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A NAC gene regulating senescence improves grain protein, *Zn*, and *Fe* content in wheat

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Abstract

Enhancing the nutritional value of food crops is a means for improving human nutrition and health. We report here the positional cloning of *Gpc-B1*, a wheat quantitative trait locus associated with increased grain protein, *Zn*, and *Fe* content. The ancestral wild wheat allele encodes a NAC transcription factor (*NAM-B1*) that accelerates senescence and increases nutrient remobilization from leaves to developing grains, whereas modern wheat varieties carry a non-functional *NAM-B1* allele. Reduction in RNA levels of the multiple *NAM* homologs by RNA interference delayed senescence by over three weeks and reduced wheat grain protein, *Zn*, and *Fe* content by over 30%.

The World Health Organization estimates that over 2 billion people have deficiencies in key micronutrients such as *Zn* and *Fe*, and over 160 million children under the age of five lack adequate protein (1); leading to an economic burden for society (2). The two major types of wheat, tetraploid wheats (2n = 28), used for pasta, and hexaploid wheats (2n = 42), used primarily for bread, account for approximately 20% of all calories consumed worldwide. Annual wheat production is estimated at 620 million tons of grain (3), translating into approximately 62 million tons of protein. Increasing grain protein content (GPC) has been hindered by environmental effects, complex genetic systems governing this trait, and a negative correlation with yield (4). Less progress has been made in increasing *Zn* and *Fe* content; the focal point of the HarvestPlus global initiatives (5).

Wild emmer wheat (*Triticum turgidum* ssp. *dicoccoides*), abbreviated hereafter as DIC, is the ancestor of cultivated pasta wheat (*T. turgidum* ssp. *durum*) and a promising source of genetic variation in protein, *Zn*, and *Fe* content (6, 7). A quantitative trait locus (QTL) for

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Materials and Methods
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GPC was mapped on chromosome arm 6BS in a population of recombinant inbred lines derived from the *T. turgidum* ssp. *durum* cultivar Langdon (LDN) and the chromosome substitution line LDN (DIC6B) (8). This locus was associated with GPC increases of approximately 14 $g*kg^{-1}$ in both tetraploid and hexaploid lines (8–10). Olmos *et al.* (11) mapped this QTL as a simple Mendelian locus, *Gpc-B1* (Fig. 1A), which was later located within a 0.3 cM interval (12). Molecular markers *Xuhw89* and *Xucw71* within this region flank a 245-kb physical contig including *Gpc-B1* (13).

Tetraploid and hexaploid wheat lines carrying this 245-kb DIC segment show delayed senescence and increased GPC and grain micronutrients (14–15). The complete sequencing of this region (DQ871219) revealed five genes (Fig. 1B) (16). A high-resolution genetic map, based on approximately 9,000 gametes and new molecular markers (table S1), was used to determine the linkage between these genes and the *Gpc-B1* locus. Three recombinant substitution lines with recombination events between markers *Xuhw106* and *Xucw109* delimited a 7.4-kb region (Fig. 1, C–D) (16). The recombinant lines carrying this DIC segment senesced on average 4 to 5 days earlier ($P<0.01$, Fig. 1, E–F) and exhibited a 10% to 15% increase in GPC (Fig. 1G), *Zn* (Fig. 1H), and *Fe* (Fig. 1I) concentrations in the grain ($P<0.01$). Complete linkage of the 7.4-kb region with the different phenotypes suggests that *Gpc-B1* is a single gene with multiple pleiotropic effects.

The annotation of this 7.4-kb region (Fig. 1C) identified a single gene encoding a NAC domain protein, characteristic of the plant specific family of NAC transcription factors (17). NAC genes play important roles in developmental processes, auxin signaling, defense and abiotic stress responses, and leaf senescence (18, 19). Phylogenetic analyses revealed that the closest plant proteins were the rice gene *ONAC010* (NP_911241) and a clade of three *Arabidopsis* proteins including No Apical Meristem (NAM) (figs. S1–2). On the basis of these similarities, the gene was designated *NAM-B1* (DQ869673). To indicate the species source we have added a two letter prefix (e.g. *Ta* and *Tt* for *T. aestivum* and *T. turgidum* genes, respectively).

Comparison of the parental *TtNAM-B1* sequences revealed a 1-bp substitution within the first intron and a thymine residue insertion at position 11, generating a frame shift mutation in the LDN allele (DQ869674, Fig. 1J). This frame shift resulted in a predicted protein having no similarity to any GenBank sequence and lacking the NAC domain.

