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The *Grain Number Increase 1* alleles *GNI-A1*-105Y and -105K increase grain number in spring wheat

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Abstract

Wheat (*Triticum aestivum* L.) has inflorescences made up of multiple spikelets arranged along a central rachis, with each spikelet producing between one and four grains. The *Grain Number Increase 1* (*GNI-A1*) gene wheat directly influences grain number per spikelet and grain size. Three naturally occurring alleles have been described previously: *GNI-A1*-105N, 105Y, and 105K. This project's goal was to characterize the impact of these alleles within hard red spring wheat cultivars in Montana, where each of the alleles is common. The 105N allele and the 105K allele were compared through analysis of an F_5 Vida by Spring-Yellowstone recombinant inbred line (RIL) population, and with near isogenic lines (NILs) derived from the same population. The 105N allele and the 105Y allele were compared with NILs derived from an F_4 Lanning by Egan RIL population. We analyzed the impact of each of the three alleles and compared their effects on inflorescence architecture, grain size, grain yield, grain quality, and milling quality under Bozeman, MT, field conditions. Data show that either loss-of-function alleles (105Y and 105K) increased grain number per spikelet by 5% when compared to the more functional allele (105N) across all years and environments tested. Overall grain size was not significantly reduced and there was also not a significant increase in overall grain yield.

1 | INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most widely cultivated crops, comprising 20% of the world's food supply (Enghiad et al., 2017; Sakuma et al., 2019). Increasing wheat yield while ensuring its nutritional composition and quality characteristics remain constant or improved is one of the foremost challenges in agriculture (Erenstein et al., 2022). Different steps in the development and maturation of a wheat plant can be manipulated via plant breeding to increase yield. Increased grain size, grain number per spike, and spike number are common metrics that breeders select for to increase yield while attempting to maintain or increase protein content (Quintero et al., 2018; Tillett et al., 2022). Manipulating grain size affects wheat quality parameters in different ways. Decreased grain size is generally associated with decreased milling yield (Ficco et al., 2020; Marshall et al., 1986). Smaller grains are also associated with an increase in grain and flour protein content. Decreased milling yield is undesirable from a miller's perspective; however, increased protein content is desirable for bread wheats because it improves end product quality and adds economic value

Abbreviations: NIL, near isogenic line; RIL, recombinant inbred line; HIF, heterogeneous inbred family.

(Baasandorj et al., 2015; Hogg & Giroux, 2019). In general, it is difficult to increase total grain yield per spike through the manipulation of any one specific gene, since grain size and grain number per spike are often inversely related (Tillett et al., 2022; Xie & Sparkes, 2021). Understanding the genetic relationship between these two specific traits is important when attempting to alter wheat spike architecture and grain traits.

Wheat and other cereal crops in the Triticeae family produce inflorescences centered around an unbranched rachis, with one to three spikelets growing from each rachis node. Each spikelet is made up of multiple florets alternately spaced along a central rachilla. (Sakuma & Schnurbusch, 2020) Each spikelet may have as many as six individual florets, but only fertile florets will produce a grain. The number of fertile florets per spikelet directly impacts grain yield (Golan et al., 2019; Sakuma et al., 2019; Tillett et al., 2022). It has generally been accepted that increasing the number of potential grains per unit area by increasing productive spikes per unit area or by increasing potential grain number per spike during plant development prior to flowering has a positive impact on yield (Ferrante et al., 2020). The number of grains per spike is fixed after flowering, so increasing grain size by increasing potential for increased grain fill has the biggest impact on yield (Reynolds et al., 2022; Slafer et al., 2023). While length and rate of grain fill is known to affect grain size and overall grain yield, longer grain fill periods are not associated with larger grain size, especially in environments that experience heat and drought stress during this period (Bruckner & Frohberg, 1987; Lizana et al., 2010; Xie et al., 2015). Other plant traits can indirectly affect these considerations. For example, plant height is often associated with higher grain number, but smaller grains and larger flag leaf size are being associated with larger grains (Ali et al., 2010; Khaliq et al., 2008). In general, increasing the number of grains decreases the ability of a plant to increase grain size, and vice versa. However, this convention has been contradicted by recent studies that have selectively upregulated expansins (expression, if so what?) in developing seeds after flowering, showing a positive impact on grain size and a direct positive impact on grain yield with no decrease in grain number (Calderini et al., 2021). These results magnify the importance of studying genes that may help to increase potential grain number, since beneficial grain number alleles could be successfully combined with alleles of other genes that increase seed size, resulting in a net positive for grain yield.

The *GNI-A1* (*Grain Number Increase-A1*) locus was first identified as a quantitative trait loci (QTL) for grain number per spike on chromosome arm 2AL (Guo et al., 2017). The underlying polymorphic gene was later discovered to be a transcription factor that plays a key role in the tradeoff relationship between grain size and number per spike by affecting floret fertility (Sakuma et al., 2019). *GNI-A1* is a HOX-1 class transcription factor that promotes floret abortion, belonging to a family of genes known as homeodomain leucine zipper (HD-Zip) transcription factors, which suppress organ development (Gonzalez-Grandio et al., 2017). This gene is directly orthologous to the more well studied gene *Vrs1* (*Six Rowed Spike-1*) in barley (*Hordeum vulgare*), and is more distantly orthologous to *Grassy Tillers 1* (*GT1*) in maize (*Zea mays*) (Bull et al., 2017; Dong et al., 2019; Klein et al., 2022; Sakuma et al., 2013, 2019; Whipple et al., 2011). Additionally, other orthologs have been described in other monocots and dicots including rice (*Oryza sativa*), *Brachypodium* (*Brachypodium distachyon*), and *Arabidopsis* (*Arabidopsis thaliana*) (Perotti et al., 2017; Sakuma et al., 2010; Shao et al., 2018).

