CROP SCIENCE

Biofortification of iron content by regulating a NAC transcription factor in maize

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Iron (Fe) deficiency remains widespread among people in developing countries. To help solve this problem, breeders have been attempting to develop maize cultivars with high yields and high Fe concentrations in the kernels. We conducted a genome-wide association study and identified a gene, *ZmNAC78* (*NAM/ATAF/CUC DOMAIN TRANSCRIPTION FACTOR 78*), that regulates Fe concentrations in maize kernels. We cultivated maize varieties with both high yield and high Fe concentrations in their kernels by using a molecular marker developed from a 42–base pair insertion or deletion (indel) in the promoter of *ZmNAC78*. *ZmNAC78* expression is enriched in the basal endosperm transfer layer of kernels, and the ZmNAC78 protein directly regulates messenger RNA abundance of Fe transporters. Our results thus provide an approach to develop maize varieties with Fe-enriched kernels.

Iron (Fe) is an essential microelement for human health. Fe deficiency occurs often in human diets and affects an estimated 2 billion people, especially infants, young children, and pregnant women (*1*, *2*). The risk of Fe deficiency is much greater in sub-Saharan Africa (*3*, *4*)—where maize is a staple food providing at least 30% of total calorie intake (*5*)—as compared with other regions. A diet high in maize, however, makes people prone to Fe deficiency, and Fe concentrations in maize endosperm are low (*6*). In Zimbabwe, for example, about 30% of pregnant and lactating women suffer from Fe deficiency, which weakens the immune system, stunts growth, and impairs cognitive development (*7*, *8*).

Although supplementation, dietary diversification, and commercial food fortification have been used to increase the micronutrient content of human diets, these measures have been unsatisfactory in developing countries because of low economic sustainability and low consumer acceptability (*9*, *10*). By contrast, biofortification through genetic modification of crops appears to be more promising (*11*). Genes related to Fe uptake and metabolism have been successfully genetically engineered to increase Fe content in edible parts of crops. For example, synergistic expression of *AtNAS1* (*NICTOTIANAMINE SYNTHASE 1*), *PvFERRITIN*, and *AfPHYTASE* increased Fe concentrations in rice endosperm (*12*); endosperm-targeted overexpression of *TaFERRITIN1-A* resulted in a 50 to 85% increase in the Fe content in wheat grain (*13*); and coexpression of a mutated *AtIRT1* (*IRON-REGULATED TRANSPORTER 1*) and *AtFERRITIN1* increased the Fe content in field-grown cassava (*14*).

Developing biofortified maize with high Fe concentrations in the kernels should be an effective way to alleviate Fe deficiency–induced anemia in sub-Saharan Africa, but the development of biofortified maize varieties has been limited. One challenge to biofortifying Fe in maize is that Fe concentrations in grain are negatively correlated with maize yield (*6*, *15*). In addition, the process of Fe loading into maize kernels is almost completely unknown. It is therefore valuable to identify genetic resources that could enhance Fe concentrations in maize kernels without reducing yield.

Results

Identification of ZmNAC78

We determined Fe concentrations in kernels of a maize natural-variation population growing in Sanya, Hainan Province, China. The population consisted of 273 maize inbred lines, including introgression lines, Chinese elite inbred lines [SPT (Sipingtou), LRC (Lvda Red Cob), PA (group A germplasm derived from modern US hybrids in China), PB (group B germplasm derived from modern US hybrids in China), Reid, Lancaster, and Iodent], and inbred lines from the US (table S1). The Fe concentrations in the kernels of this population ranged from 4.90 to 55.18 mg kg^{-1}, with a mean of 24.15 mg kg^{-1} (Fig. 1A and table S1). From this population, we randomly selected 20 inbred lines and planted them in Shunyi, Beijing, to investigate the repeatability of the Fe concentration phenotypes. Fe concentrations in maize kernels are substantially affected by soil conditions (*3*). Although soil properties differ considerably between Sanya (pH 4.9) and Shunyi (pH 8.2), the Fe concentrat in maize kernels produced in Sanya were related with those produced in Shunyi [Pearson's correlation coefficient (R) = 0.83; $P = 5 \times 10^{-6}$] (Fig. 1B).

Using 301,603 single-nucleotide polymorphisms (SNPs) with a minor allele frequency ≥0.05 and a missing rate <10.0% covering the whole maize genome, we conducted a genome-wide association study (GWAS) for Fe concentrations in maize kernels with the general linear model approach controlling population structure. On the basis of a linkage-disequilibrium region [coefficient of determination (R^2) ≥ 0.1] (*16*), a total of 11 SNPs were significantly associated with the Fe concentrations in maize kernels (Fig. 1C). All of the identified candidate genes associated with Fe concentrations in maize kernels are listed in table S2. In the population, Fe concentrations in kernels were significantly negatively correlated with 100-kernel weight (fig. S1A). To detect potential genes regulating kernel Fe concentrations in maize, we performed RNA sequencing (RNA-seq) on six inbred lines with different kernel Fe concentrations but similar 100-kernel weights to reduce bias from bioaccumulation by small kernels (fig. S1B). The RNA libraries yielded a total of >0.32 billion reads after adaptor trimming, and ~91.05% of the clean reads could be perfectly mapped to the maize B73 v4 reference genome (*17*). The abundance of each gene was determined in terms of reads per kilobase per million mapped reads (*18*). A total of 1531 genes differentially expressed between high- and low-Fe inbred lines on the basis of fold-change criteria >1.5 and $P < 0.05$ (*19*, *20*). Among the differentially expressed genes, 857 were up-regulated and 674 were down-regulated in high-Fe lines relative to low-Fe lines (fig. S1C).

