Corrected 19 December 2023. See full text.

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* (*NICOTIANAMINE 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 concentrations 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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Plant Biotechnology Journal

Plant Biotechnology Journal (2021) 19, pp. 1489–1491 doi: 10.1111/pbi.13634

Brief Communication

Mining novel kernel size-related genes by pQTL mapping and multi-omics integrative analysis in developing maize kernels

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Received 10 April 2021;
revised 8 May 2021;
accepted 10 May 2021.

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Keywords: proteomics, protein quantitative trait locus, multi-omics integration, kernel-related traits, maize.

As a sink organ for starch, protein, oil and essential nutrients, the maize (*Zea mays*) kernel is not only the main target for yield and quality improvement but also a model system for genetic and molecular biology studies. We identified many candidate genes for maize kernel quality and size quantitative trait loci (QTLs) at the genomic, transcriptomic, metabolomic and phenomic levels by genome-wide association studies (GWAS) and joint-linkage mapping (Fu *et al.*, 2013; Liu *et al.*, 2017b; Liu *et al.*, 2017a; Wen *et al.*, 2014; Yang *et al.*, 2014) using a widely adopted Chinese association panel (Yang *et al.* 2011) and five recombinant inbred line (RIL) populations (Liu *et al.*, 2017b). However, maize kernel proteomics studies at the population scale have lagged behind.

Protein QTL (pQTL) analysis has proven to be useful in the diagnosis of various human diseases and has provided genome–proteome networks for clinical applications (Suhre *et al.*, 2017). It is also necessary for elucidating the functional context of gene expression variation during modern maize breeding (Jiang *et al.*, 2019). However, how pQTLs control maize kernel traits remain to be investigated. Here, we identified 468 clear and consistent protein spots in developing kernels of 210 inbred lines by combining 2-D gel electrophoresis with LC-MS/MS. These protein spots were translated from 283 unigenes, 84 of which encode proteins with post-transcriptional/translational modifications. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis indicated that 54% of the identified proteins were annotated and enriched in carbohydrate metabolism (13%), amino acid metabolism (16%) and genetic information processing (18%) (Figure 1a). Interestingly, 46% of the identified proteins could not be assigned to known KEGG pathways, suggesting that a considerable number of proteins in developing maize kernels are uncharacterized. A total of 297 protein spots were successfully retrieved and found to differentially accumulate among inbred lines. We generated regulatory networks based on the pairwise Pearson correlation coefficients ($r^2 \geq 0.25$, $P \leq 0.05$) of transcript and protein abundance using the modularity method implemented in Gephi0.9.2 (Figure 1b, c). Analysis with both networks revealed that genes involved in the same pathway do not always appear in the same module. Additionally, we noticed moderate or low correlations between the transcript level and the protein abundance of the same gene; examples included several previously reported genes for kernel development (Dai *et al.*, 2021), GRMZM2G068506 (*Bt2*), GRMZM2G429899 (*Sh2*), GRMZM2G089713 (*Sh1*), GRMZM2G415359 (*Mdh4*), GRMZM2G306345 (*Pdk1*) and GRMZM2G097457 (*Pdk2*), among which only *Bt2* exhibited a strong correlation between the transcript and protein levels ($r = 0.78$, $P < 0.01$). These genes clustered into different subnetworks at the transcript level (Figure 1b) but into the same subnetwork at the protein level (Figure 1c). The results reveal that the transcript level alone does not always reliably predict protein abundance at the population scale, and protein abundance variation may play an important role in orchestrating the biological functions of genes involved in the same biological pathways. pQTL analysis is therefore necessary to fully elucidate the molecular basis of kernel-related phenotypes.

