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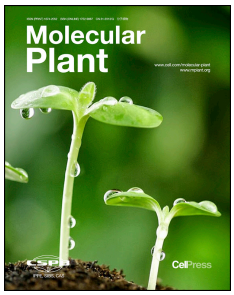

A 4bp insertion at *ZmPLA1* encoding a putative phospholipase A generates haploid induction in maize

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A 4bp 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 it is also an essential raw material for food, fuel and fodder industry. 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 used to accelerate the efficiency of breeding widely on maize and other crops (Ishii et al., 2016). Maize is a typical diploid plant (2*n*=20) with a very low rate (~0.1%) to produce haplolid (2*n*=10) naturally. *In vivo* haploid induction by inducer line Stock6 (Coe, 1959) can lead to maternal haploid with a rate of 1-2% when it is used as pollinator. 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 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. Further, *qhir1* has been narrowed down to a 243Kb 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. Other 11 genes are either low confidence genes or transposable elements related genes (Figure 1A, Supplementary Table 1). RNA sequencing of the anthers were performed for B73 and B73-inducer (with ~80% B73 background, HIR = 10%) during the different developmental stages (meiosis, one nucleus, two nuclei, three nuclei, Figure 1B). In the target region, only three transcripts were detected (FPKM >= 5) based on B73

genome including two lncRNAs and *ZmPLA1*, however, in B73-inducer, only *ZmPLA1* expressed and no any significant gene expression difference was found between B73 and B73-inducer. Thus, *ZmPLA1* is the most likely candidate (Figure 1B). *ZmPLA1* was observed in anther and not in other tissues based on the public data for B73 from MaizeGDB (www.maizegdb.org). The mRNA was detected at the second mitosis stage (two nuclei stage) and reached the highest level at the three nuclei stage (Figure 1B).

To further characterize the variation at the underlying gene, a bacterial artificial chromosome (BAC) library of the Stock6 derived haploid inducer line CAU5 was constructed. BAC clones covering the mapping region of *qhir1* were identified and one positive BAC clone was then sequenced and assembled. Sequences of the positive CAU5-BAC clone was compared with the B73 reference genome. *ZmPLA1* of CAU5 contained 11 SNPs and a 4bp (CGAG) insertion in the fourth exon compared to the B73 reference genome (Figure 1C, Supplementary Table 2). Of the 11 SNPs, the 409th base pair C-T substitution, the 421th base pair C-G substitution, and the 1210th base pair G-C substitution resulted in amino acid substitutions. In addition, the 4bp 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. The analysis of the *ZmPLA1* sequences in 50 inbred lines revealed that the three SNPs which led to amino acid substitutions also occurred in non-inducer lines; however, the 4bp insertion in the fourth exon was a unique feature of inducers. Further, we tested for the variation of 4bp InDel in more than 300 diverse maize inbred lines and 180 teosinte accessions and 5 additional inducer lines and found that the 4bp insertion was consistently present in inducer lines and absent in all other teosinte and maize lines. Thus, we proposed that the 4bp (CGAG) insertion may cause a weak or loss-of-function allele of *ZmPLA1* leading to the haploid induction phenotype. This allele is a rare mutation and may have occured after maize domestication since it was not detected in the teosinte accessions.

Next, we used the CRISPER/Cas9 gene-editing technique to knockout *ZmPLA1* to validate the gene function. Based on the sequence of the first exon (Figure C), gRNA sequence was designed and inserted into the vector pBUE411. The gene knockout efficiency of the recombinant vectors was tested and verified on 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 (Supplementary Table 3). Three knockout lines including 1bp insertion (ZmHIR1-1), 11bp deletion (ZmHIR1-2) and 1bp deletion (ZmHIR1-3) in the target region (Figure 1C) were chosen for self-pollination and also used as male to pollinate to two commercially used hybrids ZD958 and JK968 for testing the HIR of the T_1 transgenic plants.

Similar with Stock6 derived inducer lines, obvious endosperm abortion kernels were observed at 14.3% frequency in the self-pollinated knockout lines (Figure 1D&E, supplementary Table 4). When using the knockout lines as males, the hybrid F_1 ears had the endosperm abortion rate of 10.25% for ZD958 and 9.05% for JK968, (Figure 1E, Supplementary 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&E). Haploid plants are usually characterized by a phenotype of short plant height, compact-type, small anther, and sterility and can be identified directly in the field (Figure 1F&G). Flow cytometry further confirmed the haploid ploidy (Figure 1H). To determine the origin of the haploid chromosome, knockout receptor line and ZD958/JK968 were screened by using polymorphic molecular markers. We found that all the haploids were derived from 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 mutant allele families ZmHIR1-1 and ZmHIR1-2, haploids were found in the progenies of ZD958 and JK968 with a HIR of 1.85 - 3.51%. In the homozygous mutant allele family ZmHIR1-3, the HIR was 1.55% based on ZD958(Supplementary Table 5). According to our results from the two test hybrids, 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. Above all, our results suggest that *ZmPLA1* is the underlying gene of *qhir1* and the rare 4bp insertion within *ZmPLA1* is the casual allele which leads to haploid induction.

