anthesis to promote grain number. A gradient in CK concentrations along the spike was reported by Youssef et al. (2017) in barley (cv. Bowman) with the lowest levels in the basal spikelets and highest in the apical spikelets during spike differentiation. In the two HiBAP II genotypes, there was an apparent trend for CK levels to increase from the basal to apical spikelets, but the spike position effect was not significant. None of the cytokinins showed a genotype × spike position interaction at anthesis.

4.2. Relation of yield component traits with grain yield

In modern cultivars, grain yield is mainly sink-limited under favourable conditions (Clarke et al., 2012; Fischer, 1985; Slafer et al., 2023), so further increases in grain number are critical to improving wheat yields. In the present study, genetic variation in FE_{chaff} correlated strongly with grains per MS in the glasshouse (r = 0.75) and GN in the field experiment (r = 0.61). Like many previous studies (Peltonen-Sainio et al., 2007; Shearman et al., 2005), a significant positive association among genotypes between grain number and grain yield was reported in both environments (glasshouse r = 0.85, P < 0.01; field r = 0.44, P < 0.001). This is likely due to breeders increasing grain sink strength through increasing grain number, potential grain weight or both (Slafer and Savin, 1994).

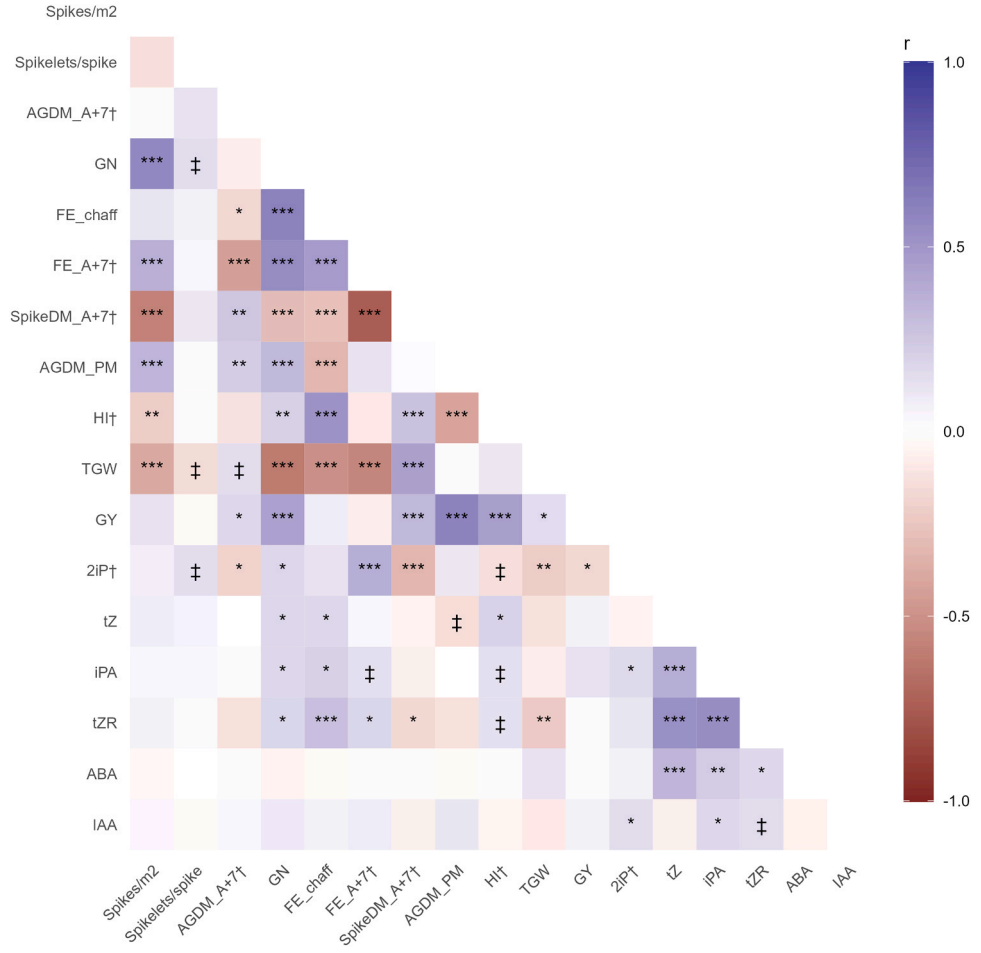

Fig. 5. Pearson's correlation coefficients between traits measured at anthesis + 7 days and physiological maturity and spike hormone analyte levels (ng/g FW) for 150 spring wheat HiBAP II genotypes measured at anthesis in 2018–19 in the field. Hormone levels are calculated as the means of 2 reps, other traits 3 reps. †: DTA (days to anthesis) added as a covariate if P < 0.05, correlation significance: ‡P < 0.10, * P < 0.05, ** P < 0.01, *** P < 0.001, 2iP: isopentenyladenine t-Z: zeatin, iPA: isopentenyladenosine, t-ZR: zeatin riboside, ABA: abscisic acid, IAA: indole-3-acetic acid (auxin), Spikes/m2: spikes per m^2, Spikelets/spike: no. of spikelets per spike, AGDM_A+ 7: above-ground dry matter (DM) at anthesis + 7 days, spikeDM_A+ 7: spike DM at anthesis + 7 days, AGDM_PM: above ground DM at physiological maturity, GN: grain number per m^2, GY: grain yield per m2, HI: harvest index, FE_A+ 7: fruiting efficiency calculated using spike DM at anthesis + 7 days, FE_chaff: fruiting efficiency calculated using chaff DM at maturity, TGW: thousand-grain weight (g).