The wild type *TtNAM-B1* allele was found in all 42 wild emmer accessions examined (*T. turgidum* ssp. *dicoccoides*) (table S2), and in 17 of the 19 domesticated emmer accessions (*T. turgidum* ssp. *dicoccum*). However, 57 cultivated durum lines (*T. turgidum* ssp. *durum*) (20) (table S3) lack the functional allele suggesting that the 1-bp frame shift insertion was fixed during the domestication of durum wheat. The wild type *TaNAM-B1* allele was also absent from a collection of 34 varieties of hexaploid wheat (*T. aestivum* ssp. *aestivum*), representing different market classes and geographic locations. Twenty-nine of these showed no PCR amplification products of the *TaNAM-B1* gene suggesting that it is deleted, while the remaining five lines have the same 1-bp insertion observed in the durum lines (table S4).

In addition to the mutant *TtNAM-B1* copy, the durum wheat genome includes an orthologous copy (*TtNAM-A1)* on chromosome arm 6AS and a paralogous one (*TtNAM-B2*) 91% identical at the DNA level to *TtNAM-B1* on chromosome arm 2BS (21) (fig. S3, table S5). These two copies have no apparent mutations. Comparisons at the protein level of the five domains characteristic of NAC transcription factors (17) revealed 98% to 100% protein identity (fig. S2) between barley, wheat, rice, and maize homologs.

Quantitative PCR (16) showed transcripts from the three *TtNAM* genes at low levels in flag leaves prior to anthesis, after which their levels increased significantly towards grain maturity (Fig. 2A). Transcripts were also detected in green spikes and peduncles. The similar transcription profiles and near identical sequences of *TtNAM-A1, B1* and *B2* suggest that the 4–5 day delay in senescence and the 10% to 15% decrease in grain protein, *Zn*, and *Fe* content observed in LDN are likely the result of a reduction in the amount of functional protein rather than the complete loss-of-function of a unique gene.

To test this hypothesis, we reduced the transcript levels of all *NAM* copies using RNA interference (RNAi). An RNAi construct (16) was transformed into the hexaploid wheat variety Bobwhite, selected for its higher transformation efficiency relative to tetraploid wheat. The RNAi construct targeted the 3′ end of the four *TaNAM* genes found in hexaploid wheat (*TaNAM-A1, D1, B2* and *D2*), outside the NAC domain, to avoid interference with other NAC transcription factors (fig. S4, table S6) (22).

We identified two independent transgenic plants (L19-54 and L23-119) with an expected stay-green phenotype. Quantitative PCR analysis of transgenic L19-54 plants showed a significant reduction in the endogenous RNA levels of the different *TaNAM* copies (22) at four and nine days after anthesis (Fig. 2, B–C, $P<0.05$) compared to control lines. Transgenic plants reached 50% chlorophyll degradation in flag leaves 24 days later than their non-transgenic sibs (Fig. 2D, $P<0.001$), and their main spike peduncles turned yellow more than 30 days later than the controls (Fig. 2, E–F).

The presence of the RNAi transgene also had significant effects on grain protein, *Zn* and *Fe* concentrations. Transgenic plants showed a reduction of over 30% in GPC ($P<0.001$), 36% in *Zn* ($P<0.01$), and 38% in *Fe* ($P<0.01$) concentration compared to the non-transgenic controls (Table 1). No significant differences were observed in grain size ($P=0.41$), suggesting that the extra days of grain filling conferred by the reduced *TaNAM* transcript level did not translate into larger grains in our greenhouse experiments (23). Similar results were obtained for the second transgenic event, L23-119 (fig. S5, table S7).

These results suggest that the reduced grain protein, *Zn*, and *Fe* concentrations were the result of reduced translocation from leaves, rather than a dilution effect caused by larger grains. This hypothesis was confirmed by analyzing the residual nitrogen (*N*), *Zn*, and *Fe* content in the flag leaves. We analyzed both transgenic events together (due to greater variability in flag leaves compared to the grains) and confirmed higher levels of *N* ($P=0.01$), *Zn* ($P<0.01$), and *Fe* ($P<0.01$) in the flag leaves of transgenic plants compared to the non-transgenic sister lines (table S8). This supports a more efficient *N*, *Zn*, and *Fe* remobilization in plants with higher levels of functional *TaNAM* transcripts.

These results confirm that a reduction in RNA levels of the *TaNAM* genes is associated with a delay in whole plant senescence, a decrease in grain protein, *Zn* and *Fe* concentrations, and an increase in residual *N*, *Zn* and *Fe* in the flag leaf. These multiple pleiotropic effects suggest a central role for the *NAM* genes as transcriptional regulators of multiple processes during leaf senescence, including nutrient remobilization to the developing grain.

The differences observed between the transgenic and non-transgenic plants for these traits were larger than those observed between the LDN and DIC alleles. The RNA interference on all functional *TaNAM* homologs may result in a larger reduction of functional transcripts than the single non-functional *TtNAM-B1* allele in tetraploid recombinant lines carrying the LDN allele.

