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Letter to the Editor

A 4-bp Insertion at *ZmPLA1* Encoding a Putative Phospholipase A Generates Haploid Induction in Maize

Dear Editor,

Maize is one of the most important crops in the world and is also an essential raw material for the food, fuel, and fodder industries. Maize hybrids are widely used today, and gain of elite inbred lines is a crucial step for hybrid breeding. Doubled haploid (DH) technology based on *in vivo* haploid induction (HI) is often used to accelerate the efficiency of breeding of maize and other crops (Ishii et al., 2016). Maize is a typical diploid plant ($2n = 20$) with a very low rate (~0.1%) in producing haploid ($2n = 10$) naturally. *In vivo* HI by inducer line Stock6 can lead to maternal haploid with a rate of 1%–2% when it is used as pollinator (Coe, 1959). Stock6-derived inducers have been considered as the most effective method for DH breeding in maize.

Although the phenomenon of Stock6-induced haploidy was discovered 50 years ago, the genetic and biological mechanism of HI is still unclear. Several quantitative trait loci (QTLs) affecting the haploid induction rate (HIR) have been mapped (Prigge et al., 2012; Dong et al., 2013; Hu et al., 2016), of which the *qhir1* QTL located in bin 1.04 had the greatest effect on HI. Furthermore, *qhir1* has been narrowed down to a 243-kb region based on the B73 reference genome, which paves the way for gene cloning (Dong et al., 2013). In addition, fine mapping of *qhir1* and functional studies have revealed that, in addition to regulating HIR, *qhir1* also affects embryo abortion rate, endosperm abortion rate, and segregation distortion (Dong et al., 2013; Xu et al., 2013).

In the region defined by fine mapping, we identified 13 genes in the B73 reference genome including GRMZM2G471240 and GRMZM2G062320, encoding phospholipase A (named as *ZmPLA1*) and thiolase, respectively. The other 11 genes are either low-confidence genes or transposable elements-related genes (Figure 1A and Supplemental Table 1). RNA sequencing of the anthers of B73 and B73-inducer (with ~80% B73 background, HIR = 10%) was performed at the different developmental stages (meiosis, one nucleus, two nuclei, three nuclei; Figure 1B). In the mapping region of *qhir1*, only three transcripts were detected (fragments per kilobase of exon per million mapped reads ≥ 5) based on B73 genome including two long noncoding RNAs and *ZmPLA1*; however, in B73-inducer, only *ZmPLA1* was expressed, and no significant gene expression difference was found between B73 and B73-inducer. Thus, *ZmPLA1* is the most likely candidate gene for *qhir1* (Figure 1B). *ZmPLA1* was observed in anther and not in other tissues based on the public data for B73 from MaizeGDB (www.maizegdb.org), and its mRNAs were detected at the second mitosis stage (two-nuclei stage) and reached the highest level at the three-nuclei stage (Figure 1B).

To characterize the variation at *ZmPLA1*, we constructed a bacterial artificial chromosome (BAC) library of the Stock6-derived haploid inducer line CAU5. BAC clones covering the mapping region of *qhir1* were identified and one positive BAC clone was then sequenced and assembled. The sequence of the positive CAU5-BAC clone was compared with that of the B73 reference genome. *ZmPLA1* of CAU5 contained 11 SNPs and a 4-bp (CGAG) insertion in the fourth exon compared with the B73 reference genome (Figure 1C and Supplemental Table 2). Of the 11 SNPs, the 409th base-pair C-T substitution, the 421st base-pair C-G substitution, and the 1210th base-pair G-C substitution resulted in amino acid substitutions. In addition, the 4-bp insertion at the fourth exon led to a frame shift causing 20 altered amino acids and a premature transcription termination that truncates the protein by 29 amino acids. Analysis of the *ZmPLA1* sequences in 50 inbred lines revealed that the three SNPs that led to amino acid substitutions also occurred in non-inducer lines; however, the 4-bp insertion in the fourth exon was a unique feature of inducers. We also tested for the variation of 4-bp InDel in more than 300 maize inbred lines and 180 teosinte accessions and five additional inducer lines, and found that the 4-bp insertion was consistently present in inducer lines and absent in all other teosinte and maize lines. Thus, we proposed that the 4-bp (CGAG) insertion may cause a weak or loss-of-function allele of *ZmPLA1*, leading to the HI phenotype. This allele is a rare mutation and may have occurred after maize domestication, since it was not detected in the teosinte accessions.

