Unleashing floret fertility in wheat through the mutation of a homeobox gene

Shun Sakuma, Guy Golan, Zifeng Guo, Taichi Ogawa, Akemi Tagiri, Kazuhiro Sugimoto, Nadine Bernhardt, Jonathan Brassac, Martin Mascher, Goetz Hensel, Shizen Ohnishi, Hironobu Jinno, Yoko Yamashita, Idan Ayalon, Zvi Peleg, Thorsten Schnurbusch, and Takao Komatsuda

*Agrogenomics Research Center, National Institute of Agrobiological Sciences, 305-8602 Tsukuba, Japan; †Independent HEISENBERG Research Group Plant Architecture, Leibniz Institute of Plant Genetics and Crop Plant Research, 06466 Gatersleben, Germany; College of Agriculture, Tottori University, 680-8553 Tottori, Japan; ‡The Robert H. Smith Institute of Plant Sciences and Genetics in Agriculture, The Hebrew University of Jerusalem, 7610001 Rehovot, Israel; §Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, 305-8518 Tsukuba, Japan; ‡Institute of Crop Science, National Agriculture and Food Research Organization, 305-8518 Tsukuba, Japan; †Research Group Experimental Taxonomy, Leibniz Institute of Plant Genetics and Crop Plant Research, 06466 Gatersleben, Germany; †Independent Research Group Domestication Genomics, Leibniz Institute of Plant Genetics and Crop Plant Research, 06466 Gatersleben, Germany; ‡Hokkaido Agricultural Experiment Station, Hokkaido Research Organization, 099-1496 Kunneppu, Japan; ‡Central Agricultural Experiment Station, Hokkaido Research Organization, 068-1395 Naganuma, Japan; and †Institute of Agricultural and Nutritional Sciences, Faculty of Natural Sciences III, Martin Luther University Halle-Wittenberg, 06120 Halle, Germany

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Floret fertility is a key determinant of the number of grains per inflorescence in cereals. During the evolution of wheat (*Triticum* sp.), floret fertility has increased, such that current bread wheat (*Triticum aestivum*) cultivars set three to five grains per spikelet. However, little is known regarding the genetic basis of floret fertility. The locus *Grain Number Increase 1* (*GNI1*) is shown here to be an important contributor to floret fertility. *GNI1* evolved in the Triticeae through gene duplication. The gene, which encodes a homeodomain leucine zipper class I (HD-Zip I) transcription factor, was expressed most abundantly in the most apical floret primordia and in parts of the rachilla, suggesting that it acts to inhibit rachilla growth and development. The level of *GNI1* expression has decreased over the course of wheat evolution under domestication, leading to the production of spikes bearing more fertile florets and setting more grains per spikelet. Genetic analysis has revealed that the reduced-function allele *GNI-A1* contributes to the increased number of fertile florets per spikelet. The RNAi-based knockdown of *GNI1* led to an increase in the number of both fertile florets and grains in hexaploid wheat. Mutants carrying an impaired *GNI-A1* allele out-yielded WT allele carriers under field conditions. The data show that gene duplication generated evolutionary novelty affecting floret fertility while mutations favoring increased grain production have been under selection during wheat evolution under domestication.

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Data deposition: Gene sequences generated in this study have been deposited in the DNA Data Bank of Japan (DDBJ) (accession nos. AB711370-AB711394 and AB711888-AB711913) and in the NCBI GenBank database (accession nos. MH134465-MH134483). The RNA-seq data have been deposited in the European Nucleotide Archive (accession nos. PRJEB25119).

1To whom correspondence may be addressed. Email: ssakuma@tottori-u.ac.jp, schnurbusch@ipk-gatersleben.de, or takaodafffr.go.jp.

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**Significance**

Grain number is a key determinant of cereal grain yield, but its underlying genetic basis in wheat remains undefined. This study demonstrates a direct association between increased floret fertility, higher grain number per spike, and higher plot yields of field-grown wheat. The *GNI1* gene, encoding an HD-Zip I transcription factor, was identified as responsible for increased floret fertility. The WT allele acts specifically during rachilla development, with its product serving to lower grain yield potential; in contrast, the reduced-function variant increased both floret and grain number. *GNI1* evolved through gene duplication in the Triticeae, and its mutations have been under parallel selection in both wheat and barley over the course of domestication.
set one or two grains per spikelet, tetraploid wheats two or three, and hexaploid wheats more than three (Fig. 1 B–H) (10).