Core Ideas

- The wheat *Grain Number Increase 1* gene increases grain number per spikelet.
- *Grain Number Increase 1* missense alleles increased grain number per inflorescence by 5%.
- *Grain Number Increase 1* missense alleles did not increase grain yield.

Across the three *GNI1* homoeologues in wheat, there is expression of *GNI-A1* and *GNI-D1* in spike tissue, but very little detectable expression of *GNI-B1*. Expression of *GNI-A1* is four times higher than that of *GNI-D1* in spike tissue. No allelic variation in *GNI-D1* among modern cultivars has been reported (Tillett et al., 2022). Three different *GNI-A1* alleles have been reported among 210 hexaploid winter wheat cultivars (Sakuma et al., 2019). The ancestral or wild-type allele is defined by the encoding of an asparagine (N) at the 105th codon. Two different missense alleles have been reported in modern wheat, each containing a single amino acid change at this codon. *GNI-A1*-105Y encodes a tyrosine, and *GNI-A1*-105K encodes a lysine at this position (Figure 1). The *GNI-A1*-105Y allele reduces floret abortion, resulting in more grains per spikelet and higher yields relative to the wild-type allele. This was shown through analysis of lines within a population where mutagenesis of the variety Kitahonami (which contains the 105Y allele) was employed to artificially recreate the 105N allele. M_4 lines were compared in full-density field experiments (Sakuma et al., 2019). An association between the 105Y allele and higher grain number per spikelet was also shown through QTL analysis of a double haploid line population derived from a cross between parents Shunyo (containing the 105N allele) and Kitahonami (containing the 105Y allele) (Mizuno et al., 2021). No prior research has been conducted to compare the 105K and the 105N alleles. Ancestral, tetraploid emmer wheat (*Triticum dicoccum)* varieties were shown to have higher expression of *GNI-A1* resulting from two gene copies in the A genome, while modern tetraploid durum wheat

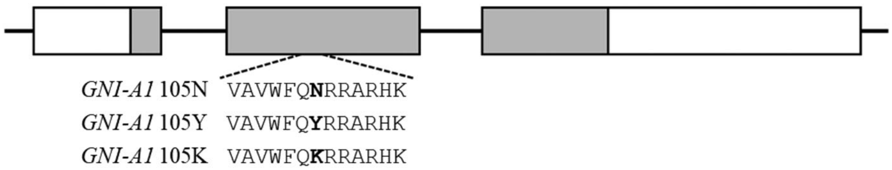

FIGURE 1 Graphical representation of polymorphism between *Grain Number Increase 1* (*GNI-A1*) alleles. The ancestral allele of *GNI-A1* encodes an asparagine (N) at the 105th codon, while missense alleles *GNI-A1*-105Y encodes a tyrosine, and *GNI-A1*-105K encodes a lysine.

(*Triticum durum*) cultivars were shown to have one gene copy on the A genome with half the expression of *GNI-A1*, and larger grains, but fewer grains per spikelet as a result (Golan et al., 2019). *GNI-A1* can be described in modern wheat cultivars as controlling the tradeoff between grain size and grain number per spikelet, with functional alleles resulting in larger but fewer grains per spikelet, and decreased function alleles resulting in smaller, but more grains per spikelet (Sakuma et al., 2019; Sakuma & Schnurbusch, 2020).

The aims of this study were to determine the distribution of *GNI-A1* 105N, 105Y, and 105K alleles in Montana-adapted wheat varieties, to investigate the impact of the 105K allele in a recombinant inbred line (RIL) population, to confirm the effects of the 105K allele along with the previously published effects of the 105Y allele using near isogenic lines (NILs), and to determine whether or not these alleles have an effect on grain yield and end use quality traits.

