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RESEARCH

Mutation of the wheat homeobox gene *Grain Number Increase 1* increases grain number and grain yield but decreases grain protein content

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Abstract Inflorescence structure affects final grain yield (GY) in wheat (*Triticum aestivum* L.). Recent breeding efforts have focused on improving grain number per spike, which is positively correlated with GY. *Grain Number Increase 1* (*GNI-A1*) encodes a homeodomain leucine zipper class I (HD-Zip I) transcription factor that controls the number of grains per spike and GY. However, how this increase in grain number affects grain quality, especially grain protein content (GPC) in wheat, remains elusive. Here we investigated within-spikelet variation in GPC using *GNI-A1* near-isogenic lines. Yield trials in two seasons and at two sites demonstrated that lines harboring a reduced-function allele, *GNI-A1* (105Y), consistently showed improved GY due to a 27% increase in grain number per spike, along with a 1.7% reduction in GPC compared with lines containing a functional allele, *GNI-A1* (105N). We confirmed the positive correlation between GY and grain number and the negative correlation between GY and GPC, but we observed no correlation between GY and thousand-grain weight. The increased grain number conferred by the 105Y allele was due to better floret fertility around the central part of the spike and whole florets. In-depth phenotypic analysis using dissected grain samples revealed that GPC was nearly uniform among spikelets and florets. These results suggest that in plants carrying a mutation in *GNI-A1*, the increase in the total number of grains is accompanied by a reduction in GPC.

Keywords Floret fertility · Grain number · Grain protein · HD-Zip I transcription factor · Wheat

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Introduction

Enhancing grain yield (GY) together with grain protein content (GPC) is a major target for wheat (*Triticum aestivum*) breeding. The GY of wheat is mainly determined by grain number and grain weight (Sakuma and Schnurbusch 2020). Grain number per plant depends on the number of spikes and the number of grains in each spike, which is itself dependent on floret fertility. The number of grains is positively correlated with GY; hence it is important to improve spike architecture (Sakuma and Koppolu 2023). The wheat spike is composed of several spikelets, with each spikelet producing three to five florets (Fig. 1).

The gene *Grain Number Increase 1* (*GNI1*) encodes a homeodomain leucine zipper class I

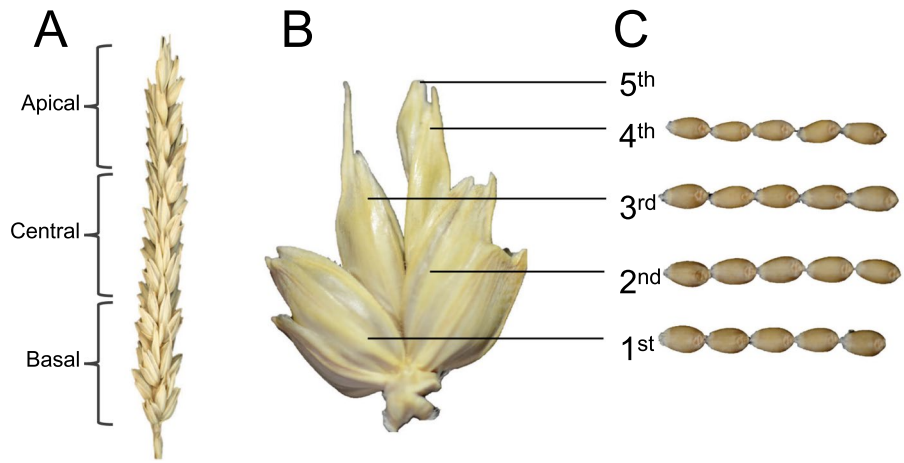

Fig. 1 Inflorescence architecture of wheat. **A** Representative spikes were harvested from each plot, and the spikelets were classified into three parts: apical, central, and basal. **B** Grains in each spikelet were subdivided according to the position of the florets (1st, 2nd, 3rd, and 4th) from the base of the spikelet. **C** Grains were collected from florets at each position

(HD-Zip I) transcription factor that regulates floret fertility (Sakuma et al. 2019). *GNI1* is mainly expressed in apical florets within the spikelet and suppresses floret development. A mutation in the homeodomain of the A-genome homoeolog, *GNI-A1* (N105Y: 105 asparagine to tyrosine), releases the suppression of floret fertility and increases the number of grains. Yield tests using TILLING mutant lines of Kitahonami, a Japanese high-yielding winter-type wheat cultivar, confirmed higher GY in plants carrying the reduced function *GNI-A1* allele (Sakuma et al. 2019). The *GNI-A1* allele had no significant effect on grain weight, although in tetraploid wheat, an increased number of grains due to the presence of the *GNI-A1* allele led to a decrease in grain weight (Golan et al. 2019). However, the effects of this allele on grain traits such as protein content remain unknown. Also, little is known about positional effects within a spike/spikelet on grain quality.