The cloning of *Gpc-B1* provides a direct link between the regulation of senescence and nutrient remobilization and an entry point to characterize the genes regulating these two processes. This may contribute to their more efficient manipulation in crops and translate into food with enhanced nutritional value.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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21. The paralogous *TtNAM-A2* copy was not detected in the LDN tetraploid BAC library nor by PCR with 2A genome specific primers derived from *T. urartu*, where the *TuNAM-A2* gene is present.
22. The Bobwhite *TaNAM-B1* gene is deleted as determined by PCR with four sets of independent *NAM-B1* specific primers (table S5). Therefore, no expression data is included for *TaNAM-B1* in the transgenic plants.
23. Field experiments including *Gpc-B1* isogenic lines showed a more variable effect of the DIC chromosome region (including *TtNAM-B1*) on grain size (14).

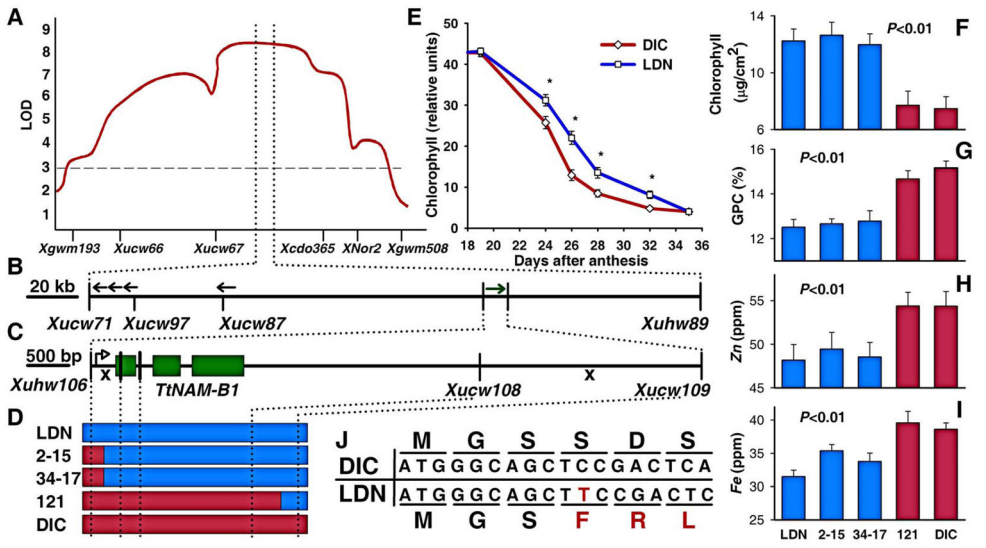

Fig. 1.

Map-based cloning of *Gpc-B1*. (**A**) QTL for grain protein on wheat chromosome arm 6BS (11). (**B**) Sequenced B-genome physical contig. The position and orientation of five genes is indicated by arrows. (**C**) Fine mapping of *Gpc-B1*. The x's indicate the positions of critical recombination events flanking *Gpc-B1*. Vertical lines represent polymorphism mapped in the critical lines. A single gene with three exons (green rectangles) was annotated within the 7.4 kb region flanked by the closest recombination events. The open arrowhead indicates the transcription initiation site. (**D**) Graphical genotypes of critical recombinant substitution lines used for fine-mapping of *Gpc-B1*. Blue bars represent LDN markers; red bars represent DIC markers. (**E**) Flag leaf chlorophyll content of recombinant substitution lines segregating for *Gpc-B1* (14). Asterisks indicate significant differences ($P<0.01$). Phenotypes of critical recombinant substitution lines: (**F**) chlorophyll at 20 days after anthesis (DAA), (**G**) grain protein, (**H**) *Zn*, and (**I**) *Fe* concentrations. Blue and red bars indicate the presence of the LDN and DIC alleles at *TtNAM-B1*, respectively. (**J**) First 18 nucleotides of DIC and LDN *TtNAM-B1* alleles and their corresponding amino acid translation. The LDN allele carries a 1-bp insertion (red T) that disrupts the reading frame (indicated by red amino acid residues). Error bars represent standard error of the means (E–I).

