Theor Appl Genet (2013) 126:2931–2941
DOI 10.1007/s00122-013-2183-9

ORIGINAL PAPER

Haploid plants carrying a sodium azide-induced mutation (*fdr1*) produce fertile pollen grains due to first division restitution (FDR) in maize (*Zea mays* L.)

Naho Sugihara · Takeyuki Higashigawa · Daiki Aramoto · Akio Kato

Received: 28 January 2013 / Accepted: 15 August 2013 / Published online: 3 September 2013

Abstract
Key message **We induced a *fdr1* mutation in maize which makes haploid plants male fertile due to first division restitution; the optimum sodium azide treatment on maize kernels has been identified.**
Abstract Sodium azide mutagenesis experiments were performed on haploid and diploid maize plants. Kernels with haploid embryos of maize inbred line B55 were induced by pollinating with RWS pollen. These kernels were treated with 0.2, 0.5, or 1.0 mM sodium azide solution for 2 h. The 0.5 mM solution was optimal for inducing numerous albino sectors on the treated plants without significant damage. Kernels of a maize hybrid, Oh43 × B55, were treated with sodium azide solutions at concentrations of 1.5, 2.0, 2.5, and 3.0 mM. Haploids were generated by pollinating RWS pollen. The highest rate of chlorophyll mutations in seedlings (15.3 % [13/85]) was recorded with the 2.5 mM concentration. A mutated haploid plant (PP1-50) with higher pollen fertility was isolated during the experiments. This haploid plant produced four kernels on the ear after selfing. These kernels were germinated and produced ears with full seed set after selfing. The haploid plants induced from PP1-50 diploids also exhibited high pollen fertility. In situ hybridization studies showed that meiocytes in PP1-50 haploid anthers underwent first division restitution at a rate of 48 % and produced equally divided dyads. We designated the genetic factor responsible for this high pollen fertility as *fdr1*. PP1-50 haploid ears exhibited high levels of sterility, as seen for regular haploids. Diploid PP1-50 meiocytes in the anther underwent normal meiosis, and all selfed progenies were normal diploids. We concluded that the *fdr1* phenotype is only expressed in the anthers of haploid plants and not in the anthers of diploid plants.

Communicated by H. Geiger.

N. Sugihara · A. Kato (✉)
Department of Life and Environmental Sciences, Kyoto Prefectural University, 1-5 Shimogamo Hangi-cho, Sakyo-ku, Kyoto, Kyoto-fu 606-0823, Japan
e-mail: katoa@kpu.ac.jp

Present Address:
T. Higashigawa
Matusaka-Nourin-Shoukou-Kankyou-Jimusho, 138 Taka-machi, Matusaka, Mie-ken 515-0011, Japan

Present Address:
D. Aramoto
Shin-Nippon Calendar Co. Ltd., 5-9 Techno park, Sanda, Hyogo-ken 669-1339, Japan

Introduction

The main purpose of this mutagenesis work was to induce a maize line with a higher rate of spontaneous chromosome doubling in the haploid state. Haploids of a maize inbred line, B73, undergo spontaneous chromosome doubling at a higher rate in both tassel branches and ovules. About a quarter of haploid B73 plants carries doubled tassel branches and produces 5–25 kernels after self-pollination. Haploid plants induced from W23 × W22 also exhibited higher rates of spontaneous chromosome doubling of both the tassel and ear (Kato 2002). There are a few reports on maize lines with higher rates of spontaneous chromosome doubling in the haploid state (Chase 1969; Kleiberab et al. 2012), and it is apparent that these higher rates are controlled genetically. Here, we screened for "highly fertile haploids" in sodium azide-treated maize lines by observing anthesis and pollen production at the haploid stage.