Using GWAS based on 1.25 M SNPs, we identified 421 independent significant SNPs for the abundance of 40 protein spots encoded by 38 unigenes using the recommended *P*-value (1/N, $P \leq 2.04 \times 10^{-6}$). Forty-six non-redundant pQTLs were defined within +/− 50 kb flanking regions of their lead SNPs based on the linkage disequilibrium of 50 kb ($r^2 \geq 0.1$) in genome-wide average of 210 inbred lines. These included 13 local pQTLs and 33 distant pQTLs that distributed unevenly across the ten maize chromosomes (Figure 1d). Chromosome 7 and 2 had the lowest and highest density of pQTLs, respectively (Figure 1e). Five protein spots, P3206, P4506, P3005, P5202 and P2111, were underlain by two or more pQTLs, and the remaining protein spots by only one pQTL. Two pQTLs were found to regulate proteins that function in post-transcriptional/translational modifications (P5315 and P6207, P6508 and P7502).

CellPress
Partner Journal

Molecular Plant
Research Article

The chimeric gene *atp6c* confers cytoplasmic male sterility in maize by impairing the assembly of the mitochondrial ATP synthase complex

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https://doi.org/10.1016/j.molp.2022.03.002

ABSTRACT

Cytoplasmic male sterility (CMS) is a powerful tool for the exploitation of hybrid heterosis and the study of signaling and interactions between the nucleus and the cytoplasm. C-type CMS (CMS-C) in maize has long been used in hybrid seed production, but the underlying sterility factor and its mechanism of action remain unclear. In this study, we demonstrate that the mitochondrial gene *atp6c* confers male sterility in CMS-C maize. The ATP6C protein shows stronger interactions with ATP8 and ATP9 than ATP6 during the assembly of F_1F_o-ATP synthase (F-type ATP synthase, ATPase), thereby reducing the quantity and activity of assembled F_1F_o-ATP synthase. By contrast, the quantity and activity of the F_1' component are increased in CMS-C lines. Reduced F_1F_o-ATP synthase activity causes accumulation of excess protons in the inner membrane space of the mitochondria, triggering a burst of reactive oxygen species (ROS), premature programmed cell death of the tapetal cells, and pollen abortion. Collectively, our study identifies a chimeric mitochondrial gene (*ATP6C*) that causes CMS in maize and documents the contribution of ATP6C to F_1F_o-ATP synthase assembly, thereby providing novel insights into the molecular mechanisms of male sterility in plants.

Key words: maize, cytoplasmic male sterility, *atp6c*, ATP synthase, mitochondria

Yang H., Xue Y., Li B., Lin Y., Li H., Guo Z., Li W., Fu Z., Ding D., and Tang J. (2022). The chimeric gene *atp6c* confers cytoplasmic male sterility in maize by impairing the assembly of the mitochondrial ATP synthase complex. Mol. Plant. **15**, 872–886.

INTRODUCTION

Since the first exploitation of hybrid heterosis in maize in the 1920s, a hybrid breeding strategy has been used in many crops and vegetables to achieve higher yields, improve quality, and strengthen disease resistance. Cytoplasmic male-sterile lines have greatly facilitated hybrid seed production in maize, rice, rapeseed, and many other agronomic and horticultural crops (Havey, 2004; Bohra et al., 2016; Kim and Zhang, 2018). New biotechnologies such as seed production technology (SPT) (Wu et al., 2016) and the multicontrol sterility system (Zhang et al., 2018) have been devised for the development of male-sterile lines. Among the numerous means of hybrid seed production, the use of male-sterile lines, especially cytoplasmic male-sterile lines, remains an important method for modern seed enterprises because it can lower the cost of seed production and improve seed purity.

Cytoplasmic male sterility (CMS) is a maternally inherited phenomenon in which pollen development is aborted as a result of genetic conflicts between the nucleus and the cytoplasm (Schable and Wise, 1998; Hanson and Bentolila, 2004). Previous work has shown that single putative mitochondrial open reading frames (ORFs) or aberrant chimeric genes generated by mitochondrial genomic rearrangement are responsible for CMS. They are often co-transcribed or interact with genes involved in the electron transport chain (ETC) or the adenosine triphosphate (ATP) synthase complex (F_1F_o-ATP synthase, F-type ATP synthase, ATPase) (Chase, 2007; Kubo and Newton, 2008; Chen and Liu, 2014; Kim and Zhang, 2018). CMS-associated genes always show constitutive mRNA expression and specific or preferential protein accumulation in the anthers (Grelon et al., 1994; Song and Hedgcoth, 1994; Duroc et al., 2005; Peng et al., 2010; Heng et al., 2018). The product of a CMS gene often affects mitochondrial functions by

