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Green Revolution reduced height (*Rht*) genes did not increase amylase/trypsin-inhibitor content in near isogenic wheat lines

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ABSTRACT

The prevalence of wheat-related disorders increased during the last 50 years and it is still under discussion if breeding is one reason for this increase. One key element of the Green Revolution was the introduction of reduced height (*Rht*) genes into wheat that decreases plant height and increases harvest index by enhancing assimilate partitioning toward the grain, thereby contributing to higher yield potential. One suggestion was that *Rht* genes increased the content of amylase/trypsin-inhibitors (ATI), which are important triggers of wheat-related disorders such as wheat allergy and sensitivity. To verify this assumption, near isogenic lines of four *Triticum aestivum* genotypes with five different *Rht* alleles/combinations and one tall control (*rht*) were analyzed for the ATI content by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The absolute total ATI content was not affected by the *Rht* genes. In contrast, the proportion of total ATI based on crude protein was decreased by extreme dwarf *Rht2+3* compared to tall *rht*, but semi-dwarf *Rht1* and *Rht2*, dwarf *Rht1+2* and extreme dwarf *Rht3* showed similar proportions compared to tall *rht*. The same was observed for the most abundant and most bioactive ATI 0.19 and CM3. The ATI distribution was mostly affected by genotype and environment and minimally by allele. The analysis of three biological replicates, which were grown in three consecutive years, strengthen the findings that the semi-dwarf *Rht* genes that are present in modern wheat do not increase the ATI content.

1. Introduction

More and more people follow a wheat- or gluten-free diet, not only because of lifestyle, but more importantly due to wheat-related disorders such as celiac disease, wheat allergy and non-celiac wheat sensitivity (NCWS) (reviewed by Shewry (2018)). One group of wheat (*Triticum aestivum* L.) proteins – the amylase/trypsin-inhibitors (ATI) – is frequently mentioned in the context of wheat-related disorders. ATI are responsible for or may play a role in almost all wheat-related disorders including NCWS (activation of TLR4-MD2-CD14 complex), celiac disease (additional antibody response) and wheat allergy (predominant allergens) (reviewed by Brouns et al. (2019), Geisslitz et al. (2021) and Kumar et al. (2024)).

In general, ATI belong to the albumin/globulin fraction and account for about 20 % of this fraction, but the content of both is only weakly correlated (Jahn et al., 2023). ATI inhibit amylase and/or trypsin from different sources (insects and mammals) and during digestion in the human body, the inhibitory activity of ATI may lead to intestinal problems due to incompletely digested starch and proteins (Gélinas and Gagnon, 2017). There are different types of ATI, which have primarily inhibitory activity against amylase (monomeric 0.28, dimeric 0.19 and 0.53) or against both amylase and trypsin (tetrameric CM1, CM2, CM3, CM16 and CM17). Other ATI exhibit inhibitory activity against trypsin (wheat trypsin inhibitor (WTI), CMX1/3 and CMX2), against chymotrypsin (wheat chymotrypsin inhibitor, WCI) or amylase and subtilisin (wheat amylase subtilisin inhibitor, WASI). The ATI 0.19 and CM3 are the two most abundant and most bioactive ones in wheat (Geisslitz et al., 2020; Sielaff et al., 2021; Zevallos et al., 2017).

The prevalence of wheat-related disorders increased during the last 50 years and one hypothesis is that breeding is one of the causes for this increase, as it may have increased the content of ATI in modern wheat. One key element during breeding was the introduction of reduced height

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(*Rht*) genes during the Green Revolution resulting in a lower tendency to lodging due to lower plant height and higher harvest index (Wuerschum et al., 2017). Today, more than 70 % of the wheat grown worldwide carries at least one of the semi-dwarf genes *Rht-B1* or *Rht-D1* (Evans, 1998; Wuerschum et al., 2017). Near-isogenic lines (NILs) have become a useful tool for evaluating the effect of particular genes by isolating genetic differences in a specific locus. NIL populations have been used to analyze the effect of dwarfing genes on plant height, agronomic properties, floral traits, response to osmotic stress (Börner et al., 1993; Flintham et al., 1997; Landjeva et al., 2008; Schierenbeck et al., 2024) and recently the grain protein composition (Geisslitz et al., 2025). The semi-dwarf genes *Rht1* (*Rht*-B1b/*Rht*-D1a) and *Rht2* (*Rht*-B1a/*Rht*-D1b) had only small effects on the protein composition, but the extreme dwarf *Rht2+3* (*Rht*-B1c/*Rht*-D1b) decreased the content of glutenins and increased the content of albumins/globulins (Geisslitz et al., 2025).

The aim of our study was to elucidate the effect of different *Rht* alleles and combinations in four genotypes on the ATI content and distribution to answer the question whether breeding and specifically the introduction of *Rht* genes during the Green Revolution increased the ATI content. As some *Rht* alleles indeed changed the protein composition, it is likely that they also had an effect on the ATI content.

2. Materials and methods

2.1. Plant material

The same samples as previously described by Geisslitz et al. (2025) were analyzed. NILs of the four winter wheat varieties 'April Bearded', 'Bersee', 'Maris Huntsman' and 'Maris Widgeon' carried the following alleles/combinations: Green Revolution semi-dwarfs *Rht1* (*Rht*-B1b/*Rht*-D1a) and *Rht2* (*Rht*-B1a/*Rht*-D1b), dwarf *Rht1+2*, (*Rht*-B1b/*Rht*-D1b), extreme dwarfs *Rht3* (*Rht*-B1c/*Rht*-D1a) and *Rht2+3* (*Rht*-B1c/*Rht*-D1b) and the tall wild-type *rht* (*Rht*-B1a/*Rht*-D1a) (Börner et al., 1993; Schierenbeck et al., 2024). The lines were developed by recurrent backcrossing over six generations as described in Youssefian et al. (1992). Field trials were conducted at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany (11°160 LE; 51°490 LN). The experimental design is described in Schierenbeck et al. (2024). A split-split plot design with three blocks in three consecutive growing seasons (2020/2021, 2021/2022, 2022/2023) had three biological replicates per year. Each plot was 1.2 m^2 (1 m long by 1.2 m wide) comprising six rows. The environments and biological replicates (main plots) were considered as random effects and alleles (subplots) and genotypes (sub-subplots) as fixed effects. Standard agronomic practices for managing insects, fungal diseases and weeds were implemented. The mean annual temperature was 9.6 °C in 2021, 10.8 °C in 2022 and 11.0 °C in 2023 (https://wetter.ipk-gatersleben.de). The cumulative precipitation was 437 mm in 2021, 394 mm in 2022 and 782 mm in 2023. Soil parameters are available in Geisslitz et al. (2025).