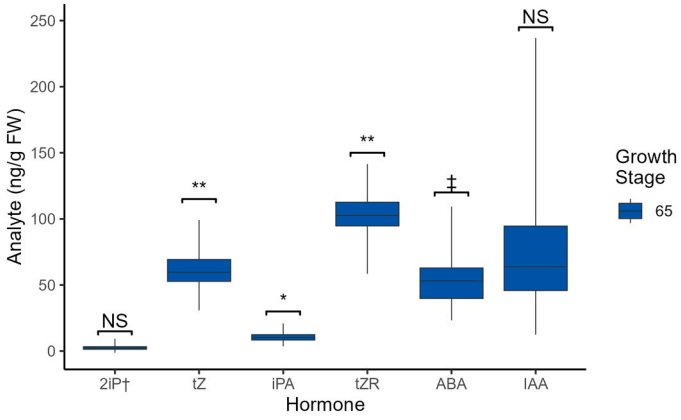

Fig. 6. Boxplots showing genetic ranges for 150 HiBAP II genotypes in spike hormonal analyte levels at anthesis (GS65) in the field in 2018–19. Values represent means of 2 replicates. The line within the box represents the mean, bottom and top of boxes represent upper and lower quartiles and lines below and above box minimum and maximum values, respectively, Significance values indicate if genotype is significant ** $P < 0.01$, * $P < 0.05$, ‡$P < 0.1$, NS: not significant. †: DTA added as a covariate if $P^{<}0.05$, 2iP: isopentenyladenine, t-Z: zeatin, iPA: isopentenyladenosine, t-ZR: zeatin riboside, ABA: abscisic acid, IAA: indole-3-acetic acid (auxin).

There was some consistency between the expression of yield component traits in the subset of genotypes in the glasshouse experiments and the field expression of these traits in the HiBAP II. For the subset of 10 cultivars in the glasshouse experiments, the HI in the glasshouse experiments was significantly positively correlated with HI in the field experiment for these cultivars ($r = 0.68$, $P < 0.05$); similarly, among these 10 cultivars, the correlation was significant for grain yield ($r = 0.77$, $P < 0.01$) and above-ground DM at physiological maturity ($r = 0.64$, $P < 0.05$). Scaling genetic variation in trait expression from the glasshouse to field experiments can be difficult due to differences in interplant competition, so the general consistency between both environments in the present study is encouraging. There was a temperature peak above 35 °C during the sensitive period for grain number determination from GS49 to GS65 in one year, 2019, potentially affecting floret fertility and/or pollen production (Farhad et al., 2023). However, this did not appear to explain the significant year × genotype interaction for grain yield. Indeed, there was no significant year × genotype interaction for grains per MS or for fruiting efficiency for the MS (measured either based on spike DM at anthesis or the chaff). Furthermore, the mean grain number per MS was lower in 2018 than in 2019 or 2017. In the field experiment, there were small differences in plants per m^2 of 132–214, but there was no significant association among genotypes between plant establishment and GN or associated grain-number traits. So the genetic differences in GN and the role of cytokinins in the present experiment were not associated with variation in plants per m^2.