2 | MATERIALS AND METHODS

2.1 | Variety screen methods

A nested reaction was required to sequence the entire gene. First, the whole gene was amplified with primers *GNI*-A1F and *GNI*-A1R, as published in Sakuma et al. (2019). Each 25 µL pre-amplification reaction consisted of 1.2 µL of genomic DNA at a concentration of approximately 2 µg/µL, 13.67 µL of ultrapure nuclease-free water, 5 µL of 5x Green GoTaq Flexi Buffer (Promega), 2 µL of $MgCl_2$ at a concentration of 25 mM, 2 µL of dNTP at a concentration of 2 mM, 0.5 µL of 20 µm Primer *GNI*-A1F, 0.5 µL of 20 µm Primer *GNI*-A1R, and 0.13 µL of GoTaq G2 Flexi DNA Polymerase (Promega). Polymerase chain reaction (PCR) conditions for *GNI-A1* pre-amplification were 40 cycles of 30 s at 96˚C, 30 s at 59˚C, and 2 min at 72˚C, with an expected amplicon size of about 1800 base pairs. The results of the *GNI-A1* pre-amplification reaction were diluted 1:100 with deionized water to be used as template DNA for the internal amplification reactions. The first half of the *GNI-A1* gene was internally amplified from the diluted results of the pre-amplification reaction, utilizing primers *GNI*-A1F and *GNI*-A1E3R (Table S1). The reaction mixture followed the same formula as stated above, with pre-amplification dilutions used in place of genomic DNA. PCR conditions for the *GNI-A1* first half internal amplification were 40 cycles of 30 s at 96˚C, 30 s at 59˚C, and 1 min at 72˚C, with an expected amplicon size of about 1000 base pairs. Amplicons were sequenced using primer *GNI*-A1E3R by Azenta/GENEWIZ (Azenta US, Inc.). The second half of the *GNI-A1* gene was internally amplified from the diluted results of the pre-amplification reaction, utilizing primers *GNI*-A1E2F and *GNI*-A1R (Table S1). The reaction mixture followed the same formula as stated above, with pre-amplification dilutions used in place of genomic DNA. PCR conditions for the GNI-A1 first half internal amplification was 40 cycles of 30 s at 96˚C, 30 s at 59˚C, and 1 min at 72˚C, with an expected amplicon size of about 1100 base pairs. Amplicons were sequenced using primer *GNI*-A1E2F by Azenta/GENEWIZ (Azenta US, Inc.).

To rule out the possibility of additional *GNI-1* variation resulting from different alleles of the *GNI-D1* homoeologue, an additional screen was performed across the 32 hexaploid wheat varieties. Two additional primers and a protocol were designed to aid in sequencing of *GNI-D1*. Sequence differences between the three *GNI1* homoeologues were analyzed manually to come up with two new *GNI-D1*-specific primers: *GNI*-DexF 5′-AACTGAACTTTATCGACCG-3′ and *GNI*-DexR 5′-TGGGAGAAACGATTTAGC-3′ (Table S1). The previously reported primer *GNI*-A1EPF was also used (Sakuma et al., 2019). A nested PCR was performed in which pre-amplification of the entire gene was followed by a dilution of products to be used as the DNA in the subsequent internal amplification of specific regions. PCR products were then Sanger sequenced and screened for polymorphisms. Each 25 µL pre-amplification reaction consisted of 1.2 µL of genomic DNA at a concentration of approximately 2 µg/µL, 13.67 µL of ultrapure nuclease-free water, 5 µL of 5x Green GoTaq Flexi Buffer (Promega), 2 µL of $MgCl_2$ at a concentration of 25 mM, 2 µL of dNTP at a concentration of 2 mM, 0.5 µL of 20 µm Primer GNI-DexF, 0.5 µL of 20 µm Primer GNI-DexR, and 0.13 µL of GoTaq G2 Flexi DNA Polymerase (Promega). PCR conditions for *GNI-D1* pre-amplification were 40 cycles of 30 s at 96˚C, 30 s at 60˚C, and 2 min at 72˚C, with an expected amplicon size of about 1600 base pairs. The GNI-D1 pre-amplification did not produce enough product for proper Sanger sequencing, so an internal amplification was utilized to increase products to a concentration adequate for sequencing. The results of the

GNI-D1 pre-amplification reaction were diluted 1:100 with deionized water to be used as template DNA for the internal amplification reaction. The internal amplification of *GNI-D1* targeted all exons of the gene, utilizing primers *GNI*-A1EPF and *GNI*-DexR (Table S1; Sakuma et al., 2019). The reaction mixture followed the same formula as stated above, with pre-amplification dilutions used in place of genomic DNA. PCR conditions for the *GNI-D1* internal amplification were 40 cycles of 30 s denaturation at 96˚C, 30 s of annealing at 55˚C, and 2 min of extension at 72˚C, with an expected amplicon size of about 1500 base pairs. Sanger sequencing using both *GNI*-A1EPF and *GNI*-DexR as sequencing primers was performed by Azenta/GENEWIZ (Azenta US, Inc.). Sequences were aligned and analyzed in SeqMan Pro Version 17 (DNASTAR), with variant nucleotide peak discovery threshold set to 30%.

Once it was determined that only previously reported *GNI-A1* alleles were present among the screened Montana cultivars, all populations created by intercrossing varieties containing the different known alleles were genotyped by sequencing across the *GNI-A1*-105th codon position. (see Figure 1 for a representation of amino acid polymorphism between alleles.)

2.2 | Development of plant material

To determine if there were phenotypic differences in lines containing the *GNI-A1*-105K allele compared to the *GNI-A1*-105N allele, an RIL population segregating for the respective alleles was genotyped and grown in the field in Bozeman, MT, in 2021 and 2022. The RIL population in this study was derived from a cross between cultivars hard red spring Vida (PI642366) and a spring habit version of the hard red winter variety Yellowstone (PI 643428). The spring habit Yellowstone (PI 643428) was created by marker-assisted backcrossing of the spring habit allele of the *VRN-A1* gene into Yellowstone (Bruckner et al., 2007; Cook et al., 2018; Lanning et al., 2006). Vida contains the 105K allele and Spring-Yellowstone contains the 105N allele. Note that 146 different lines were advanced by single seed decent to the F_5 generation and grown in head rows. Bulk samples of $F_{5:6}$ grain from these head rows were grown and genotyped. Seventy-six of these lines were fixed for the *GNI-A1*-105N allele, 66 were fixed for the *GNI-A1*-105K allele, and four were heterogeneous lines.