Phospholipase A (*PLA*) gene family is common and is present in rice, Arabidopsis,

sorghum and many other plants (Wang, 2001). The conserved sequences in the *PLA* gene from different plants may indicate conservation of function. Thus, *PLA* gene knockout might be used to create haploid inducer lines in other crops such as sorghum and rice in addition to maize. *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 haploid induction. During the preparation of this manuscript we bacame aware of a paper by Kelliher et at (Kelliher et al., 2017) also implicating the phospholipase A gene in haploid inductiuon." Further detailed studies may pave the way for understanding the molecular and genetic mechanisms of maize haploid induction thus facilitate increasing breeding efficiency in other important crops as well as maize.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at Molecular Plant Online.

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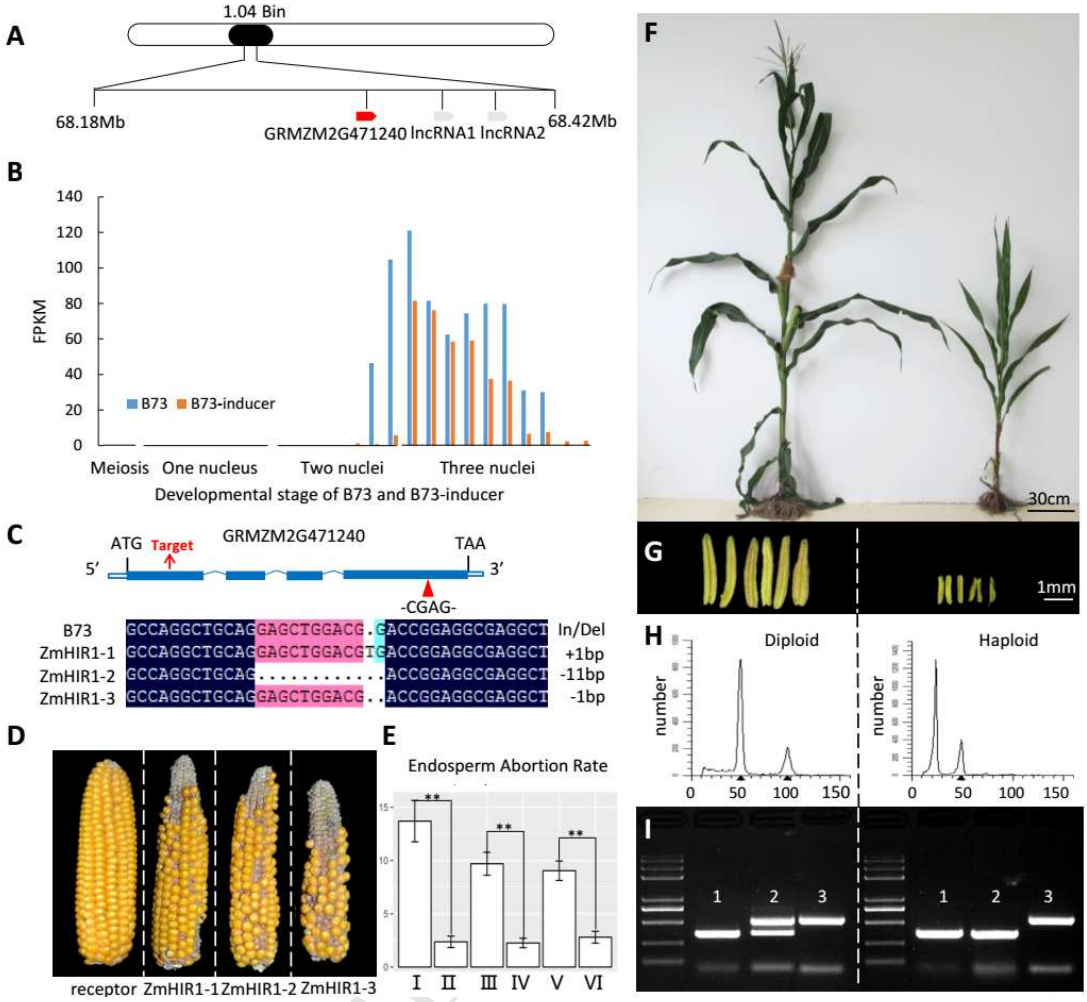

Figure 1. *ZmPLA1* (GRMZM2G471240) mutation causes haploid induction in maize.

(A) Genetic mapping for *qhir1*. Fine mapping identified a 243kb region containing three expressed genes, a coding gene GRMZM2G471240 (in red) and two lncRNAs (in grey).

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

(C) The structure of GRMZM2G471240 with 4bp insertion (in red triangle) were shown. The red arrow showes the CRISPER 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 significant difference between wild type, T_1 generation knockout plants and also in their hybrid plants with ZD958 and JK968. Ⅰ: slefing-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 SSR 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 could be found in Supplementary Table 3.