The kernels were milled to wholemeal flours with a tube mill (IKA, Staufen, Germany). About 20 g of kernels were milled three times for 1 min with a break of 1 min in between. The flour was stored at least for two weeks before analysis, but not longer than for six months.

2.2. Crude protein content

The crude protein content of the flours (nitrogen × 5.71) was determined by the Dumas method using a Dumatherm Nitrogen analyzer (Gerhardt Instruments, Königswinter, Germany) following ICC Standard No. 167. All determinations were done in triplicates (three technical replicates) for each biological replicate (*e.g.*, $n = 9$ for April Bearded *rht* from 2021; $n = 27$ for April Bearded *rht*). The crude protein content is already reported in Geisslitz et al. (2025).

2.3. ATI sample preparation for absolute quantitation

ATI were extracted according to Geisslitz et al. (2020) and Jahn et al. (2025) with very slight modifications. In brief, flour was extracted two times with ammonium bicarbonate solution (50 mmol/L, pH 7.8). After stirring (30 min at 22 °C) and centrifugation (25 min at 3550 rcf and 22 °C), the supernatants were combined and the solvent was removed by evaporating to dryness in a rotational vacuum concentrator (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). The residue was redissolved in 320 µL each of Tris-HCl (0.5 mol/L, pH 8.5) and 1-propanol and a mixture of 19 internal standards was added (Table S1). In contrast to Geisslitz et al. (2020), the qualifier peptide of CM2 (EYVAQQTCGVGIVGSPVSTEPGNTPR) was not included as internal standard due to poor MS properties. Further, in contrast to Jahn et al. (2025), the qualifier for CMX1/3 (GSLLQDMSR) and the quantifier for 0.19 (EHGAQEGQAGTGAFPR) were not included. The content of 0.19 and that of 0.53 were determined and calculated as described in Geisslitz et al. (2020). Reduction was performed using tris(2-carboxyethyl) phosphine for 30 min at 60 °C and alkylation using chloroacetamide for 45 min at 37 °C in the dark. The solvent was removed by evaporation in a rotational vacuum concentrator (8 mbar, 40 °C). Tryptic digestion (enzyme-to-substrate ratio: 1:50) was performed overnight at 37 °C and the reaction was stopped with trifluoroacetic acid (5 µL). After evaporation to dryness, the residue was dissolved in water (1 mL) containing 2 % acetonitrile and 0.1 % formic acid. Three separate extraction experiments were carried out (three technical replicates) for each biological replicate (*e.g.*, $n = 9$ for April Bearded *rht* from 2021; $n = 27$ for April Bearded *rht*).

2.4. Absolute quantitation of ATI

The method described in Geisslitz et al. (2020) was adapted to a Q Exactive Plus Orbitrap (ThermoFisher Scientific, Waltham, MA, USA) mass spectrometry system coupled to a Vanquish UHPLC system (ThermoFisher Scientific, Waltham, MA, USA). Exactly the same targeted LC-MS/MS method as described in Jahn et al. (2025) was used for the detection of the 20 peptides and 19 internal standards. For the response lines, two solutions (25–100 µg/mL of each peptide), solution 1 with all 20 peptides and solution 2 with all 19 internal standards, were prepared from the stock solutions. An aliquot of each solution 1 and 2 was reduced and alkylated as described in Geisslitz et al. (2020). Alkylated solutions 1 and 2 (0.3–1.3 µg/mL of each peptide) were mixed in molar ratios n(peptide)/n(internal standard) between 9.1 and 0.1 ($9 + 1, 7 + 1, 5 + 1, 3 + 1, 1 + 1, 1 + 3, 1 + 5, 1 + 7$ and $1 + 9$) for calibration. The injection volume was 2 µL for the response curves and for the samples. Skyline (version 23.1, MacCoss Lab Software, University of Washington, Seattle, WA, U.S.A. (MacLean et al., 2010)) was used to evaluate the MS data. Data analysis was performed in the same way as described by Geisslitz et al. (2020).

2.5. Statistics

Statistical analysis was conducted with OriginPro 2023 (OriginLab Corporation Northampton, MA, USA). Differences of *Rht1*, *Rht2*, *Rht3*, *Rht1+2* and *Rht2+3* to *rht*, respectively, were tested by two-sample *t*-test ($n = 27$), whereas equal variance was not assumed (Welch correction). Pearson correlation coefficients (r) were calculated using individual values of biological replicates ($n = 216$), without averaging. Principal component analysis (PCA) was conducted with averaged values for each genotype and allele combination (e.g., April Bearded *rht*). Three-way analysis of variance (ANOVA) with the factors genotype, allele and environment was performed at a significance level of $p \leq 0.05$ without considering repeated measurements.

3. Results

The four NILs (April Bearded, Bersee, Maris Huntsman and Maris Widgeon) had six alleles, whereas *rht* was the tall wild-type and five *Rht* alleles showing different dwarfing degree (*Rht1*, *Rht2*, *Rht3*, *Rht1+2* and *Rht2+3*) were compared to the wild-type. Three biological replicates were cultivated in three subsequent harvest years (2021, 2022 and 2023) and analyzed separately for the ATI content by LC-MS/MS. To eliminate environmental factors, only the mean of three biological replicates and three years is discussed ($n = 27$). All detailed data are summarized in Table S2 (absolute ATI content), Table S3 (ATI proportion based on protein content), Table S4 (ATI distribution based on total ATI content) and Table S5 (ATI proportion based on albumin/globulin content).

3.1. Changes in the total ATI content

The mean total ATI content varied between 3.6 mg/g and 4.7 mg/g. There was no difference between the tall wild-type (*rht*) and the extreme dwarfs *Rht3* and *Rht2+3* for all four NILs (Fig. 1A). With the exception of Bersee (−7 % to −8 %), this was also true for the comparison between *rht* (tall) and the semi-dwarfs *Rht1* and *Rht2* and dwarf *Rht1+2*.