We then investigated the mRNA abundances of the 11 candidate genes identified by GWAS in these RNA libraries. Because its expression level was low in all six inbred lines, *Zm00001d027400* was excluded from our analysis. Among the remaining 10 candidate genes, only *Zm00001d027395* [*ZmNAC78* (*NAM/ATAF/CUC DOMAIN TRANSCRIPTION FACTOR 78*)] had consistently higher expression in high-Fe lines compared with low-Fe lines (fig. S2A), and the expression levels of *ZmNAC78* were significantly positively correlated with Fe concentrations in the kernels of 30 randomly selected inbred lines (11 with high Fe concentrations, 4 with medium Fe concentrations, and 15 with low Fe concentrations) (fig. S2B). We therefore inferred that *ZmNAC78* might regulate Fe concentrations in maize kernels.

ZmNAC78 regulates Fe concentrations in maize kernels

We investigated the expression patterns of *ZmNAC78* in the Maize eFP Browser RNA-seq

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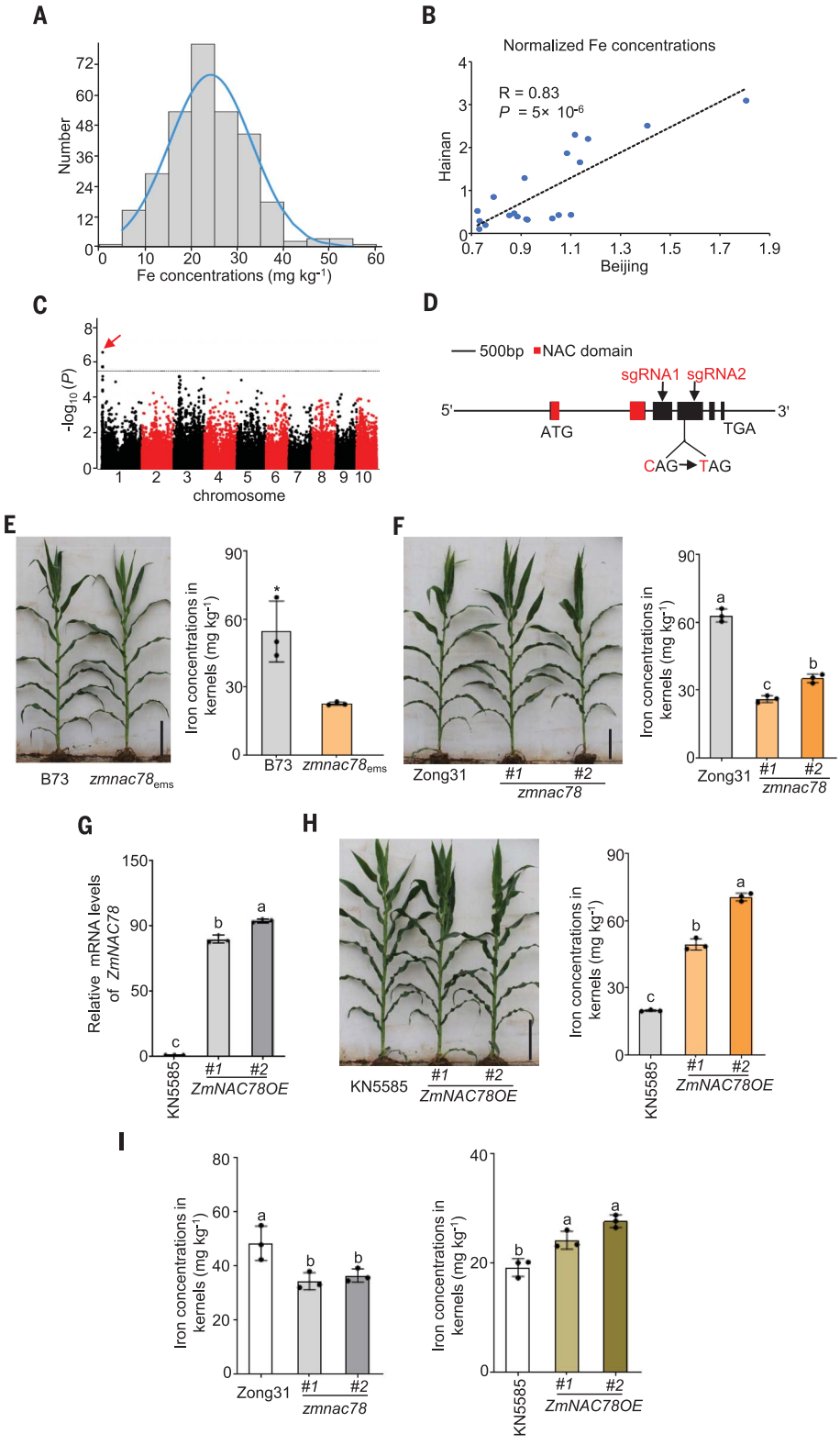

