

RESEARCH PAPER

Gametophytic and zygotic selection leads to segregation distortion through *in vivo* induction of a maternal haploid in maize

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Abstract

Production of maternal haploids via a male inducer can greatly accelerate maize breeding and is an interesting biological phenomenon in double fertilization. However, the mechanism behind haploid induction remains elusive. Segregation distortion, which is increasingly recognized as a potentially powerful evolutionary force, has recently been observed during maternal haploid induction in maize. The results present here showed that both male gametophytic and zygotic selection contributed to severe segregation distortion of a locus, named *segregation distortion 1 (sed1)*, during maternal haploid induction in maize. Interestingly, analysis of reciprocal crosses showed that *sed1* is expressed in the male gametophyte. A novel mapping strategy based on segregation distortion has been used to fine-map this locus. Strong selection for the presence of the *sed1* haplotype from inducers in kernels with haploid formation and defects could be detected in the segregating population. Dual-pollination experiments showed that viable pollen grains from inducers had poor pollen competitive ability against pollen from normal genotypes. Although defective kernels and haploids have different phenotypes, they are most probably caused by the *sed1* locus, and possible mechanisms for production of maternal haploids and the associated segregation distortion are discussed. This research also provides new insights into the process of double fertilization.

Key words: Defective kernel, haploid, maize, male gametophyte, pollen competitive ability, segregation distortion.

Introduction

Double fertilization is a complex mechanism that has evolved in flowering plants (angiosperms). Successful double fertilization involves two sperm cells and two female gametes, the egg cell and the central cell, progenitors of the embryo and the endosperm, respectively. Disruption of any part of the process will lead to a block or abnormality in double fertilization.

Haploids can be one of the outcomes after failure of double fertilization. While in most sexually reproducing plant species, the life cycle alternates between a diploid sporophytic phase

and a highly reduced gametophytic haploid phase (Walbot *et al.*, 2003), it is sometimes possible to obtain haploid plants containing the same number of chromosomes in their somatic cells as do normal gametes of the species (Dunwell, 2010). Two methods are generally used to produce haploids. One approach is to produce haploids from male or female gametic cells, but many species and genotypes are recalcitrant to this process (Forster *et al.*, 2007). Another approach for inducing haploids in some genera is ‘wide crossing’, in which one

parental genome is selectively eliminated after fertilization (Kasha and Kao, 1970; Bennett et al., 1976; Sanei et al., 2011). Ravi and Chan (2010) found that haploids in *Arabidopsis thaliana* can be generated through uniparental chromosome elimination by manipulating a single centromere protein, the centromere-specific histone CENH3. In addition to these two approaches, two other methods exist in maize: haploids can be induced either by the *ig1* mutant (Kermicle, 1969; Evans, 2007) as the female parent or by Stock6-derived inducers (Coe, 1959; Sarkar and Coe, 1966) as the male parent. The latter method is referred to as *in vivo* induction of maternal haploids and has become the method of choice in many maize breeding programmes worldwide (Prigge and Melchinger, 2012).

In a recent quantitative trait locus (QTL) mapping study on the *in vivo* haploid induction rate (HIR) in maize (Prigge et al., 2012), significant segregation distortion (SD) against the inducer haplotype was observed in bins 1.03–1.05. The SD level reached a maximum value in bin 1.04, where one QTL for haploid induction (*qhir1*), explaining up to $p = 66\%$ of the genetic variance for HIR, was detected. In another study, too, the same region on chromosome 1 was associated with HIR as well as with SD (Barret et al., 2008). Although considerable progress has been made in the genetic analysis of *in vivo* haploid induction, the mechanisms behind SD and the genetic characteristics of the *qhir1* locus remain unknown.

Segregation distortion is defined as the deviation of genotype frequencies from Mendelian expectations. It can originate from double fertilization failure events that include competition among gametes or from abortion of the gamete or zygote (Faris et al., 1998; Fishman et al., 2005). Similar to other QTLs, SD loci are cryptic, but may carry an important evolutionary function because they control the viability of individuals facing natural selection (Xu, 2008). If a cluster of closely linked markers displays SD, one or several of them or tightly linked genes become candidate genes for viability selection and, thus, are no longer selectively neutral. Grini et al. (1999) reported that by using five visible recessive markers on chromosome 1, seven gametophytic mutations that were induced by ethyl methanesulphonate (EMS) could be recognized by the SD of the nearby markers. Moreover, gametophytic mutants affecting various aspects of pollen development and function in *Arabidopsis* were identified through genetic screens for SD (Lalanne et al., 2004a, b).

In this study, SD at the *qhir1* locus was given top priority. First, the genomic region responsible for SD, subsequently referred to as the *segregation distortion 1* (*sed1*) locus, was narrowed down to a 450 kb region. It was found that both gametophytic and zygotic selection are involved in haploid induction and these two mechanisms together lead to strong SD of *sed1*. Furthermore, the role of gametophytic expression of *sed1* in the fate of the zygote was discussed to shed light on the mechanism underlying maternal haploid induction in maize.

Materials and methods

Plant materials

The four inducer lines CAUHOI (Li et al., 2009), CAU5, CAU079, and UH400 (Prigge et al., 2012) with HIRs of ~2, 10, 9, and 8%,

respectively, all carrying the *R1-nj* marker gene, and seven elite maize inbred lines with null HIR (1680, B73, Jing 24, Z58, C7-2, BY815, and GY923) were used. Hybrid ZD958, which is one of the most popular maize cultivars grown in China, and shows good purple pigmentation in the endosperm and embryo when crossed with inducers carrying the *R1-nj* marker gene, was used as a tester for determining the HIR. For pollen competitive ability tests, three female parents were used: hybrid ND5598 with long ears, sweet corn inbred CS1, and waxy corn inbred CW2. For production of crosses, standard practices in genetic field experiments with maize were followed to ensure that no contaminating pollen reached the silks: (i) all tassels of female parents were removed and the ears of seed parents were covered with paper bags prior to silk emergence; and (ii) silks were cut back the day before pollination, so that all exposed silks were of the same length, making it easier to receive the pollen.

Haploid identification

Two methods were employed for determination of the HIR. The first relied on self-pollinated ears and was called S-HIR. Here, all normal kernels obtained by selfing were planted in the field to infer their ploidy status. Haploid plants can be distinguished from diploid plants by their shorter stature, slender weak stems, erect and narrow leaves, and reduced growth rate. The S-HIR was calculated by the following formula: $S\text{-HIR} = (\text{number of haploid plants} / \text{total number of plants}) \times 100\%$. The second method relied on test crosses with hybrid ZD958 to evaluate the HIR of inducers and was called T-HIR. Here, testcross seeds with purple endosperm (indicating a regular triploid endosperm resulting from successful fertilization with inducer pollen) and a colourless embryo (indicating a haploid embryo of ZD958 origin) were designated as putative haploid seeds following the guidelines published by Li et al. (2009). To verify the effectiveness of the *R1-nj* colour marker classification and to account for misclassification of seeds (Prigge et al., 2012), all putative haploids were grown in Beijing and visually scored for plant characteristics as described above. The following formula was applied to calculate T-HIR: $T\text{-HIR} = (\text{number of putative haploids} / \text{total number of purple endosperm kernels}) \times K \times 100\%$, where K is a correction factor given by the ratio $K = (\text{number of true haploids} / \text{number of putative haploids})$.