The RIL population in this study varied for many other traits, so to isolate variability to the gene of interest, NILs from different heterogeneous inbred families (HIFs) (Tuinstra et al., 1997) were generated and grown in the field in Bozeman, MT, in 2022. To create NILs to directly compare the *GNI-A1*-105K and *GNI-A1*-105N alleles, two F_5 Vida/Spring-Yellowstone RILs segregating for *GNI-A1* were selected as HIFs. These families are identified as Vida/Spring-Yellowstone 34 and Vida/Spring-Yellowstone 87. From these F_5 families, several F_6 single plants were grown and genotyped to identify individual plants that were heterozygous for *GNI-A1*-105N and *GNI-A1*-105K alleles. Twenty F_7 seeds from each of the two F_6 heterozygous plants representing the two HIF families were grown. Three plants homozygous for *GNI-A1*-105N and three plants homozygous for *GNI-A1*-105K within each of the two HIF families were randomly selected as NILs. F_8 seed from these individual F_7 plants was increased in Yuma, AZ. $F_{7:9}$ seed was used to plant experimental plots in Bozeman in 2022.

Another set of NILs was generated to confirm phenotypic differences between lines containing the *GNI-A1*-105N and *GNI-A1*-105Y alleles within populations with isolated variability. A Lanning (PI 676978) by Egan (PI 671855) F_4 RIL population was screened for *GNI-A1* alleles. Lanning and Egan are both Montana-adapted hard red spring wheat lines (Blake et al., 2014; Heo et al., 2016). Lanning contains *GNI-A1*-105N, and Egan contains *GNI-A1*-105Y. Four F_4 RIL were found to be segregating for *GNI-A1* and were selected as HIF. These families are identified as Lanning/Egan 44, Lanning/Egan 68, Lanning/Egan 71, and Lanning/Egan 82. NILs were derived from each of these families, as in the Vida/Spring-Yellowstone lines. Three F_6 plants homozygous for each allele were randomly selected from each family. After increase, $F_{6:8}$ seed was used to plant experimental plots in Bozeman in 2022.

This RIL population was grown in 2022 and 2023, while the two groups of NILs were grown in 2022 only. Vida, Spring Yellowstone, Lanning, and Egan, from which these populations were developed, are all modern, high-yielding cultivars adapted to a Montana environment, so while these lines vary morphologically, any linkage effects associated with any of these cultivars would not be considered to have a negative effect on overall grain yield.

2.3 | Field experiment methods

All experiments were grown at the Montana State University Post Agronomy Farm near Bozeman, MT. The Vida/Spring-Yellowstone RIL population was planted in a randomized complete block design with two replications. The RIL trial was planted in two separate but adjacent, rainfed and irrigated experiments in 2021 and 2022. The NILs from Vida/Spring-Yellowstone and Lanning/Egan were grown in a randomized complete block design with two replications in rainfed and irrigated trials. Seeds were sown to a depth of 5 cm. The RIL population plots consisted of two 3-m rows spaced 30 cm apart, and the NIL population plots consisted of four 3-m rows spaced 30 cm apart. Seeding rate for all experiments was 3.3 g of seed per meter of row.

In the 2021 growing season, the irrigated RIL trial was planted on April 21, and the rainfed trial was planted on April 22. Before planting, 289 kg/ha of urea (46-0-0) was applied. Between May 1 and August 31, the post farm received 17.2 cm of precipitation. The highest recorded air temperature across the growing season was 37.2˚C on August 8, and the lowest recorded air temperature was −4.4˚C on May 22 (NOAA, https://www.ncdc.noaa.gov/cdo-web/datasets/GHCND/stations/GHCND:USC00241047/). On June 22 and 28 and July 5, approximately 5 cm of irrigation water was applied using hand line sprinklers. On June 6, 1.75 L/ha of Vendetta (31.7% 3,5-dibromo-4-hydroxybenzonitrile, 34% 2-ethylhexyl ester of 2-methyl-chlorophenoxyacetic acid; Wilbur-Ellis Co.) and 0.77 L/ha of Parity (11.3% fenoxaprop-P-ethyl; Tenkoz Inc.) were applied for disease/weed control.

In the 2022 growing season, the RIL population irrigated and rainfed trials were planted on May 6. The irrigated NIL population trial was planted on May 18, and the rainfed NIL trial was planted on May 27. From May 1 to August 31, the research station received 19.8 cm of precipitation. The highest recorded air temperature across the growing season was 33.8˚C on August 1, and the lowest recorded air temperature was −3.8˚C on May 9. (NOAA, https://www.ncdc.noaa.gov/cdoweb/datasets/GHCND/stations/GHCND:USC00241047/detail). On June 21 and 26 and July 2, approximately 5 cm of irrigation water was applied using hand line sprinklers. Before planting, 174 kg/ha of urea (46-0-0) was applied. On June 3, 0.05 L/ha of Affinity TankMix (40% thifensulfuron-methyl, 10% tribenuron methyl; E.I. DuPont de Nemours and Co.), 0.50 L/ha MCPE (68.7% 2-methyl-4-chlorophenoxyacetic acid isocytl (2-ethylhexyl) ester; Agriliance, LLC), and 1.17 L/ha of Discover (6.4% clodinafop-propargyl; Syngenta Crop Protection LLC) were applied for weed and disease control.