4.3. *Effects of spike cytokinin levels on grain number traits*

At late booting, genetic variation in spike *t*-Z level was correlated with each of grains per MS and FE_{chaff} and variation in spike 2iP level was positively correlated with fertile florets per spike in the glasshouse experiments. These results are consistent with those in winter wheat reported by Wang et al. (2001) on cultivar YM 158, that showed Z increased grains per spike when applied exogenously at each of five stages during floret development, but particularly when applied at anther lobe formation (approximately mid stem extension) and meiosis (approximately early booting). When Z was applied during floret degradation after meiosis a smaller increase was reported (Wang et al., 2001). Similarly, in Chinese winter wheat, an exogenous application of synthetic CK 6-BA during booting reduced the floret abortion rate by 77% (Zheng et al., 2016), thereby increasing grain number. Floret abortion occurs between the onset of booting (GS41) and flowering (Guo et al., 2018), so the present correlations of *t*-Z and 2iP at GS49 with fertile floret number, GN and FE_{chaff} are likely to be associated with enhanced floret survival throughout the phase from early to late booting. Spike *t*-Z levels at GS49 were also found to be negatively associated with TGW (MS) in the present study in the glasshouse experiments likely reflecting the negative trade-off between grain number and TGW ($r = -0.62$, $P < 0.01$, data not shown); this trade-off has been frequently observed in other studies in wheat (Ferrante et al., 2015; Slafer et al., 2015). In summary, spike *t*-Z concentration was correlated with grain number and FE_{chaff} in the glasshouse experiments, indicating it as a target trait for breeders for enhancing grain number.

Statistically significant correlations were also found among the 150 genotypes between spike *t*-Z level at anthesis and GN and FE_{chaff} in the field experiment, although the correlations were less strong than those in the glasshouse experiments. This may partly reflect that the correlations in glasshouse experiments were based on mean values over three experiments, whereas in the field the correlations were based on one experiment. Also, there may have been more 'within experiment' environmental variation in the field experiment than in the glasshouse experiments. Although the spike *t*-Z showed positive correlations with grain number in both the glasshouse and field experiments, there was a difference in the timing of the assessment, i.e., late booting in the glasshouse experiments and anthesis in the field experiment. Due to the large amount of time and resources involved in the spike hormone phenotyping, the spike *t*-Z assessment was only made at one stage (anthesis) in the field experiment on the 150 genotypes. It is possible if the assessment had been carried out at both late booting and anthesis there would also have been a positive correlation with grain number at late booting in the field experiment. The single assessment at anthesis in the field experiment also raises some uncertainty as to whether the genetic differences in hormone content were the cause of the greater number of grains or vice versa. From the present results, the optimum time for assessment of spike *t*-Z during the booting to anthesis window to detect the strongest correlations with grain number is not certain. Therefore, further work is required with assessments at more frequent stages during the booting to anthesis development window to investigate both the time course of spike *t*-Z and the strength of the correlations with grain number and associated traits.

In the present experiments, statistically significant correlations were found between *t*-Z and GN and FE_{chaff}, but the r values were relatively modest in the range 0.60–0.77 in the glasshouse experiments and 0.17–0.19 in the field experiments. This may reflect that it is not only the spike *t*-Z level that is influencing the grain number and fruiting efficiency but also CK signalling mechanisms (i.e., the sensitivity to CK). Cytokinin is perceived via a two-component phosphorelay system, in which type-A response regulators and pseudo phosphotransfer proteins (PHPs) act as inhibitors. Recent work has shown that rice lines in which all three PHPs were knocked out showed elevated CK response, changes in CK homeostasis and fewer filled seeds per panicle (Vaughan-Hirsch et al., 2021). Those results suggest a more complex interaction between CK response and grain number, in which manipulation of CK signalling can have major effects. They suggest grain number in wheat is regulated by both CK homeostasis affecting analyte levels (ng per g) and the perception of CK via downstream signalling mechanisms in which CKs are transduced to activate transcription factors or repressors modulating gene expression. Thus, manipulating the genetic regulation of CKs to enhance grain number in plant breeding will require an understanding of both the genes controlling the spike CK levels and the downstream signalling mechanisms affecting the sensitivity to CK, and future work should also focus on elucidating these signalling mechanisms. In addition, since the genes regulating cytokinins are temporally and spatially regulated (Ogonowska et al., 2019), an improved understanding of this regulation is required so that CK levels and responses can be enhanced in the spike during floret development without leading to global effects in