Published by the Molecular Plant Shanghai Editorial Office in association with Cell Press, an imprint of Elsevier Inc., on behalf of CSPB and CEMPS, CAS.

nature communications

Article

https://doi.org/10.1038/s41467-024-46289-6

Copine proteins are required for brassinosteroid signaling in maize and Arabidopsis

Received: 28 February 2023

Accepted: 21 February 2024

Published online: 08 March 2024

Check for updates

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Copine proteins are highly conserved and ubiquitously found in eukaryotes, and their indispensable roles in different species were proposed. However, their exact function remains unclear. The phytohormone brassinosteroids (BRs) play vital roles in plant growth, development and environmental responses. A key event in effective BR signaling is the formation of functional BRI1-SERK receptor complex and subsequent transphosphorylation upon ligand binding. Here, we demonstrate that BONZAI (BON) proteins, which are plasma membrane-associated copine proteins, are critical components of BR signaling in both the monocot maize and the dicot Arabidopsis. Biochemical and molecular analyses reveal that BON proteins directly interact with SERK kinases, thereby ensuring effective BRI1-SERK interaction and transphosphorylation. This study advances the knowledge on BR signaling and provides an important target for optimizing valuable agronomic traits, it also opens a way to study steroid hormone signaling and copine proteins of eukaryotes in a broader perspective.

Brassinosteroids (BRs) are an important group of growth-promoting hormones found throughout the plant kingdom[1]. Genetic studies demonstrated that BRs play essential roles during nearly all phases of plant growth and development, as BR biosynthetic or signaling mutants display multiple developmental defects, such as short hypocotyls in the dark[2–5], dwarfism[6,7], abnormal leaf angle[8], and decreased crop yields[9]. Over the past two decades, tremendous progress has been made toward the understanding of the BR signaling pathway, making it one of the best understood signaling pathways in plants[1,10,11]. BRs are perceived outside of the cell by the plasma membrane-localized receptor BRASSINOSTEROID-INSENSITIVE1 (BRI1)[12,13]. In the absence of BRs, BRI1 remains in an inactive state via interaction with the inhibitory protein BRI1 KINASE INHIBITOR1 (BKI1) or BOTRYTIS-INDUCED KINASE1 (BIK1)[14,15]. Upon BR perception, BRI1 phosphorylates BKI1, leading to its dissociation from BRI1[14]. The released BRI1 then interacts with its co-receptor BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1, also named SERK3), which works redundantly with the three other SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASEs (SERKs) BAK1-LIKE1 (BKK1, also named SERK4), SERK1 and SERK2 to cause trans-phosphorylation between BRI1 and SERKs[16–19]. The activated BRI1–SERKs receptor complex directly phosphorylates BR SIGNALING KINASEs (BSKs) and CONSTITUTIVE DIFFERENTIAL GROWTH1 (CDG1)[20,21]. BSKs and CDG1 further activate a family of phosphatases, called BRI1 SUPPRESSOR1/BSU-LIKEs (BSU1/BSLs)[21,22], which then dephosphorylate and inactivate BRASSINOSTEROID INSENSITIVE2 (BIN2)[21–23]. BIN2 typically targets

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Plant Biotechnology Journal

Plant Biotechnology Journal (2024) 22, pp. 1269–1281 doi: 10.1111/pbi.14263

A P-type pentatricopeptide repeat protein ZmRF5 promotes 5′ region partial cleavages of *atp6c* transcripts to restore the fertility of CMS-C maize by recruiting a splicing factor

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Received 29 July 2023;
revised 31 October 2023;
accepted 26 November 2023.
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Keywords: maize, cytoplasmic male sterility, *ZmRf5*, cleaving complex, restoration.