Although heading date, maturity date, flag leaf length, and plant height are not known to be affected by the *GNI1* gene, these traits were measured since they can directly affect grain size and grain number (Ali et al., 2010; Khaliq et al., 2008; Xie et al., 2015). Heading date was recorded for all plots as the number of days after January 1 on which approximately 50% of the primary spikes were emerged. Maturity date was recorded as the number of days after January 1 on which approximately 50% of the peduncles in each plot turned brown. Flag leaf length from the stem to the tip of the leaf, and width at the widest part of the leaf were recorded for three random primary flag leaves in each plot and averaged. Plant height from ground level to the top of the tallest spike (excluding awns) was recorded in two different places in each plot and averaged.

Spike morphology analysis was conducted on primary spikes, and grain traits were analyzed on bulk grain samples. Spike length from the base of the first spikelet to the top of the apical spikelet (excluding awns) was recorded for three spikes in each plot and averaged. Primary spikes were collected randomly from each plot for postharvest analysis. The selection of primary spikes was based on picking representative spikes that exhibited greater height and maturity when compared to other spikes in the plot. Five spikes were collected per plot from the NIL populations, and three spikes were collected per plot from the RIL population. Total spikelet count and sterile spikelet count were recorded for each of these spikes. After all spikelets were counted, the spikes from each individual plot were hand threshed together and grains were collected in a single envelope. A total weight and total grain count were measured for the spikes from each plot. From this data, average grains per primary spike, average grain yield per primary spike, average primary spike single grain weight, average fertile spikelets per primary spike, average grains per fertile spikelet, and average grain yield per fertile spikelet were calculated. Plots were harvested with a Wintersteiger Nurserymaster small plot combine and grain yield was recorded.

2.4 | Grain quality analysis methods

For all samples, whole grain protein and moisture content were measured by near-infrared transmittance using a Foss Infratec 1241 Grain Analyzer. In the RIL population, individual grain weight was calculated using a seed counter and a sample of 200 grains. For the two NIL populations, individual grain weight, grain diameter, and kernel hardness index were measured on a Single Kernel Characterization System 4100 (Perten; AACCI Method 55-31.01). For the two NIL populations, grain samples were analyzed for flour quality. After moisture content analysis on the Foss Infratec 1241 Grain Analyzer, grain was tempered to 14.5% moisture (AACC Method 26-10.02) before milling in a Brabender Quadromat Senior grain mill (C.W. Brabender Instruments) to obtain straight grade flour. Flour ash (FASH) was obtained using the AACC Approved Method 08-01.01. Percent flour yield was calculated by dividing total flour yield by the sum of the total flour, bran, and shorts/middlings × 100. Percent bran was calculated by dividing the total bran yield by the sum of the total flour, bran, and shorts/middlings × 100.

2.5 | Statistical analysis

All response variables for the Vida/Spring-Yellowstone RIL population were analyzed via mixed model analysis of variance using the lme4 package (Bates et al., 2015) in R (R Foundation for Statistical Computing, Version 4.0.5). Each year by experiment within year combination was treated as an environment. The model included environment, block within year, *GNI-A1* allele class, lines within *GNI-A1* allele class, and their interactions with environment. All factors were considered fixed except lines within *GNI-A1* allele class and its interaction with environment which were considered as random effects. The Vida/Spring-Yellowstone NILs and Egan/Lanning NILs were analyzed separately within each of the two backgrounds, but averaged across the HIFs within each background using a mixed linear model with the lme4 package in R. The model for each NIL population included environment, block within environment, HIF, *GNI-A1* allele class, lines within each HIF by *GNI-A1* allele class combination, and interaction with environment except for those with replication within environment. All factors were fixed except for lines within family by *GNI-A1* class combination, and its interaction with environment, which were each considered random effects.

3 | RESULTS

3.1 | Variety screen results

Out of 17 spring wheat varieties, four contained the *GNI-A1*-105N allele, nine contained the *GNI-A1*-105Y allele, and four contained the *GNI-A1*-105K allele. Out of the 15 winter wheat varieties screened, 11 contained the *GNI-A1*-105N allele, four contained the *GNI-A1*-105Y allele, and none contained the *GNI-A1*-105K allele. Durum wheat (*T. durum*) lines were also screened. Two related durum varieties, Carpio (PI 670039) and MT Blackbeard (PI number pending), contained the *GNI-A1*-105N allele, while all other screened durum varieties carried the *GNI-A1*-105Y allele (Elias et al., 2015). (see Table S2 for a list of screened varieties). Sequencing of the *GNI-D1* homeologue across all screened hexaploid varieties did not detect any polymorphism.

3.2 | RIL results

Allelic class means (105N and 105K) combined over all environments are reported for traits in the Vida/Spring-Yellowstone RIL population (Table 1). In the RIL population, plant height, spike length, yield, bulk single grain weight, and primary spike single grain weight all had significant genotype by environment interactions. These interactions were often the result of one of the four environments showing no difference between genotype classes (see Table S3 for data from each separate environment).

Averaged across all environments, lines with the *GNI-A1*-105K allele had longer flag leaves, more grains per spikelet, more grains per primary spike, higher yield per primary spike, and smaller primary spike single grain weight when compared to the *GNI-A1*-105N allele. Heading date and maturity date were similar between the two allele classes.