the rest of the plant which may have detrimental effects.

The number and arrangement of each spikelet on the spike are under strong hormonal control (Dixon et al., 2018; McSteen, 2009; Poursarebani et al., 2015), but, in the present experiments, there were no associations between spike plant hormone levels and total spikelet number among the genotypes (data not shown). It is possible that the lack of correlation between spikelet number and the levels of spike CKs may have been related to the timing of the assessments in the present study at late booting and anthesis being later than the phase of spikelet primordia production from floral initiation to terminal spikelet when spikelet number is principally determined, and further experiments with earlier measurements at around the terminal spikelet stage would be justified to investigate this further. It can be speculated that the spike hormones have a role in regulating the development of the spike vascular architecture (Slafer et al., 2023), especially the main conducting elements, i.e., the sieve tube elements, and their architectural configurations in the spikelet/floret. However, there are no published studies on the elucidation of spike vascular architecture and its association with CKs and other plant hormones in wheat to date.

The exogenous application of CKs (kinetin) stimulated the growth of tillers in oats (Harrison and Kaufman, 1980) and rice (Yeh et al., 2015), and when endogenous CKs were increased in transgenic plants because of the downregulation of one of the eleven CKX (catabolic) enzymes in rice (Yeh et al., 2015) or with a silenced *HvCKX1* gene in barley (Morris et al., 1993) tillers increased. However, in our field experiment, we found no association between any of the four CKs and spikes m^{-2} in the field experiment. Thus, the effect of the CKs on grains m^{-2} in the present study was driven by increased grains per spike rather than spikes m^{-2}.

4.4. Effects of spike ABA and IAA levels on grain number traits

In the present study, there were no associations between genetic variation in spike ABA and grain number or associated traits in either the glasshouse or field experiments. For spike IAA, the only correlation with grain number traits was a negative correlation between IAA at anthesis and HI in the glasshouse experiments. Exogenous application of IAA to winter wheat cv. YM 158 at all floret development stages resulted in grain loss in all spikelet positions (Wang et al., 2001). High auxin activity is upregulated from the double ridge stage in bread wheat, most likely contributing to the generation of new axillary meristems. However, there is a negative correlation between auxin concentration and the number of fertile florets at the abortion stage (Li et al., 2018b). The presently observed negative correlation between IAA and HI is therefore consistent with the direction of effects reported in this previous work. Overall, the genetic range for spike IAA was relatively greater than for the CKs and ABA in our study, particularly at GS65, possibly indicating that IAA levels in wheat spikes were more sensitive to small fluctuations in environmental conditions according to the differences in date of reaching GS65 among genotypes than was the case for the CKs and ABA.

ABA had a consistently lower spike analyte level at anthesis than at late booting. Endogenous ABA concentration is reported to decrease sharply during mid stem elongation from anther-lobe formation to meiosis in wheat (Cao et al., 2000), and it has been suggested that ABA concentration should be maintained at low levels to maximise potential grain number (Wang et al., 2001). However, present results were inconclusive as we found no association among genotypes between spike ABA levels and grain number or FE in either the glasshouse or field experiments. Levels of ABA previously reported by McWha (1975) in wheat (cvs. Arawa and Aotea) were higher (100–125 ng/g FW) than the levels reported in the present study (1.29–44.9 ng/g FW).