Because the protein content varied both due to harvest year and genotype (Geisslitz et al., 2025), total ATI was expressed as proportion based on crude protein content. Except for April Bearded (−4 %), the extreme dwarf *Rht2+3* had a significantly decreased proportion of total ATI in all lines (−12 % to −26 %) compared to the tall wild-type (Fig. 1B). The dwarf *Rht1+2* had a decreased ATI proportion only in Maris Huntsman (−17 %). No difference was found between *rht* (tall) and the Green Revolution semi-dwarfs (*Rht1* and *Rht2)* and the extreme dwarf *Rht3*.

Taken together, the absolute amount of ATI was similar for all combinations of the *Rht* alleles (Fig. 1A) and a decreasing trend was observed for the proportion of total ATI based on crude protein content only when comparing the tall wild-type (*rht*) and extreme dwarf *Rht2+3* (Fig. 1B).

3.2. Changes of the two most bioactive ATI 0.19 and CM3

The ATI 0.19 and CM3 are the most abundant and most bioactive ATI in wheat. The absolute content of 0.19 varied between 751 μg/g and 1146 μg/g and that of CM3 between 561 μg/g and 795 μg/g, confirming that these ATI were the two most abundant ones. For most *Rht* genes no difference to the tall wild-type (*rht*) was detected, but the content of 0.19 was significantly reduced only in April Bearded, semi-dwarf *Rht2* (−22 %), dwarf *Rht1+2* (−21 %) and extreme dwarf *Rht2+3* (−19 %) (Fig. 2A) and that of CM3 only in Maris Huntsman, dwarf *Rht1+2* (−15 %) and extreme dwarf *Rht2+3* (−17 %) (Fig. 2B).

Similar to the proportion of total ATI, a slightly decreasing trend was observed for the proportion of 0.19 and CM3 based on crude protein, when comparing *rht* (tall) to extreme dwarf *Rht2+3*. With the exception of April Bearded (−17 %), the decrease in the proportion of 0.19 (Fig. 2C) was significant for Bersee (−22 %), Maris Huntsman (−23 %) and Maris Widgeon (−24 %) and that of CM3 (Fig. 2D) for Maris Huntsman (−38 %) and Maris Widgeon (−16 %), whereas the reduction in April Bearded (−3 %) and Bersee (−18 %) was not significant. Further, the decrease of the proportion of CM3 was also significant for extreme dwarf *Rht3* (−18 %) and dwarf *Rht1+2* (−24 %) in Maris Huntsman. In contrast to that, it has to be stated that the semi-dwarfs *Rht1* (15 %) and *Rht2* (17 %) increased the proportion of CM3 significantly in April Bearded compared to the tall wild-type (*rht*).

3.3. Changes of individual ATI

The distribution of ATI (*i.e.*, proportion based on total ATI, Table S4, Fig. S1) was similar within each genotype. Because no effect of *Rht* genes was observable on the ATI distribution, detailed values are not discussed here, but their correlations are detailed in section 3.4.

The absolute content of individual ATI varied in a big range between the three years which can be seen in the high standard deviation for the three years (Table S2, Fig. S2). The reason for this was the lower crude protein content in 2022 (mean 8.6 %) compared to 2021 (10.8 %) and 2023 (11.8 %) (Geisslitz et al., 2025). To compensate for the varying protein content, the content of individual ATI was expressed as proportion based on crude protein content (Table S3, Fig. 3). Following 0.19 (0.70–1.03 %) and CM3 (0.52–0.78 %), CM17 (0.38–0.58 %), 0.28 (0.32–0.56 %), CM16 (0.30–0.48 %), 0.53 (0.15–0.23 %), CM1 (0.19–0.27 %) and CM2 (0.28–0.42 %) were more abundant (Table S3) than CMX1/2/3 (0.10–0.19 %), WCI (0.07–0.13 %), WASI (0.06–0.11 %) and WTI (0.03–0.12 %).

The proportion of all individual ATI was significantly reduced in the extreme dwarf *Rht2+3* of Maris Huntsman (−14 % to −42 %) compared to the tall wild-type (*rht*). A decrease in the proportion of 0.28 (−18 %), WASI (−12 %) and WCI (−25 %) was also seen for the extreme dwarf *Rht2+3* in Maris Widgeon, as well as for CMX1/2/3 in Bersee (−12 %). Extreme dwarf *Rht3* had a decreased proportion of CMX1/2/3 in Maris Huntsman (−19 %). Further, in Maris Huntsman, dwarf *Rht1+2* had a decreased proportion of 0.53 (−20 %), CM16 (−25 %), CMX1/2/3 (−24 %) and WCI (−22 %).

In contrast, some alleles had an increased proportion of individual ATI. A significant increase was observed in the proportion of WTI (19 %) in April Bearded and of CMX1/2/3 (20 %) in Maris Widgeon for the extreme dwarf *Rht2+3* compared to the tall wild-type (*rht*). The extreme dwarf *Rht3* had an increased proportion of WTI in April Bearded (26 %) and Maris Widgeon (23 %). There were further significant increases in the proportion due to semi-dwarf (*Rht1* and *Rht2*) and dwarf (*Rht1+2*): Semi-dwarf *Rht2* had an increased proportion of 0.28 (18 %), CM1 (17 %), CM16 (13 %), CM17 (16 %), WASI (15 %) and WTI (35 %) in Maris Widgeon. Further, semi-dwarf *Rht2* had an increased proportion of 0.28 (16 %), WCI (30 %) and WTI (28 %) in April Bearded. The semi-dwarf *Rht1* had an increased proportion of 0.28 (10 %), CMX1/2/3 (15 %) and WTI (29 %) in April Bearded and of CMX1/2/3 (12 %) in Maris Widgeon. The dwarf *Rht1+2* had an increased proportion of 0.28 (14 %) and WTI (31 %) in April Bearded, of WASI (13 %) and WTI (21 %) in Maris Widgeon and of WTI (28 %) in Bersee.

Because ATI are part of the albumin/globulin fraction, the proportion based on this fraction was also taken into account (Table S5, Fig. S3). The same significant decreasing effect of *Rht* genes was observed as for the proportion based on crude protein with the exception of 0.53 in extreme-dwarf *Rht2+3* of Maris Huntsman.

In contrast, the increasing effect of different *Rht* genes was less pronounced for the proportion based on albumin/globulin compared to the proportion based on crude protein. Except for a significantly higher proportion of WASI due to *Rht1* in Maris Huntsman, all additional significant differences to the wild-type were characterized by a lower proportion based on albumin/globulin.