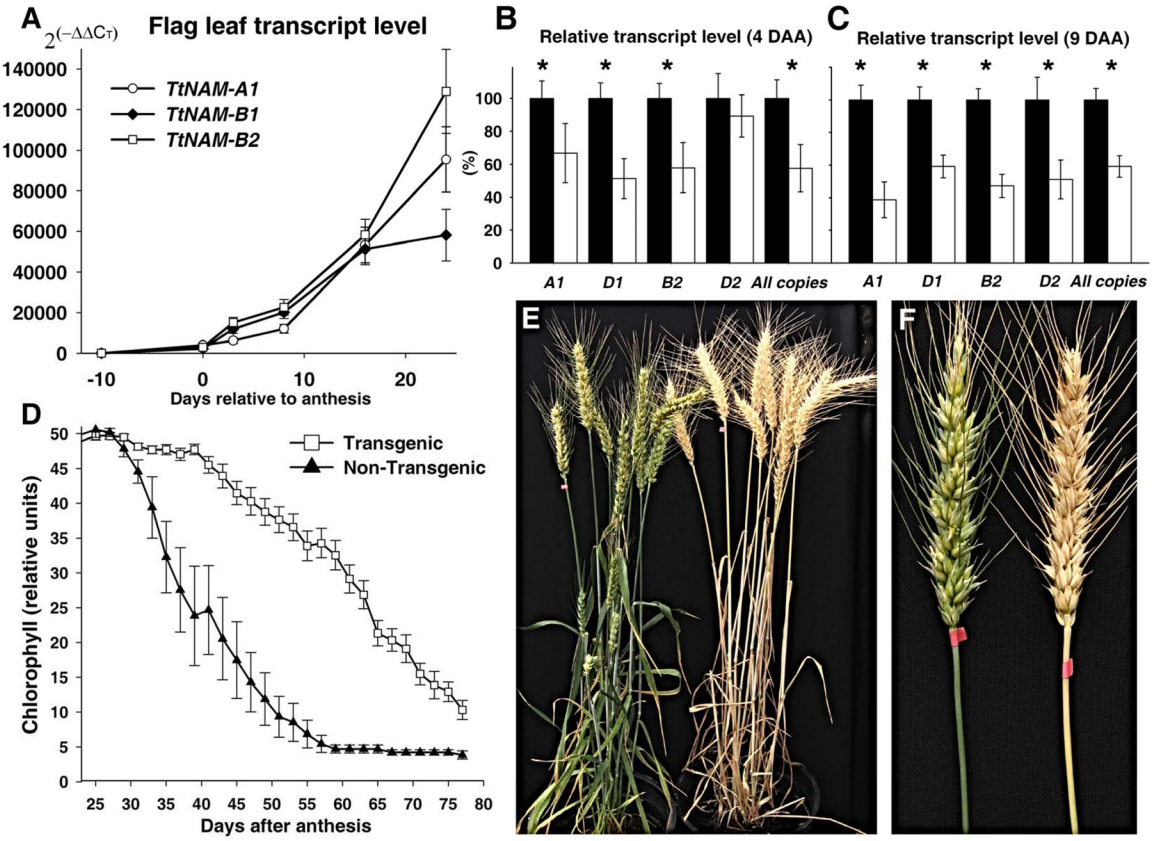

Fig. 2.

(**A**) Expression profile of the different *TtNAM* genes relative to *ACTIN* in tetraploid wheat recombinant substitution line 300 carrying a functional *TtNAM-B1* gene. Units are values linearized with the $2^{(-\Delta\Delta C_T)}$ method, where C_T is the threshold cycle. (**B**) Relative transcript level of endogenous *TaNAM* genes in T_2 plants (L19-54) segregating for transgenic (n = 12, white) and non-transgenic (n = 11, black) *TaNAM* RNAi constructs at 4 and (**C**) 9 days after anthesis. Asterisks indicate significant differences ($P<0.05$). (**D**) Flag leaf chlorophyll content profile of transgenic (n = 22 T_1 plants) and non-transgenic controls (n = 10 T_1 plants). (**E**) Representative transgenic (left) and non-transgenic (right) plants 50 DAA. (**F**) Main spike and peduncles of representative transgenic and non-transgenic plants 50 DAA. Error bars represent standard error of the means.

Table 1

Characterization of grain and senescence related traits of transgenic Bobwhite T_1 plants (event L19-54) segregating for the presence (transgenic, n = 22 plants) or absence (non-transgenic, n = 10 plants) of the *TaNAM* RNAi construct.

	GPC (%)	***Zn* (ppm)**	***Fe* (ppm)**	**TKW* (g)**	**Dry Peduncle (DAA)***	**Dry Spike (DAA)**
Transgenic	13.27	52.45	37.40	30.23	72.5	53.0
Non-transgenic	19.08	82.50	60.83	31.27	38.4	37.2
Difference	−5.81	−30.09	−23.42	−1.04	+ 34.1	+ 15.8
P value	<0.001	<0.01	<0.01	0.41	<0.001	<0.001

* TKW = Thousand kernel weight, DAA = Days after anthesis.