With regard to correlations between CKs, ABA and IAA there was a negative correlation at GS49 between IAA and the CK iPA in the glasshouse experiments. Crosstalk between CKs and IAA has been previously reported to affect tillering, e.g., CKs regulating *PIN* transport proteins, resulting in increased auxin efflux and affecting shoot branching (Waldie and Leyser, 2018). However, in the present study, there was no association between either spike IAA or iPA levels and fertile tillers per plant.

4.5. Implications for plant breeders

Measuring endogenous spike hormone levels is presently labour-intensive and expensive and not high-throughput. To sample and process a spike for hormonal analysis required freezing the spike in liquid nitrogen in the field, and sub-sampling 100 mg of material in the laboratory which took approximately five minutes per sample. The subsequent analysis of the hormones through UHPLC/ESI-MS/MS took approximately four person-days per 100 samples. Therefore, for the endogenous hormone traits to be deployed in plant breeding, molecular markers need to be developed for use in marker-assisted selection. To develop molecular markers, the trait in question needs to be heritable (Hayward et al., 2015). In the glasshouse experiment, there was a significant year × genotype interaction for the four CKs assessed at late booting and anthesis, apart from iPA and *t*-ZR at anthesis. Encouragingly though for several of the spike CK levels in the glasshouse experiments heritability was relatively high such as *t*-Z and *t*-ZR at GS49, H^2 was 0.79 and 0.77, respectively (Table S5), indicating that the year × genotype interactions were not sufficiently strong to impede selection in plant breeding programmes and suggesting these traits would be good candidates for molecular markers. Marker-trait associations could be identified by conducting a genome-wide association study (GWAS) and subsequently identifying candidate genes and developing Kompetitive allele-specific PCR (KASP) markers from sequence information on the candidate genes. Such GWAS studies on spike *t*-Z and *t*-ZR using endogenous plant hormonal data should be a priority in future work.

In the glasshouse experiments, genotype 5 (F2SR2–69//Yangling Shaanxi/Pastor) had very high spike cytokinin levels at anthesis (Table S9) and also high values for FE and GN (Fig. 1). This is an advanced line of elite origin but not a released cultivar and may be of value in developing future crosses for enhancing spike CK levels in wheat breeding. It is important to state that the present results from the glasshouse experiments are supported by only one field experiment, and therefore present findings on the spike CK expression of individual genotypes require further validation in field experiments and must be interpreted cautiously. The glasshouse results were only on a subset of 10 genotypes, so it is encouraging that the association between spikes CKs and grain number was also observed in the much larger number of genotypes in the HiBAP II in the field environment, confirming the potential for spike CKs as a trait to be targeted by wheat breeders for enhancing grain number and grain yield. In addition, a better understanding of the crosstalk of CKs with other plant hormones will be needed to understand fully their influence on grain number traits in breeders' trials, building on the present understanding of the interactions between cytokinins and auxins (Youssef and Hansson, 2019).

The HiBAP II is comprised of genotypes from not only elite backgrounds, but also landrace- and synthetic-derived backgrounds. Many breeding programs may have less genetic variation available within their elite germplasm than in the present study, potentially leading to limited variation for traits such as spike hormone levels. Therefore, to deploy these traits, breeders may need to screen a wider range of germplasm. Although no high-throughput screen currently exists for CKs, favourable sources of expression can be identified at low throughput among potential parents, an exercise that will be a prerequisite to genetic studies and the identification of molecular markers. Relevant wider germplasm for screening could be publicly available landrace panels (Wingen et al., 2014) or primary synthetics panels curated at CIMMYT (Reynolds et al., 2015).

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CRediT authorship contribution statement

Bethany Love: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Gemma Molero**: Conceptualization, Writing – review & editing. **Carolina Rivera-Amado:** Investigation, Writing – review & editing. **Maren Müller:** Investigation, Writing – review & editing. **Sergi Munné-Bosch**: Investigation, Writing – review & editing. **Matthew P. Reynolds**: Conceptualization, Funding acquisition, Writing – review & editing. **M. John Foulkes:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

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

Data will be made available on request. The data that support the findings of this study are openly available in the CIMMYT Enterprise Breeding System (EBS) database at https://www.cimmyt.org/tag/enterprise-breeding-system/.

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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.eja.2023.127011.

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