Fig. 1. *ZmNAC78* regulates Fe concentrations in maize kernels. (**A**) Fe concentrations in kernels are consistent with a normal distribution in a maize natural-variation population. (**B**) Pearson's correlation coefficient of Fe concentrations in kernels of randomly selected maize grown in Hainan Province versus randomly selected maize grown in Beijing. n = 20 inbred lines. (**C**) Manhattan plot for the GWAS. The dashed line represents the Bonferroni-adjusted significance threshold ($P = 3.3 \times 10^{-6}$). (**D**) Diagram illustrating the EMS-mutated site and synthetic guide RNAs (sgRNAs). The gene model is from MaizeGDB (*41*). (**E**) Effects of *ZmNAC78* EMS mutation on maize growth and Fe concentrations in kernels. (**F**) Effects of *ZmNAC78* loss of function on maize growth and Fe concentrations in kernels. (**G**) Detection of *ZmNAC78* mRNA abundance in *ZmNAC78OE* transgenic maize. (**H**) Effects of *ZmNAC78* overexpression on maize growth and Fe concentrations in kernels. (**I**) Fe concentrations in kernels of *ZmNAC78* loss-of-function mutants and *ZmNAC78OE* transgenic maize in Beijing. Scale bars in (E), (F), and (H), 30 cm. Error bars in (E) through (I) represent the standard deviation of three biological replicates. Asterisk in (E) indicates significant difference at $P < 0.05$ according to t tests. Means with the same letter in (F) through (I) are not significantly different at $P < 0.05$ according to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

database (*21*) and found that *ZmNAC78* is preferentially expressed in the endosperm at 16 to 24 days after pollination (DAP) (fig. S3A), when nutrients rapidly accumulate in maize kernels. The expression patterns of *ZmNAC78* were verified by reverse transcription quantitative polymerase chain reaction (RT-qPCR) (fig. S3, B and C).

We obtained an ethyl methanesulfonate (EMS) mutant of *ZmNAC78* in the B73 background from the Maize EMS-induced Mutant Database (mutant ID: EMS4-16d24b) (*22*). EMS4-16d24b contains a C/T substitution in the fourth exon of *ZmNAC78*, which leads to a premature stop codon in the gene (Fig. 1D). We designated the mutant as $zmnac78_{ems}$. The mutation in $zmnac78_{ems}$ did not affect maize development (Fig. 1E). However, the Fe concentration in

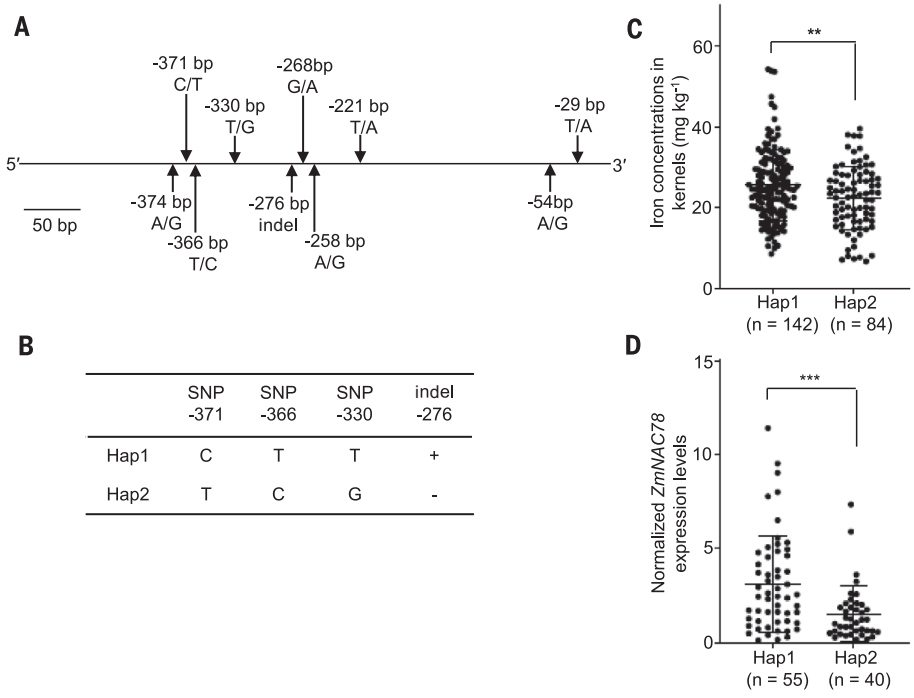

Fig. 2. Natural variation in the *ZmNAC78* core promoter is associated with Fe concentrations in maize kernels. (**A**) Diagram illustrating the differences in the core promoter between five high-Fe and five low-Fe inbred lines. (**B**) Identification of *ZmNAC78* haplotypes (Hap) on the basis of consistent variations in a maize natural-variation population. n = 226 inbred lines. (**C**) Fe concentrations in kernels in Hap1 and Hap2. (**D**) Expression levels of *ZmNAC78* in the kernels of Hap1 and Hap2. Asterisks in (C) and (D) indicate significant differences according to t tests. $^{**}P < 0.01$, $^{***}P < 0.001$.

the kernels of the $zmnac78_{ems}$ mutant was 22.69 mg kg^{-1}, which was 40% lower than that found in the kernels of the control line, B73 (Fig. 1E).