Kernel classification

Kernels obtained from self-pollinated and cross-pollinated ears could be readily classified into three categories according to their morphological features: (i) normal kernels, with normal embryo and endosperm, including normal diploids and haploids (Fig. 1B1, B2); (ii) endosperm abortion (EnA) kernels, with shrunken endosperm and kernel size smaller than that of normal haploids (Fig. 1B4–B8); or (iii) embryo abortion (EmA) kernels, with normal endosperm but without an embryo (Fig. 1B3). Moreover, some embryo sacs did not show any development, which might be caused by early seed abortion or lack of fertilization, leading to a poor seed set rate (Fig. 1A2, A3).

Evaluation of ear traits

In Experiment 1, 11 maize inbred lines listed in Table 1, comprising four inducers and seven normal maize lines, were used for morphological analysis of the progeny produced by self-pollination. In addition, the progeny of five populations from UH400 and 1680 were also analysed (Table 1). Based on kernel classification, besides S-HIR, the following traits were determined for all ears: normal kernel number (NKN), normal seed set rate (NSSR), endosperm abortion rate (EnAR), and embryo abortion rate (EmAR) using the formulae described in the footnote of Table 1. All traits were evaluated for at least three ears for each entry.



Fig. 1. Morphological differences in maize ears and seeds from various progeny. (A) Selfing ears of inbreds B73 and UH400 and ears from their reciprocal cross. (A1) Selfing ear of B73 showing good seed set; (A2) ear of the cross B73×UH400 showing poor seed set with many EnA kernels; (A3) selfing ear of UH400 with poor seed set and many EnA kernels; (A4) ear of the cross UH400×B73 showing good seed set. (B) Different kernel types after maternal haploid induction. (B1) diploids; (B2) haploids; (B3) EmA kernels; (B4–B8) different types of EnA kernels. From B4 to B8, the kernel size and volume progressively decreased, reflecting an impairment of kernel development at different developmental stages.

Mapping strategy for *sed1*

In Experiment 2, three F_2 populations derived from the cross 1680×UH400 were used to narrow down the *sed1* region responsible for SD in bins 1.03–1.05. In all cases, SD of individual markers in the target region on bin 1.04 was evaluated by a χ^2 test based on the three genotypes within each $F_{2,3}$ family derived from recombinant F_2 plants. First, nine recombinants in the QTL confidence interval of *qhir1* described in a previous study (Prigge *et al.*, 2012) were identified in a sample of 186 plants. By analysing the genotype of the offspring from three recombinant F_2 plants (R1, R2, and R3), the SD level for each $F_{2,3}$ family could be assessed, which allowed delimitation of the *sed1* locus to the region between markers *umc1917* and *bnlg1811*.

In an attempt to narrow down the region further, a second and third sample of 2000 and 6000 F_2 plants, respectively, were generated to obtain more recombinants. This led to the identification of 48 and 125 recombinants, respectively, by using the markers *umc1917* and *bnlg1811*. Moreover, 12 new markers (primer sequences for each of these markers are given in Supplementary Table S1 available at JXB online) were used to genotype the recombinants in order to confirm their exchange boundary. These markers represented the subset of polymorphic markers between both parents from a set of 250 newly developed markers with primers designed on the basis of the B73 genome sequence, which spans a physical distance of 7.85 Mb between markers *umc1917* and *bnlg1811*. Each confirmed recombinant F_2 plant was evaluated for T-HIR; moreover, each recombinant with a heterozygous genotype in this region

was self-pollinated to obtain a large number of seeds for evaluating the SD level in each resulting $F_{2,3}$ family. The genotypes of at least 50 plants in each $F_{2,3}$ family were determined to ascertain that SD could be detected with sufficient power. PCR-based marker development and genotyping were conducted following the protocol of Yang *et al.* (2010).

$F_{2,3}$ families originating from 10 independent F_2 recombinants were tested for their SD level at the *sed1* locus. Pollen of the three most important recombinant F_2 plants (R1, R4, and R5) in F_2 was used to pollinate the female parent 1680. The segregation ratios for each haplotype in the resulting backcross progeny were calculated by using either of the markers *umc1917* or *bnlg1811*, depending on which one segregated in the particular cross.

SD detection in different cross combinations

In Experiment 3, the 15 populations listed in Table 2, each with at least 50 plants, were analysed for SD in the *sed1* region. Again, SD from expected Mendelian segregation ratios was assessed by a χ^2 test using either marker *X18* or *umc1917*.

Endosperm DNA extraction

In Experiment 4, endosperm DNA from different types of kernels on self-pollinated ears harvested from F_1 plants of cross 1680×UH400 was extracted according to the following protocol: small pieces of endosperm sampled from single seeds without destruction of the

Table 1. Kernel traits on selfing and cross-pollinated ears of different maize germplasm with and without haploid induction ability of the pollinator.

Parent		Ear and kernel traits ^a				Haploid induction	
Female	Male ^b	NKN (per ear)	NSSR (%)	EnAR (%)	EmAR (%)	S-HIR (%)	T-HIR (%)
Male parents with haploid induction ability							
CAU5	⊗	73	24.33	65.71	9.59	11.89	10.9
CAUHOI	⊗	125	60.19	8.76	1.54	1.31	1.94
CAU079	⊗	142	45.08	38.53	8.45	8.80	9.84
UH400	⊗	95	29.32	44.77	6.32	10.43	9.26
1680×UH400	⊗	373	71.45	24.40	1.87	1.93	2.53
1680	UH400	290	43.11	48.78	8.98	8.50	ND ^c
1680×UH400	UH400	210	39.91	51.86	10.47	8.86	ND ^c
Average		186.86	44.77	40.40	6.75	7.39	6.81
Male parents without haploid induction ability							
1680	⊗	320	93.14	1.84	0	0	0
B73	⊗	357	91.16	1.11	0	0	0
Jing 24	⊗	287	91.77	0	0	0	0
Z58	⊗	320	91.65	0	0	0	0
C7-2	⊗	386	96.44	0	0	0	0
BY815	⊗	350	95.45	1.02	0	0	0
GY923	⊗	392	93.32	0	0	0	0
UH400	1680	287	91.40	0	0	0	ND ^c
1680×UH400	1680	467	93.40	0	0	0	ND ^c
Average		351.78	93.08	0.44	0	0	0

^a NKN, normal kernel number; NSSR, (number of normal kernels/number of embryo sacs)×100%; EnAR, (number of EnA kernels/number of embryo sacs)×100%; EmAR, (number of EmA kernels/number of normal kernels)×100%;

^b ⊗, self pollination

^c ND, not determined.

Table 2. Analysis of segregation distortion in selfing or crossing progeny of different combinations of crosses between female and male parents with high (H), low (L), and no (N) endosperm abortion rated. Parents with induction ability are underlined.

Female	Male ^a	Genotype ^b			χ ² test			
		A	H	B	A:H:B=1:2:1	(A+B):H=1:1	A:H=1:1	B:H=1:1
Cross combination N×H; marker <i>X18</i>								
1680× UH400	⊗	67	102	17	28.62***	1.55		
GY923× UH400	⊗	84	111	32	23.93***	0.07		
1680× UH400	1680	19	28	0			1.36	
1680× UH400	UH400	11 ^c	41	46				0.18
1680	1680× UH400	32	4	0			20.25***	
Cross combination N×L; marker <i>X18</i>								
1680× CAUHOI	⊗	31	24	9	19.13***	3.52		
1680× CAUHOI	1680	44	49	0			0.17	
1680× CAUHOI	CAUHOI	3 ^c	33	28				0.09
1680	1680× CAUHOI	38	0	0			36.03***	
Cross combination L×H; marker <i>umc1917</i>								
CAUHOI × UH400	⊗	68	111	42	6.12*	0		
CAUHOI × UH400	CAUHOI	71	53	1 ^c			2.33	
CAUHOI × UH400	UH400	4 ^c	51	53				0.01
1680	CAUHOI × UH400	85	84	0			0	
Cross combination H×H; marker <i>umc1917</i>								
CAU5 × CAU079	⊗	11	19	14	1.23	0.57		
CAU5 × CAU079	CAU079	7 ^c	24	35				4.34

^a ⊗, self pollination.