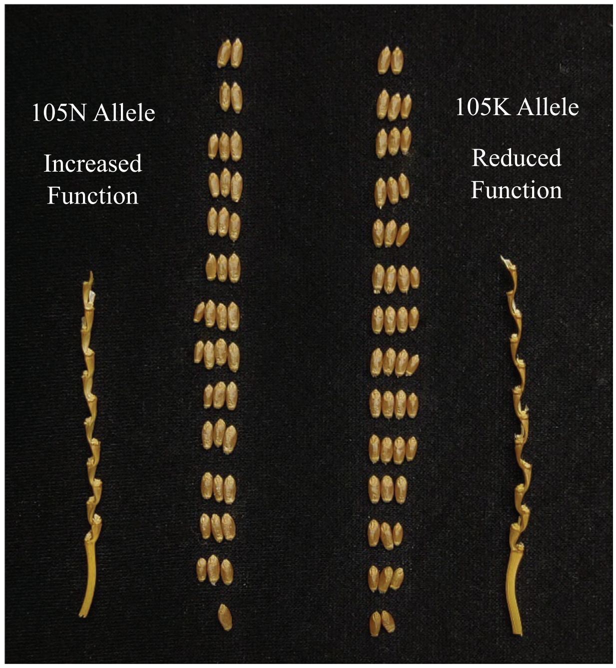

FIGURE 2 Representative spikes from the family Lanning/Egan 68, the heterogeneous inbred family (HIF) family with the largest average difference in spike size between *Grain Number Increase 1* (*GNI-A1*) 105N and 105K allele classes. Primary spikes from different allele classes did not vary in spikelets per spike but did vary in seeds per spikelet resulting in an overall increase in seeds per primary spike in lines containing the *GNI-A1*-105K allele.

3.3 | NIL results

Allelic class means combined over all environments are reported for the two HIF derived NIL populations (Tables 2 and 3; see Table S4 for data from each separate environment). The magnitude of difference between allele classes of some traits is different among the different NIL populations, indicating that genetic background has an influence on the phenotypic differentiation between alleles of this gene. The HIF Lanning/Egan 68 showed the largest difference in grains per spikelet between allele classes (Figure 2).

In the NIL populations comparing the *GNI-A1*-105N and *GNI-A1*-105K alleles, the *GNI-A1*-105K allele conferred a 2.8% increase in height ($p < 0.0001$), a 5.7% increase in flag leaf length ($p < 0.05$), a 5.1% increase in grains per spikelet ($p < 0.05$), and a 3.3% decrease in primary spike single grain weight ($p < 0.1$). Heading date and maturity date were similar between genotypes within each NIL population. There was a significant genotype by environment interaction for primary spike single grain weight. These data are consistent with findings in the RIL population. The *GNI-A1*-105K allele conferred an increase in milling yield by 1.2% ($p < 0.001$) and a decrease in bran percent by 0.8% ($p < 0.1$). There was no difference in flour ash content between the two alleles.

TABLE 1 Vida/Spring-Yellowstone recombinant inbred line (RIL) population data, averaged across 2 years and two environments in each year.

GNI-A1 allele	*N*[a]	Height (cm)	Flag leaf length (cm)	Flag leaf width (cm)	Yield (kg/ha)	Protein content (%)	Bulk SGW (mg)
105N	76	87.9 ± 0.65	**17.0 ± 0.34**	1.34 ± 0.02	6213 ± 86	13.9 ± 0.06	30.4 ± 0.3
105K	66	88.9 ± 0.69	**17.7 ± 0.35**	1.33 ± 0.03	6218 ± 89	13.8 ± 0.07	29.9 ± 0.3
Pr(> *F*)		0.26	**0.024**	0.67	0.96	0.10	0.22

GNI-A1 allele	*N*[a]	Spike length (cm)	Fertile spikelets (No./spike)	Grains/spikelet (No./spikelet)	Primary spike SGW (g)	Grains/primary spike (No./spike)	Yield/primary spike (g)
105N	76	8.88 ± 0.1	16.9 ± 0.1	**2.73 ± 0.02**	**33.6 ± 0.3**	**46.1 ± 0.5**	**1.54 ± 0.02**
105K	66	9.02 ± 0.1	17.0 ± 0.1	**2.88 ± 0.02**	**32.7 ± 0.3**	**48.9 ± 0.6**	**1.59 ± 0.02**
Pr(> *F*)		0.20	0.55	**<0.0001**	**0.041**	**<0.001**	**0.020**

Note: Values represent the average for each genotype ± standard error. RILs with the *GNI-A1*-105K allele had more grains per spikelet, and slightly reduced primary spike single grain weight, although this did not translate to an overall grain yield increase. Bold values indicate *Pr* > *F*.

Abbreviation: SGW, single grain weight.

[a]The number of lines in each genotype class.

TABLE 2 Vida/Spring-Yellowstone heterogeneous inbred family (HIF)-derived near isogenic line (NIL) population data, averaged across two environments.