Next, we used the CRISPR/Cas9-mediated genome editing technology to knock out *ZmPLA1* for gene function validation. Based on the sequence of the first exon (Figure 1C), we designed and synthesized the sequence of multiple guide RNAs and inserted into the plant gene expression vector pBUE411. The gene editing efficiency of the recombinant vectors was tested and verified in maize protoplasts. The recombinant vector with high knockout efficiency was selected and used to transform the receptor line. In the T_0 generation, more than 10 heterozygous transgenic plants were screened by both bialaphos (bar) strip test and sequencing analysis. The transgenic plants showing sequence variations in the target region were self-pollinated to generate T_1 generations and genotyped using the primers flanking the target region (Supplemental Table 3). Three lines including 1-bp insertion (ZmHIR1-1), 11-bp deletion (ZmHIR1-2), and 1-bp deletion (ZmHIR1-3) in the target region (Figure 1C), which are putative knockout alleles of *ZmPLA1*, were chosen for self-pollination and used as male to pollinate two commercially

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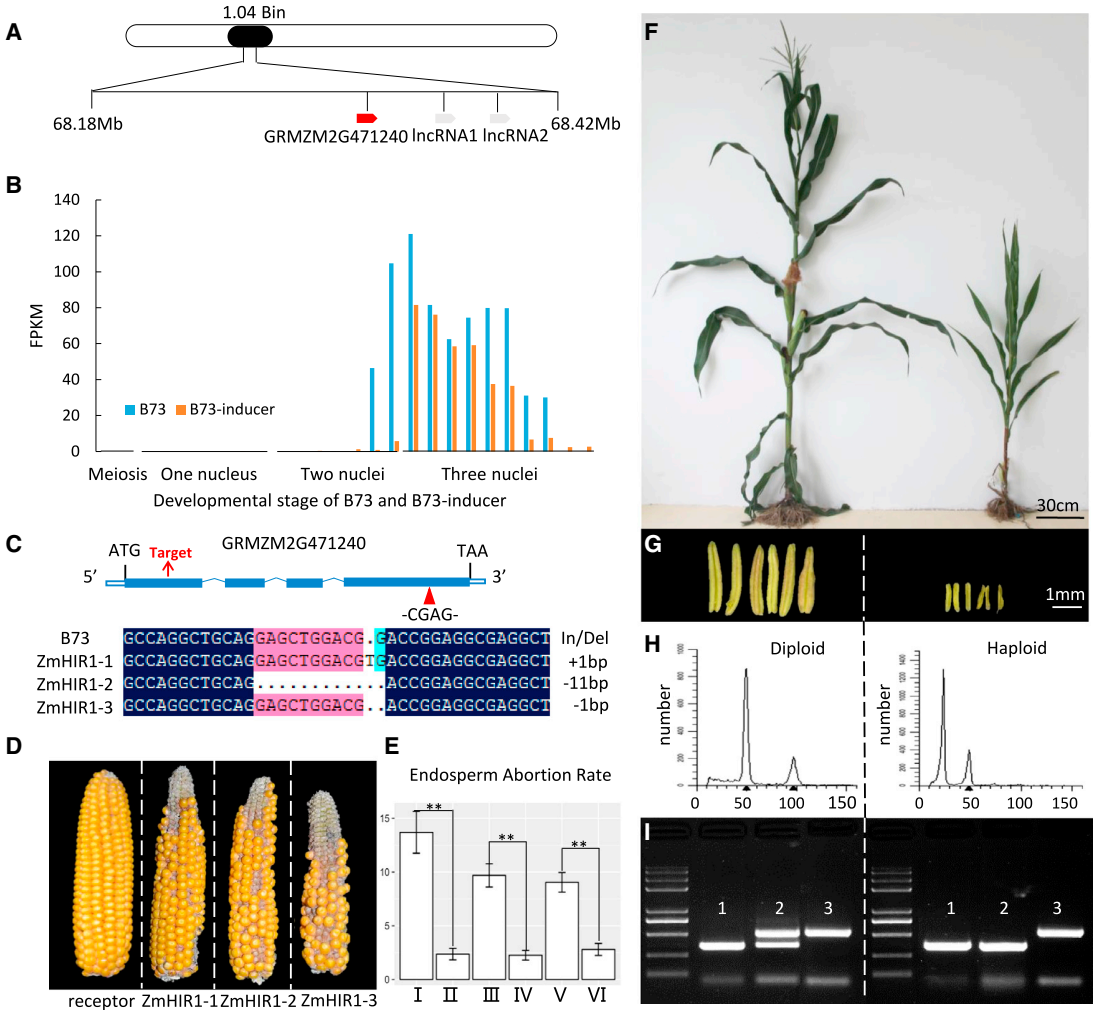

Figure 1. *ZmPLA1* (GRMZM2G471240) Mutation Causes Haploid Induction in Maize.

(A) Genetic mapping for *qhir1*. Fine mapping identified a 243-kb region containing three expressed genes, a coding gene GRMZM2G471240 (in red), and two long noncoding RNAs (lncRNA1 and 2; in gray).