We generated *ZmNAC78* null mutants (*zmnac78*) in the Zong31 background with CRISPR-Cas9. Two guide RNAs were designed that targeted the sequence at nucleotides 677 to 695 and 1185 to 1203 after the ATG codon (Fig. 1D). The *zmnac78* mutants exhibited deletions of 35–base pair (bp) or 71-bp fragments in the coding sequence, which resulted in frameshifts (fig. S4). Consistent with observations in $zmnac78_{ems}$, the *zmnac78* mutant was similar to the control line Zong31 during vegetative development (Fig. 1F). Although the kernel length, kernel width, and 100-kernel weight were reduced in the *zmnac78* mutants (table S3), the Fe concentrations in kernels were still much lower in *zmnac78* mutants than in Zong31 (Fig. 1F). *ZmNAC78* is closely related to *ZmNAC57* in maize (fig. S5A), but the cDNA sequence and mRNA abundance of *ZmNAC57* were not altered in *zmnac78* mutants (fig. S5, B and C). This indicated that CRISPR-Cas9 did not produce off-target effects and that the reduction of Fe concentrations in kernels of the *zmnac78* mutants was due to *ZmNAC78* loss of function.

We constructed transgenic maize lines overexpressing *ZmNAC78* in the KN5585 background. We chose two transgenic lines (*OE#1* and *OE#2*) for further analysis (Fig. 1G). Overexpression of *ZmNAC78* did not affect the growth or 100-kernel weight of maize but significantly increased the Fe concentrations in kernels (Fig. 1H and table S3). The Fe concentrations in the kernels of *ZmNAC78*-overexpressing transgenic maize ranged from 49.4 to 70.5 mg kg^{-1}, which was 2.5 to 3.6 times the range in KN5585 (Fig. 1H).

All of the results presented to this point were from $zmnac78_{ems}$, *zmnac78* mutants, and *ZmNAC78OE* transgenic maize grown in Sanya, Hainan Province. The *zmnac78* mutants and *ZmNAC78* OE transgenic maize were also grown in Nankou (soil pH 7.5), Beijing. Compared with the Fe concentrations in the wild-type (WT) maize, those in kernels were reduced in *zmnac78* mutants and increased in *ZmNAC78OE* transgenic maize grown in Nankou, Beijing (Fig. 1I). These results demonstrated that *ZmNAC78* regulates Fe concentrations in maize kernels and that the regulation is independent of maize genotype and growing site.

Promoter natural variation affects ZmNAC78 mRNA abundance

In the natural-variation population, the expression levels of *ZmNAC78* were significantly positively associated with Fe concentrations in kernels (fig. S2B). We cloned the coding sequence (CDS) and the core promoter of *ZmNAC78* in five randomly selected high-Fe lines and five randomly selected low-Fe lines. The core promoter was defined as the ~500-bp region upstream of the transcription start site (TSS), which is where *cis*-regulatory elements accumulate (*23*). The CDS sequences of *ZmNAC78* were identical between high- and low-Fe lines (fig. S6). By contrast, the core promoter of *ZmNAC78* in high- and low-Fe lines differed at nine SNPs and one indel-276 (Fig. 2A and fig. S7).

We resequenced the core promoter of *ZmNAC78* from 226 maize inbred lines. SNP-330, SNP-366, SNP-371, and indel-276 showed consistent variations among the inbred lines. We grouped lines into two major haplotypes (Hap1 and Hap2) on the basis of these consistent variants (Fig. 2B). Fe concentrations in kernels were significantly higher in Hap1 than in Hap2 (Fig. 2C). We also determined the abundance of *ZmNAC78* mRNA in 55 randomly selected Hap1 inbred lines and 40 randomly selected Hap2 inbred lines. The abundance of *ZmNAC78* mRNA was much higher in Hap1 kernels than in Hap2 kernels (Fig. 2D), consistent with the high Fe concentrations in Hap1 kernels. These results suggested that natural variation in the core promoter region affects the expression levels of *ZmNAC78*.

Molecular marker–assisted selection of maize varieties with Fe-enriched kernels

To determine whether *ZmNAC78* underwent selection during maize breeding, we sequenced the core promoter of *ZmNAC78* from a previously reported maize population (*24*). The population included 60 public US inbred lines (Public-US), 83 USA elite commercial lines with expired Plant Variety Protection Act Certificates (Ex-PVP), 28 Chinese inbred lines released during 1960 to 1979 (CN1960&70s), 87 Chinese inbred lines released during 1980 to 1999 (CN1980&90s), and 20 Chinese inbred lines released after 2000 (CN2000&10s). We found that the frequency of the Hap1 allele increased over time, which was consistent with the inference that *ZmNAC78* was selected for during modern breeding in both the US and China (Fig. 3A). We further analyzed the frequency of the Hap1 allele in 168 Chinese elite inbred lines and found that the frequency of the Hap1 allele was high in SPT, Reid, and Lancaster (Fig. 3B). Reid × Lancaster is a very important heterotic combination in temperate regions (*25*). This suggested that the favorable Hap1 allele could be used for modern breeding.

To test this hypothesis, we conducted a hybrid cross between B73 (Hap1) and KN5585 (Hap2) to construct an $F_{2:3}$ population with 75 families. We randomly selected three Hap1 homozygous

families and three Hap2 homozygous families for analysis of Fe concentrations in kernels. Consistent with observations in our natural-variation population, the Hap1 families in the $F_{2:3}$ population had higher Fe concentrations and higher *ZmNAC78* mRNA abundances in kernels than were found in Hap2 families (fig. S8).