3.4. Changes of ATI distribution

PCA was conducted using the absolute ATI content, proportion based on total ATI (*i.e.*, ATI distribution) and ATI proportion based on crude protein (Fig. 4). Similar correlations were observed for the PCA of absolute content (Fig. 4A) and for that of ATI distribution (Fig. 4B), because the loadings of CM2, CM3, CM1 and CM17 pointed in the same direction, while those of WCI, WASI and WTI together pointed to a different direction. The high correlation between the aforementioned ATI was proven by Pearson correlation matrices (Fig. S4 and Fig. S5). It is evident that the genotypes exhibited greater clustering in comparison to the different *Rht* alleles in both PCA plots. In Fig. 4A, the NILs of Maris Widgeon clustered close to the loadings of CM3, CM2 and WCI and in Fig. 4B, close to the ones of CM3, CM17 and CM1. Maris Widgeon was characterized by the highest CM3 and CM2 content and the highest

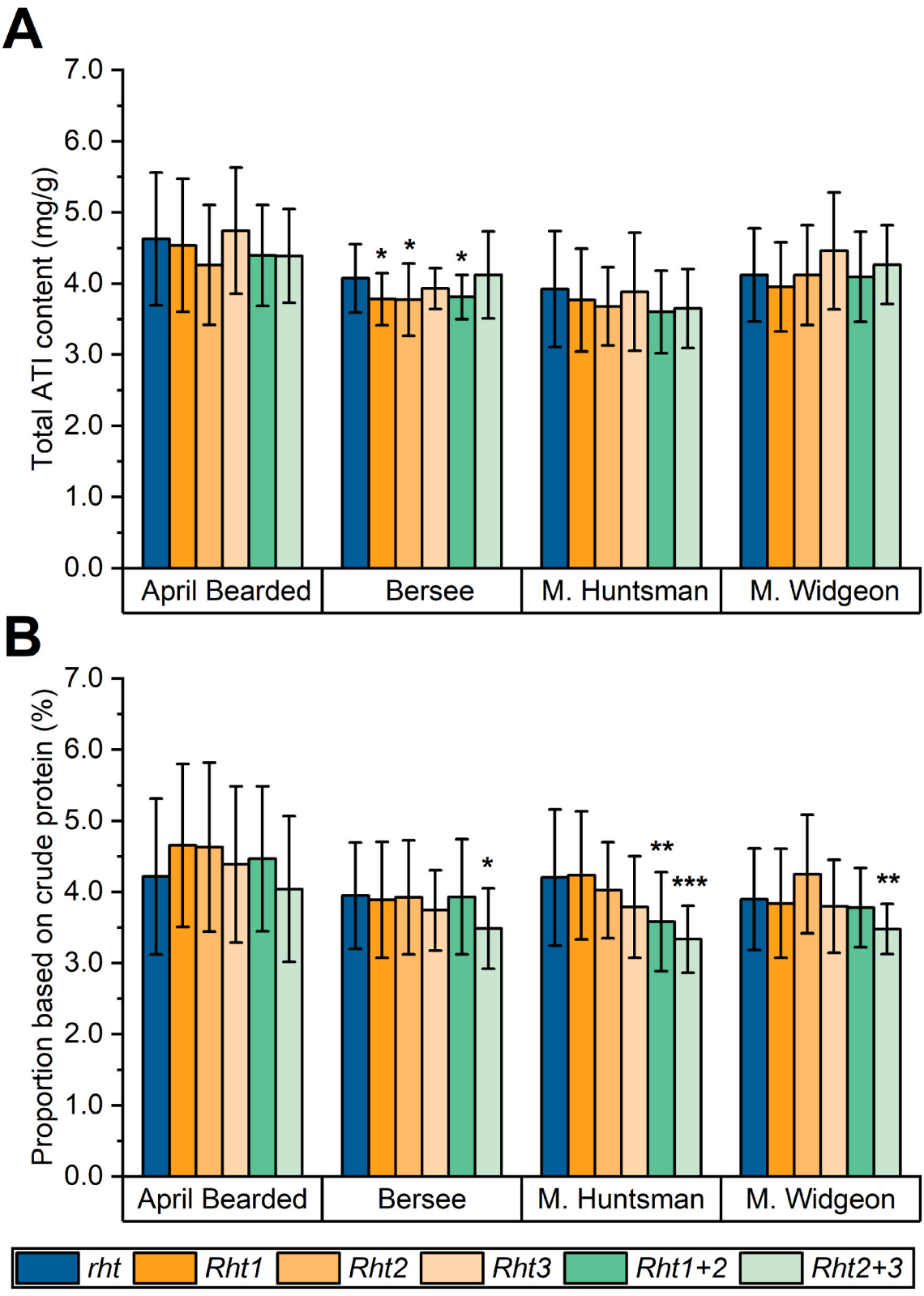

Fig. 1. Total ATI content (A) and ATI proportion based on crude protein (B) of NILs of the four genotypes April Bearded, Bersee, Maris (M.) Huntsman and Maris (M.) Widgeon with different *Rht* alleles. *Rht1* (semi-dwarf), *Rht2* (semi-dwarf), *Rht3* (extreme dwarf), *Rht1+2* (dwarf) and *Rht2+3* (extreme dwarf) are compared to wild-type *rht* (tall). Mean of three years, three biological replicates per year and three technical replicates ($n = 27$). Significant differences to *rht* are marked with asterisks (*t*-test, $^{*}p \leq 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$).