Wheat grain supplies approximately 20% of human nutritional protein worldwide (FAOSTAT, 2020). As nitrogen (N) is mainly stored in wheat grains in the form of proteins, GPC strongly affects flour quality. GY is often negatively correlated with GPC among different genotypes (Asseng and Milroy 2006; Kramer 1979). GPC is influenced by crop management practices such as N supply and irrigation (Debaeke et al. 1996; Pan et al. 2023; Farrer et al. 2006; Fischer et al. 1993). However, simply increasing the rate of N application does not increase GPC. On the contrary, excessive N application leads to reduced N uptake and/or N use efficiency due to N loss, which increases the risk of environmental pollution. Also, considering the recent increases in global N prices, understanding the genetic and physiological basis of GPC is crucial for further wheat improvement.

The *Grain Protein Content-B1* (*Gpc-B1*) gene, encoding a NAC (NAM, ATAF, and CUC) transcription factor, regulates protein levels in wheat grain (Uauy et al. 2006). The *Gpc-B1* allele from wild emmer wheat (*Triticum turgidum* ssp. *dicoccoides*) increases GPC by accelerating senescence and improving nutrient remobilization to the developing grain compared to cultivated wheat lines with non-functional alleles of this gene. A recent study showed that a modified wheat spike architecture known as paired spikelets enhances GPC without altering GY or grain number per spike (Dixon et al. 2022). The higher protein content is associated with an increase in the hydraulic conductivity of the spike and peduncle and a greater supply of amino acids to the rachis. However, no reports describe the effects of improved grain number per spike on GPC, and the within-spike/spikelet distribution of N remains elusive.

To better explore the relationship between GY and GPC, it would be useful to examine lines carrying the *GNI-A1* mutation. Therefore, in this study, we conducted yield trials in multiple environments to investigate the effects of the *GNI-A1* allele on GY and GPC. To gain insights into the distribution of N among grains, we investigated within-spike/spikelet variation in N content. Our findings lay the foundation for improving GY and GPC in wheat.

Materials and methods

Plant materials

The bread wheat (*Triticum aestivum*) used in this study was previously selected by TILLING (Sakuma et al. 2019). The genotypes with a functional *GNI-A1* allele (105N) and a reduced-function *GNI-A1* allele (105Y) were derived from Japanese high-yielding winter wheat cv. 'Kitahonami'. The plants in the M4 generation and M5 generation were used in 2016/2017 and 2017/2018 seasons, respectively. M4 seeds were collected from ~10 M3 plants selected as either homozygous for the 105Y or for the 105N allele. M5 seeds were collected from M4 plants.

Yield trials

Field experiments were conducted at Kitami Agricultural Experiment Station (43° 44′ N, 143° 43″ E, Kitami, Hokkaido, Japan) from 2016 to 2018 and at Central Agricultural Experiment Station (43° 3′ N, 141° 45″ E, Naganuma, Hokkaido, Japan) from 2016 to 2018, for a total of four environments. Yield tests were conducted in a randomized complete block design with four replications at 5.4 m^2/plot (200 grains sown per m^2) at Kitami Agricultural Experiment Station and with three to four replications at 4.8 m^2/plot at Central Agricultural Experiment Station (200 grains sown per m^2). Fertilizers were supplied before planting at Kitami (5.6 kg/10a N, 17.5 kg/10a P, and 7.0 kg/10a K) and at Naganuma (4.0 kg/10a N, 12.5 kg/10a P, and 5.0 kg/10a K). At Kitami, 5.0 kg/10a N was applied during the stem elongation stage and at the flag leaf stage. At Naganuma, 6.0 kg/10a N was applied during the stem elongation stage, and 4.0 kg/10a N was applied at the flag leaf stage. Disease control was carried out by spraying fungicides at both sites in a timely manner.