We therefore developed an indel marker to perform molecular marker–assisted selection of maize varieties with Fe-enriched kernels (fig. S9). The parents originated from Reid and/or Lancaster. Zhengdan958 was selected as the control because it has been grown in 13% of the maize planting area in China since 2004 (*26*). Five self-breeding varieties, including three from Hap1 (variety 1, 2, and 3) and two from Hap2 (variety 4 and 5), were planted alongside Zhengdan958 in Yuanyang, Henan Province (soil pH 8.5). Compared with Zhengdan958, variety 1 had both higher grain yield and higher Fe concentrations in kernels, and variety 2 had similar grain yield and higher Fe concentrations in kernels (Fig. 3, C and D). The self-breeding varieties and Zhengdan958 were also grown in Nanning, Guangxi Province (soil pH 6.4). In the subtropics (Nanning), grain yield and Fe concentrations were higher in variety 1 than in Zhengdan958 (Fig. 3, E and F). In both locations, the average Fe concentration was higher in Hap1 varieties than in Hap2 varieties (Fig. 3, C and E). These results suggested that *ZmNAC78* is a useful gene resource for achieving Fe biofortification in maize without reducing yield.

ZmNAC78 is enriched in the basal endosperm transfer layer

To clarify the molecular pathways by which *ZmNAC78* regulates Fe concentrations in maize kernels, we first determined the subcellular localization of ZmNAC78 protein. ZmNAC78-GFP and GFP-ZmNAC78 signals were detected only in the nucleus of maize leaf protoplasts (Fig. 4A). The localization was confirmed by colocalization with the nuclear marker gene, NFYA4 (NUCLEAR FACTOR Y, SUBUNIT A 4) (*27*).

ZmNAC78 contains a NAC domain, indicating that ZmNAC78 should have transcription-regulation activity. To test this hypothesis, pGBKT7-ZmNAC78 fusion plasmid was generated and cotransformed with the pGADT7 vector into the yeast strain Y2HGold. The yeast could survive on selective medium [synthetic defined (SD)/-Leu/-Trp/-His] along with an α-galactosidase activity (Fig. 4B). These results indicated that ZmNAC78 protein might have transcriptional-activation activity.

We then investigated the expression patterns of *ZmNAC78* by performing mRNA in situ hybridization using the 15-DAP B73 kernels. A clear signal was detected in the basal endosperm transfer layer (BETL) during the filling stage, and signals were also detected in