^b A refers to the homozygous genotype of the first parental line, B to the homozygous genotype of the second parental line, and H to the heterozygous genotype.

^c Haploid.

*, **, ***Significant at probability levels 0.05, 0.01, and 0.001, respectively.

embryo germination capacity were placed directly in a 96-well PCR plate. Subsequently, 100 µl of sodium hydroxide (0.1 M) was added to each well and the plate was incubated at 99.9 °C for 10 min. After 1 min of centrifugation with a centrifugal force of 168 g, 100 µl of 1× TE buffer (pH=2.0) was added to each well and mixed, and the plate was centrifuged again with a centrifugal force of 671 g for 2 min. The clear DNA-containing solution was then used for further PCR analysis.

Pollen competitive ability tests

In Experiment 5, three maize genotypes (CS1, CW2, and ND5598) were chosen as female parents. The four inducers (CAUHOI, CAU5, CAU079, and UH400) and the three inbred lines 1680, CS1, and CW2 were used as pollen sources to evaluate their pollen competitive ability (PCA).

PCA was assayed by applying two different kinds of pollen to the same ear at several time intervals. The exact process was as follows (Supplementary Fig. S1 at JXB online): first, for hybrid ND5598, pollen from the four inducers and non-inducer 1680 was applied separately to the different ears. Then 0, 0.5, 1, 1.5, 2, 2.5, 3.5, and 6 h later, the ears pollinated first with pollen from the inducer lines were pollinated again with pollen from line 1680. Vice versa, the ears initially pollinated with pollen from line 1680 were pollinated with pollen from the four inducer lines. In order to ensure that an equal amount of pollen was allocated to each ear, newly collected pollen was divided into equal parts by volume by using 1.5 ml tubes as a measuring cup. On the basis of the purple pigmentation of the endosperm, it was possible to distinguish between the two types of pollen applied by the dual pollination method. For the female parents CS1 or CW2, the same protocol was employed except that pollen from line 1680 was replaced by pollen from lines CS1 or CW2. At least five ears were used as replicates for each treatment.

In Experiment 6, pollen germination speed and pollen tube growth were compared between UH400 and 1680. First, fresh pollen of inbreds UH400 and 1680 was collected separately at 09:00 h on the same day. Then, the pollen was divided into a number of equal parts by volume and pollinated separately on the silks of the tester hybrid ZD958. After intervals of 1, 2, 4, and 8 h, silk tissue from ears pollinated with excess pollen of UH400 or 1680 were fixed in 9:1 v/v ethanol:acetic acid at 4 °C. The aniline blue staining protocol described by Lausser *et al.* (2010) was adopted to observe pollen tube germination. Specimens were mounted with fresh staining solution on a slide with a coverslip and squashed by gentle squeezing; subsequently, they were examined on a fluorescence microscope (OLYMPUS BX51) with near-UV excitation, using wavelengths ranging from 315 nm to 380 nm. In order to guarantee high accuracy of the experiment, each treatment (three ears) and each ear (five silks) were analysed using aniline blue staining.

Genetic analysis of haplotype transmission through male and female gametes

To gain a better understanding of the gametophytic transmission of *sedl*, the process from the parental F_1 to the F_2 generation was taken as an example (Supplementary Fig. S2 at JXB online). Several possibilities affecting SD were taken into consideration. Let p and q represent the frequency of wild-type haplotypes (defined as '+') in the male and female gametes involved in fertilization, respectively, then $1-p$ and $1-q$ represent the frequency of '*sedl*' haplotypes in the male and female gametes, respectively. The survival rate of zygotes with genotype '+/++', '+/sedl', 'sedl/+', and 'sedl/sedl' is denoted by r_1 , r_2 , r_3 , and r_4 , respectively. Moreover, the common notation A, B, and H was used to describe the genotypes '+/++', 'sedl/sedl', and '+/sedl' or 'sedl/+', respectively. Taken together, the final frequency of each genotype in the F_2 population can be described by the following formulae:

$$A: F_A = q \times p \times r_1 / T \times 100\% \quad (1)$$

$$H: F_H = [q \times (1-p) \times r_2 + (1-q) \times p \times r_3] / T \times 100\% \quad (2)$$

$$B: F_B = (1-q) \times (1-p) \times r_4 / T \times 100\% \quad (3)$$

where $T = q \times p \times r_1 + q \times (1-p) \times r_2 + (1-q) \times p \times r_3 + (1-q) \times (1-p) \times r_4$ is the survival rate of the zygotes summed over all three genotypes.

To obtain estimates for each parameter, the populations derived from the two parents 1680 and UH400 were used as a model. First, the mode of *sedl* transmission in the backcross population (1680×UH400)×1680 from Experiment 3 was analysed for estimating q . Secondly, the ear and kernel traits of crosses from Experiment 1, where inducers served as female parents (*sedl/sedl*) and non-inducers (+/+) as male parents, were analysed to estimate r_1 and r_3 . Thirdly, EnAR of kernels from the backcross (1680×UH400)×UH400 from Experiment 1 were analysed to obtain estimates for r_2 and r_4 . Finally, a maximum likelihood estimator for p was calculated based on the above formula using a numerical algorithm implemented in software R 13.0 (<http://www.r-project.org>).

Results

The haploid induction process entails both haploid and defective kernels

In Experiment 1, estimates for S-HIR ranged from 1.31% to 11.89% and for T-HIR from 1.94% to 10.9% when the pollen parent was an inducer line, whereas S-HIR and T-HIR were null for all combinations in which the pollen parent was a non-inducer line (Table 1). The first group averaged only half the number of normal kernels (NKN) per ear of the second group, but had a high rate of defective kernels, as reflected by means for EnAR (40.4%) and EmAR (6.75%). Moreover, S-HIR for individual male parents in this group was tightly associated with T-HIR and defects in kernel traits of their progeny (Fig. 2). In comparison, materials without induction ability had a mean of EmAR and EnAR of exactly or close to zero. Ears of the cross 1680×UH400 and the backcross (1680×UH400)×UH400 had high values for both S-HIR (8.50% and 8.86%, respectively) and EnAR (48.78% and 51.86%, respectively), similar to those of the self-pollinated ear of UH400. In contrast, the reciprocal crosses UH400×1680 and (1680×UH400)×1680 showed null S-HIR and almost no defective kernels. In further crosses with various inducers as the female parent and normal lines as the male parent, no haploids and few defective kernels were found (Supplementary Fig. S3A at JXB online) and the ear phenotype resembled that of the F_1 of cross UH400×1680.