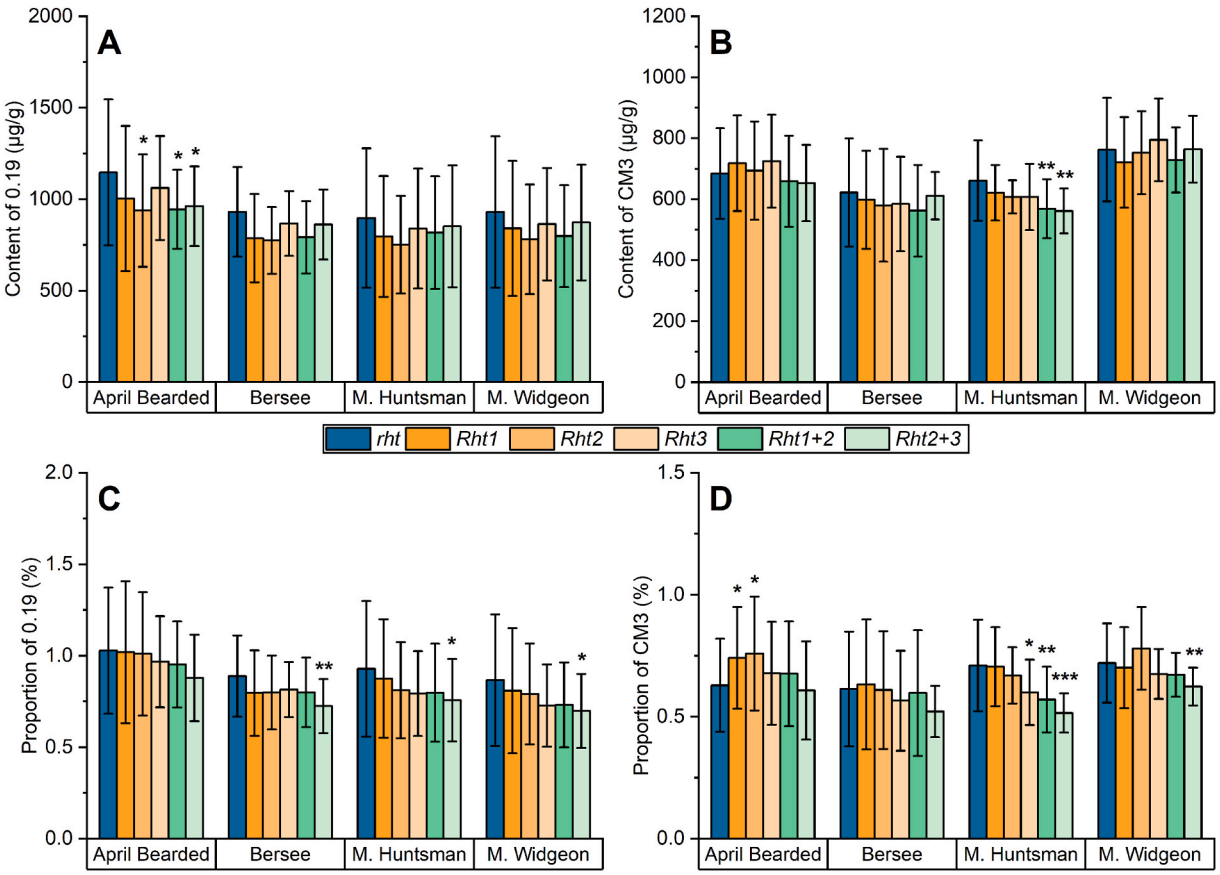

Fig. 2. Most abundant and most bioactive ATI 0.19 and CM3 of NILs of the four genotypes April Bearded, Bersee, Maris (M.) Huntsman and Maris (M.) Widgeon with different *Rht* alleles. *Rht1* (semi-dwarf), *Rht2* (semi-dwarf), *Rht3* (extreme dwarf), *Rht1+2* (dwarf) and *Rht2+3* (extreme dwarf) are compared to wild-type *rht* (tall). Content of 0.19 (A) and CM3 (B) as well as proportion of 0.19 (C) and CM3 (D) based on crude protein. Mean of three years, three biological replicates per year and three technical replicates ($n = 27$). Significant differences to *rht* are marked with asterisks (*t*-test, $*p \leq 0.05$; $**p < 0.01$; $***p < 0.001$).

proportion of CM3, CM2 and CM17 based on total ATI compared to the other three genotypes. The CM1 and WCI content was comparable to April Bearded and Maris Huntsman, respectively, and further, the proportion of WCI was in the middle of all genotypes and may be the reason for the different loadings in Fig. 4A and B. The NILs of Maris Huntsman clustered close to the loadings of WASI and WTI (highest content) in Fig. 4A and close to the ones of WASI, WTI and WCI (highest proportion) in Fig. 4B. In contrast to the PCA of total ATI content (Fig. 4A), in which a distinction of April Bearded and Bersee was possible, both NILs clustered close to the loadings of 0.53, 0.28 and CM16 in Fig. 4B and a distinction was not observed. This is because the proportions based on total ATI (Fig. S1, Table S4) were very similar between April Bearded and Bersee, but the content of individual ATI (Table S2) varied between the genotypes and with the exception of WASI, all ATI had a higher content in April Bearded compared to Bersee.

In contrast to the other PCA plots, the genotypes clustering was not as clear in the PCA considering the ATI proportion based on crude protein (Fig. 4C). With the exception of WASI, all loadings pointed to the right. The high correlation between most individual ATI was confirmed by a Pearson correlation matrix (Fig. S6) and only WCI, WASI and WTI showed a lower number of correlations compared to all other ATI. One might conclude that the extreme dwarf *Rht2+3* had an effect on the ATI proportion based on protein content, because these samples were located at the left within each cluster of the NILs, but again the genotypes clustered more than the alleles.

Based on the PCA results, it seems that the genotype had a higher influence on ATI than the allele. Thus, a three-way ANOVA should reveal the influence of the genotype and *Rht* allele as well as that of the environment.

3.5. Influence of genotype, allele and environment on ATI content and distribution

The results of the three-way ANOVA display the effect of genotype, *Rht* allele and environment on the total ATI content, on individual ATI and on the ATI proportion based on crude protein (Fig. 5, Table S6).

In general, the absolute total ATI content and that of individual ATI was predominantly affected by the environment, except for the content of 0.28 and WTI, where the effects of the environment and the genotype were almost equal. The ATI proportion based on crude protein was mainly affected by the interaction of genotype and environment (G*E), except for 0.28, 0.53, CM16 and CMX1/2/3 where the environment had the largest effect. Only the proportion based on crude protein of total ATI (6 %) and CM3 (7 %) were partly affected by the allele, whereas the percentage was below 5 % for all other parameters.

The proportions of WASI and WTI were most affected by G*E, but the genotype had a high effect on the proportion of WASI and the environment on the proportion of WTI, as well.

4. Discussion

The prevalence of wheat-related disorders increased during the last 50 years (Bradauskiene et al., 2021) and possible reasons for this are still under discussion. To gain more insights into whether breeding, specifically the incorporation of *Rht* genes, might be a cause of the increasing prevalence, NILs with different *Rht* alleles and conferring contrasting levels of dwarfism were analyzed for the ATI content and distribution by LC-MS/MS.