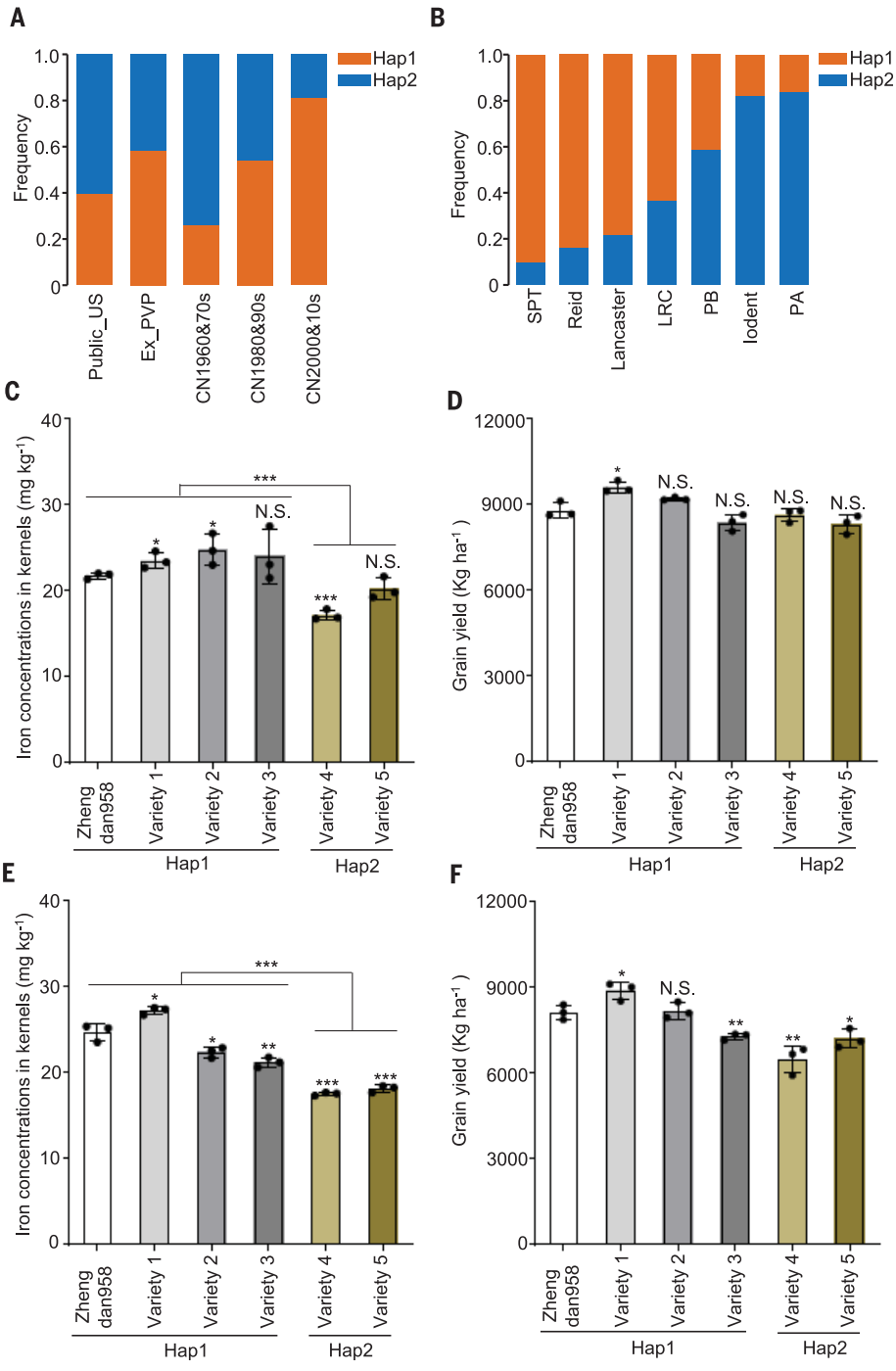

Fig. 3. Molecular marker–assisted breeding of maize with both increased Fe concentrations in the kernels and high yield. (**A**) Haplotype (Hap) frequency changes during breeding in the USA and China. (**B**) Hap frequency changes in Chinese elite inbred lines. The orange and blue colors in (A) and (B) represent Hap1 and Hap2, respectively. (**C**) Fe concentrations in kernels of Zhengdan958 and self-breeding varieties planted in Yuanyang, Henan Province. (**D**) Grain yields of Zhengdan958 and self-breeding varieties planted in Yuanyang, Henan Province. (**E**) Fe concentrations in kernels of Zhengdan958 and self-breeding varieties in Nanning, Guangxi Province. (**F**) Grain yields of Zhengdan958 and self-breeding varieties in Nanning, Guangxi Province. Error bars in (C) through (F) represent the standard deviation of three biological replicates. Asterisks in (C) through (F) indicate significant differences according to *t* tests. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; N.S., not significant.

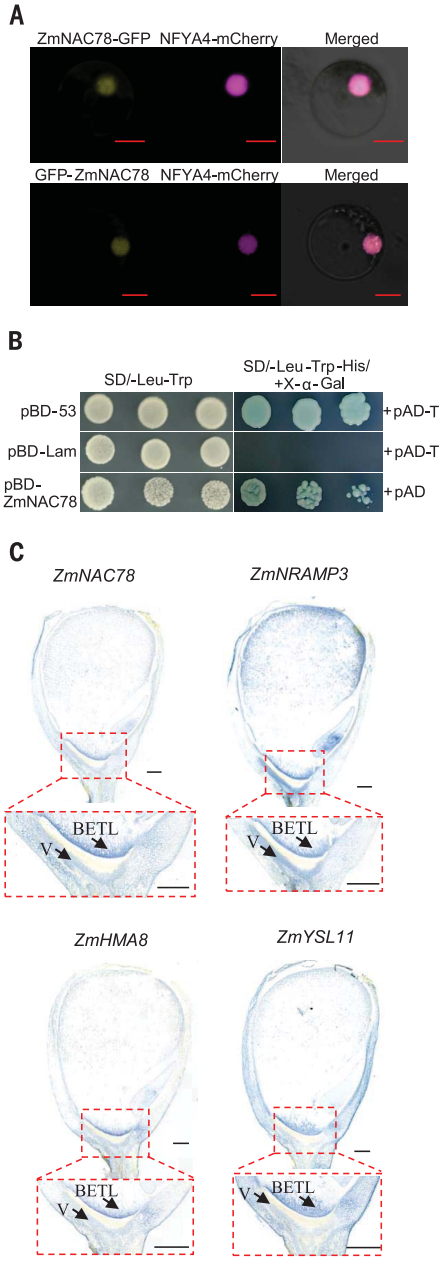

Fig. 4. Expression patterns of *ZmNAC78*. (**A**) Subcellular localization of ZmNAC78 protein. ZmNAC78-GFP (yellow) and GFP-ZmNAC78 (yellow) constructs were transiently coexpressed with the nuclear marker NFYA4-mCherry (magenta) in maize-leaf protoplasts. Scale bars, 10 μm. (**B**) Transcriptional-activation assay of ZmNAC78 protein in yeast. pBD-53 and pBD-Lam were used as a positive control and a negative control, respectively. (**C**) Expression patterns of *ZmNAC78*, *ZmNRAMP3*, *ZmHMA8*, and *ZmYSL11* in 15-DAP (days after pollination) B73 kernels as determined by in situ hybridization analysis. Scale bars in the whole kernels, 500 μm. Scale bars in the magnified views of the regions marked by the dashed red boxes, 100 μm. V, vascular; BETL, basal endosperm transfer layer.