The genetic diversity of grass inflorescences determines its reproduction and therefore, the resulting number of branches, flowers, and grains (11). Grass inflorescences take the form of either racemes (a single central monopodial axis), panicles (primary and secondary branches), or spikes (lacking a pedicel). Following the domestication of the cereals, inflorescence architecture has been improved by encouraging the formation of a higher number of reproductive branches (spikelets) (12). Since inflorescence architecture is a target for selection, a better understanding of the genetic mechanisms underlying spikelet development may help increase cereal grain yield. Floret number per spikelet is a major determinant of spikelet architecture. The spikelets of rice (Oryza sativa), barley, sorghum (Sorghum bicolor), and maize (Zea mays) are classified as determinate. They produce one floret each in rice and barley, but two in sorghum and maize. On the other hand, an indeterminate number of florets per spikelet are produced by both wheat and oat (Avena sativa). Sterile florets are a common feature, independent of spikelet determinacy; thus, there are two lateral florets formed in two-rowed barleys, a lower floret in maize and sorghum, and several apical florets in wheat and oat.

Recent studies have suggested that wheat grain yield is affected more by variation in grain number per spike than by variation in grain size (13, 14). A number of quantitative trait loci (QTL) affecting grain number per spike have been mapped in wheat; however, the gene(s) underlying these loci have yet to be identified (15–18). Genome-wide association analyses of European winter bread wheats have revealed a QTL responsible for an enhanced grain number per spikelet on chromosome arm 2AL (19); however, the underlying gene is unknown. The present study investigated natural variation for grain number per spikelet in polyploid wheats and their wild relatives and identified a gene underlying floret fertility and grain number. Additionally, the evolutionary trajectory of floret fertility in wheats was explored.

Results

Cloning of a QTL for Grain Number per Spikelet. To reveal the genetic basis of the number of fertile florets formed per spikelet, a population of recombinant inbred substitution lines (RISLs), derived from a cross between durum wheat cultivar (cv.) Landon (LDN) and the line DIC-2A, were characterized. DIC-2A harbors a copy of chromosome 2A inherited from the wild emmer wheat accession ISR-A in the genetic background of LDN (20); it produced an average of two grains per spikelet whereas LDN produced 2.4 (Fig. 2A). The increased grain number per spikelet in LDN was largely driven by the higher number of grains set in the basal and central parts of the spike (Fig. 2B). A single major QTL, associated with a log_{10} odds (LOD) score of 18.71 was mapped to chromosome 2AL; it accounted for 61% of the phenotypic variance (Fig. 2C). To further narrow the target genomic region, a backcross recombinant line population was developed, which allowed the locus to be mendelized as the gene Grain Number Increase 1-A (GNI-A1) (Fig. 2D). Fine mapping located GNI-A1 within a 5.4-Mbp region which harbors 26 putative genes, including one encoding an HD-Zip I transcription factor, the closest wheat homolog to the barley Six-rowed spike 1 gene (vrs1) (SI Appendix, Table S1) (21). A sequence comparison of the two parental GNI-A1 alleles revealed a polymorphism responsible for a single amino acid substitution (N105Y: 105 asparagine to tyrosine) within the highly conserved homeodomain (Fig. 2E). The recombinant plants carrying the LDN allele (generating the 105Y variant) displayed a significantly higher grain number per spikelet than those carrying the DIC-2A allele (105N variant, ancestral) (Fig. 2F). Notably, the mutation in LDN was identical to that found in the barley six-rowed spike mutant Int-d-41 allele at vrs1 (21), suggesting that the function of the resulting HD-Zip I protein was lost or attenuated in LDN.

To verify the inhibitory role, GNI-A1 was silenced using RNA interference (RNAi). The relevant RNAi construct was transformed into two hexaploid wheats, which carries the 105N allele. Four independent transgenic events were obtained: All of the plants produced a higher number of florets and of grains per spikelet on average than did sibling construct-negative plants (Fig. 2 G and H). A decreased abundance of GNI1 transcript was associated with the presence of each of the four transgenes (Fig. 2H). These results supported the hypothesis that a functional copy of GNI-A1 inhibits floret development in wheat. No significant effect of the transgene was observed for plant height, spike number, spike length, spikelet number, or grain size, indicating that the gene’s function is likely spatially specific (SI Appendix, Fig. S1).