SD and its features in different genetic populations is controlled by *sedl*

By screening for SD in $F_{2,3}$ progeny of 10 (heterozygous) recombinant F_2 plants in Experiment 2, the region of *sedl* between the two markers *X22* and *X93* could be narrowed down (Fig. 3) with a physical distance of 455 kb, based on the B73 reference sequence. Moreover, estimates of T-HIR for each of these 10 recombinants were closely associated with the level of SD (Fig. 4). Recombinants with significant SD

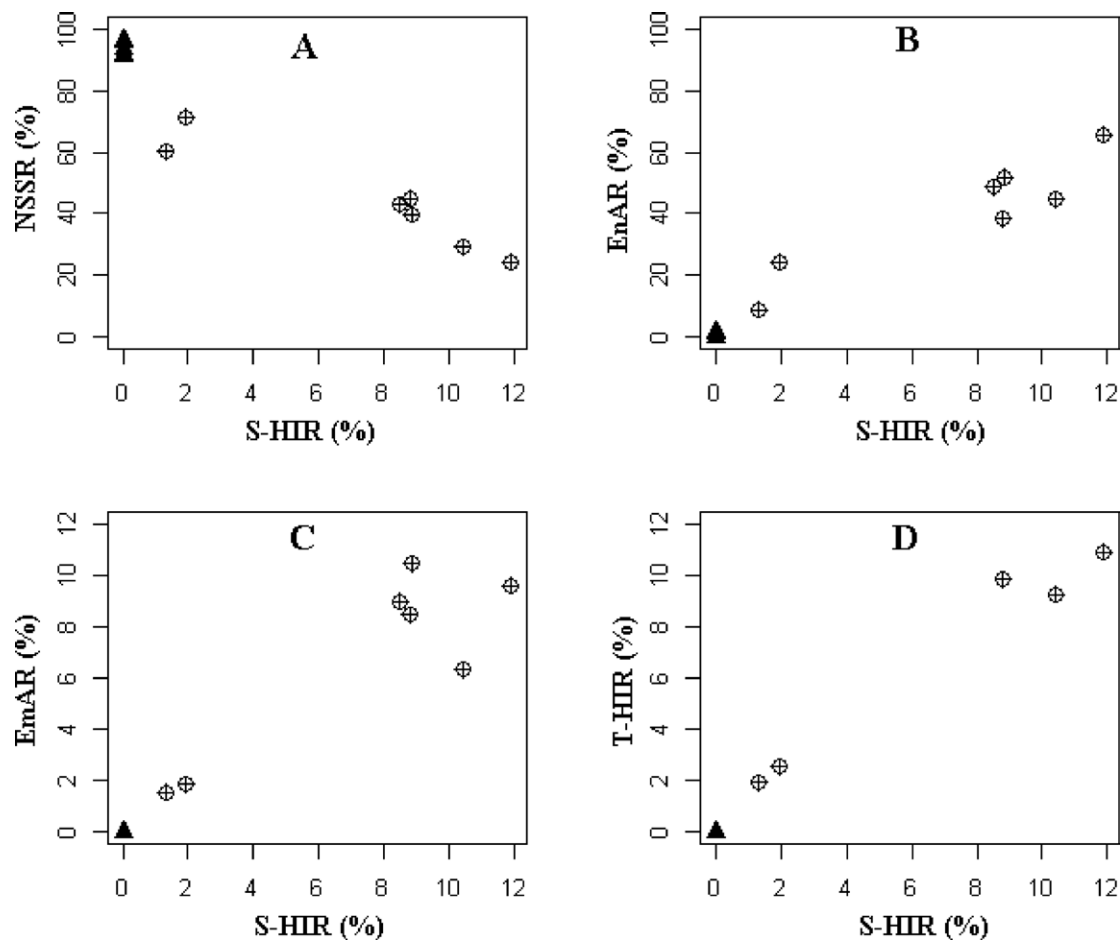


Fig. 2. Plots of S-HIR against different ear and kernel traits. (A–D) Plots of S-HIR against NSSR, EnAR, EmAR, and T-HIR in 11 maize inbred lines (four inducers and seven normal maize lines) and five progeny derived from crosses between line 1680, inducer UH400, or their single cross (see Table 1). Open circles with a cross, pollen contains *sed1* male gametes; filled triangles, pollen that did not contain *sed1* male gametes.

had a mean T-HIR of $1.64 \pm 0.19\%$, whereas those without SD had a significantly ($P < 0.001$) smaller mean T-HIR of $0.26 \pm 0.09\%$.

Based on the EnAR determined in Experiment 1 (Table 1), the four inducers and two common maize lines were classified into three groups: 1680 and GY923 were non-abortion (N) lines; CAUHOI was a low (L) abortion line (8.76%); and UH400, CAU5, and CAU079 were high (H) abortion lines with an EnAR of 44.77, 65.71, and 38.53%, respectively. In Experiment 3, different crosses among these groups (N×L, N×H, L×H, H×H) were used to investigate the universality and characteristics of SD in *sed1*. In general, F₂ populations derived from crosses of type N×L and N×H showed significant ($P < 0.01$) SD. In the backcross populations, if the *sed1* haplotype was present in the female gametes of crosses of type (*sed1*/+)(+/+), the segregation ratios at markers *X18* or *umc1917* were in agreement with normal Mendelian expectations. However, if the male gametes carried the *sed1* haplotype, for example in the cross (+/+)(*sed1*/+), high significant ($P < 0.01$) SD was observed. In F₂ populations derived from crosses of type L×H, significant ($P < 0.05$) SD against the *sed1* haplotype derived from the H parent was found, but,

when the F₁ hybrid CAUHOI×UH400 of type L×H was used as male parents to pollinate the female parent 1680, no SD was found in the progeny ($n=169$). Moreover, no significant SD was detected in this region for the backcross, when L×H hybrid CAUHOI×UH400 was used as female parent. In the H×H crosses, no significant SD was detected for both the F₂ populations and CAU079 backcross populations.

Genotypic analysis of the different kernel types in segregating population

High HIR was always associated with a high rate of defective kernels and SD (Figs 2, 4). For investigating the association between the latter two traits, 7544 kernels from 35 ears of the F₂ population 1680×UH400, segregating for the *sed1* locus, were classified into different kernel classes (Table 3). Randomly sampled kernels in each class were assayed for their endosperm genotype. In addition, the ploidy level for all putative haploid kernels was determined by planting and genotyping each plant for 20 markers polymorphic between UH400 and 1680. In the 74 putative haploids, 86.5% of the kernels were finally determined to be haploids. After