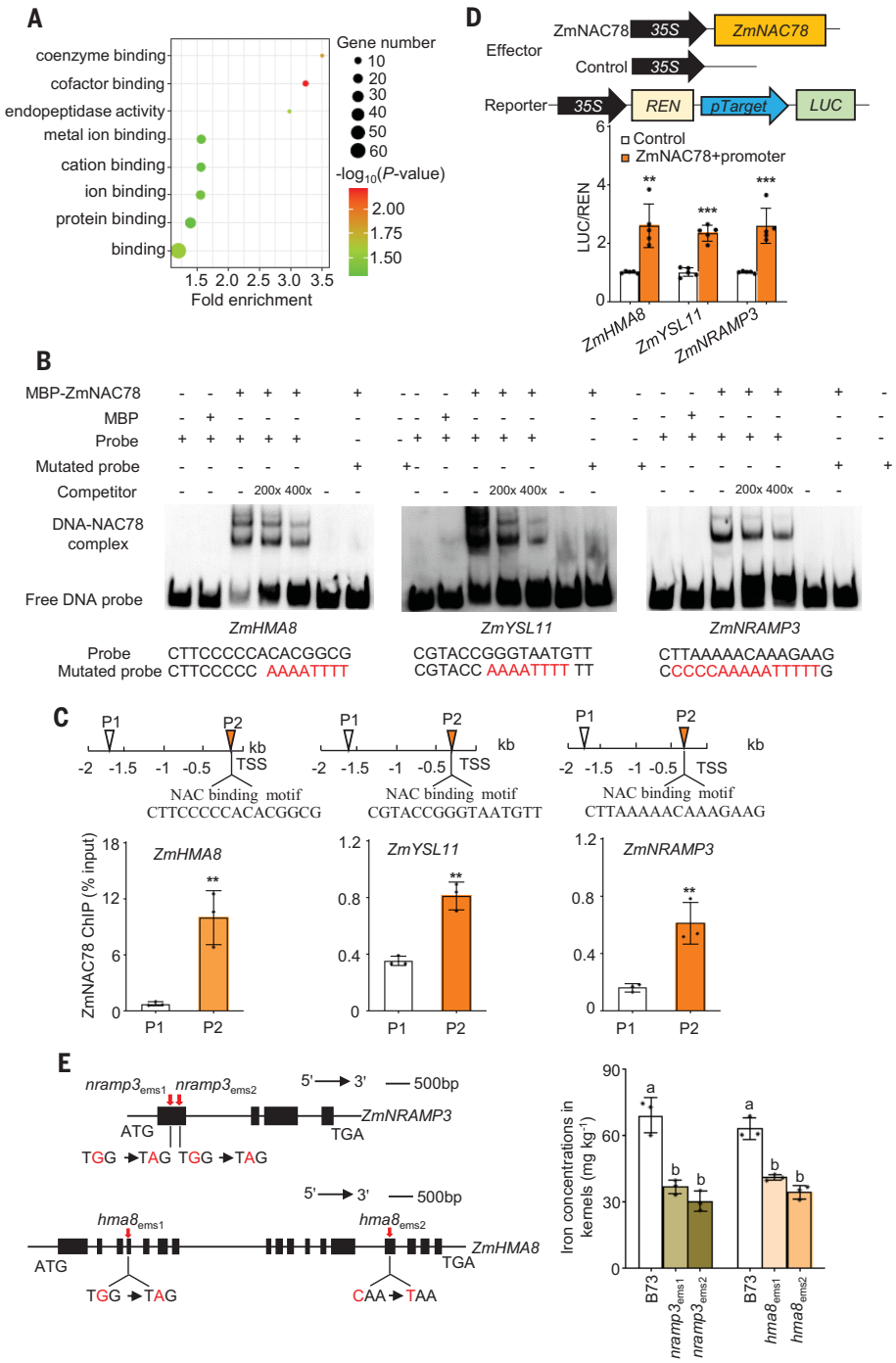

Fig. 5. ZmNAC78 regulates the expression of genes related to Fe uptake. (**A**) GO classifications of genes positively affected by *ZmNAC78* abundance. The point size represents the number of genes in the terms; the point color represents $-\log_{10}$ (*P* value). (**B**) EMSA with ZmNAC78 protein performed with the probes derived from the *ZmHMA8*, *ZmYSL11*, and *ZmNRAMP3* promoter. Competition for the labeled sequences was tested by adding an excess of unlabeled probes as indicated. Red represents the mutated sequence. The gene annotation is from MaizeGDB (*41*). (**C**) ChIP-qPCR assays verified the in vivo binding of ZmNAC78 to the *ZmHMA8*, *ZmYSL11*, and *ZmNRAMP3* promoter. (**D**) Transient-transactivation assay of ZmNAC78 protein with the *ZmHMA8*, *ZmYSL11*, and *ZmNRAMP3* promoter in maize mesophyll protoplasts. Relative REN (*Renilla* luciferase) activity was used as an internal control, and the relative LUC (Firefly luciferase)/REN ratios are shown. (**E**) Effects of *ZmNRAMP3* or *ZmHMA8* EMS mutation on Fe concentrations in maize kernels. Error bars represent the standard deviation of three biological replicates in (C) and (E) and of five biological replicates in (D). Asterisks in (C) and (D) indicate significant differences according to *t* tests. $^{**}P < 0.01$, $^{***}P < 0.001$. Means with the same letter in (E) are not significantly different at $P < 0.05$ according to one-way ANOVA followed by Tukey's multiple comparison test.

the vascular end of the pedicel, the pericarp, other endosperm cells, and the embryo (Fig. 4C and fig. S10).

ZmNAC78 regulates Fe uptake–related genes

To gain insight into the molecular events in the *ZmNAC78*-mediated signaling pathway, we compared the whole-transcriptome profiles of kernels of the *ZmNAC78OE* line, the *zmnac78* mutant, and their corresponding WT kernels at 15 DAP. The total of 12 RNA libraries yielded more than 0.25 billion reads after adaptor trimming, and ~89.70% of the clean reads could be mapped to the maize genome. We identified the genes directly affected by *ZmNAC78* on the basis of the following criteria: (i) fold-change > 1.5, (ii) P value < 0.05, and (iii) expression levels showing opposite trends between *ZmNAC78OE* maize and the *zmnac78* mutant. The expression levels of 99 genes were positively affected, and 1132 genes were negatively affected by *ZmNAC78* mRNA abundance (fig. S11 and table S4). Gene Ontology (GO) analysis showed that the 99 genes were related to metal ion binding (GO:0046872, P = 0.048), ion binding (GO:0043167, P = 0.044), and cation binding (GO:0043169, P = 0.049) (Fig. 5A).

