Loss of Function of an RNA Polymerase III Subunit Leads to Impaired Maize Kernel Development1[OPEN]

Hailiang Zhao,a Yao Qin,1 Ziyi Xiao,a Qi Li,b Ning Yang,c Zhenyuan Pan,a Dianming Gong,a Qin Sun,a Fang Yang,a Zuxin Zhang,2 Yongrui Wu,b Caoy, and Fazhan Qiu*a,2,3

aNational Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China
bNational Key Laboratory of Plant Molecular Genetics, Chinese Academy of Science Center for Excellence in Molecular Plant Sciences, Institute of Plant Physiology & Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China
cState Key Laboratory of Plant Genomics, National Center for Plant Gene Research, Institute of Genetics and Developmental Biology, The Innovative Academy of Seed Design, Chinese Academy of Sciences, Beijing 100101, China

ORCID IDs: 0000-0001-9093-711X (H.Z.); 0000-0002-0709-9635 (F.Q.).

Kernel size is an important factor determining grain yield. Although a number of genes affecting kernel development in maize (Zea mays) have been identified by analyzing kernel mutants, most of the corresponding mutants cannot be used in maize breeding programs due to low germination or incomplete seed development. Here, we characterized small kernel7, a recessive small-kernel mutant with a mutation in the gene encoding the second-largest subunit of RNA polymerase III (RNAPIII; NRPC2). A frame shift in ZmNRPC2 leads to a premature stop codon, resulting in significantly reduced levels of transfer RNAs and SS ribosomal RNA, which are transcribed by RNAPIII. Loss-of-function nrpc2 mutants created by CRISPR/CAS9 showed significantly reduced kernel size due to altered endosperm cell size and number. ZmNRPC2 affects RNAPIII activity and the expression of genes involved in cell proliferation and endoreduplication to control kernel development via physically interacting with RNAPIII subunits RPC53 and AC40, transcription factor class C1 and Floury3. Notably, unlike the semidominant negative mutant floury3, which has defects in starchy endosperm, small kernel7 only affects kernel size but not the composition of kernel storage proteins. Our findings provide novel insights into the molecular network underlying maize kernel size, which could facilitate the genetic improvement of maize in the future.

Increasing grain yield in maize (Zea mays) is required to meet the rapidly expanding demands for maize-derived foods, feed, and fuel. Kernel size, one of the most important factors determining grain yield, has been selected during maize domestication and improvement. Breeders enhance grain productivity in maize hybrids by pyramiding genes for desirable characteristics such as larger kernels. Maize kernel size is regulated by many factors, such as the supply of energy or material, cell proliferation and division, and important intracellular biological processes. To date, several defective kernel (dek) genes and empty pericarp genes have been cloned in maize. However, most of these genes encode pentatricopeptide repeat proteins and cannot be used in maize breeding programs due to incomplete seed development of the corresponding mutants (Xiu et al., 2016; Cai et al., 2017; Chen et al., 2017; Qi et al., 2017; Ren et al., 2017).

Unlike pentatricopeptide repeat proteins, Dek44 encodes a mitochondrial ribosome protein that regulates maize kernel development by affecting the expression of chain-related proteins (Qi et al., 2019). Dek15 encodes a homolog of SISTER CHROMATID COHESION PROTEIN4. The chromosome separation error in dek15 results in reduced endosperm size and embryo lethality (He et al., 2019). The varied kernel size1 mutant contains a mutation in Zmkin11 that causes impaired spindle assembly, sister chromatid separation, and phragmoplast formation, leading to varying degrees of defective cell proliferation, ultimately resulting in the formation of seeds of different sizes (Huang et al., 2019).

1This work was supported by the National Key Research and Development Program of China (grant no. 2016YFD0101803) and the National Natural Science Foundation of China (grant nos. 31971951 and 31860382).
2Author for contact: qiufazhan@mail.hzau.edu.cn.
3Senior author.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Fazhan Qiu (qiufazhan@mail.hzau.edu.cn).

F.Q., Y.W., Z.Z., and H.Z. conceived the original screening and research plans; F.Q. supervised the experiments; H.Z., Y.Q., and Z.X. designed the experiments and analyzed the data; F.Q., Z.Z., F.Y., Y.W., C.X., and H.Z. drafted and critically revised the article; H.Z., Y.Q., Z.X., Q.L., N.Y., Z.P., D.G., and Q.S. performed the research.
The retinoblastoma-related (RBR) pathway controls maize endosperm development by regulating the cell cycle (Sabelli et al., 2013). Several important intracellular biological processes, such as post-transcriptional regulation, also play important roles in maize kernel development. These processes have been uncovered by studying mutants *rbm48* (Bai et al., 2019), *ubi1* (Li et al., 2017b), and *urb2* (Wang et al., 2018). These findings have enriched our knowledge of the regulatory mechanisms behind maize kernel development. However, most of these mutants exhibit significantly reduced kernel weight and defective seedling development, preventing them from producing normal offspring. Therefore, identifying and characterizing new genes controlling kernel size is still needed to provide genetic and molecular knowledge about kernel development that could be used to enhance breeding efforts for improving grain yield.

RNA polymerase III (RNAPIII) is the largest of the three eukaryotic RNA polymerases. The RNAPIII transcriptional complex consists of a 17-subunit enzyme and three transcription factors (TFIIFIA, TFIIFB, and TFIIFIC; Hoffmann et al., 2015; Abascal-Palacios et al., 2018). Early studies have shown that RNAPIII is responsible for the transcription of small noncoding RNAs, such as transfer RNAs (tRNAs), 5S ribosomal RNA (rRNA), U6 small nuclear RNA, and 7SL RNA (RN7SL1 RNA) in eukaryotes (Paule and White, 2000; Geudseck and Kassavetis, 2001; Dieci et al., 2007). These small noncoding RNAs play widespread roles in basic biological activities, including protein synthesis (tRNAs), ribosome biogenesis (5S rRNA; Ciganda and Williams, 2011), mRNA splicing (U6 small nuclear RNA), membrane targeting of newly translated proteins (7SL RNA; Dieci et al., 2002), and selective mRNA export (SINE RNAs; Poncsan et al., 2010; Karijolich et al., 2017) and they also act as cell-type–specific enhancers (Alu elements; Hardeland and Hurt, 2006; Zhang et al., 2019). These findings suggest that RNAPIII is required for growth, development, and reproduction (Goodfellow and White, 2007; Filer et al., 2017). However, most studies of RNAPIII subunit enzyme and transcription factors have been performed in yeast (*Saccharomyces cerevisiae*) and animals (Mann et al., 1992; Marshall and White, 2008; Pavon-Eternod et al., 2009), and few have focused on plants, especially studies involving RNAPIII subunits. In plants, a mutation of RNAPIII transcription-related plant AT-rich sequence and zinc binding protein suppressed the transcriptional activity of RNAPIII, leading to decreased grain length in rice (*Oryza sativa*) and floury endosperm in maize (Li et al., 2017c; Wang et al., 2019). Despite these findings, the components and mechanisms that regulate RNAPIII-mediated transcription and how the target genes transcribed by RNAPIII regulate endosperm development in plants have not been uncovered.