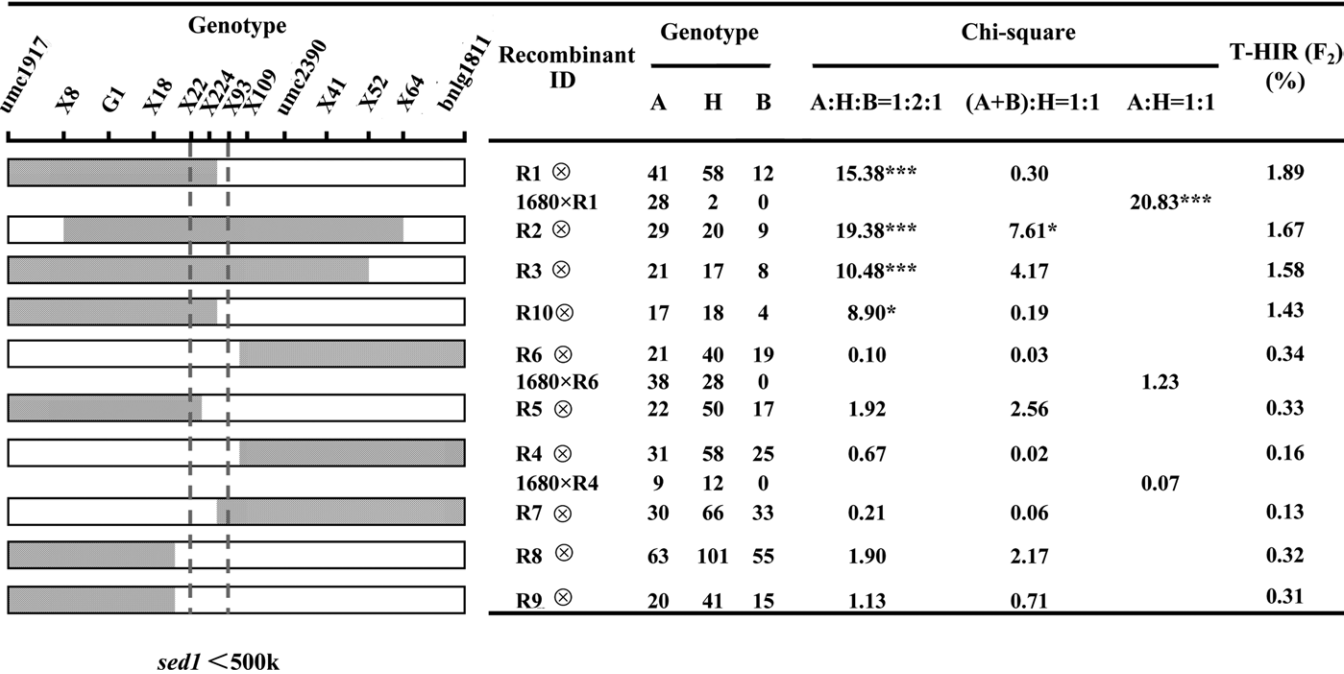


Fig. 3. Fine-mapping of *sed1* and segregation distortion (SD) in F₃ progeny or backcross progeny to 1680 of F₂ plants recombinant for the *sed1* region. Left side: the physical position of markers mapping to bin 1.04 is shown on top of the bars. The bars reflect the genotype of the F₂ plants (open bars, homozygous for the 1680 haplotype; shaded bars, heterozygous for the UH400 haplotype). Right side: number of F₃ progeny or backcross progeny in each genotype class and χ^2 test for deviation from expected Mendelian segregation ratios. The S-HIR of the parental recombinant F₂ plants is also given. *, **, *** Significant at probability levels 0.05, 0.01, and 0.001, respectively.

correcting for misclassification in determination of putative haploids, strong selection was found for the presence of the *sed1* haplotype from the inducer in the endosperm of haploids or defective kernels (Table 3). Notably, all haploids (*n*=64) and EmA kernels (*n*=66) harboured at the UH400 haplotype marker locus *X18* in their endosperm.

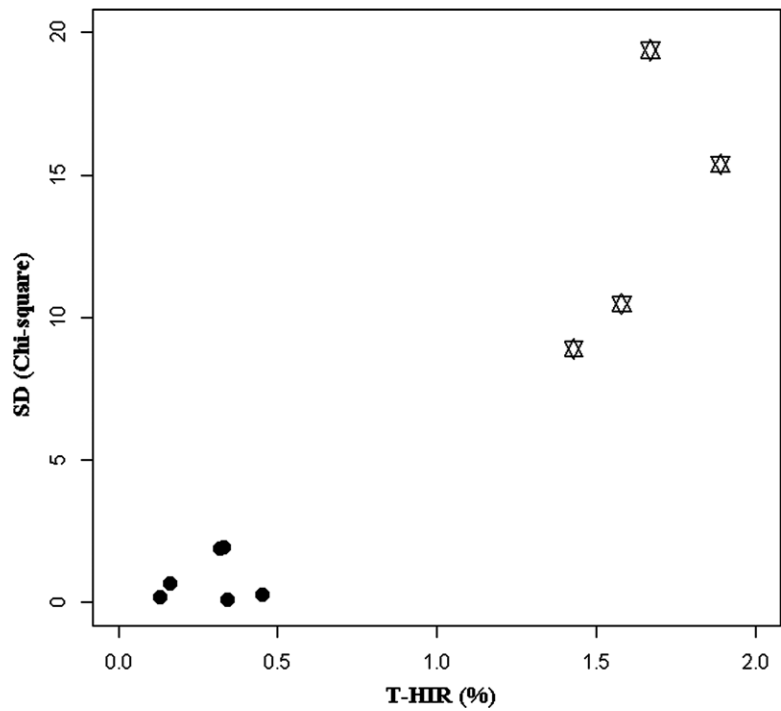


Fig. 4. Plot of T-HIR of 10 recombinants in the F₂ generation against χ^2 test statistic for SD. Stars, recombinants with significant SD; filled circles, recombinants without SD.

Table 3. Genotypes at marker X18 of different kernel types in the F₂ population from 1680×UH400.

Type	Selected kernel no.	Genotyped kernel no.	Genotype ^a (endosperm)			Genotype ^a (seedling)		
			A	H	B	A	H	B
Normal kernel	5892	5499	2031	2847	621	ND ^c	ND ^c	ND ^c
Haploid ^b	64	64	0	33	31	33	0	31
EnA kernel	1438	183	2	96	85	ND ^c	ND ^c	ND ^c
EmA kernel	150	66	0	38	28	ND ^c	ND ^c	ND ^c

^a Genotype of different classes: A=*Sed1* homozygous, H= heterozygous, B= *sed1* homozygous.
^b Confirmed as haploids by planting and genotyping of the seedlings.
^c ND, not determined.

Pollen competitive ability (PCA) of *sed1* pollen

A potential reason for SD could be differences in PCA (Xu et al., 1997; Lu et al., 2002). The dual-pollination method used in Experiment 5 allows the PCA of haploid inducers and common (non-inducer) lines to be distinguished (Table 4). When female parents were first crossed with a non-inducer line, followed by application of pollen from an inducer at different time intervals (0–2 h), up to 98.86% of the kernels showed colourless endosperm, indicating successful pollination by the non-inducer lines after an interval of 0.5 h. If inducer pollen was first applied, followed by pollination with a non-inducer at different time intervals (0–6 h), up to 88.2% of the resulting kernels had colourless endosperm, as was also the case after simultaneous pollination with a 1:1 mixture of pollen from both sources.

From ears first pollinated with pollen of UH400 and afterwards pollinated with pollen from 1680 at different time intervals (0–2.5 h), most of the kernels with purple endosperm (indicating fertilization with inducer pollen) were found in the upper part of the ear (Fig. 5D) and the number of kernels with purple endosperm significantly ($P < 0.05$) differed between the upper and lower parts of the ear (Table 4). The

same results were observed if pollen from CS1 or CW1 was used instead of pollen from 1680 (data not shown).

Results from *in vivo* pollen germination in Experiment 6 showed that most pollen grains from line 1680 germinated within 1 h (Fig. 5A1), whereas most of the UH400 pollen grains did not germinate within this time frame (Fig. 5B1). However, the latter began to germinate after 1 h, and most of them eventually germinated like the pollen of 1680 (Fig. 5B2).

Genetic characterization of *sed1* pollen mutants

The crosses (1680×UH400)×1680 and (1680×CAUHOI)×1680 belong to the (*sed1*/+)(+/+) type of cross. For both, the frequencies of '*sed1*/+' and '+/+' genotypes were according to the χ^2 test in agreement with the expected Mendelian segregation ratio of 1:1 (Table 2). Consequently, $q=1-q=0.5$ and the *sed1* haplotype from the female parent did not contribute to SD.

For the reciprocal crosses 1680×(1680×UH400) and 1680×(1680×CAUHOI), which belong to the (+/+)(*sed1*/+) type of cross, severe SD from the expected 1:1 genotypic frequencies of *sed1*/+ and +/+ was found (Table 2). Thus, the *sed1* haplotype from the male parent was the reason for the SD. Considering that defective kernels and haploids could be

Table 4. Pollen competitive ability test for normal and haploid inducing lines after pollination with pollen from Male 1 at 09:00 h and sequent pollination with pollen from Male 2 at different time intervals from 0 (=equal parts by volume) to 6 h.

