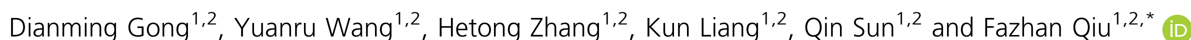
Plant Biotechnology Journal

Plant Biotechnology Journal (2023) **21**, pp. 451–453 doi: 10.1111/pbi.13957

Brief Communication

Overexpression of *ZmKL9* increases maize hybrid hundred kernel weight

Dianming Gong[1,2], Yuanru Wang[1,2], Hetong Zhang[1,2], Kun Liang[1,2], Qin Sun[1,2] and Fazhan Qiu[1,2,*]



[1]*National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, China*
[2]*Hubei Hongshan Laboratory, Wuhan, China*

Received 21 June 2022;
revised 7 September 2022;
accepted 28 October 2022.
**Correspondence* (Tel +86-02787286870; fax +86-02787280016; email qiufazhan@mail.hzau.edu.cn)

Keywords: maize, *ZmKL9*, transposon element, kernel and ear yield.

Increasing maize yield remains the primary goal in maize breeding in the world. Kernel and ear morphology are two important yield-determining traits in maize (Simmons *et al*., 2020). However, maize kernel shape and ear size are complex quantitative traits, for which a few functional genes with potential breeding applications have been reported. Overexpression of *vacuolar protein sorting 29* (*ZmVPS29*) can improve the yield per plant in different maize genetic backgrounds (Chen *et al*., 2020). *Kernel number per row 6* (*KNR6*) can also increase the grain yield of hybrid maize either through the introduction of useful allelic variants or by raising *KNR6* transcript levels (Jia *et al*., 2020). Moreover, genome editing of *1-aminocyclopropane-1-carboxylate oxidase 2* (*ZmACO2*) promoted maize ear development, resulting in an ~13.4% increase in grain yield per ear in hybrid lines (Ning *et al*., 2021). In a previous study, we preliminarily evaluated the potential breeding value of the quantitative trait locus *kernel length 9* (*qKL9*), which controls kernel length and kernel weight in various populations (Gong *et al*., 2021). We wished to clone the causal gene responsible for the quantitative variation in maize grain yield associated with *qKL9* and explore the causal polymorphisms. Our ultimate goal is to develop new functional markers and evaluate the breeding potential of the causal gene behind *qKL9* to create new avenues for improving maize yield.

To further fine-map *qKL9*, we developed a backcross population of about 20 000 individuals to screen for recombinants. Despite the size of the population, the low recombination rate over the candidate region harbouring *qKL9* resulted in an ~736-kb region flanked by markers IN6706 and N23, corresponding to 19 annotated genes, based on the B73 RefGen v4 genome (Figure 1a, Tables S1 and S2). An analysis of published gene expression data (Yi *et al*., 2019) and transcriptome deep sequencing (RNA-seq) data of seed at 6, 9 and 14 DAP (days after pollination) of the maize inbred Mc and the BC_4F_2 line Mc^{qKL9} revealed *Zm00001d046718* as the only differentially expressed gene in 6-DAP seeds between Mc and Mc^{qKL9}. Another 13 genes were expressed at similar levels or at low levels in seeds (Figure 1b and Figure S1). Based on this result, we selected *Zm00001d046718*, which encodes a basic leucine zipper (bZIP) transcription factor, as the key candidate gene for *qKL9*, prompting us to name it *ZmKL9*.

To identify the polymorphic sites in *ZmKL9*, we determined the genomic sequence of *ZmKL9* in Mc and Mc^{qKL9}, which revealed single nucleotide polymorphisms (SNPs) and insertion/deletions (InDels) in the promoter and coding region, as well as the insertion of a 288- and 557-bp transposable element (TE) in the 5′ untranslated region (5′ UTR) and the first intron, respectively, named TE1 and TE2, in Mc relative to Mc^{qKL9} (Figure 1c, Table S3). Moreover, we genotyped the recombinants (Figure 1a) for the presence of TE1 and TE2 and established that their presence cosegregates with the kernel length phenotype (Figure 1d). We also sequenced *ZmKL9* in a set of 149 diverse maize inbred lines and determined that TE1 is significantly associated with kernel length by association mapping (Figure 1e). Indeed, inbred lines harbouring TE1 (TE+) were characterized by shorter kernels and lower hundred kernel weight (HKW), on average than the lines lacking TE1 (TE–), although kernel width was not affected (Figure 1f). We investigated the possible effect of the presence of TE1 on *ZmKL9* expression by performing a transient luciferase (LUC) transcriptional assay in maize protoplasts. Accordingly, we placed the firefly *LUC* reporter gene under the control of the *ZmKL9* promoter from Mc (with TE1) or Mc^{qKL9} (without TE1) and detected lower LUC activity derived from the construct containing TE1 (TE+ construct) relative to the construct lacking the TE (TE− construct; Figure 1g). We concluded that the TE1 in the *ZmKL9* 5′ UTR is a key polymorphism that influences the transcriptional output of *ZmKL9*.