Summary

A fast evolution within mitochondria genome(s) often generates discords between nuclear and mitochondria, which is manifested as cytoplasmic male sterility (CMS) and fertility restoration (*Rf*) system. The maize CMS-C trait is regulated by the chimeric mitochondrial gene, *atp6c*, and can be recovered by the restorer gene *ZmRf5*. Through positional cloning in this study, we identified the nuclear restorer gene, *ZmRf5*, which encodes a P-type pentatricopeptide repeat (PPR) family protein. The over-expression of *ZmRf5* brought back the fertility to CMS-C plants, whereas its genomic editing by CRISPR/Cas9 induced abortive pollens in the restorer line. ZmRF5 is sorted to mitochondria, and recruited RS31A, a splicing factor, through MORF8 to form a cleaving/restoring complex, which promoted the cleaving of the CMS-associated transcripts *atp6c* by shifting the major cleavage site from 480th nt to 344 th nt for fast degradation, and preserved just right amount of *atp6c* RNA for protein translation, providing adequate ATP6C to assembly complex V, thus restoring male fertility. Interestingly, ATP6C in the sterile line CMo17A, with similar cytology and physiology changes to YU87-1A, was accumulated much less than it in NMo17B, exhibiting a contrary trend in the YU87-1 nuclear genome previously reported, and was restored to normal level in the presence of ZmRF5. Collectively these findings unveil a new molecular mechanism underlying fertility restoration by which ZmRF5 cooperates with MORF8 and RS31A to restore CMS-C fertility in maize, complemented and perfected the sterility mechanism, and enrich the perspectives on communications between nucleus and mitochondria.

Introduction

Cytoplasmic male sterility (CMS) is a phenomenon genetically controlled by the factors in the cytoplasm in plant and exhibits normal development except for male organs. CMS was first reported in maize (Rhoades, 1931) and is found in more than 200 plant species (Hu *et al.*, 2012). Molecular evidence shows that, in all instances till now, CMS is associated with abnormal open reading frames (ORFs) located in mitochondrial genomes (Chase, 2007; Chen and Liu, 2014). These ORFs can be processed by specific nuclear genes (*Rfs*), which rescue the male sterile phenotype (Kim and Zhang, 2018). Therefore, CMS and the corresponding *Rf*(s) build an ideal model system for understanding the mechanism of co-evolution and cross-talk between the nucleus and mitochondria in plants. The *CMS/Rf* systems have been widely employed for hybrid seed production in many crops, such as maize, rice, rapeseed, sorghum, radish, and Chinese cabbage (Kim and Zhang, 2018).

Recently, a bunch of *Rf* genes have been cloned and identified in various crops and other plants (Kim and Zhang, 2018). *Rf* genes annihilate the detrimental effects associated with CMS at various levels. At the genomic level, *Fr* gene restores fertility of CMS-Sprite common bean through altering the 25-kb *PVS* (*Phaseolus vulgaris* sterility)-associated mitochondrial genomic sequence (Janska *et al.*, 1998). At the post-transcriptional level, the CMS-associated transcripts are processed by the restorer either through RNA editing and degrading (Gagliardi, 1999; Qin *et al.*, 2021; Tang *et al.*, 1999), or through cleaving and degrading (Jiang *et al.*, 2022; Kazama *et al.*, 2008; Kennell and Pring, 1989; L'Homme *et al.*, 1997; Menassa *et al.*, 1999; Wang *et al.*, 2006) or through direct degrading (Luo *et al.*, 2013). *Rf* genes also function by translational or post-translational mechanism to suppress CMS (Dewey *et al.*, 1991; Luo *et al.*, 2013; Sarria *et al.*, 1998; Uyttewaal *et al.*, 2008; Wang *et al.*, 2021). The *Rf2* gene in maize CMS-T may functions at the metabolic level because it encodes a detoxification enzyme and has no effects on *urf13-orf221* transcripts and URF13 protein (Liu *et al.*, 2001). In previous studies, more than half of the cloned *Rf* genes belong to the pentatricopeptide repeat (PPR) family, most of which perform their best at the post-transcriptional or translation/post-translational levels (Chen and Liu, 2014; Kim and Zhang, 2018).

The PPR family has been greatly expanded with more than 400 members in different plant genomes. They are subdivided into two major subfamilies (P and PLS) according to the structures of the three basic PPR motif (P, L and S; Barkan and Small, 2014).