Phenotypic analysis

GY was estimated by measuring the harvested grain weight per area from each plot. GPC for whole grains from each plot was determined using an Infratec NOVA grain analyzer (Foss, Japan). Ten representative spikes were randomly selected from each plot, and the number of grains per spike and the number of grains per spikelet were measured. The spikes were manually dissected and divided into the apical, central, and basal parts (Fig. 1A). Grains on each spikelet were subdivided according to the position of the florets (1st, 2nd, 3rd, 4th, and 5th from the base of the spikelet; Fig. 1B and C). After classifying the grains, the grain weight was measured using an electronic balance. Grain size (grain surface area, length, and width) was measured using Smart Grain analysis software (Tanabata et al. 2012). The grain carbon and N concentrations were measured based on the Dumas-Pregl method using a CN elemental analyzer (JM1000CN, J-SCIENCE LAB CO, Kyoto, Japan). GPC was calculated by multiplying the grain N concentration by 5.69 according to published methods (Fujihara et al. 2008). Data on GY, spike number m^{-2}, and TGW in the 2016/17 season at Naganuma and Ktami were obtained from Sakuma et al. (2019)

Data analysis

Data analyses, including descriptive statistics, Pearson correlation analysis, analysis of variance (ANOVA), and Student *t*-test were performed using Prism 10.1.1 software (GraphPad Software, LLC). A two-way ANOVA with Tukey's multiple comparison tests was conducted for each trait. Phenotypic correlation analysis and positional effect analysis within spike and spikelet were done using combined data from all environments. The broad-sense heritability (H^2_{Cullis}) (Cullis et al. 2006) was estimated for each trait using H2cal function with the inti package version 0.6.4 in R software (https://CRAN.R-project.org/package=inti).

Results

Effects of genotype and environment

The analysis of variance revealed that the genotype effect was significant for all traits except spike number m^{-2}, and the environment effect was significant for all traits. The genotype and environment interaction effect were not significant for all traits except floret fertility, thousand-grain weight (TGW), grain area size, and GPC (Table 1).

A broad-sense heritability (H^2) ranging from 0 to 0.89 was observed for each trait (Table 1). Grain number per spike, grain number per spikelet, floret

Table 1 Analysis of variance (ANOVA) and broad-sense heritability of the traits

Traits	Genotype	Environment	G×E	Heritability
Grain yield	2.27.E−10	2.09.E−13	0.050	0.70
Grain number spike^{-1}	2.21.E−12	7.02.E−12	0.529	0.72
Grain number spikelet^{-1}	8.71.E−09	9.98.E−05	0.214	0.82
Floret fertility	6.51.E−10	7.43.E−07	0.007	0.73
Spikelet number spike^{-1}	1.57.E−04	1.63.E−08	0.137	0.47
Spike number m^{-2}	0.787	2.83.E−09	0.827	0.00
Thousand grain weight	2.27.E−04	3.92.E−10	0.025	0.33
Grain weight	4.10.E−04	9.25.E−07	0.725	0.63
Grain area size	0.001	5.44.E−04	0.001	0.00
Grain protein content	1.00.E−15	3.00.E−15	0.002	0.75
Grain carbon content	4.47.E−06	0.036	0.358	0.89

fertility, GPC, and grain carbon content exhibited particularly high heritability (0.72–0.89). These findings suggest that these traits are robust in different environments and could be targets of breeding selection. Low heritability of spike number m^{-2}, grain area size, and TGW indicate that *GNI-A1* alleles have no or less effects for these traits.

Relationships between GY and grain number

In all four environments, plants carrying the 105Y allele consistently produced higher GY (592–895 kg 10a^{-1}) compared to plants carrying the 105N allele (467–792 kg 10a^{-1}; Fig. 2A). On average, the 105Y genotype had 26.8% more grains per spike (54.33–73.22) than the 105N genotype (40.67–59.33; Fig. 2B). Little or no difference in spike number, spikelet number, or TGW was observed between genotypes in all environments (Fig. 2C–E). There was a positive correlation between GY and both grain number per spike ($P = 0.0002$) and spike number ($P < 0.0001$), but none between GY and TGW ($P = 0.0734$) or spikelet number ($P = 0.1565$; Table 2). Therefore, the improved GY in the 105Y genotype was mainly due to an increased grain number per spike, which