In maize, ionizing radiation is not an effective method for mutagenesis, because ionizing radiation frequently induces chromosomal breaks instead of point mutations (Bird and Neuffer 1987). EMS (methane sulfonic acid ethyl ester) has been utilized extensively as a mutagen for maize. Pollen grains suspended in mineral oil were treated with EMS (Neuffer and Coe 1978), and oil-suspended pollen was used to pollinate the recipient silks. The resulting point mutation frequencies were quite high (24 % seedling mutation rate, Bird and Neuffer 1987). Although EMS is a potent mutagen, it is volatile and carcinogenic. Great care has to be taken in the handling, storage, and disposal of this chemical: wearing a gasmask and a coverall are mandatory (Kato 2000) and the treatment has to be done in an isolated area. EMS can be neutralized in a 10 % sodium thiosulfate solution, and in this sense the chemical is easier to handle than a radioactive substance. Many universities are located in highly populated areas, and the use of EMS put students, staffs, and surrounding residents at risk. In many cases, there is no highly specialized or isolated facility suitable for the use of EMS.

Consequently, we attempted to use sodium azide (NaN_3) to treat haploid maize kernels to induce mutations. Sodium azide is a solid, non-volatile salt that has been used as a mutagen in plants and is known to induce point mutation by a base change (Conger and Carabia 1977; Olsen et al. 1993), although far less often than EMS. Before conducting large-scale mutagenesis experiments, we evaluated the potency of sodium azide as a mutagen in maize.

Recently, the application of mutagens to haploids and subsequent chromosome doubling have been attempted in many plant species for the rapid fixation of mutations (Szarejko and Forster 2007). Haploids also offer advantages for evaluating the potency of certain mutagens in a very short period. In maize, there are good haploid inducers available, e.g., Stock 6 (Coe 1959; Sarkar and Coe 1966), WS14 (Lashermes and Beckert 1988) and RWS (Rober et al. 2005). The haploid inducing character is controlled by a few QTL loci (Barret et al. 2008; Prigge et al. 2012; Xu et al. 2013). By using these inducers, the generation of dry kernels with haploid embryos is relatively easy. Maize is a monoecious plant species, and its male and female flowers emerge at different positions. Because of this trait, two rounds of self-pollination are necessary to reveal recessive mutations. The use of haploids in mutation work in maize is highly advantageous, and in this study, we utilized maize haploids in various ways to facilitate the progression of our research.

In recent decades, to facilitate the production of doubled haploid maize lines for breeding purpose, attempts have been made to double the haploid chromosome number using colchicine or nitrous oxide gas (Eder and Chalyk 2002; Kato 2002; Kleiberab et al. 2012). However, the success rates of obtaining DH progenies have been in the range of 10–20 %. The main problem in haploid chromosome doubling in maize arises from its monoecious nature and the single shoot plant form. The chromosome sets of both male and female flowers have to be doubled without injury, and once the single shoot is injured, successful self-pollination becomes impossible. In nitrous oxide gas treatment on maize haploids, the doubling efficiency is strongly influenced by the genotype. A discussion of the current situation in the production of doubled haploid maize was made by Geiger and Gordillo (2009). The authors of this study believe that improving the rate of spontaneous chromosome doubling through mutagenesis is another possibility for the successful production of doubled haploids in maize. The objective of this work was to induce mutated line that haploids produce fertile pollen grains without any chemical treatments, and we have successfully induced such a mutation.

Materials and methods

Haploid induction

We obtained a haploid inducer RWS line (Rober et al. 2005) from H.H. Geiger (Germany), and we introduced *C1-I* (a dominant color inhibitor gene) by backcrossing for six times. A maize inbred line, B55 (*R1-scm2*/*R1-scm2*), or a hybrid line, Oh43 × B55 (*R1-scm2*/*R1-scm2*), were pollinated with RWS pollen. The gene *R1-scm2* induces a deep purple pigmentation in both the aleurone and scutellum. The dominant gene *C1-I* inhibits expression of the purple color in both the scutellum and aleurone, resulting in colorless kernels. Haploid kernels were separated as kernels with purple embryos and a colorless aleurone. The rates of haploid induction were 3–5 %.