In this study, we characterized the maize small kernel7 (*smk7*) mutant. This mutant has small kernels, good germination, good seedling development, and normal reproduction; small kernel size does not influence the grain yield or nutrition value of maize. We cloned *Smk7* and determined that it encodes the second-largest subunit of RNAPIII (NRPC2); *Smk7* is therefore referred to hereafter as *ZmNRPC2*. *smk7* contains a mutant version of *ZmNRPC2* that carries a frame shift and premature stop codon, resulting in small kernels due to impaired RNAPIII-mediated transcription. This mutation affects the transcription of genes involved in the mitotic cell cycle and DNA replication, which further inhibits cell proliferation and endoreduplication during endosperm development. Finally, we uncovered a regulatory pathway of *ZmNRPC2* and its effects on maize kernel development.

### RESULTS

#### The *smk7* Mutant Produces Small Kernels

The *smk7* mutant was originally isolated from ethyl methanesulfonate-mutagenized Mo17 progeny (Fig. 1A). Compared with the wild type, mature *smk7* kernels were smaller, in terms of length, width, and thickness (Supplemental Fig. S1, A and B), and had a lower 100-kernel weight (~70% that of wild type), but no significant difference in the test weight (Fig. 1B). These differences made it easy to distinguish mutant *smk7* kernels from wild-type kernels by size, even on genetically segregating F1 ears as early as 8 d after pollination (DAP; Supplemental Fig. S2, B and C). The *smk7* kernels showed no germination defects and the resulting seedlings developed and reproduced normally, despite exhibiting weaker kernel growth than wild type (Supplemental Fig. S1, D and E).

Cytological observation revealed that fewer *smk7* endosperm cells stained positive for starch in *smk7* kernels than in wild-type kernels at different development stages, suggesting that they accumulated less starch, although *smk7* kernels produced normal endosperm and embryos (Fig. 1, C and D; Fig. S2C). The BETL cells in *smk7* kernels were smaller and lacked extensive cell wall ingrowths (Fig. 1, E and H). Moreover, the CZ cells were compacted lengthways (Fig. 1, E and F) and endosperm cells located in the area immediately internal to the AL were more similar to AL cells than endosperm cells, because they were smaller and accumulated less starch (Figs. 1, I and K). These results indicate that *smk7* is a small-kernel mutant with normal embryonic structures, but the cell shape in BETL, CZ, and peripheral of endosperm is different from wild type.

#### Loss of Function of *ZmNRPC2* Is Responsible for the Mutant Phenotypes

To identify the *Smk7* locus, the *smk7* mutant was crossed to the B73 inbred line and the progeny were selfed. The segregation ratio of wild-type to mutant

---

Zhao et al.
kernels on the self-crossed ears was 1,477:490, which is ~3:1 (χ²-test, P > 0.05), indicating that the smk7 mutant is a monogenic recessive mutation (Supplemental Fig. S2A). Bulk-sequence analysis was then performed and the smk7 was mapped to a 63.64-Mb interval on chromosome 6 (from 19,352,816 to 83,002,397 bp; Fig. 2A). Using 4,300 mutant individuals, smk7 was fine-mapped to a 2.61-Mb region (from 61,269,234 to 63,875,645 bp) that was flanked by two developed insertion/deletion markers (ID51 and ID76; Fig. 2B). The 2.61-Mb mapping interval contained 22 genes that were annotated on the B73 reference genome version 4 (B73 RefGen V4; Jiao et al., 2017). Among these, a G-to-T substitution located at the splice acceptor in the 36th exon of Zm00001d035960 was detected and confirmed (Fig. 2C). This G/T mutation resulted in an alternatively spliced transcript that retained a 26-bp intron, which caused a frame-shift that generated a premature stop codon. The alternatively spliced transcript can be translated to generate a protein lacking three amino acids and containing 13 changed amino acids. We estimated the expression of Zm00001d035960 transcripts and observed that the alternatively spliced transcript accounted for 75% of the total mRNA, suggesting the smk7 mutant phenotype might be attributed to a decrease in wild-type transcripts of Zm00001d035960 (Fig. 2D).

The Zm00001d035960 protein showed high sequence similarity to the DNA-directed RNAPIII subunit II (NRPC2) of well-studied species such as Homo sapiens, Mus musculus, and S. cerevisiae (Supplemental File S1); thus, it is hereafter referred to as ZmNRPC2. Phylogenetic analysis showed that ZmNRPC2 clustered within the monocot NRPC2 clade (Fig. 3A). The defective protein from the alternative transcript showed a loss of the conserved RNA_pol_Rpb2_7 domain, which contains a region termed “switch 4” (Fig. 3B; Supplemental Fig. S3A). The switch domains within the polymerase are thought to signal different stages of transcription (Cramer et al., 2001) and interact with the “clamp domain” of NRPC1 (Supplemental Fig. S3C; Cramer et al., 2001). The alternative smk7 protein also lacked an α-helix and a β-folding domain (Supplemental Fig. S3B), which formed 14 hydrogen bonds in the predicted three-dimensional structure (Supplemental Fig. S3D), suggesting that a defective ZmNRPC2 protein was present in the smk7 mutant.