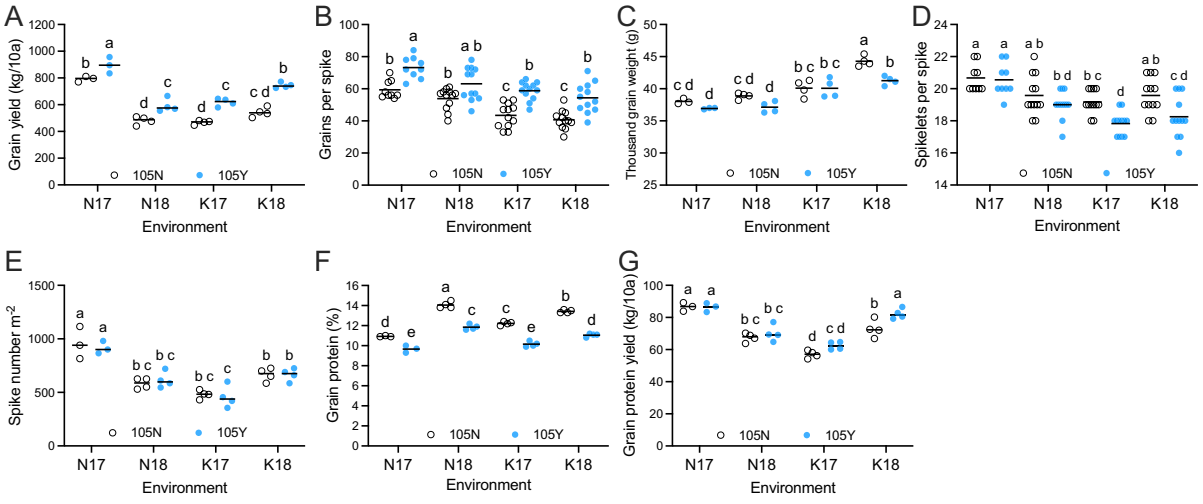

Fig. 2 Phenotypic variation of the two *GNI-A1* genotypes under field conditions. The performance of the plant lines was compared at two locations (N: Naganuma, K: Kitami) and two seasons (17: 2016–2017, 18: 2017–2018). 105N: the functional *GNI-A1* allele, 105Y: the reduced-function allele. The letters are used to indicate where mean values differed from one another significantly ($P < 0.05$) as determined by a two-way ANOVA with Tukey's multiple comparison test

Table 2 Pearson correlation coefficients among observed traits

	Grain yield	Grain number spike^{-1}	Thousand grain weight	Spikelet number spike^{-1}	Spike number m^{-2}	Grain protein content
Grain yield	–					
Grain number spike^{-1}	0.634**	–				
Thousand grain weight	−0.332	−0.737**	–			
Spikelet number spike^{-1}	0.265	0.294	−0.267	–		
Spike number m^{-2}	0.724**	0.414*	−0.270	0.553**	–	
Grain protein content	−0.759**	−0.580**	0.368*	0.094	−0.246	–

*,**Significant at $P<0.05$ and $P<0.01$

in turn was due to better grain setting around the basal and central parts of the spike (Fig. 3A). Interestingly, the 105Y genotype showed improved floret fertility not only in apical florets (3rd–5th floret) but also in basal florets (1st–2nd floret; Fig. 3B). These results demonstrate that the enhanced GY in the 105Y genotype is due to improved floret fertility.

A trade-off between GY and GPC

The GPC of whole grains harvested from each plot was significantly lower (by an average of 1.7%) in the 105Y genotype (9.7–11.9%) compared to 105N (10.9–14.1%) in all four environments (Fig. 2F). A negative correlation was detected between GY and GPC ($P<0.0001$) and between grain number per spike and GPC ($P=0.0008$; Table 2), indicating a clear phenotypic trade-off. Although GPC was consistently reduced in 105Y vs. 105N, grain protein