GNI-A1 allele	*N*[a]	Height (cm)	Flag leaf length (cm)	Flag leaf width (cm)	Yield (kg/ha)	Protein content (%)	Bulk SGW (mg)
105N	6	**82.4 ± 0.4**	17.6 ± 0.3	1.30 ± 0.02	4465 ± 127	16.6 ± 0.09	30.0 ± 0.5
105K	6	**84.7 ± 0.3**	18.6 ± 0.3	1.28 ± 0.02	4543 ± 124	16.5 ± 0.10	29.7 ± 0.5
Pr(> *F*)		**<0.0001**	0.039	0.59	0.60	0.42	0.69

GNI-A1 allele	*N*[a]	Spike length (cm)	Fertile spikelets (No./spike)	Grains/spikelet (No./spikelet)	Primary spike SGW (cm)	Grains/primary spike (cm)	Yield/primary spike (g)
105N	6	8.88 ± 0.1	13.5 ± 0.1	**2.53 ± 0.03**	30.5 ± 0.3	**34.4 ± 0.6**	1.05 ± 0.02
105K	6	9.02 ± 0.1	13.9 ± 0.1	**2.66 ± 0.03**	29.9 ± 0.3	**36.9 ± 0.6**	1.10 ± 0.02
Pr(> *F*)		0.20	0.089	**0.011**	0.062	**0.011**	0.13

GNI-A1 allele	*N*[a]	Milling yield (%)	Flour protein (%)	Flour ash (%)	Bran (%)	Kernel diameter (mm)	Kernel hardness (KHI)
105N	6	68.1 ± 0.3	15.2 ± 0.08	0.50 ± 0.004	27.7 ± 0.27	2.65 ± 0.03	79.0 ± 1.2
105K	6	69.3 ± 0.2	15.0 ± 0.08	0.50 ± 0.004	26.9 ± 0.27	2.62 ± 0.03	77.4 ± 1.2
Pr(> *F*)		<0.001	0.14	0.52	0.07	0.47	0.36

Note: Values represent the average for each genotype ± the standard error. Height, grains per spikelet, grains per primary spike, and milling yield were all increased in Near Isogenic Lines containing the *GNI-A1*-105K allele when compared to the *GNI-A1*-105N allele. Bold values indicate *Pr* > *F*.

Abbreviation: KHI, kernel hardness index; SGW, single grain weight.

[a]The number of lines in each genotype class.

In the NIL populations comparing the *GNI-A1*-105N and *GNI-A1*-105Y alleles, the *GNI-A1*-105Y allele conferred a 5.5% increase in grains per spikelet ($p < 0.01$) and a 1.9% decrease in primary spike single grain weight ($p = 0.1$). However, this population did not show differences in plant height or leaf length. There were no significant genotype by environment interactions for the NIL populations comparing the *GNI-A1*-105N and the *GNI-A1*-105Y alleles. The 105Y allele conferred a decrease in milling yield by 0.5% ($p = 0.01$) and an increase in bran percent by 0.4% ($p < 0.05$). The 105Y allele also conferred a 2% increase in kernel hardness ($p < 0.1$). There was no difference in flour ash content between the two alleles.

4 | DISCUSSION

Previous research using ethyl methanesulfonate mutagenesis populations has shown that *GNI-A1*-105Y confers an increase in grains per spikelet, but there has not been conclusive research on whether similar increases are conferred by the *GNI-A1*-105K allele (Golan et al., 2019; Mizuno et al., 2021;

TABLE 3 Lanning/Egan heterogeneous inbred family (HIF)-derived near isogenic line (NIL) population data, averaged across two environments.

GNI-A1 allele	*N*[a]	Height (cm)	Flag leaf length (cm)	Flag leaf width (cm)	Yield (kg/ha)	Protein content (%)	Bulk SGW (mg)
105N	10	82.0 ± 0.3	18.3 ± 0.3	1.35 ± 0.02	4794 ± 77	16.9 ± 0.09	30.6 ± 0.3
105Y	10	82.7 ± 0.3	18.2 ± 0.3	1.37 ± 0.02	4864 ± 81	16.7 ± 0.09	30.2 ± 0.3
Pr(> *F*)		0.20	0.83	0.38034	0.60	0.18	0.33

GNI-A1 genotype	*N*[a]	Spike length (cm)	Fertile spikelets (No./spike)	Grains/spikelet (No./spikelet)	Primary spike SGW (mg)	Grains/primary spike (No./spike)	Yield/primary spike (g)
105N	10	8.88 ± 0.1	14.5 ± 0.1	**2.90 ± 0.03**	31.4 ± 0.3	**42.1 ± 0.6**	1.32 ± 0.02
105Y	10	9.02 ± 0.1	14.6 ± 0.1	**3.06 ± 0.04**	30.8 ± 0.3	**44.5 ± 0.7**	1.36 ± 0.02
Pr(> *F*)		0.20	0.64	**0.0094**	0.10	**0.025**	0.14

GNI-A1 allele	*N*[a]	Milling yield (%)	Flour protein (%)	Flour ash (%)	Bran (%)	Kernel diameter (mm)	Kernel hardness (KHI)
105N	10	**69.79 ± 0.08**	15.4 ± 0.1	0.47 ± 0.004	26.5 ± 0.1	2.67 ± 0.1	80.7 ± 0.6
105Y	10	**69.31 ± 0.09**	15.4 ± 0.1	0.48 ± 0.004	26.9 ± 0.1	2.68 ± 0.1	82.3 ± 0.6
Pr(> *F*)		**0.010**	0.44	0.20	0.018	0.91	0.083

Note: Values represent the average for each genotype ± the standard error. Grains per spikelet and grains per primary spike were increased, and milling yield was slightly reduced in Near Isogenic Lines containing the *GNI-A1*-105K allele when compared to the *GNI-A1*-105N allele. Bold values indicate $Pr > F$.

Abbreviation: KHI, kernel hardness index; SGW, single grain weight.

[a]The number of lines in each genotype class.