Parent			Time interval (h)	Traits ^a				P-value
Female	Male 1	Male 2		Kernel no.		PEKR (%)		
				Up	Down	Up	Down	
ND5598	1680	UH400	0.5	248	278	1.44	1.31	ND ^b
ND5598	1680	UH400	0	225	255	16.89	7.05	0.0004**
ND5598	UH400	1680	0.5	193	189	17.1	5.82	0.0002**
ND5598	UH400	1680	1	201	152	36.81	28.95	0.024*
ND5598	UH400	1680	1.5	215	160	88.37	37.5	0**
ND5598	UH400	1680	2.5	189	194	97.88	45.36	0**
ND5598	UH400	1680	3.5	191	118	95.81	94.07	0.2446
ND5598	UH400	1680	6	122	104	95.08	97.12	0.7828

^a Kernel no. number of normal kernels in different parts of the ear; PEKR, purple endosperm kernel rate=(number of purple endosperm kernels/total number of normal kernels)×100%
^b ND, not determined.
*, **Significant at probability levels 0.05 and 0.01, respectively.

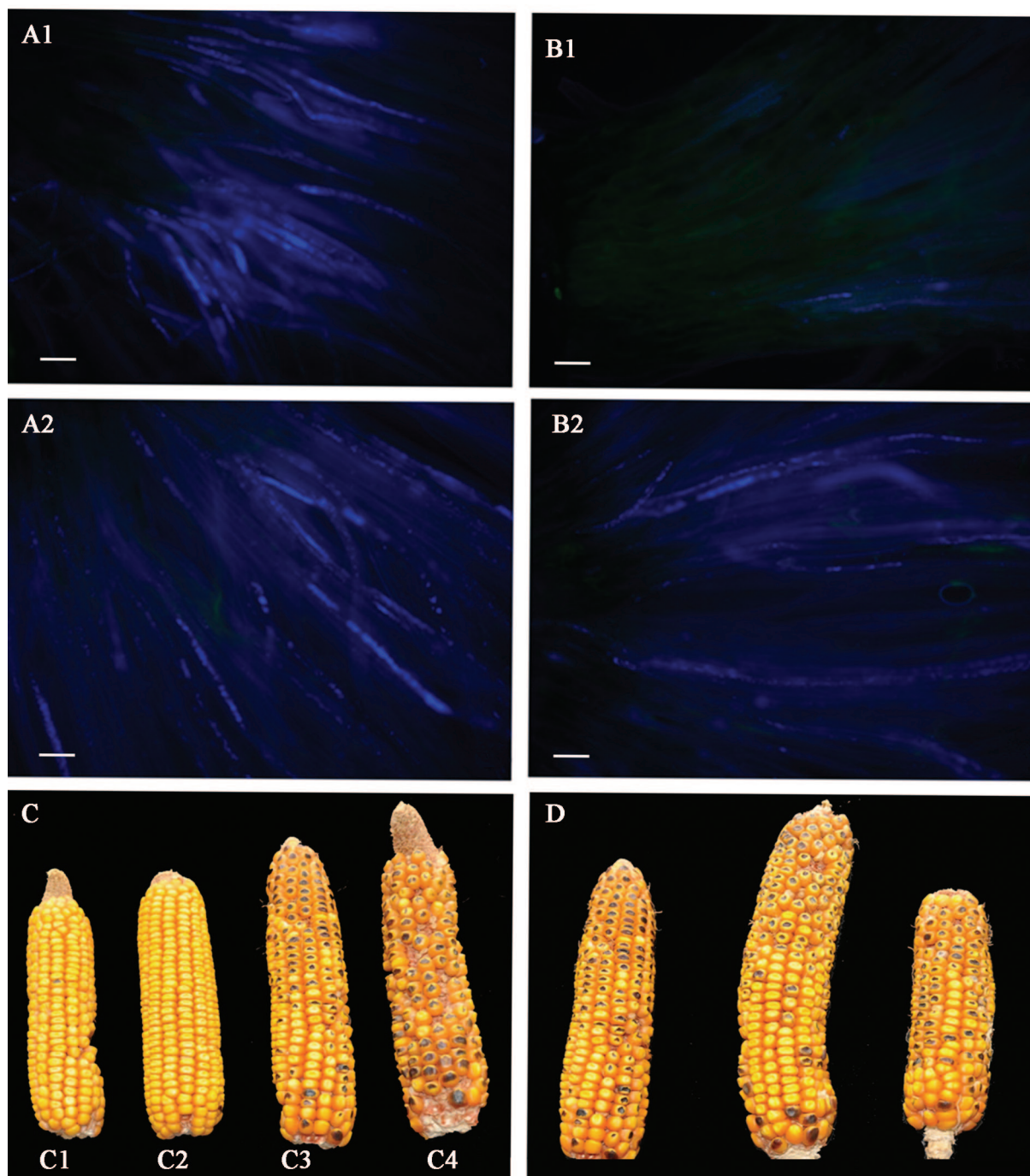


Fig. 5. Pollen competitive ability (PCA) in haploid inducers and non-inducer maize inbreds. (A1) and (A2) Germination of 1680 pollen on ZD958 silks; (B1) and (B2) germination of UH400 pollen on ZD958 silks (scale bars are 100 μ m). (A1 and B1) Pollen tubes were stained with aniline blue 1 h after pollination. (A2 and B2) Pollen tubes were stained with aniline blue 8 h after pollination; the pollen tube is shown in blue colour. (C) PCA tested by dual pollination, from left to right: (C1) ear of ND5598 first pollinated with 1680 pollen, then pollinated with UH400 pollen after 1 h; (C2) ear of ND5598 pollinated with a mixture of equal amounts of UH400 and 1680 pollen at the same time; (C3) ear of ND5598 first pollinated with UH400, then pollinated with 1680 after 1 h; (C4) ear of ND5598 first pollinated with UH400, then pollinated with 1680 after 2 h; (D) ears of ND5598 first pollinated with UH400, then pollinated with 1680 after 1 h, showing clusters of purple endosperm kernels in the distal part of the ear, and colourless endosperm kernels concentrated mainly at the base of the ear.

detected only rarely when inducers served as female parents (*sed1/sed1*) and non-inducers (+/+) served as male parents, it follows that $r_1=r_3=1$. The χ^2 tests for a ratio of 1:1 between homozygous (A+B) versus heterozygous (H) genotypes at

the *sed1* locus in the (*sed1*+/+) \times (*sed1*+/+) type of F_2 population indicated no significant deviation from the expected Mendelian segregation ratios (Table 2), leading to the conclusion that $r_2=r_4=r$. In the cross (1680 \times UH400) \times UH400,

EnAR was 51.8%, from which it is concluded that r was $\sim 50\%$. Hence, the maximum likelihood estimator for p obtained from Equations 1–3 was 0.6 when inserting $q=0.5$, $r_1=r_3=1$, and $r_2=r_4=r=0.5$ (Fig. 6). Based on this value, for normal kernels ($n=5499$), the genotype frequencies in the F_2 population of cross 1680×UH400 were A:H:B=0.375:0.500:0.125, which were in close agreement with the observed frequencies of 36.9, 51.8, and 11.3%, respectively (Table 3). In the self-pollinated ears of F_1 plants from cross 1680×UH400, the EnAR amounted to $\sim 24.40\%$ (Table 1), which was again in excellent accordance with the predicted value of 25% based on the value of the parameters given above (Fig. 6).