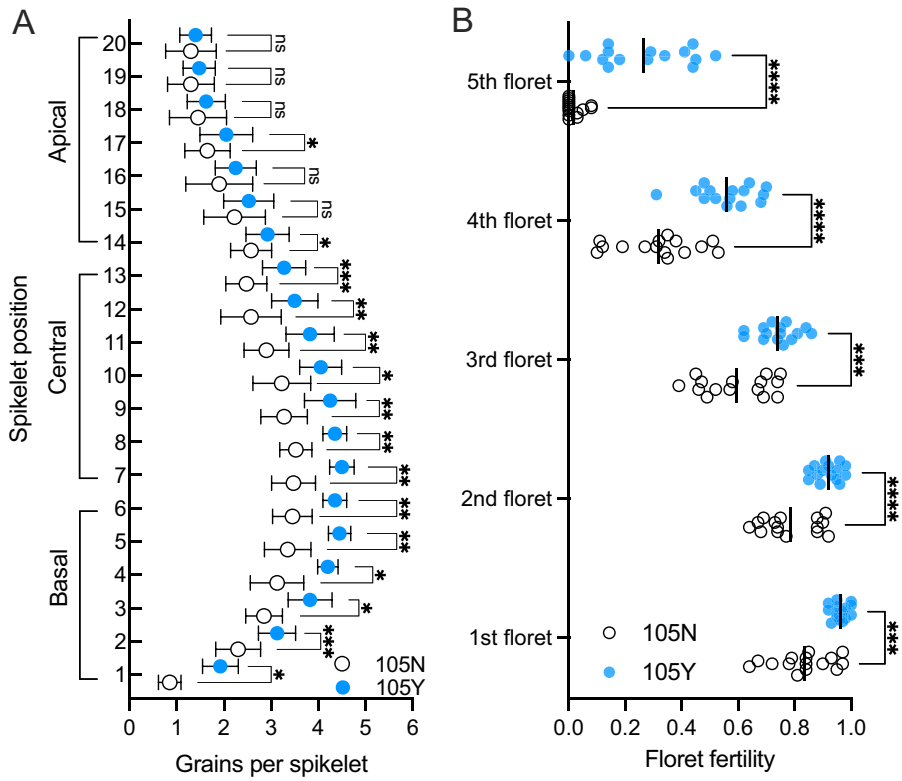

Fig. 3 The *GNI-A1* genotype affects grain setting. **A** Grain number per spikelet across spikelet positions. **B** Floret fertility within a spikelet. 105N: the functional *GNI-A1* allele, 105Y: the reduced-function allele. The significance of differences between trait means was determined by the Student's *t*-test. *,**,*** Significant at $P<0.05$, $P<0.01$, and $P<0.001$, respectively; ns, not significant at $P>0.05$

yield (GY x GPC) did not significantly differ between genotypes at Naganuma station (105N: 86.8 and 67.5 kg/10a; 105Y: 86.4 and 70.1 kg/10a) and Kitami station 2016/17 season (105N: 57.0 kg/10a; 105Y: 62.4 kg/10a). By contrast, grain protein yield was significantly higher in the Kitami station 2017/18 season (105Y: 82.3 kg/10a; 105N: 72.9 kg/10a) (Fig. 2G). A weak positive correlation between TGW and GPC ($P = 0.0456$) was also observed. To study the positional effects of *GNI-A1*, we measured the variation in GPC across spikelet and floret positions in the two genotypes. Unexpectedly, there was little difference in GPC across spike positions (apical, central, and basal) and floret positions (1st floret–4th floret) in either genotype (Fig. 4A–C). These results suggest that grain protein is evenly distributed within a spike, no matter the *GNI-A1* genotype.

The grain carbon content, an indicator of grain size and TGW, was conserved between the 105N and 105Y genotypes (Fig. 4D–F). This result was further supported by the finding that there was almost no difference in grain weight or grain area size between the 105N and 105Y genotypes (Fig. 5A–F). Within the spikelet, the second florets were heaviest among the five florets, with their weights in the following order: second florets > first florets > third florets > fourth florets > fifth florets. This trend was conserved between the two genotypes. Taken together, these results indicate that plants carrying the 105Y genotype produce more grains without reducing grain size; thus, the protein content is diluted evenly in each grain.