We further examined the expression pattern of the ZmNRPC2 gene via reverse transcription quantitative
PCR (RT-qPCR), which showed that it was constitutively expressed in diverse wild-type maize tissues (Fig. 2E), with a relatively high expression level in the tassel and ovule. The level of the ZmNRPC2 expression decreased throughout endosperm development from 10 to 15 DAP, increased from 15 to 21 DAP, and then gradually decreased. To investigate the effect of the nrpc2 mutation, we examined the ZmNRPC2 transcript level in immature endosperm of wild type and smk7 at 10, 12, and 15 DAP. Surprisingly, an increased level of ZmNRPC2 transcripts was detected at these three developmental stages in the smk7 mutant.

To identify the function of ZmNRPC2 in maize kernel development, we generated three independent ZmNRPC2 knock-out transgenic lines that contained either a 901-bp deletion including the first to the third exons of ZmNRPC2 (nrpc2-KO1), a 1-bp deletion in the 36th exon (nrpc2-KO2), or two single-nucleotide polymorphisms (SNPs) in the 36th exon (nrpc2-KO3; Fig. 4A). The self-crossed ears of heterozygous plants showed a segregation in kernel size, with 161 wild-type:63 mutant kernels in nrpc2-KO2/1 and 186 wild-type:68 mutant kernels in nrpc2-KO3/1, which was not statistically significantly different from a 3:1 segregation ratio ($\chi^2$-test, $P > 0.05$; Fig. 4B). In the self-crossed ears of nrpc2-KO1/+, homozygous s mutant kernels were developmentally arrested, with blocks in embryogenesis and endospermogenesis (Fig. 4B), which might be explained by the zmnrpc2 mutation that caused a deletion within the protein. The nrpc2-KO2 and nrpc2-KO3 mutated proteins were generated by mutations within the 36th exon of ZmNRPC2, which
created two weaker alleles of zmnrpc2. These results indicated that ZmNRPC2 is required for kernel development in maize (Figs. 1A and 4B). Allelism tests were performed by crossing three independent heterozygous mutated alleles with smk7. The hybrid ears showed defective phenotypes similar to those obtained by self-crossing nrpc2-KO1/1, nrpc2-KO2/1, and nrpc2-KO3/1 ears (Supplemental Fig. S4), indicating that ZmNRPC2 is the mutated gene in smk7.

ZmNRPC2 Interacts with Subunits of the RNAPIII Complex and Regulates Expression of tRNAs and 5S rRNA

A search of the maize protein database via BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) with sequences of subunits of the RNAPIII complex of S. cerevisiae and H. sapiens identified the amino acid sequences of maize RNAPIII subunits. The full-length open reading frames (ORFs) of subunit-encoding genes were constructed into pGADK7 and the full-length ORF of ZmNRPC2 and dNRPC2 (defective ZmNRPC2, representing the mutation in smk7) were separately cloned into pGBKT7. The yeast two-hybrid (Y2H) assay and bimolecular luciferase complementation (BiLC) assays revealed that RNAPIII complex subunits, including RPC53, the AC40, and the TFIIIC subunit TFC1, and the key FL3 protein in endosperm development, physically interacted with ZmNRPC2 and dNRPC2 (Fig. 5, A and B), indicating that ZmNRPC2 is important for the function of the RNAPIII transcription complex. Furthermore, ZmNRPC2 and dNRPC2 were localized to the cytoplasm and the nucleus (Supplemental Fig. S5), and the G/T mutation in smk7 did not affect their subcellular localization. These results indicated that the peptide that was absent from the mutant Smk7 protein was not essential for protein–protein interaction, and suggest that dNRPC2 competitively binds with subunits of the RNAPIII complex to counteract ZmNRPC2 function in vivo.

To elucidate the effects of ZmNRPC2 on transcription, we analyzed the biogenesis of tRNAs and 5S rRNA and observed that the transcript levels of 5S rRNAs and 17 tRNAs were more than 2-fold lower in smk7 endosperm cells than in wild type; in particular, the expression of four tRNAs (tRNA-Trp, tRNA-Pro, tRNA-Arg, and tRNA-Gly) was reduced by more than 10-fold relative to that in wild-type endosperm cells (Fig. 5C), suggesting that ZmNRPC2 regulates the transcription of 5S rRNAs and tRNAs in kernel endosperm cells.

ZmNRPC2 Genetically Interacts with FL3

To test the interaction between ZmNRPC2 and FL3, we crossed smk7 to fl3 and identified single and double

---

**Figure 3.** Phylogenetic analysis of NRPC2.

A, Neighbor-joining sequence similarity analysis of ZmNRPC2 and its homologs in other organisms. The phylogenetic reconstruction was conducted in the program MEGA7.0 (https://www.megasoftware.net/). The numbers next to the branches represent the percentage support from 1,000 bootstraps. Scale bar = mean number of amino acid substitutions per site. B, The RNA_pol_Rpb2_7 domain in NRPC2 is conserved. If the score is greater than global score of similarity, it will be rendered as colored characters (red characters on a white background by default and red characters on a red background if residues are strictly conserved in the column) with blue frames. Bold blue frame in Z. mays represent mutation residues.
mutant kernels on self-crossed F1 ears by genotyping. We observed an opaque and soft endosperm phenotype for \( fl3 \) kernels, which differed from the small-kernel phenotype of \( smk7 \); the double \( smk7;fl3 \) mutant exhibited cumulative features of the two single mutants and possessed small and opaque kernels (Figs. 6A and 7A). The 100-kernel weight of \( smk7 \) and the \( fl3 \) was 47.5% and 54.2% lower, respectively, whereas the 100-kernel weight of the double mutants was even lower (76.2%) relative to that of wild type (Fig. 6B), suggesting that the effect of both \( smk7 \) and \( fl3 \) on kernel weight was cumulative.

Consistent with the decreased kernel weight phenotype, the size of the endosperm of \( smk7 \) and \( smk7;fl3 \) kernels was dramatically reduced by 47.2% and 60%, respectively, at 10 DAP (Supplemental Fig. S6D), and decreased by 26.9% and 48.3% in 15 DAP kernels, respectively, relative to wild type (Fig. 6D). However, the endosperm of \( fl3 \) was 10.9% smaller than that in wild-type kernels (Fig. 6D), showing a difference in endosperm size between \( fl3 \) and \( smk7;fl3 \) that was even lower (76.2%) relative to that of wild type (Fig. 6B), suggesting that the effect of both \( smk7 \) and \( fl3 \) on kernel weight was cumulative.