Discussion

Gametophytic and zygotic selection

Several studies reported that gametophytic selection (Mangelsdorf and Jones, 1926; Lavery and James, 1987; Lin *et al.*, 1992) or zygotic selection (Rick, 1963; Gadish and Zamir, 1986) might be the mechanism underlying SD. However, no report indicated that both selections together influence haplotype transmission. In the present study, the endosperm genotype of kernels with haploid formation or defects in the self-pollinated ears of F_1 plants from cross 1680×UH400 showed strong selection for the presence of the *sed1* haplotype from the inducer. In all haploid and EmA kernels, the endosperm consistently showed the UH400 haplotype (Table 3). In EnA kernels, too, the endosperm genotype of all ($n=183$) kernels except two indicated the presence of the *sed1* haplotype. These two exceptions are most probably due to nutritional competition among sporophytes (Sheridan

et al., 1980), because a low frequency of EnA kernels (0.44%) was also found in the common (non-inducer) maize lines. In Experiment 5, PCA of inducers was lower than that of common materials, suggesting that the *sed1* haplotype is selected for not only in male gametophytes but also in the sporophytes after fertilization. One explanation for the pleiotropic effect of the *sed1* locus on both gametophytic and sporophytic development might be that it plays a central role in cell development.

The estimates for the survival rates r_1 – r_4 and parameter p were based on F_2 and backcross populations of cross 1680×UH400. Obviously, the strength of gametophytic and zygotic selection may depend on the parental lines, as indicated by the variation in EnAR and EmAR values for progeny produced by self- and cross-pollination (Table 1).

Embryo–endosperm interactions

Defective kernels caused by defects in endosperm development, embryogenesis, or both were found in *dek* mutants (Neuffer and Sheridan, 1980; Scanlon *et al.*, 1994) and inter-ploidy-intraspecific crosses (Johnston *et al.*, 1980; Birchler, 1993). It was observed that defective kernels occur concomitantly with haploids in the haploid induction process. Compared with haploid kernels (Fig. 1B2), defective kernels differed in their appearance and showed a continuous distribution in size (Fig. 1B3–B8). In particular, the embryo aborted in EmA kernels (Fig. 1B3), but their endosperm developed quite well and resulted in ‘germless’ kernels. This corroborates that the embryo phenotype is not dependent on the endosperm genotype (Scanlon, 1997). Since most EnA kernels lacked an embryo, it is speculated that defective kernels and haploids are

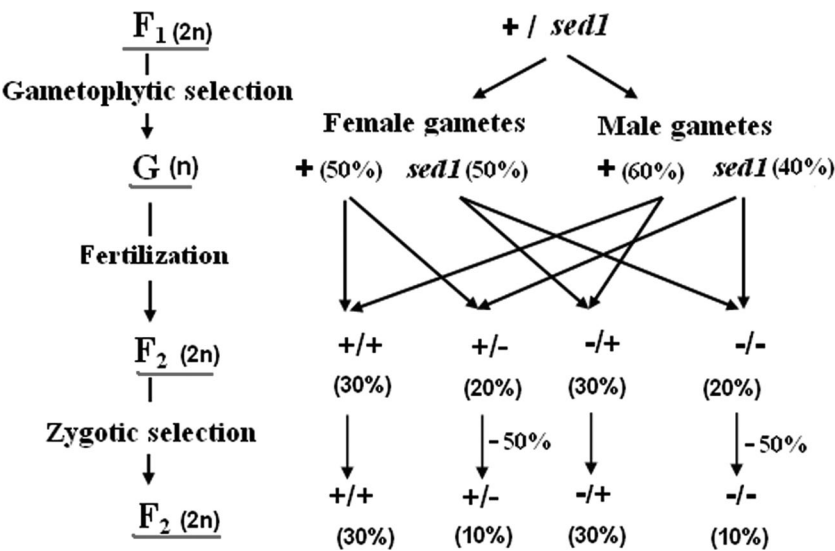


Fig. 6. Genetic transmission of *sed1* in the F_2 population 1680-UH400. For the $+ / sed1$ F_1 plants, there is no distortion in the female gametes, and transmission of both haplotypes occurs at a frequency of 50%. Because of the poor PCA of *sed1* gametes, male gametes face competition and selection in the gametophytic stage, and the transmission of *sed1* gametes is reduced to 40%. The male and female gametes then fuse together, and one sperm of the pollen fuses with the egg cell to produce the embryo while the second fuses with the central cell to form the endosperm. Because *sed1* is a male gametophyte-expressed gene and will influence the fate of the zygote, the kernel survival rate will be 50% for both ‘+ / -’ and ‘- / -’ zygotes.

caused by the same genetic mechanism. Thus, defective kernels might also be haploids that suffered from an early disruption of embryogenesis, leading to a disruption of endosperm development at different stages. An additional mechanism leading to zygote suspension during seed development might also be involved, but this warrants further research.

Mechanism for induction of maternal haploids and defective kernels

There are two main hypotheses to explain the maternal haploid induction in maize. One is that the haploid embryo can be formed from an unfertilized egg cell (Sarkar and Coe, 1966; Barret *et al.*, 2008) and the other is chromosome elimination. A third hypothesis, named semigamy (Turcotte and Feaster, 1967), suggests failure of karyogamy in the fertilized egg cell, which results in chimeras of haploid maternal and paternal tissue that develop into the proper embryo tissue and the suspensor cells, respectively.

Many male gametophytic mutations have been studied well and several of them such as *cdka-1*, *fbl17*, Chromatin Assembly Factor-1 (*CAF-1*), and meiosis pathway mutants (*fas1*, *fas2*, and *msi1*) were described, which cause the formation of bicellular pollen harbouring a single germ cell due to failure of germ cell division. The single sperm of mutants *cdka-1*, *fbl17*, and *msi1* can still fertilize the egg cell or central cells, leading to single fertilization events (Iwakawa *et al.*, 2006; Nowack *et al.*, 2006; Chen *et al.*, 2008; Kim *et al.*, 2008; Aw *et al.*, 2010). In contradiction to the hypotheses of single sperm-like fertilization and semigamy, Sarkar and Coe (1966) reported that all pollen grains produced by inducer lines are tricellular and, in previous studies (Fischer, 2004; Li *et al.*, 2009), genome fragments from the inducer were found in a few haploid plants. Furthermore, except for some extreme examples such as B chromosomes, it is known that each of the two sperm cells can fertilize either the egg cell or the central cell (Chen *et al.*, 2008; Frank and Johnson, 2009; Aw *et al.*, 2010). Consequently, if the single sperm that carried the *sed1* haplotype fused with either the egg cell or the central cell and eventually resulted in a haploid kernel or a defective kernel, respectively, then the endosperm of aborted kernels should not always carry the *sed1* haplotype. However, in contradiction to the single fertilization hypothesis, all but two defective kernels (including haploids, EnA kernels, and EmA kernels) in the F₂ population of cross 1680×UH400 carried the *sed1* haplotype. Therefore, the present results corroborate that double fertilization also happens in maternal haploid induction, and chromosome elimination in the embryo is the underlying cause of maternal haploid induction in maize. Because the endosperm of all defective kernels contains the paternal haplotype of *sed1* in the mature stage, chromosome elimination seems not to occur in the endosperm.

Several hypotheses have been proposed to explain uniparental chromosome elimination in interspecific crosses (Gernand *et al.*, 2005). For example, when CENH3 null mutants in *Arabidopsis* expressing altered CENH3 proteins are crossed with the wild type, chromosomes from the mutant are eliminated, and haploid progeny are produced (Ravi and

Chan, 2010). In maize, maternal haploid induction is most probably not caused by a mutation in the CENH3 gene, because the maize CENH3 gene is located on chromosome 6 (<http://www.maizesequence.org>) and no CENH3 homologue exists in maize bin 1.04.