Discussion

In this study, we detected a trade-off between the number of grains and protein content in wheat. Yield tests in four environments demonstrated that the reduced-function mutation of *GNI-A1* significantly contributes to GY by improving grain number per spike without a penalty on TGW; however, we detected reduced GPC in each grain. Interestingly, we observed an almost equal distribution of GPC within spikes and spikelets. We suggest that the lower GPC in 105Y plants occurred because the supply of N in the experiments was not sufficient to generate a GPC matching that of the 105N genotype. The application of N fertilizer at the late stage of growth is effective

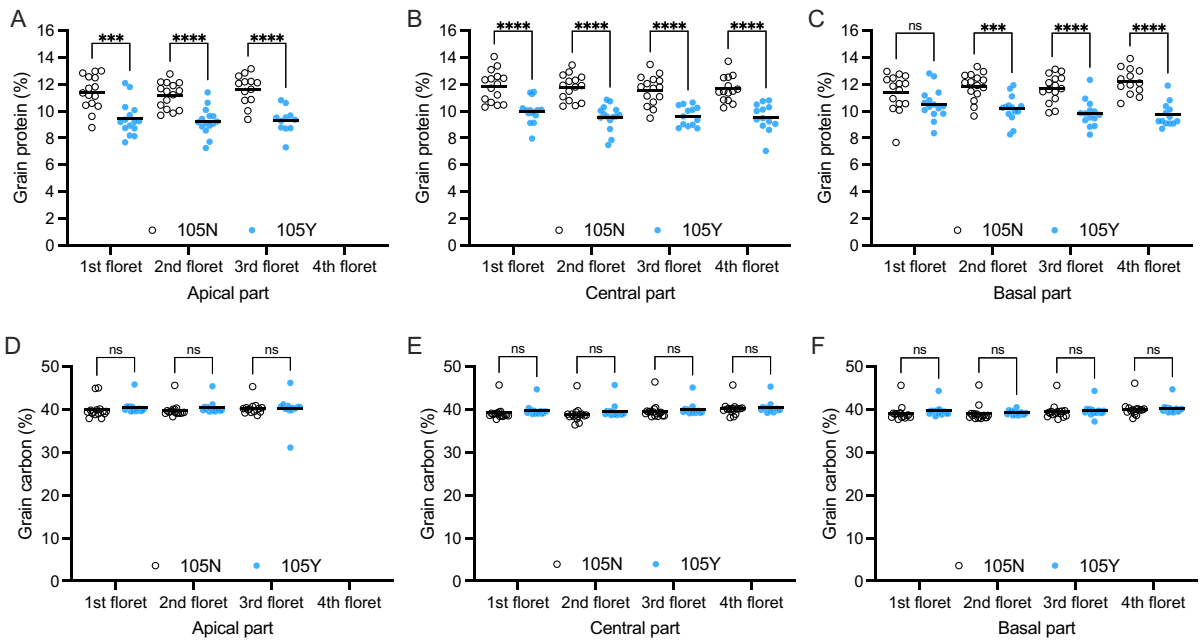

Fig. 4 Grain protein content and carbon content. **A**–**C**) Grain protein content at different spike part. **D**–**F** Grain carbon content at different spike part. 105N: the functional *GNI-A1* allele, 105Y: the reduced-function allele. The significance of differences between trait means was determined by the Student's *t*-test. *,**,*** Significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively; ns, not significant at $P > 0.05$