(B) The expression pattern of GRMZM2G471240 in B73 and B73-inducer line at meiosis and one-nucleus, two-nuclei, and three-nuclei stages of pollen development.

(C) The structure of GRMZM2G471240 with 4-bp insertion (at red triangle). The red arrow shows the CRISPR target site in the first exon of GRMZM2G471240. The insertion and deletion sites of three allelic mutations (ZmHIR1-1, ZmHIR1-2, ZmHIR1-3) are shown in the alignment comparison with B73 sequence.

(D) The phenotype of wild (receptor line) and three T_1 generation (ZmHIR1-1, ZmHIR1-2, ZmHIR1-3) self-cross ears. Endosperm abortion kernels were detected in the three T_1 generation knockout ears.

(E) The rate of endosperm aborted kernels showed a significant difference between wild-type and T_1 generation knockout plants, and also in their hybrid plants with ZD958 and JK968. I, selfing-T_1; II, receptor inbred line (wild-type); III, ZD958 × T_1; IV, ZD958 × receptor inbred line (wild-type); V, JK968 × T_1; VI, JK968 × receptor inbred line (wild-type). $^{**}p < 0.01$.

(F) Field performance of diploid (left) and haploid (right) plants from the progeny of ZD958 pollinated by using T_1 knockout plants as male.

(G) The anther phenotypes of diploid (left) and haploid (right) plants from the progenies of ZD958 pollinated by T_1 knockout plants as male.

(H) Flow cytometry results of diploid (left) and haploid (right) DNA (signal intensity values indicated).

(I) PCR testing of diploid (left) and haploid plants (right) using polymorphic simple sequence repeat markers. Left-1, JK968 as female; left-2 (diploid), F_1 between JK968 and knockout T_1 plant; left-3, knockout T_1 plant as male. Right-1, JK968 as female; right-2 (haploid), F_1 between JK968 and knockout T_1 plant; right-3, knockout T_1 plant as male. Primer information is provided in Supplemental Table 3.

available hybrids, ZD958 and JK968, for testing the HIR of the T_1 transgenic plants.

Similar to Stock6-derived inducer lines, obvious endosperm abortion kernels were observed at 14.3% frequency in the self-pollinated knockout lines (Figure 1D and 1E; Supplemental Table 4). When using the knockout lines as males, the hybrid F_1 ears had an endosperm abortion rate of 10.25% for ZD958 and 9.05% for JK968 (Figure 1E and Supplemental Table 4). Kernels from self-pollinated ears and kernels from crossing ears

with ZD958 and JK968 were selected randomly to test haploid frequency (Figure 1D and 1E). Haploid plants are usually characterized by phenotypes of short plant height, compact type, small anther, and sterility, which can be easily identified in the field (Figure 1F and 1G) and further confirmed by flow cytometry analysis for their haploid ploidy (Figure 1H). To determine the origin of the haploid chromosome, we screened the knockout receptor line and ZD958/JK968 using polymorphic molecular markers. We found that all the haploids were derived from the maternal genome (Figure 1I). In the self-pollinated knockout lines, the putative HIR was 3.7% for ZmHIR1-2 and 6.67% for ZmHIR1-3. In the heterozygous mutants of ZmHIR1-1 and ZmHIR1-2, haploids were found in the progenies of ZD958 and JK968 with an HIR of 1.85%–3.51%. In the homozygous mutant allele of ZmHIR1-3, the HIR was 1.55% based on ZD958 (Supplemental Table 5). Our results from the two test hybrids showed that the average HIR of different mutants was approximately 2%, which is close to the HIR of Stock6 (Coe, 1959), indicating that the effect of *ZmPLA1* knockout lines on HIR in our tested plants may be similar to that of gene mutation in the ancestry inducer line Stock6. Collectively, our results suggest that *ZmPLA1* is the casual gene responsible for the HI in *qhir1* and that the 4-bp insertion within *ZmPLA1* results in a rare allele causing HI.

Members of the *Phospholipase A* (*PLA*) gene family is common and present in rice, *Arabidopsis*, sorghum, and many other plants (Wang, 2001). The high sequence similarity of PLAs from different plants may indicate certain conservation of their function. Thus, editing of *PLA* genes might represent a very promising approach to create haploid inducer lines in many other crops such as sorghum and rice. It was reported that *PLA* is involved in phospholipid degradation and linolenic acid production, which is required for jasmonic acid biosynthesis (Zheng and Zhang, 2015). However, little is known about the functions of *PLA* in HI. During the preparation of this work, a recent study by Kelliher et al. (2017) also showed that *ZmPLA1/MTL* is required for in HI in maize. Further studies toward understanding the molecular and genetic mechanisms of *ZmPLA1/MTL*-mediated HI may facilitate to enhance breeding efficiency not only in maize but also in other important crops.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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