The Reduced-Function Allele of GNI-A1 Enhances Yield. The Japanese high yielding bread wheat cv. Kitahonami carries the GNI-A1 allele which encodes the 105Y variant; it sets on average 4.26 grains per spikelet (Fig. 2I). Pedigree analysis revealed that cultivars carrying the 105Y variant produced a significantly higher number of grains per spikelet than did those encoding either the 105N or the 105K variant (4.03 vs. 3.21 and 3.07 grains per spikelet, respectively) and that the 105Y allele in cv. Kitahonami probably arose from the United Kingdom bread wheat cv. Norman (SI Appendix, Fig. S2). TILLING of cv. Kitahonami generated a number of heterozygous M2 plants harboring a Y105N mutation. As predicted, 105N homozygous progeny of these plants produced significantly fewer florets per spikelet and grain per spike than did 105Y homozygous progeny, as well as a lower grain weight per plant (Fig. 2I). The change in grain number per spikelet was mainly confined to the basal and central parts of the spike, as was also the case in tetraploid wheat. No significant variation was associated with the GNI-A1 allele for other traits (SI Appendix, Fig. S3). Thus, the N105Y mutation appears to contribute to a high number of grains per spikelet due to its ensuring a lower rate of apical floret abortion.

Yield tests were conducted to investigate the effect of the GNI-A1 allele on grain yield in the field (SI Appendix, Fig. S4). The performance of M2-derived cv. Kitahonami TILLING (encoding either the 105N or the 105Y variants) was compared at a site in both Kitami and Naganuma (Hokkaido, Japan), with four replications at the former site and three at the latter. Plants carrying the 105Y allele enjoyed a yield advantage of 10 to 30% at both sites. Their grain number per spike was slightly increased, but there was no change with respect to either grain size (1,000
grain weight) or the number of spikes per plant (SI Appendix, Fig. S4). The biomass of plants carrying the 105N variant was significantly lowered at Kitami. The indication was that the GNI-A1 allele made a positive contribution to grain yield.

**GNI1 Transcript Accumulates in the Distal End of the Spikelet and the Rachilla.** GNI1 mRNA was localized using in situ hybridization in the spikelet meristem of einkorn wheat (*T. monococcum*), which generates floret meristems on its lower flank (Fig. 3 A–F). Einkorn wheat was selected for this experiment because it exhibits a particularly high abundance of the GNI1 transcript (Fig. 3G). Following the differentiation of the second and third floret primordia during the terminal spikelet stage, GNI1 transcripts were detected in the spikelet meristem and the rachilla bearing the florets and floret primordia, except for the first floret, which is usually fertile (Fig. 3 C–E). Thus, as development progressed, GNI1 transcription was diminished in the more basal florets within a spikelet. These observations implied that GNI1 expression inhibited apical floret...
development at the distal end of the spikelets and in part rachilla growth and development.

Quantitative real-time PCR (qRT-PCR) analysis revealed that GNI-A1 was predominantly transcribed in immature spikes (Fig. 3 G–I). Transcript abundance peaked between the white and the green anther stages in tetraploid and hexaploid wheats, corresponding to the presence of the maximum number of floret primordia (6). A minor difference in GNI-A1 transcript level was observed between tetraploid wheats carrying the 105N and those carrying the 105Y allele. The result suggests that the N105Y change is a causal mutation and not linked with regulatory change (Fig. 3H). A slightly higher expression in the 105Y allele could be the result of a mild negative autoregulation. The abundance of the GNI-B1 transcript was negligible in the floral organs of both tetraploid and hexaploid wheat, despite its nucleotide sequence being very similar to that of GNI-A1 (Fig. 3 H and I and SI Appendix, Fig. S5). The abundance of the GNI-D1 transcript was lower than that of GNI-A1 (Fig. 3I).

RNA-seq profiles were generated from contrasting allelic forms selected in the cv. Kitahonami TILLING population to gain a better understanding of the molecular basis of the effect of the GNI-A1 mutation. The abundance of the GNI-A1 transcript was slightly higher in plants carrying the 105Y than in those carrying the 105N allele (SI Appendix, Fig. S6B), supporting the notion that suppression of grain number by the 105N allele is not caused by the expression abundance of GNI-A1. The results revealed that genes involved in nitrogen and sucrose metabolism, as well as in G protein beta/gamma-subunit complex binding, were more strongly transcribed in plants carrying the 105Y variant, consistent with the increased grain number associated with this allele (SI Appendix, Fig. S6D and Datasets S1 and S2). Consistent also with the improved floret fertility shown by the 105Y variant, there was a greater abundance of transcript generated from the Flowering locus T homolog FT-D1 in the 105Y spike although there was no evidence for the differential transcription of either of its homoeoalleles FT-A1 and FT-B1, either at white or green anther stages (SI Appendix, Fig. S6C). Inspection of a public RNA-seq database showed that similar profiles have been reported elsewhere (SI Appendix, Fig. S7). Given that the product of FT1 likely acts as a floral promoting factor during early floret development, the differential transcription of FT-D1 may represent the consequence of a greater level of floral activity occurring in plants carrying the GNI-A1 105Y allele. These plants reached heading on average 3 to 5 d earlier than those carrying the GNI-A1 105N allele, but there was no difference evident in the days required to reach maturity.