The opaque and soft endosperm phenotype of \( fl3 \) kernels (Li et al., 2017c) was not observed in the \( smk7 \) mutant and the \( nrpc2;KO \) lines. To identify the basis for this difference in endosperm phenotype between \( fl3 \) and the \( smk7 \), we tested the content of starch and storage proteins in the dry powder of mature kernels. The starch content did not differ significantly among the three mutants (Supplemental Fig. S6C). Compared with wild-type kernels, the levels of zeins in kernels of \( fl3 \) and \( smk7;fl3 \) were 23.8% and 24.4% lower than those in wild type, respectively, whereas the level of nonzein proteins significantly increased by 56.4% and 109.7%, respectively (Fig. 7D). However, the levels of zeins and nonzein proteins did not differ significantly between \( smk7 \) and wild type (Fig. 7D). Similarly, the levels of most free amino acids showed no significant difference among the mutants, except for Arg, Lys, and Met, which were slightly less abundant in \( smk7 \) kernels than in wild-type kernels. Notably, the levels of free amino acids dramatically increased in \( fl3 \) and \( smk7;fl3 \) kernels (Fig. 7E). Scanning electron microscopy revealed that the starch granules in the endosperm were tightly embedded within the storage protein body matrix in wild-type and \( smk7 \) kernels, but were naked in \( fl3 \) and \( smk7;fl3 \) (Fig. 7, B and C). These results demonstrate that the ratio of zeins/nonzeins was altered in \( fl3 \) kernels but not in \( smk7 \).
Transcriptomic Alterations in the smk7 Endosperm

To reveal the function of smk7, we performed RNA sequencing (RNA-seq) using endosperm cells from smk7, fl3, smk7;fl3, and wild-type kernels at 15 DAPS on the same F1 ears. Differentially expressed genes (DEGs) were identified as those with a threshold fold change greater than two times at $P_{0.01}$. Compared with wild type, 3,009 and 7,927 DEGs were identified in smk7 and fl3, respectively. Notably, 1,478 (49.1%) DEGs were common to smk7 and fl3 (Fig. 8A), indicating that smk7 and fl3 share overlapping functions. Gene ontology (GO) analysis showed that these DEGs were mainly related to DNA-dependent replication (GO:0006261, false discovery rate [FDR]: 3.21E-06), DNA replication initiation (GO:0006270, FDR: 2.97E-03), and recombinational repair (GO:0000725, FDR: 1.89E-03; Fig. 8B). According to the Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.kegg.jp/) analysis, these DEGs were primarily related to DNA replication, fatty acid biosynthesis, and metabolism (Fig. 8C). Multiple key genes involved in DNA replication and the cell cycle were differentially expressed in the three mutants, such as those belonging to the minichromosome maintenance (MCM) 2-7 gene family, the proliferating cell nuclear antigen (PCNA) subunit of DNA polymerase $\delta$, RBR protein, and cyclins. The MCM2-7 complexes bind to the origin region to form the DNA prereplication complex (Kusunoki and Ishimi, 2014). The expression of 10 MCM2-7 genes increased by more than 2-fold in smk7 and smk7;fl3 and five MCM2-7 genes were upregulated in fl3 (Fig. 8D). The PCNA protein functions as the hub protein in the replication fork (Boehm et al., 2016), and PCNA1 and PCNA2 were upregulated in all three mutants (Fig. 8D). However, RBR3, which plays a positive role in E2F-dependent gene expression and DNA replication, was upregulated in the three mutants (Fig. 8D; Sabelli et al., 2009). Previous study showed that RBR3, PCNA, and MCM2-7 positively regulate endoreduplication in maize and Arabidopsis (Arabidopsis thaliana; Park et al., 2005; Sabelli et al., 2013). In smk7, all flow cytometry peaks examined were substantially less pronounced than in wild type, suggesting that the mean ploidy of the endosperm nuclei was higher in mutant endosperm and that endoreduplication is affected by zmnrpc2 (Supplemental Fig. S7). These results demonstrate that both fl3 and smk7 affect DNA replication.
To investigate the different functions of smk7 and fl3 in maize endosperm development, GO and KEGG analyses were performed using the smk7 DEGs (1,531) and fl3 DEGs (6,449). GO categories that were related to cell proliferation, such as mitotic cell cycle process, mitotic nuclear division, cell population proliferation, and DNA replication, as well as MCM complex and DNA packaging complex, were highly enriched among smk7 DEGs (Fig. 8B), and were shared by DEGs in double mutants. Furthermore, 12 cyclins were upregulated in smk7 and smk7;fl3 (Fig. 8D), confirming that smk7 affects cell proliferation. However, fl3 DEGs were mainly related to small molecule metabolic process, lipid biosynthetic process, organic acid metabolic process, oxoacid metabolic process, and peroxisome categories (Fig. 8B). Some key genes involved in protein biosynthesis were significantly downregulated in fl3 and smk7;fl3, including Opaque5 (O5), O11, O7, Oxalyl-CoA Decarboxylase1 (OCD1), Naked endosperm1 (Nkd1), Pyruvate Orthophosphate Dikinase1, and Pyruvate Orthophosphate Dikinase2 (Fig. 8D) and strikingly, five CYCLIN genes were upregulated in fl3 (Fig. 8D). These results indicated that FL3 plays a major role in zein synthesis to control seed quality and might also affect cell proliferation.

**DISCUSSION**

**Mutation of ZmNRPC2 Affects Maize Endosperm Development**

We have described a recessive mutant that represses endosperm cell proliferation, causes defects in the endosperm peripheral regions and BETL cell wall in-growths, and compacts the length of the CZ cells (Fig. 1,
To date, most maize kernel mutants that have been characterized show either defects in storage reserve accumulation (e.g. the opaque and floury mutants), or incomplete seed development, which affects germination, such as *dek* or *empty pericarp* mutants (Zhang et al., 2016; Cai et al., 2017; Chen et al., 2017; Feng et al., 2018). Unlike those mutants, *smk7* affected neither kernel storage protein composition nor germination, but delayed endosperm development and in turn, led to lower kernel weight and smaller kernels. The BETL in the kernel is composed of two to three strata of highly elongated transfer cells with invaginated cell walls (McCurdy and Hueros, 2014), and transports nutrients from phloem termini in the pedicel to the BETL in the kernel is composed of two to three strata of highly elongated transfer cells with invaginated cell walls (McCurdy and Hueros, 2014), and transports nutrients from phloem termini in the pedicel to the BETL, CZ, and the growth and function of the BETL, the CZ, and the central starchy endosperm; however, *FL3* is exclusively expressed in the central starchy endosperm (Supplemental Fig. S8; Zhan et al., 2015; Li et al., 2017c). Taken together, we suggest that ZmNRPC2 controls endosperm development by regulating the BETL, CZ, and the growth and function of starchy endosperm cells, providing a novel entry point to understand seed development.