We showed that the total ATI content and the ATI proportion based

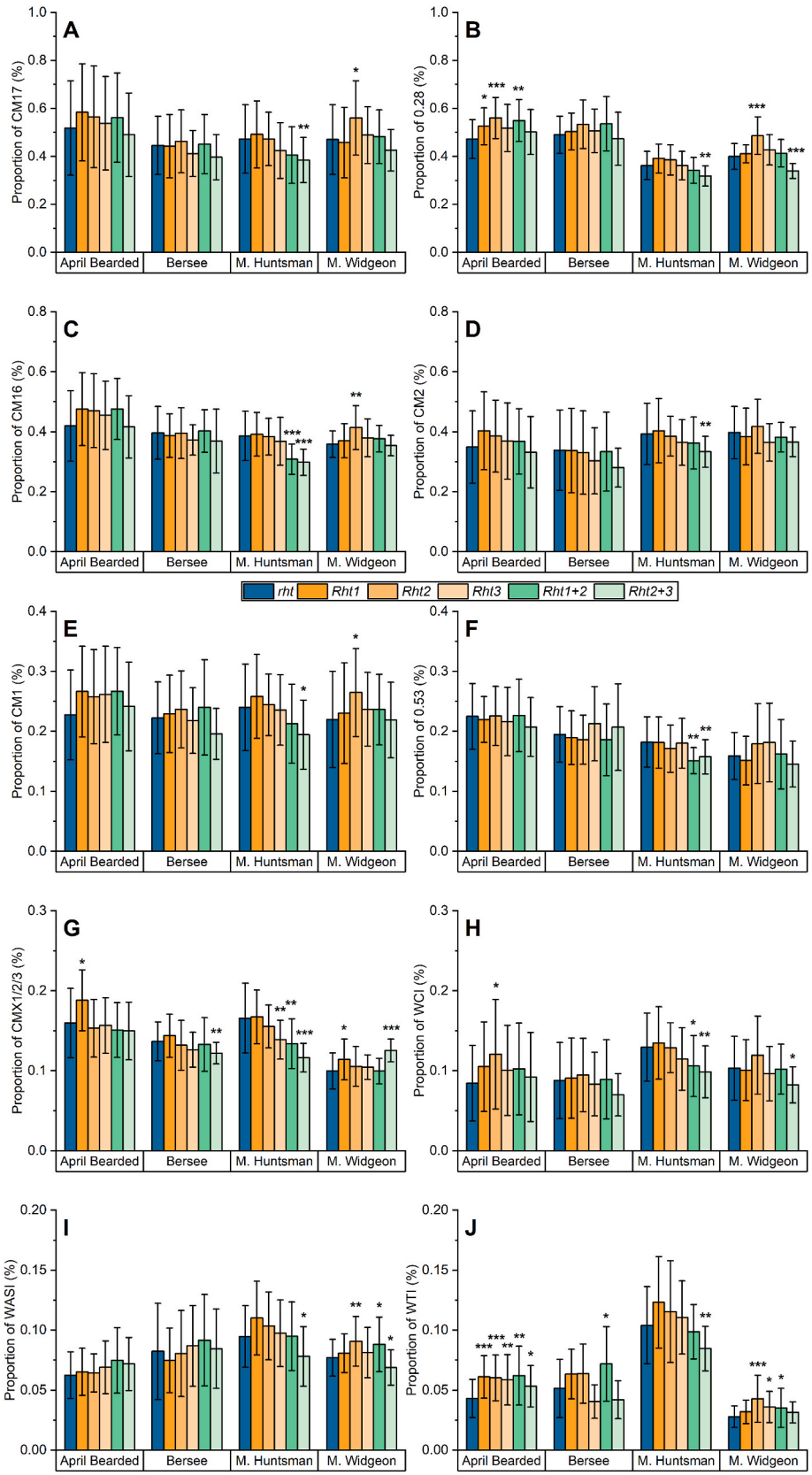

Fig. 3. Individual ATI of NILs of the four genotypes April Bearded, Bersee, Maris (M.) Huntsman and Maris (M.) Widgeon with different *Rht* alleles. *Rht1* (semi-dwarf), *Rht2* (semi-dwarf), *Rht3* (extreme dwarf), *Rht1+2* (dwarf) and *Rht2+3* (extreme dwarf) are compared to wild-type *rht* (tall). Proportion based on crude protein of CM17 (A), 0.28 (B), CM16 (C), CM2 (D), CM1 (E), 0.53 (F), CMX1/2/3 (G), WCI (H), WASI (I), and WTI (J). Data are displayed as in Fig. 2.