Allelic Variation of GNI-A1 in Wheat. Natural variation within the GNI-A1 locus was investigated by resequencing the allele present in a set of 72 tetraploid wheats, including both wild and domesticated emmer and durum wheat entries. The analysis revealed nine haplotypes (Fig. 4A and SI Appendix, Table S2). The 105Y variant was restricted to the durum wheat entries, which featured a significantly higher number of grains per spikelet than the emmer wheat entries (Fig. 4B). When the number of grains per spikelet was measured in plants grown in three different environments it was clear that lines carrying the 105Y allele consistently produced a higher number: the broad-sense heritability of the trait was 0.8, and the trait was stably expressed, even in the relatively low-yielding environment of Ruhama (Fig. 4C). Resequencing among a panel of 210 European winter bread wheat cultivars (19) revealed three haplotypes: Hap1 and -2 included the 105N variant while Hap3 included the 105Y allele (Fig. 4D and SI Appendix, Table S3). The number of florets present at the green anther stage, indicative of the potential maximum grain number, did not differ significantly among the three haplotypes (Fig. 4E). Cultivars carrying Hap3 produced more grains per spikelet at apical and central positions of the spike, as well as on average (2.97, 3.95, and 3.52, respectively), compared with cultivars carrying Hap1 (2.45, 3.58, and 3.18, respectively) and Hap2 (2.53, 3.64, and 3.22, respectively) (Fig. 4F). Hap3 cultivars produced more grains per spike and exhibited a higher spike fertility index. Their ratio of spike dry weight to stem dry weight was also higher, due to their investment of less biomass in the production of stem and leaf tissue (SI Appendix, Fig. S8).

Evolution of GNI1 in Triticeae. To clarify the evolution of GNI1 (i.e., HOX1) in the Triticeae, the gene was resequenced in a diverse collection of wild species. All 14 genera examined harbored a HOX2 homolog, in line with the known conservation of this gene among the grasses (22). Only Hordeum, Dasypyrum, Secale, Taeniatherum, Aegilops, Amphilopsyn, and Triticum species...
The function of in diploid, tetraploid, and hexaploid Triticeae
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suggests that, in S genome Aegilops species, these genes are likely to have been converted to pseudogenes whereas those in the diploid Triticum (A genome) and Hordeum (H genome) species have remained functional (27). The GNII/Vrs1-mediated floral changes are consistent with the idea of the genetic hotspot hypothesis, in which evolutionary relevant mutations tend to accumulate in specific genes and at specific positions within genes (29). In the case of GNII/Vrs1, the coding sequence changes and loss-of-function alleles were selected in parallel. This observation can have important contributions to morphological evolution in opposition to the prevailing regulatory evolution view that regulatory changes, which minimize pleiotropic effects while simultaneously promoting adaptation, are important (30).

The single amino acid substitution (N105Y) present in a conserved domain of GNI-A1, which has led to a reduction in functionality, was selected post the divergence of durum wheat. A previous study has demonstrated that six-rowed barley originated from the domesticated two-rowed type via mutations in Vrs1 (21). Together, these observations suggest that mutations for increased grain number in wheat and barley have undergone parallel selection post domestication (31). The very high allele frequency (96%) of the 105Y allele among durum wheats reflects strong selection pressure toward increased grain number; meanwhile, its absence in both wild and domesticated emmer wheat germplasm implies that the mutation has some negative effect on fitness. During the evolution and domestication of wheat, the mutant allele probably became increasingly favored in the farming environment since it delivers increased grain yield. In European winter bread wheat germplasm, the 105N and 105Y alleles were represented in a 2:1 ratio, suggesting that the latter allele probably entered breeding populations only relatively recently. The high heritability and stability of the 105N allele (Fig. 4C and SI Appendix, Fig. S4) underlie the potential utility of the 105Y allele to increase grain yield in wheat breeding programs around the world.

Materials and Methods
details on plant materials, QTL mapping, fine mapping, transformation, tillering, yield trials, quantitative RT-PCR, in situ hybridization, RNA-seq, haplotype analysis, and phylogenetic analysis are in SI Appendix and Materials and Methods. Gene sequences generated in this study are available from the DNA Data Bank of Japan (DDBJ) under accession numbers AB711394 (http://getentry.ddbj.nig.ac.jp/getentry/na/AB711394) and from the NCBI GenBank under accession numbers MH134165–MH134483. The RNA-seq data have been submitted to the European Nucleotide Archive under accession number PRJEB25119.

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