**ZmNRPC2 Is Essential for RNAPIII Function**

The two largest polypeptides in RNAPIII, NRPC1, and NRPC2 form the binding cleft for DNA and harbor the active site of the enzyme, which fosters catalytic phosphodiester bond formation during the transcription of genes encoding small noncoding RNA molecules (Vannini and Cramer, 2012). In mice, mutation of NRPC2 reduced the cell proliferation in neonatal intestinal epithelium; in zebra fish, mutation of NRPC2 caused cell cycle abnormal in highly proliferative tissues, such as the liver, retina, and terminal branchial arches; in Arabidopsis, mutation of NRPC2 caused female gametophyte developmental arrest (Yee et al., 2007; Onodera et al., 2008; Kieckhaefer et al., 2016). In this study, *smk7*, *nrpc2-KO2*, and *nrpc2-KO3* defects of three different positions in the conserved RNA-pol_Rpb2_7 domain of ZmNRPC2 suppressed the cell proliferation in endosperm and caused the small kernel.
phenotype; in addition, in nrpc2-KO1, deletion of this protein can result in kernel developmental arrest. Meanwhile, zmnrpc2 affects kernel development more seriously than seedling growth. All the above results indicate that normal ZmNRPC2 protein is essential during the kernel rapid development stage. Similar phenomena have been observed in Treacher–Collins syndrome, in which RNA polymerases mutation affects the proper formation of the first and second branchial arches at weeks 5 to 8 of fetal development (Kadakia et al., 2014).

RNAPIII comprises 17 subunits that include the conserved 10-subunit core, the stalk, and two additional subcomplexes. The composition of RNAPIII is conserved throughout eukaryotes (Ream et al., 2015). The NRPC2 protein is associated with the largest subunit of RNAPIII and provides a platform for the assembly of other subunits/subcomplexes (Hardeland and Hurt, 2006; Cieślak et al., 2015). The data here confirmed the functions of homologous NRPC2 in maize and showed that ZmNRPC2 interacts with three critical RNAPIII subunits, RPC53, AC40, and TFC1 (Fig. 5, A and B). The RPC53–RPC37 heterodimer is positioned within the lobe of RNAPIII, close to the active site, the stalk, and the heterotrimer, and plays an important role in RNAPIII transcription termination, reinitiation, and elongation, as well as promoter accessibility (Wu et al., 2011, 2012; Arimbasseri et al., 2014). The AC40 protein is a component of the 10-subunit enzymatic core (Vannini and Cramer, 2012). TFC1 is a subunit of TFIIIC, which binds to the internal promoter elements and intragenic regions of tRNAs and 5S rRNAs for recruitment of TFIIIB and RNAPIII, and is required for transcription of tRNAs and 5S rRNAs (Male et al., 2015). These RNAPIII subunits might be degraded when they are not assembled or are misassembled into the complex, as was observed for mutants defective in the assembly of the RNAPIII complexes, in which the largest RNAPIII subunit underwent nuclear degradation (Garrido-Godino et al., 2013). ZmNRPC2 also interacted with FL3, which functions as a regulator within the RNAPIII transcription machinery and controls tRNAs and 5S rRNA levels (Li et al., 2017c).
These results indicate that NRPC2 is the central core of RNAPIII in maize.

RNAPIII Affects Cell Proliferation and Endoreduplication in Maize Endosperm

The normal transcription of 5sRNA and tRNAs is critical to maintain efficient running of the protein synthesis system, which guarantees the need for protein during DNA replication. The process of cell proliferation and endoreduplication, which relies on DNA replication, ensure the normal development of endosperm (Lopes and Larkins, 1993; Park et al., 2005; Sabelli et al., 2009). Our data indicates that the decreased expression level of ZmNRPC2 or loss of FL3 function suppressed the transcription of 5sRNA and tRNAs (Supplemental Fig. S9), which affects protein synthesis thus influencing DNA replication, the mitotic cell cycle, and endoreduplication (Fig. 8B), eventually leading to defective endosperm development and small kernel size. This differs from several signaling pathways that determine seed size, such as the Leu-rich repeat kinase pathway, the ubiquitin–proteasome pathway, and G-protein signaling (Li and Li, 2016; Li et al., 2019), and represents a novel pathway in the control of seed size. Maize endosperm development undergoes two major mitotic cell cycles—the coenocytic and cellularization stages. Nuclei of the coenocytic endosperm divide rapidly but no cytokinesis occurs (until ~2–4 DAP), resulting in a single multinucleate cell. This phase is dependent on rapid DNA replication (Lopes and Larkins, 1993; Brown et al., 2003; Olsen, 2004). At cellularization, mitotic cell division causes the number of endosperm cells to increase dramatically, to generate the vast majority of starchy endosperm cells, but the total number of cells is also influenced by the coenocytic phase (Kowles and Phillips, 1985). Therefore, the number of endosperm cells might be influenced by the level of DNA replication. In smk7 and fl3, there were considerably fewer endosperm cells, whereas the expression levels of many genes involved in DNA replication and the mitotic cell cycle increased. Furthermore, the relationship between the low transcriptional activity of RNAPIII and cell proliferation defects have been confirmed in mice and zebrafish (Danio rerio; Yee et al., 2007; Kieckhaefer et al., 2016).