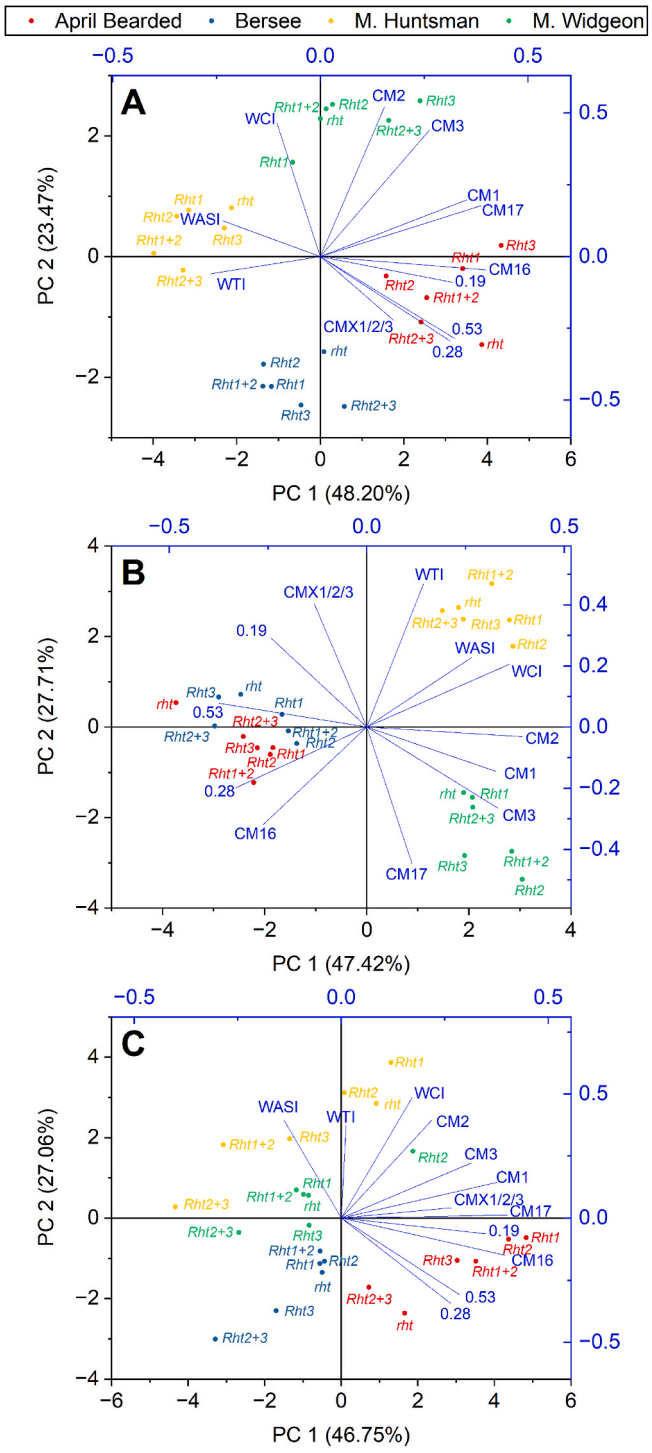

Fig. 4. Principal component analysis (PCA) of ATI in NILs of the four genotypes April Bearded, Bersee, Maris (M.) Huntsman and Maris (M.) Widgeon with different *Rht* alleles: *rht* (tall), *Rht1* (semi-dwarf), *Rht2* (semi-dwarf), *Rht3* (extreme dwarf), *Rht1+2* (dwarf) and *Rht2+3* (extreme dwarf). PCA based on absolute ATI content (A), proportion of individual ATI based on total ATI (B) and ATI proportion based on crude protein (C).

on crude protein was similar between the tall wild-type and semi-dwarf *Rht1* and *Rht2*, which are the *Rht* variations present in modern wheat. Thus, our findings support previous studies that breeding did not increase the ATI content. Jahn et al. (2025) compared modern wheat cultivars and landraces and revealed that there was no difference between both groups in the total ATI content and the ATI proportion based on crude protein. Geisslitz et al. (2023) observed a constant total ATI content for cultivars registered between 1890 and 1970 and then, a slightly decreasing trend for cultivars introduced between 1970 and 2010. At the same time, the crude protein content also slightly decreased and thus, the proportion of total ATI based on crude protein was constant between all decades. The semi-dwarf *Rht1* and *Rht2* showed exactly the same behavior in our study, suggesting that the introduction of *Rht* alleles did not increase the ATI content. The total ATI content did not increase either in 149 wheat cultivars from 1921 to 2013 and a very high variability within each breeding period was observed. The authors concluded that the selection of cultivars with low ATI content appears to be difficult (El Hassouni et al., 2021). Further, Austrian wheat cultivars from breeding periods between 1850 and 2016 and cultivars from Minnesota released between 1915 and 2020 also showed no increasing trend in the ATI content determined by RP-HPLC (Bajgain et al., 2023; Call et al., 2020). Nevertheless, it has to be mentioned that it is currently unknown, if there is link between the expression of ATI and the genetic location of *Rht* genes or if ATI expression is regulated by genes close to *Rht* genes.

Even though there was no difference in the total ATI content between the tall wild-type (*rht*) and extreme dwarf *Rht2+3*, the ATI proportion based on crude protein was lower in *Rht2+3* than in *rht* due to the higher protein content of *Rht2+3*. Further, the content of albumins/globulins was significantly higher in *Rht2+3* than in *rht* (11–15 %) (Geisslitz et al., 2025), but the increase was not due to ATI, but due to other proteins within this fraction. Subsequent untargeted LC-MS/MS measurements are required to identify these proteins.

In contrast to other studies (El Hassouni et al., 2021; Geisslitz et al., 2023), the total ATI content was not correlated (Table S7) to the content of crude protein (Pearson correlation coefficient $r = 0.062$) or albumins/globulins ($r = -0.073$). The reason for this is probably because of the low genetic variations, as we analyzed only four genotypes instead of 60 (Geisslitz et al., 2023) or 149 cultivars (El Hassouni et al., 2021), respectively.

The extreme dwarf *Rht2+3* is almost absent in modern wheat cultivars and we recently suggested that *Rht2+3* should not be included in wheat breeding programs due to its lower glutenin content and predicted inferior baking quality compared to *rht* and the semi-dwarfs *Rht1* and *Rht2* (Geisslitz et al., 2025). However, the findings in the current study suggest that the incorporation of extreme dwarf *Rht2+3* might have the potential to decrease the total ATI content. However, in the context of ATI, not only the absolute content of total ATI and individual ATI have to be taken into account, but also the inhibitory activity against amylase and trypsin and the bioactivity. The total ATI content is not correlated to the inhibitory activity against amylase (Jahn et al., 2023, 2025) and trypsin (Call et al., 2020). Further, other findings indicate that this is also true for the correlation between the total ATI content and bioactivity (Neerukonda et al., 2024). Thus, there is a strong need for research on the effect of breeding on the inhibitory activity and bioactivity.

It is well known that the protein composition of wheat is strongly affected by the environmental growing conditions, biotic and abiotic stresses (Call et al., 2020; Filip et al., 2023; Geisslitz et al., 2019, 2025; Nagy-Réder et al., 2022; Pronin et al., 2020; Ronga et al., 2020). This is further true for the ATI content (El Hassouni et al., 2021; Geisslitz et al., 2023; Jahn et al., 2025). As already discussed in detail in our previous study (Geisslitz et al., 2025), the three years varied in their climatic conditions and in short, 2021 was cool and dry, 2022 was warm and dry and 2023 was warm and wet. Due to the uncontrolled field conditions, it is difficult to draw definite conclusions, as to whether and to what extent temperature and precipitation affect ATI content. Thus, further investigations with controlled growing conditions are strongly required. El Hassouni et al. (2021) observed a significant genotypic effect on the absolute ATI content and also a considerable interaction between genotype and environment, especially for the total ATI content. In our study, the interaction between genotype and environment was high for