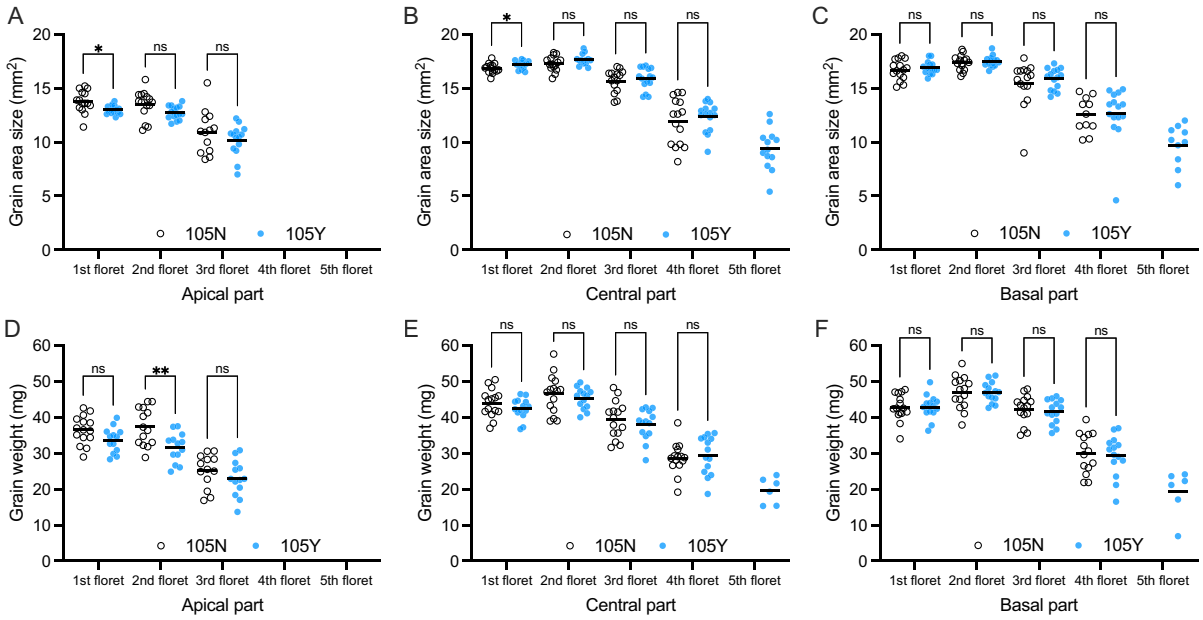

Fig. 5 Dissection of grain traits. **A–C** Grain area size at different spike part. **D–F** Grain weight at different spike part.105N: the functional *GNI-A1* allele, 105Y: the reduced-function allele. The significance of differences between trait means was determined by Student's *t*-test. *,** Significant at $P < 0.05$ and $P < 0.01$; ns, not significant at $P > 0.05$

in increasing GPC (Pan et al. 2023). In this study, N fertilizer was applied at both the stem elongation stage and flag leaf stage. Since the N treatment used in this study is the general practice widely used at experimental stations, it would not be desirable to drastically change the cultivation method to evaluate genotypic effects. Also, increasing the sustainability of agriculture requires reduced N fertilizer use (Li et al. 2018). Nonetheless, the mutation of *GNI-A1* had no negative effect on the total grain protein yield. The effect of *GNI-A1* under a lower N supply remains to be investigated. The impact of decreases in protein content varies depending on the use of the flour. Since flour from the wheat cultivar Kitahonami used in this study is used to make noodles, the effect of a 1–2% decrease in GPC would not be severe. On the other hand, as changes in protein content significantly affect the quality of wheat flour for making bread and confectioneries, it is important to obtain a sufficient protein content without yield penalty.

Several QTLs controlling GPC have been reported. Four stable QTLs with positive effects on GPC have been identified on wheat chromosomes 2A, 3A, 4D, and 7D (Groos et al. 2003). A recent genome-wide association study also identified GPC-associated markers on chromosomes 1D, 3A, 3B, 3D, 4B, and 5A (Kartseva et al. 2023). *QGpc.2B-yume*, a major QTL for GPC, was identified on the short arm of chromosome 2B (Terasawa et al. 2016). Notably, *QGpc.2B-yume* has no negative effects on yield. The effects of pyramiding *GNI-A1* and *QGpc.2B-yume* need to be investigated. A mutation of *Homeobox domain-2* (*HB-2*) in wheat leads to the generation of more spikelets (an effect called "paired-spikelets") and enhances GPC (Dixon et al. 2022). However, plants carrying this mutation produce nearly similar grain number per spike and lower TGW than the wild type. Perhaps the lower TGW contributes to the increased GPC. On the other hand, mutants of *GNI-A1* maintain TGW with increased grain number and reduced GPC. It would be interesting to test whether the combination of the *GNI-A1* and *HB-2* mutations would improve or maintain GPC and enhance grain number. Following the identification of important alleles regulating GY, further research is needed to improve or maintain protein quality in wheat.

In conclusion, we showed that a mutation in *GNI-A1* (N105Y) enhances GY by improving floret fertility without a grain size penalty but is associated with the production of grains with reduced protein

content (by 1.7%), although total grain protein yield did not decrease. These findings lay the foundation for improving GY and GPC in wheat via breeding.

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Author contributions S.S. and H.T. designed research. N.R., S.O., H.J., and Y.Y. performed phenotyping and data collection. S.S. and N.R. analyzed data. The first draft of the manuscript was written by S.S. and H.T. All authors read and approved the final manuscript.

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Data availability The data generated the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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