Endoreduplication is a specialized type of cell cycle, in which one or more rounds of DNA synthesis are performed without mitosis, resulting in polyploid cells (Larkins et al., 2001). A strong correlation between endoreduplication and cell size has been observed in the maize endosperm (Sabelli et al., 2013; He et al., 2019). In smk7, endoreduplication was affected and the endosperm cells were larger; furthermore, expression of MCM2-7, RBR3, PCNA1, and PCNA2 increased considerably in mutant kernels. In maize and Arabidopsis, RBR3, PCNA, and MCM2-7 positively regulate endoreduplication (Park et al., 2005; Sabelli et al., 2013); and RBR1 negatively regulates these DNA replication factors to stimulate nuclear and cell enlargement and control endosperm development in maize (Sabelli et al., 2013). These findings indicated that RNAPIII affects cell cycle and endosperm development in maize (Fig. 9).

RNAPIII Affects Storage Reserve Filling in Maize Kernel

Intriguingly, several well-characterized opaque endosperm genes such as O7, O11, OCD1, and NKD1...
were significantly downregulated in fl3 and smk7/fl3 endosperm. The o5, o11, ocd1, and ndl1 mutants have kernels with an opaque endosperm, which is consistent with the fl3 and smk7/fl3 phenotype (Myers et al., 2011; Yi et al., 2015; Feng et al., 2018; Yang et al., 2018). O7 expression was significantly upregulated in the fl3 and smk7/fl3 endosperm, and o7 also has an opaque endosperm (Wang et al., 2011). Notably, OCD1 acts downstream of O7 and encodes oxalyl-CoA decarboxylase, which catalyzes the breakdown of oxalyl-CoA into formyl-CoA and CO2 (Yang et al., 2018). We observed that genes involved in oxoacid metabolism were significantly enriched in fl3 DEGs (Fig. 8). Similarly, fl3 and smk7/fl3 possess an opaque endosperm with a reduced zein protein content, but smk7 has a normal endosperm. This might be caused by defects in different subunits of RNAPIII (Fig. 9) and suggests that RNAPIII potentially plays a role in affecting maize kernel development and storage reserve filling.

MATERIALS AND METHODS

Plant Material

All maize (Zea mays) plants were grown either in plastic greenhouses in Wuhan, China or in the field in Huanggang and Sanya, China. The temperature in the greenhouse was 25°C to 32°C during maize pollination and the early developmental stage. The pollen of the Mo17 inbred line was collected and mutagenized with ethyl methanesulfonate for 30 min and was then applied to Mo17 female ears. All seeds were planted and self-pollinated and a single ear in the B73 genetic background.

Subcellular Localization of ZmNRP2C

To generate a translational ZmNRP2C-GFP and dNRP2C-GFP fusion, we amplified the full-length ORFs of ZmNRP2C and dNRP2C lacking the stop codons by PCR using cDNA from the inbred line Mo17 and smk7, respectively, and cloned them into pCM99-GFP under control of the cauliflower mosaic virus 35S promoter. Maize mesophyll protoplasts were isolated from etiolated leaves by digestion with an enzyme solution (1.5% [w/v] cellulose, 0.3% [w/v] pectolyase Y23, 20 mU of MES at pH 5.7, 0.4 mM mnnitol, 20 mM KCl, 10 mM CaCl2, and 0.1% [w/v] BSA). The plasmids containing the ZmNRP2C-GFP and dNRP2C-GFP fusion constructs were transfected into the protoplasts using established protocols (Yoo et al., 2007). The transfected protoplasts were cultured at 25°C overnight and GFP and Tracker red signals were detected with a FluoView FV1200 confocal microscope (Olympus).

Cytological Observation

Kernels at 8, 10, 12, 15, 18, and 21 DAP were collected and cut along the longitudinal axis. The cut kernels were fixed overnight in 4% (v/v) paraformaldehyde (Sigma-Aldrich), dehydrated in an ethanol gradient series (30%, 50%, 70%, 85%, 95%, and 100% [v/v] ethanol), and embedded in Paraplast Plus (Sigma-Aldrich). The sample blocks were sectioned into 8-μm slices using a model no. RM2265 microtome (Leica Microsystems) and stained with 0.5% (w/v) toluidine blue O. Images were captured using a model no. MZFIII microscope (Leica Microsystems). Additionally, for transmission electron microscopy analysis, endosperms at 15 DAP were cut into 1-mm3 pieces. Fresh tissues were fixed overnight in 2.5% (w/v) glutaraldehyde in 0.1 M of phosphate buffer at pH 7.4, fixed in 2% (w/v) OsO4 in the same buffer, and then dehydrated and embedded in epoxy resin and SPI-812 (Structure Probe). Ultrathin sections obtained using a model no. UC6 ultra microtome (Leica Microsystems) were stained with uranyl acetate and subsequently with lead citrate. The observations and recording of images were performed using a model no. H-7650 transmission electron microscope (Hitachi) at 80 kV and a 802-CCD camera (Gatan). The procedures were performed as described by Yi et al. (2010). For scanning electron microscopy analysis, mature wild-type and mutant kernels were excised with a razor, sputter-coated with gold in an E-100 ion sputtering process, and observed with a scanning electron microscope (S3400N; Hitachi).

Vector Construction for Maize Transformation

We performed CRISPR/Cas9–based gene editing of Zm00001d035960, with three guide RNAs to target the insertion, using pCPB-ZmUbi-hspCas9 (Li et al., 2017a). For CRISPR/Cas9–edited plants, sequencing was used to identify the editing sites near the target position.

Measurement of Proteins, Soluble Sugars, Starch, and Soluble Amino Acids

Mature mutant and wild-type kernels were collected from the same segregating ears. A total of 30 mutant and wild-type endosperms from the same ear were pooled as a single replicate, the seed coats and embryos were removed from the kernels after soaking in water for 10 min and were ground into a fine powder in liquid nitrogen. Six biological replicates were used for subsequent analysis. A 50-μg sample was incubated overnight in 1 mL of lysis buffer (12.5 mM of sodium borate, 1% [w/v] SDS, 2% [v/v] β-mercaptoethanol, 1% [w/v] cocktail [Merck], and 1% [w/v] phenylmethylsulfonyl fluoride) in a 37°C shaker. The mixture was centrifuged for 15 min at 13,000 g after which 300 μL of supernatant was carefully transferred into two different 1.5-mL centrifuge
Flow Cytometry

The endosperm was finely chopped using a razor blade in 1 mL of ice-cold Galbraith’s buffer (45 mM of MgCl₂, 20 mM of MOPS, 30 mM of sodium citrate, and 0.1% [v/v] Triton X-100, adjusted to pH 7.0 using 1 s of NaOH, and filtered through a 0.22-mm filter) in a glass petri dish. The homogenate was filtered through a 42-mm nylon mesh into a 1.5-mL sample tube. Propidium iodide and RNase were added to a final concentration of 50 mg mL⁻¹, after which the tubes were shaken gently. The homogenate was measured immediately using a flow cytometer (MoFlo XDP, Beckman Coulter), with an argon-ion laser tuned to 488 nm. A total of 15,000 particles were collected and analyzed using the software Summit v.5.2 (http://www.cyto.purdue.edu/cdnroms/cyto5/sporrons/cytometer/summit.html).