The sed1 haplotype in male gametophytes affects seed development

There is evidence for an extensive overlapping of sporophytic and gametophytic gene expression in higher plants. For example, Tanksley *et al.* (1981) examined isozyme profiles of nine enzyme systems in tomato (*Solanum lycopersicum*) and found that 60% of the structural genes coding for them in the sporophyte were also expressed in the gametophyte. Studies on synthetics in maize also showed that PCA due to pollen tube growth rate and kernel development are largely controlled by genes expressed in both tissues (Ottaviano *et al.*, 1980). In contrast, comparative analyses of the *Arabidopsis* pollen transcriptome revealed that nearly 40% of the genes were exclusively expressed in the pollen (Honys *et al.*, 2003). Based on the results from both ear trait evaluation and SD detection of the plants from the cross combinations (+/+)×(*sed1*/+ or *sed1*/*sed1*), both defective kernels and significant SD were always detected; however, in the reciprocal crosses, almost no defective kernels and SD were found. This clearly demonstrates that the gene(s) underlying the *sed1* locus are expressed only in the pollen.

When inducer lines were used for pollinating the female parents, some ovaries develop normally, resulting in normal diploid kernels, some ovaries stop developing at different stages, leading to defective kernels, while a small proportion of kernels develop as haploids (Fig. 1B). There might be two explanations: (i) the *sed1* locus was heterozygous in the inducer and the lethality of homozygous embryos maintains heterozygosity over generations; or (ii) the gene(s) underlying *in vivo* haploid induction display gametophytic expression with incomplete penetrance. The progeny-test method was employed to examine the HIR of the selfing progeny from inducer line UH400 (Supplementary Table S2 at JXB online). Since no bimodal distribution for T-HIR was observed in the offspring, it is concluded that the *sed1* locus was homozygous for the UH400 haplotype, which rules out the first explanation.

Epigenetic regulation can be one cause of incomplete penetrance (Petronis, 2001). Epigenetic modification of the DNA of centromeric chromatin influences the precise deposition of the CENH3 protein in centromeres (Zhang *et al.*, 2008). Since binding of the CENH3 protein to centromeric chromatin is crucial for normal functioning of the centromere in proper segregation of the sister chromatids (Birchler *et al.*, 2011), it is hypothesized that the *sed1* locus exerts an epigenetic, dosage-dependent modification of the chromosomes (Fig. 7). This model assumes that the expression of *sed1* differs between the pollen of plants homozygous for the *sed1* haplotype. Therefore, the epigenetic modification of the chromosomes caused by *sed1* can differ between pollen grains. A strong modification of the sperm cell chromosomes will lead to kernel abortion or haploid formation. If the pollen is

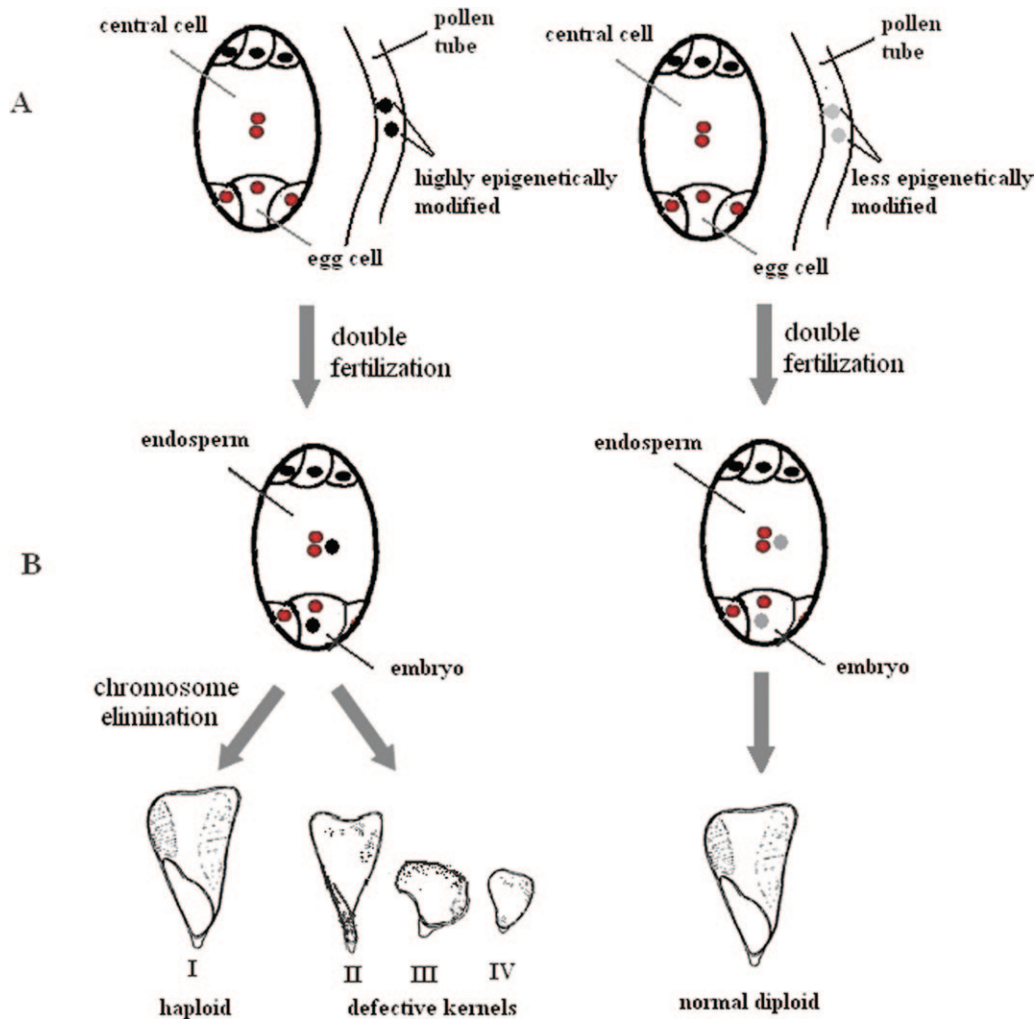


Fig. 7. Model for *sed1* as a male gametophyte-expressed gene(s) that influence the fate of the zygote. (A) *sed1* is expressed only in the male gametophyte, and not in the female gametophyte or in the sporophyte. The gene product can epigenetically modify chromosomes of the sperm cell, because the dosage of *sed1* can differ between pollen grains; in some grains, the chromosomes are highly modified (in black), whereas, in others, the chromosomes are less modified (in grey). The *sed1* gene product could reduce pollen competitive ability but not affect pollen viability. (B) The high degree of modification of the paternal chromosomes in the sperm cell leads to kernel abortion or chromosome elimination. The remaining, less modified pollen will not affect the double fertilization process, thus leading to normal kernel formation. (This figure is available in colour at *JXB* online.)

less epigenetically modified, this will not affect the double fertilization process and this finally leads to normal kernel formation. Therefore, a small proportion of the kernels become haploid but the majority develop into normal diploid kernels. Further research is needed to test this hypothesis. In particular, cloning of the gene(s) underlying the *sed1* and *qhir1* locus may help to uncover the genetic and functional basis of *in vivo* haploid induction in maize, especially whether only a single gene or a cluster of genes is involved, and this should also reveal its relationship to SD.

Supplementary data

Supplementary data are available at *JXB* online

Figure S1. Schematic diagram for the pollen competitive ability (PCA) test procedure for lines 1680 and UH400.

Figure S2. Punnett square for genetic transmission of the *sed1* haplotype through male and female gametes in F_2 populations with different survival rates r_i for the different genotypes.

Figure S3. Ear phenotype in reciprocal crossing and selfing ears.

Table S1. Oligonucleotide primers for newly developed molecular markers for fine-mapping *sed1*.

Table S2. Progeny testing of T-HIR for UH400 plants by two normal inbred lines.

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