Sakuma et al., 2019). This research confirms published grain number increase effects of the 105Y allele using NIL populations, although the differences observed were relatively small, and no significant yield increase was observed. *GNI-A1*-105K lines had more grains per spikelet when compared with *GNI-A1*-105N across all environments and populations. This indicates that the *GNI-A1*-105K allele is most likely a decreased *GNI-A1* functional allele that results in lower instances of floret abortion resulting in more grains per spikelet, similar to the *GNI-A1*-105Y allele, although analysis of *GNI-A1* transcriptional activity between alleles would need to be done to confirm the genetic basis of these findings.

There was an observed difference in the single grain weight of primary wheat spikes between lines with different *GNI-A1* alleles. However, there was a much smaller difference observed in bulk sample single grain weight, representing grains from all harvested spikes. The bulk grain size results are consistent with (Sakuma et al., 2019) which showed that *GNI-A1* RNAi induced knockouts increased grain number per spike but did not show a decrease in grain size. Inconsistencies between primary head and bulk sample grain size indicate a limitation to this study, as the differences caused by *GNI-A1* variation may not be as strongly realized in secondary spikes as opposed to primary spikes (Tables 2 and 3). A more complete analysis of secondary spikes may be needed to determine the impacts of *GNI-A1* more generally. No significant yield differences were seen in these experiments, although the natural mutant alleles may be associated with a slight upward trend in yield. The lack of significant overall yield increase, despite an increase in grain number per spike without a large reduction in grain size, could be because of a smaller change in grains per spikelet in secondary spikes, or could be because of a slight decrease in productive tiller number which was not measured. In addition, the variety screen showed that these three naturally occurring alleles are all relatively evenly distributed across different spring wheat varieties in Montana, which indicates that this locus has not been subjected to selection pressure. Thus, it would make sense that none of these alleles would confer a significant yield advantage in the studied environment.

In general, single grain weight is negatively correlated with protein content (Joppa et al., 1997; Tabbit, 2017). However, this correlation was not observed between allele classes in this study. Although a downward trend in single grain weight was detected when comparing the *GNI-A1*-105N allele to either the 105Y or 105K allele, an increase in protein content was not observed. This suggests that manipulating grain number through the *GNI-A1* gene may not affect protein content. Milling results varied between different NIL populations and are most likely due to different linkages between populations containing the two alleles and do not seem to correlate with changes in grain size. This could be a result of variability in milling and grain quality between the parent lines from which these populations are derived.

Small differences in overall plant architecture (height and leaf length) were also detected between genotypes in this study. Lines with the 105K allele have longer flag leaves by up to 1 cm when compared against lines with the 105N allele (Tables 1 and 3). Longer flag leaves in lines with the 105K allele is somewhat consistent with the finding that barley lines with lower functioning *VRS-1* alleles have more leaf veins and larger flag leaves (Thirulogachandar et al., 2017). However, the observed architectural differences could be an artifact of genetic linkage.

No detected polymorphism in the *GNI-D1* homoeologue is consistent with other screens, and QTL studies have not detected a phenotypic effect originating from the *GNI-D1* locus (Li et al., 2023; Lin et al., 2021). However, *GNI-D1* is expressed at around one-fourth the levels of *GNI-A1* in developing spikes, indicating that expression of the *GNI-D1* homoeologue on the D genome of spring wheat could also result in enough remaining GNI protein function to mask any large differences. If the natural mutant alleles of *GNI-A1* do result in decreased function and not a total loss of function, full knockout of function alleles of *GNI-A1* and potentially *GNI-D1* resulting from mutation breeding or suppressing function of the gene using RNA interference (RNAi) may be necessary to realize the maximum grain number advantages as a direct result of the manipulation of this gene.

This study, along with a QTL study by Cao et al. (2020), shows that the *GNI-A1* locus is associated with changes in grains per spike, but does not alter yield in elite backgrounds (Cao et al., 2020). *GNI-A1*, along with other genes that increase yield potential by increasing grain number per spike, are most likely subject to epistatic interactions that cancel out yield benefits, especially in environments that commonly experience terminal drought conditions during grain fill when this yield benefit would be potentially realized (Reynolds et al., 2022; Serrago et al., 2013; Slafer et al., 2023; Snowdon et al., 2021). However, combining alleles of *GNI-A1* that increase grain number with alleles of other genes that positively impact grain size may be the next step in increasing overall grain yields (Calderini et al., 2021; Tillett et al., 2022).

5 | CONCLUSION

In spring wheat, both the 105K and the 105Y alleles of *GNI-A1* confer more grains per wheat spikelet versus the 105N ancestral allele. This increase comes with a slight decrease in single grain weight, and no significant increase in overall grain yield. These alleles do not have a consistent effect on grain protein and milling traits, and thus can be selected for without decreasing grain quality.

AUTHOR CONTRIBUTIONS

C. O. Hale: Conceptualization; data curation; formal analysis; investigation; methodology; writing—original draft; writing—review and editing. **B. J. Tillett**: Conceptualization; formal analysis; investigation; writing—review and editing. **J. M. Martin**: Data curation; formal analysis; investigation; methodology; supervision; writing—review and editing. **A. C. Hogg**: Methodology; writing—review and editing. **J. P. Cook**: Methodology. **M. J. Giroux**: Conceptualization; formal analysis; funding acquisition; methodology; project administration; resources; supervision; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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