To elucidate the biological function of *ZmKL9*, we generated two overexpression lines (OE-*ZmKL9*-1 and OE-*ZmKL9*-2) and two *ZmKL9* knockout lines (CR-*zmkl9-1* and CR-*zmkl9*-2) using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease 9 (Cas9) genome editing (Figures S2 and S3). Compared with their respective nontransgenic control lines, the two *ZmKL9* overexpression lines (OE-*ZmKL9*-1 and OE-*ZmKL9*-2) had longer kernels, higher HKW, and longer and heavier ears (Figure 1h,i). Conversely, the two knockout lines CR-*zmkl9-1* and CR-*zmkl9*-2 produced shorter kernels and ears, and had a lower HKW and ear weight than the corresponding wild-type lines (Figure 1j,k). These results strongly supported the notion that *ZmKL9* is the causal gene underlying *qKL9* and positively controls kernel and ear yield in maize.

To evaluate the ability of *ZmKL9* to control maize yield in different genetic backgrounds, we crossed one *ZmKL9*-overexpressing line and its nontransgenic control line (OE-*ZmKL9*-1 and WT1) to four different elite inbred lines (Chang7-2, Zheng58, PH6WC, and PH4CV). We confirmed that *ZmKL9* expression is higher in F_1 hybrids between each inbred and the *OE* line relative to the nontransgenic F_1 hybrids. We also established that kernel

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Figure 1 ZmKL9 regulates maize kernel and ear yield. (a) Fine mapping of *qKL9*. (b) Expression levels of the candidate genes in 6/9/14–DAP seeds in the inbred Mc and the near-isogenic line Mc^{qKL9}. (c) Schematic diagram of the *ZmKL9* locus, showing all polymorphisms and the transposable elements (TEs, shown as triangles) in the 5′ UTR and the first intron of *ZmKL9*. TE structures were predicted using CENSOR. (d) Genotyping results of the recombinant lines used for fine mapping for the presence of TE1 and TE2 using specific primers. (e) Local association between 123 SNPs at the *ZmKL9* locus and kernel length in a set of 149 diverse maize inbred lines. (f) Distribution of kernel-related traits in 149 diverse inbred lines as a function of the presence/absence of TE1 at *ZmKL9*. (g) TE1 decreases transcription from the *ZmKL9* promoter in transient transfection assays in leaf protoplasts. (h, i) Kernel and ear traits in OE-*ZmKL9* and WT. (j, k) Kernel and ear traits in KO-*zmkl9* and WT. (l) Phenotypes of kernel and ear traits in F_1 hybrids between OE1-*ZmKL9* and four inbred lines. The values in (h–m) are mean ± SD. Numbers below each column indicate sample size. *P*-values were obtained from two-tailed, two-sample Student’s *t*-tests.

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length and HKW of the F_1 hybrids derived from the *OE* line displayed greater values, while kernel width was not affected, compared with F_1 hybrids derived from the wild-type control lines (Figure 1l). Moreover, in addition to kernel-related traits, multiple ear-related traits increased significantly in F_1 hybrids derived from the *OE* lines, including ear length, kernel numbers per ear, and ear weight relative to the F_1 hybrids derived from the wild-type control line (Figure 1m). We concluded that the overexpression of the *ZmKL9* can positively affect the grain and ear yield of maize in different genetic backgrounds.

Here, we report that the cloning of *ZmKL9* controls the quantitative variation of kernel and ear length in maize. We demonstrate that the presence of a transposon element in the 5′ UTR of *ZmKL9* is an important functional site that may be used as a molecular marker for marker-assisted selection. Furthermore, we propose that the introduction of a transgene, overexpressing *ZmKL9*, into different maize inbred lines will have great potential for maize yield improvement. Additional details of the research are described in Appendix S1.

Acknowledgements

This work was supported by the science and technology major program of Hubei Province (2021ABA011), the Wuhan Major Project of Key Technologies in Biological Breeding and New Variety Cultivation (2022021302024852), the National Natural Science Foundation of China (U2106230), and the National Natural Science Foundation of China (31860382).

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

FQ and DG designed the project. DG conducted the experiments. YW, HZ, KL, and QS participated in some experiments and conducted the field trials. DG analysed the data and wrote the manuscript. FQ revised the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1 Supplemental Methods.

Figure S1 Expression pattern of candidate genes in the *qKL9* region.
Figure S2 Overexpression of *ZmKL9*.
Figure S3 Sequences of two homozygous CRISPR-Cas9 knock-out lines with deletions in the target site (CR-*zmkl9*-1 and CR-*zmkl9*-2).

Table S1 Putative genes in the 736-kb *qKL9* region.
Table S2 Primers for the markers for further fine mapping *qKL9*.
Table S3 Primers for the markers for the amplification of *ZmKL9*.
Table S4 Primers for the markers for LUC activity assay.

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