Y2H Assays and BiLC

For Y2H assays and BiLC, the ZmNRPC2 coding sequence was cloned into the pGBK7 plasmid and transformed into the yeast strain Y2HGold, whereas the coding sequence encoding the other RNA Pol subunit and FL3 were inserted into the pGADT7 plasmid and transformed into the yeast strain Y187. All yeast experiments were referenced in Clontech’s Yeast Protocols Handbook.

To create constructs for BiLC experiments, the ZmNRPC2 coding sequence was cloned into JW772 (C-terminal half of luciferase [CLUC]) to yield ZmNRPC2-CLUC and sequences encoding the other RNA PolIII subunit and FL3 were cloned into JW771 (N-terminal half of luciferase). The appropriate pairs of CLUC and N-terminal half of luciferase fusion constructs were used for the luciferase complementation assay, following the protocol described in Gou et al. (2011). Agrobacterium tumefaciens strain GV3101 harboring the above constructs was infiltrated into leaves from 3-week-old Nicotiana benthamiana plants using a needleless syringe. After incubating for 72 h under 12 h-light:8 h-dark, leaves were injected with 0.8 μM of luciferin and the resulting luciferase signals were captured using a T anon-5200 system (Tanon Science and Technology). These experiments were repeated at least three times with similar results. Quantitative analysis was performed using the software ImageJ (http://rsb.info.nih.gov/ij/).

RNA-Seq Analysis

Total RNA was extracted from pooled wild-type and mutant endosperm obtained from the same F2 ears derived from the cross nrpc2 × fl3 at 15 DAP (10 kernels per sample) using TRIzol reagent, followed by cleanup and DNase I treatment using an RNeasy Mini Kit (Qiagen), according to the manufacturer’s protocol. The different types of immature kernels were identified by genotyping using SNP62,672,025 and SNP6585. Three independent biological replicates from three different ears were performed. cDNA libraries were constructed following the standard Illumina protocol and sequenced on the Illumina NovaSeq platform by Novogene. The sequence reads were trimmed using the tool Trimmomatic (v.0.33; http://www.usadellab.org/cms/?page=trimmomatic) and were mapped to the B73 RefGen_v4.34 reference genome using the program HISAT2 (v.2.1.0; http://ccb.jhu.edu/software/hisat2/index.shtml). StringTie(v.1.3.3b) was employed to reconstruct the transcripts and to estimate gene expression levels (Pertea et al., 2016). The program HTSeq (v.0.6.1; https://hyypi.org/project/HTSeq/) was used to count the number of reads per gene. Significant DEGs were identified by DESeq2 (http://www.bioconductor.org/packages/devel/bioc/html/DESeq2.html) as those with a P value of differential expression above the threshold (P < 0.01, absolute value of log[fold change] > 1). The joint GO and KEGG term enrichment results were generated using the software “gProfiler” (http://bit.io/utee/gprofiler/).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers XP_008648749 for NRPC2, NM_001152112 for FL3, XM_008669830 for RPC35, XM_008663438 for TFC1, and NP_001130839 for AC40. Sequences used for phylogenetic analysis are as follows: XP_003570586.1, Brachypodium distachyon; XP_015632810.1, Oryza sativa Japonica Group; XP_004951189.1, Setaria italica; XP_021305150.1, Sorghum bicolor; XP_008648749.1, Z. mays; XP_026427527.1, Maizea triticaula; XP_006835357.1, Glycine max; XP_017613530.1, Coccopum arboruen; XP_010690064.1, Vitis vinifera; NP_001332061.1, Arabidopsis thaliana; XP_020773981.1, Haplopodia burtornii; NP_014880.1, Saccharomex cerevisiae; XP_01232450.1, Donia ervis; XP_01426402.1, Musculus zebra; NP_011154108.1, Homo sapiens; XP_00406743.1, Ovis ares; NP_081690.1, Mus musculus; XP_002904335.1, Photophora infestans, and PRJNA63972 for RNA-seq data.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Phenotype of smk7 kernels and plants.

Supplemental Figure S2. Kernel phenotypic inheritance and a comparison of developing wild-type and smk7 kernels at different development stages.

Supplemental Figure S3. Protein structure and three-dimensional structure of NRPC2.

Supplemental Figure S4. Allelism test between smk7 and ZmNRPC2 knockout alleles.

Supplemental Figure S5. Subcellular localization of NRPC2 in maize leaf cells.

Supplemental Figure S6. Kernel phenotype of smk7β3.

Supplemental Figure S7. Flow cytometry profiles of wild-type and smk7 endosperm at 10 and 12 DAP.

Supplemental Figure S8. Spatial expression pattern of ZmNRPC2 and FL3 in maize kernels.

Supplemental Figure S9. Expression level of 17 tRNAs and 5S rRNA genes in 15-DAP endosperm of the wild type, fl3, smk7, and smk7β3.

Supplemental File S1. The full alignment file for estimating the NRPC2 protein sequences phylogenies used in this study.

Supplemental File S2. Primers used in this study.

ACKNOWLEDGMENTS

We thank Suhua Yang (Plant Science Facility of the Institute of Botany, Chinese Academy of Sciences) for her excellent technical assistance of flow cytometry. We also thank Plant Editors (https://planteditors.com/) for manuscript editing.

Received April 27, 2020; accepted June 15, 2020; published June 26, 2020.