We found that some genes or homologous genes have been previously reported to be essential for Fe distribution in maize kernels or the mobilization of vacuolar Fe stores in *Arabidopsis* seeds, such as genes that encode yellow stripe-like family (YSL) proteins (*28*) and natural resistance-associated macrophage protein (NRAMP) family proteins (*29*, *30*). In addition, proteins encoded by some of the genes have been reported to be involved in iron uptake, such as heavy-metal adenosine triphosphatases (HMAs) (*31*). We chose *ZmHMA8* (*Zm00001d027884*), *ZmYSL11* (*Zm00001d025888*), and *ZmNRAMP3* (*Zm00001d048129*) for further research. RT-qPCR data verified that these genes were up-regulated in *ZmNAC78OE* maize and down-regulated in the *zmnac78* mutant (fig. S12A). In agreement with the expression patterns of *ZmNAC78*, these three genes were preferentially expressed in the early stage of kernel development (fig. S12B). The promoter regions (2,000-bp region upstream of TSS) of *ZmHMA8*, *ZmYSL11*, and *ZmNRAMP3* contain one, one, and five NAC binding motifs, respectively (fig. S13). Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) analysis revealed that ZmNAC78 can directly bind to the promoters of *ZmHMA8*, *ZmYSL11*, and *ZmNRAMP3* (Fig. 5, B and C). Transient-expression assays in maize-leaf protoplasts further demonstrated that ZmNAC78 could activate the expression of the three genes (Fig. 5D).

We investigated the expression patterns of *ZmHMA8*, *ZmYSL11*, and *ZmNRAMP3* by performing mRNA in situ hybridization using 15-DAP B73 kernels. Although the expression patterns of *ZmHMA8*, *ZmYSL11*, and *ZmNRAMP3* varied in maize kernels, each of them could be detected in the BETL (Fig. 4C and fig. S10), which is at least partly consistent with the location of *ZmNAC78*. We then searched the EMS mutant collections and obtained two EMS mutants of *ZmNRAMP3* (mutant ID: EMS4-obfcc9 and EMS4-1cb309) and two EMS mutants of *ZmHMA8* (mutant ID: EMS4-1ce8be and EMS4-0c5a57). We could not obtain EMS mutants of *ZmYSL11*. The substitution in the exon of *ZmNRAMP3* and *ZmHMA8* leads to a premature stop codon in the two genes (Fig. 5E). Stop-gained *ZmNRAMP3* and *ZmHMA8* significantly reduced Fe concentrations in maize kernels (Fig. 5E). These results suggest that *ZmNRAMP3* and *ZmHMA8* are involved in loading of Fe into maize kernels.

Discussion

Micronutrient deficiency, also known as "hidden hunger," reduces nutritional security worldwide. The United Nations Sustainable Development Goal 2 is to end global hunger and reduce all kinds of malnutrition by 2030 (*4*). This goal could be partly achieved by the biofortification of staple food crops. Even though Fe-enriched rice, wheat, and cassava have been reported (*12*–*14*), Fe-enriched maize varieties have not been popular because of the negative correlation between Fe concentrations in kernels and grain yield (*6*, *15*). In this study, we eliminated the trade-off between kernel Fe concentration and grain yield by using molecular-assisted breeding to develop maize varieties with both high yield and high Fe concentrations in kernels.

There is some evidence that the NAC gene family may affect Fe homeostasis in plants. *OsNAP* is connected to Fe remobilization associated with senescence in rice (*32*). Additionally, Uauy *et al*. reported that *NAM-B1* accelerates senescence and increases Fe remobilization from leaves to developing grains in wheat (*33*). However, the adverse effects of senescence have limited the use of *NAM-B1* to increase Fe concentrations in grain. Our data suggest that the NAC family could directly regulate Fe uptake–related genes and enhance Fe concentrations in maize kernels. However, excessive Fe is toxic to plant cells, so it remains a possibility that higher *ZmNAC78* expression might suppress crop growth.

Transfer cells are located in the maize BETL, which is the only exchange surface between maternal and filial tissues in maize (*34*–*36*). By comparison, there is no morphologically distinct endosperm transfer region in rice, indicating that Fe loading may operate differently in rice as compared with maize (*37*, *38*). Although substantial progress has been made in elucidating the pathway of iron loading into rice grains (*39*, *40*), Fe biofortification in maize has not been well explored. Our results contribute to understanding the process of Fe loading into crop kernels.

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Raw sequence data generated during this study have been deposited in the BIG sub (*42*) with accession nos. CRA011946 and CRA011945 for RNA-seq. All other data needed to evaluate the conclusions in the paper are present in the paper or the supplementary materials.

SUPPLEMENTARY MATERIALS

science.org/doi/10.1126/science.adf3256
Materials and Methods
Figs. S1 to S13
Tables S1 to S7
References (*43*–*57*)
MDAR Reproducibility Checklist

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Biofortification of iron content by regulating a NAC transcription factor in maize

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Editor's summary

Fortifying crops with micronutrients could be a good way to improve population health. Maize is a staple for many in sub-Saharan Africa, but the edible portions are typically low in iron. By investigating a population of natural genetic maize variants, Yan *et al*. identified a transcription factor that regulates iron content in the kernels. The authors found that some maize lines exhibited different sequences in the NAC78 promoter, and the presence of these promoter variants was correlated with the expression of NAC78 in the endosperm transfer cells. In these cells, iron transporters are up-regulated, suggesting that more iron is transferred into the kernel. This work opens a route to enhancing maize iron content, which may help to address iron deficiency where it is prevalent. —Madeleine Seale