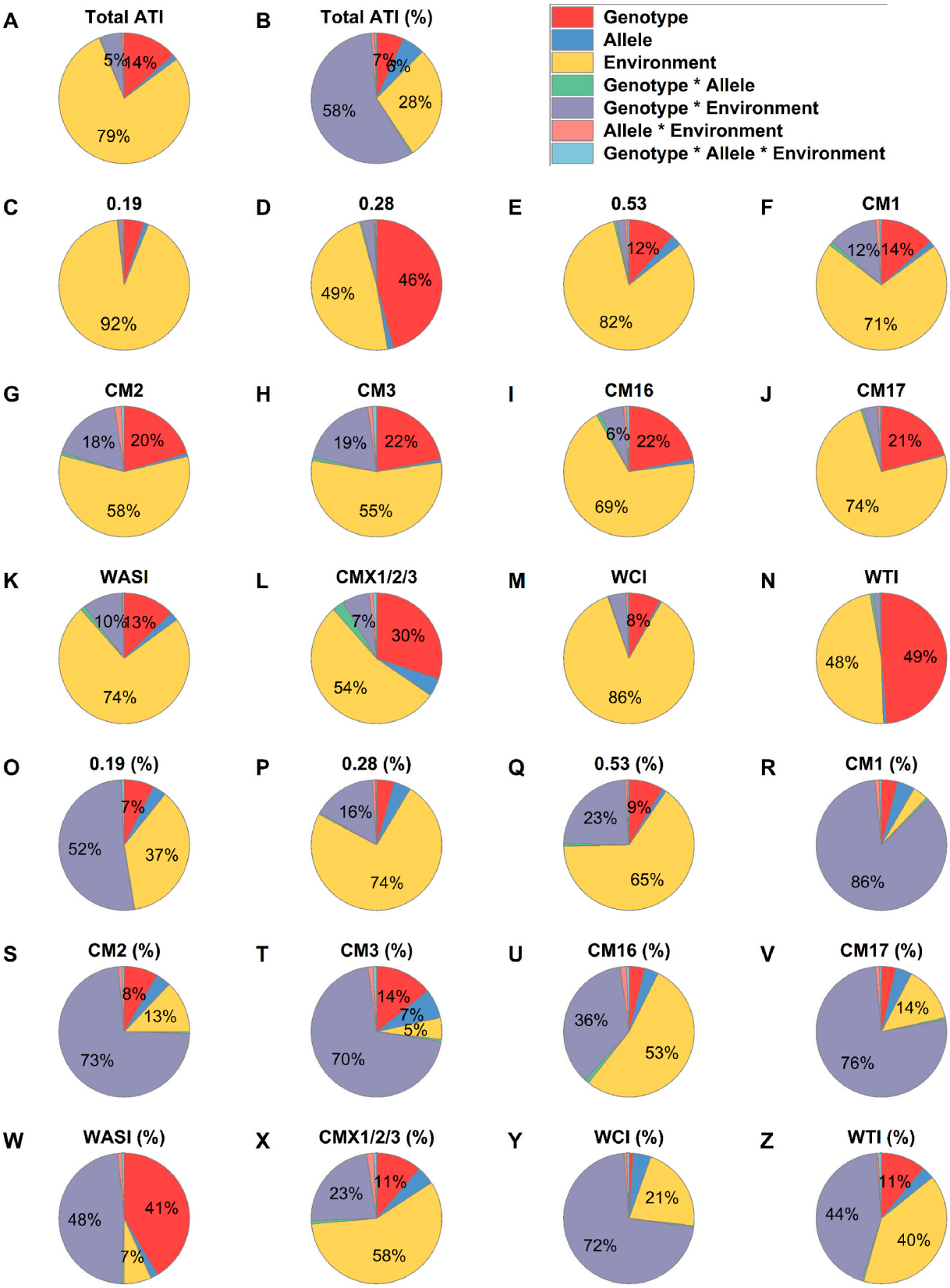

Fig. 5. Influence of genotype, allele and environment on the content of total ATI and individual ATI, as well as the ATI proportion based on crude protein (three-way ANOVA). Content of total ATI (A); proportion of total ATI based on crude protein (B). Content of individual ATI: 0.19 (C); 0.28 (D); 0.53 (E); CM1 (F); CM2 (G); CM3 (H); CM16 (I); CM17 (J); WASI (K); CMX1/2/3 (L); WCI (M); WTI (N). Proportion of individual ATI based on crude protein: 0.19 (O); 0.28 (P); 0.53 (Q); CM1 (R); CM2 (S); CM3 (T); CM16 (U); CM17 (V); WASI (W); CMX1/2/3 (X); WCI (Y); WTI (Z). Values lower than 5 % are not displayed. Detailed *F*-values and significance levels are summarized in Table S6.

the proportion of ATI based on crude protein. The absolute amount was mostly influenced by environment and genotype and to a lesser extent by their interaction. Overall, our study supports the conclusions drawn by El Hassouni et al. (2021) that classical selection and breeding are challenging strategies to obtain cultivars with low ATI content.

5. Conclusion

By analysing four NILs carrying five different *Rht* alleles/combinations, we investigated the impact of these genes on ATI content and distribution. While the absolute ATI content remained largely unaffected by the introduction of *Rht* genes, a reduction in ATI proportion based on crude protein was observed only in the extreme dwarf *Rht2+3* lines compared to the tall wild-type. However, this *Rht* combination also showed an unusually high gliadin-to-glutenin ratio previously limiting its relevance for breeding purposes. We therefore suggest not including it in further breeding programs. Importantly, the semi-dwarf alleles *Rht1 and Rht2*, which are widespread in modern wheat cultivars, did not significantly alter ATI content and distribution. In line with other studies investigating the impact of breeding on the ATI content, our findings do not support a direct link between breeding – specifically the introduction of *Rht* genes – and increased ATI content. Our study confirms and strengthens previous research suggesting that breeding is not the predominant cause of the increasing prevalence of wheat-related disorders, particularly NCWS. A strong environmental influence and interactions between genotype and environment were observed for ATI content and distribution, highlighting the challenge of selecting cultivars with consistently less ATI.

CRediT authorship contribution statement

Sabrina Geisslitz: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Matías Schierenbeck:** Writing – review & editing, Resources, Conceptualization. **Andreas Börner:** Writing – review & editing, Resources, Conceptualization. **Katharina Anne Scherf:** Writing – review & editing, Resources, Conceptualization.

Informed consent

Not applicable.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcs.2025.104325.

Data availability

Mass spectrometry data are publicly available on Panorama Public (https://panoramaweb.org/B7LfHQ.url). All detailed values presented can be found in the Supplementary Material.

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