LITERATURE CITED

Abascal-Palacios G, Ramsay EP, Beuron F, Morris E, Vannini A (2018) Structural basis of RNA polymerase III transcription initiation. Nature 553: 301–306

Arimbasseri AG, Rijal K, Maria RJ (2014) Comparative overview of RNA polymerase II and III transcription cycles, with focus on RNA polymerase III termination and reinitiation. Transcription 5: e27639

Bai F, Corll J, Shodja DN, Davenport R, Feng G, Mudunkothge J, Becraft PW, Li K, Dey N, Asuncion-Crabb Y (2001) Cell fate specification in early maize development. Plant Cell Dev Biol 12: 387–394

Becraft PW, Li K, Dey N, Asuncion-Crabb Y (2002) The maize dek1 gene functions in embryonic pattern formation and cell fate specification. Development 129: 5217–5225

Boehm EM, Gildenberg MS, Washington MT (2016) The many roles of PCNA in eukaryotic DNA replication. Enzymes 39: 231–254
Ponicsan SL, Kugel JF, Goodrich JA (2010) Genomic gems: SINE RNAs regulate mRNA production. Curr Opin Genet Dev 20: 149–155
Qi W, Lu L, Huang S, Song R (2019) Maize Dok4 encodes mitochondrial ribosomal protein L9 and is required for seed development. Plant Physiol 180: 2106–2119
Qi W, Yang Y, Feng X, Zhang M, Song R (2017) Mitochondrial function and maize kernel development requires Dek2, a pentatricopeptide repeat protein involved in nad1 mRNA splicing. Genetics 205: 239–249
Ream TS, Haag JR, Pontvianne F, Nicora CD, Norbeck AD, Paša-Tolić L, Pikaard CS (2015) Subunit compositions of Arabidopsis RNA polymerases I and III reveal Pol I- and Pol III-specific forms of the AC40 subunit and alternative forms of the CS3 subunit. Nucleic Acids Res 43: 4163–4178
Regvar M, Eichert D, Kaulich B, Gianoncelli A, Pongrac P, Vogel-Mikus (2011) New insights into globoids of protein storage vacuoles in wheat aleurone using synchrotron soft x-ray microscopy. J Exp Bot 62: 3929–3939
Ren X, Pan Z, Zhao H, Zhao J, Cai M, Li J, Zhang Z, Qiu F (2017) EMPTY PERICARP11 serves as a factor for splicing of mitochondrial nad1 intron and is required to ensure proper seed development in maize. J Exp Bot 68: 4571–4581
Sabelli PA, Hoerster G, Lizarraga LE, Brown SW, Gordon-Kamm WJ, Larkins BA (2009) Positive regulation of minichromosome maintenance gene expression, DNA replication, and cell transformation by a plant retinoblastoma gene. Proc Natl Acad Sci USA 106: 4042–4047
Sabelli PA, Liu Y, Dante RA, Lizarraga LE, Nguyen HN, Brown SW, Klingler JP, Yu J, LaBrant E, Layton TM, et al (2013) Control of cell proliferation, endoreduplication, cell size, and cell death by the retinoblastoma-related pathway in maize endosperm. Proc Natl Acad Sci USA 110: E1827–E1836
Vannini A, Cramer P (2012) Conservation between the RNA polymerase I, II, and III transcription initiation machineries. Mol Cell 45: 439–446
Wang A, Hou Q, Si L, Huang X, Luo J, Lu D, Zhu J, Shangguan Y, Miao J, Wang H, Wang K, Du Q, Wang Y, Fu Z, Guo Z, Kang D, Li WX, Tang J (2018) Maize Urb2 protein is required for kernel development and vegetative growth by affecting pre-ribosomal RNA processing. New Phytol 218: 1233–1246
Wu CC, Herzog F, Jennebach S, Lin YC, Pai CY, Aebersold R, Cramer P, Chen HT (2012) RNA polymerase III subunit architecture and implications for open promoter complex formation. Proc Natl Acad Sci USA 109: 19232–19237
Wu CC, Lin YC, Chen HT (2011) The TFIIH-like Rpc37/53 dimer lies at the center of a protein network to connect TFIIIC, Bdp1, and the RNA polymerase III active center. Mol Cell Biol 31: 2715–2728
Xiu Z, Sun F, Shen Y, Zhang X, Jiang R, Bonnard G, Zhang J, Tan BC (2016) EMPTY PERICARP16 is required for mitochondrial nad2 intron 4 cis-splicing, complex I assembly and seed development in maize. Plant J 85: 507–519
Yang J, Fu M, Ji C, Huang Y, Wu Y (2018) Maize oxalyl-CoA decarboxylase1 degrades oxalate and affects the seed metabolome and nutritional quality. Plant Cell 30: 2447–2462
Yee NS, Gong W, Huang Y, Lorent K, Dolan AC, Maraia RJ, Pack M (2007) Mutation of RNA Pol III subunit rpc2/polr3b leads to deficiency of Subunit Rpc11 and disrupts zebrinii digestive development. PLoS Biol 5: e312
Yi B, Zeng F, Lei S, Chen Y, Yao X, Zhu Y, Wen J, Shen J, Ma C, Tu J, et al (2010) Two duplicate CYP704B1-homologous genes BnMs1 and BnMs2 are required for pollen exine formation and tapetal development in Brassica napus. Plant J 63: 925–938
Yi G, Neelakandan AK, Gontarek BC, Vollbrecht E, Becraft PW (2015) The naked endosperm genes encode duplicate INDETERMINATE domain transcription factors required for maize endosperm cell patterning and differentiation. Plant Physiol 167: 443–456
Yoo SD, Cho YH, Sheen J (2007) Arabidopsis mesophyll protoplasts: A versatile cell system for transient gene expression analysis. Nat Protoc 2: 1565–1572
Zhan J, Thakare D, Ma C, Lloyd A, Nixon NM, Arakaki AM, Burnett WJ, Logan KO, Wang D, Wang X, et al (2015) RNA sequencing of laser-capture microdissected compartments of the maize kernel identifies regulatory modules associated with endosperm cell differentiation. Plant Cell 27: 513–531
Zhang XO, Gingeras TR, Weng Z (2019) Genome-wide analysis of polymerase III-transcribed Alu elements suggests cell-type-specific enhancer function. Genome Res 29: 1402–1414
Zhang Y, Zheng X, Yang J, Messing J, Wu Y (2016) Maize endosperm-specific transcription factors O2 and PBF network the regulation of protein and starch synthesis. Proc Natl Acad Sci USA 113: 10842–10847