Sodium azide treatment

Five grams of kernels with haploid embryos (24–30 kernels) were soaked in ice-cold water for 1 day, soaked kernels were then placed on a thick, moist cloth and covered with another moist cloth at room temperature for 14–16 h. Next, the kernels were placed in a plastic beaker. Ten milliliters of buffer solution (100 mM potassium phosphate, pH 3.0, with 0.1 % Tween-20 or 0.1 % detergent) and 20–100 μl of 100 mM sodium azide were added to the beaker. The beaker was placed on a shaker (approximately 100 rpm) for 2 h. A thin plastic film was used to cover the beaker to prevent evaporation. Then, the solution was removed and 20–30 ml of tap water was added. The kernels were washed twice for 1 h each on the shaker, with the tap water changed between washes. The treated kernels

were coated with the fungicide captan and embedded in moist vermiculite at 30 °C for 2–3 days to induce germination. The germinated plants were transplanted to small pots (7.5 cm diameter) and grown for a few weeks in a greenhouse. The seedlings were then planted in a field to reach maturity.

The hybrid kernels of B55 × Oh43 (*R1-scm2*/*R1-scm2*) were treated in a similar way but with increased quantities. For the hybrid, 50 g of kernels (about 200–250 kernels) and 100 ml of buffer solution were used. A shallow plastic tray was used. The hybrid seeds were more tolerant to sodium azide than B55 haploids and they were treated with 1.5, 2.0, 2.5, and 3.0 mM sodium azide solutions for 2 h. The treated plants (125 plants in each treated plot) were planted in a field, and after silk emergence, they were pollinated with pollen collected from the RWS (*C1-I*/*C1-I*) line to induce haploids to evaluate the rates of chlorophyll mutation.

Slide preparation

Meiocytes were collected from the anthers of pre-emergence tassels of B55 haploid, PP1-50 haploid, PP1-50 diploid, and KYS diploid plants. Two plants were used in each genotype. They were fixed in 90 % acetic acid for 1 h and then stored in 70 % ethanol at −20 °C until examination. Anthers were collected from glumes, and one anther was stained with acetic orcein and examined using a light microscope. Once the stage of the anther was determined, the remaining two anthers were treated with 10 μl pectolyase Y-23 (1 %) (Kikkoman Co., Tokyo, Japan) and cellulase Onozuka R-10 (2 %) (Yakult Honsha Co., Ltd., Tokyo, Japan) in citric buffer (5 mM sodium citrate, 5 mM EDTA, pH 5.5). The tube was transferred to a water bath at 37 °C, and the anthers were digested for 45–60 min. After digestion, the anthers were washed in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.6). After aspiration of the TE buffer, 50 μl of 70 % ethanol was added to the tube. Then, the tube was agitated several times to disperse the digested cells into a suspension of single cells. The tube was centrifuged at 5,000 rpm for 1 min to collect the cells. The 70 % ethanol was discarded and the cells were washed with 100 % ethanol to remove any remaining moisture. The cells were again spun down and the ethanol was aspirated. The cells were resuspended in 30 μl of 100 % acetic acid.

Clean glass slides were placed in a humid chamber consisting of a small open top cardboard box (10 × 10 × 20 cm) with moistened paper towels at the bottom and with two wooden rods inside. Four droplets (0.5 μl each) of the acetic acid cell suspension were dropped onto a clean glass slide in two lines of two droplets. The slides were allowed to dry slowly in the humid chamber. The meiocyte spreads on the slides were fixed with a 10 % formaldehyde solution for 5 min by dropping the solution onto the slide and covered with parafilm. The 10 % formaldehyde solution on the slides was washed away using drops of 70 % ethanol followed by 100 % ethanol.

Chromosome number determinations of PP1-50 haploids were achieved by the nitrous oxide air-drying method (Kato 1999) on root tips of germinating kernels followed by acetic orcein staining. For the ploidy determinations of the anthers of haploid PP1-50, the FISH procedure described below was utilized. In haploid cells, the fluorescent signal numbers are half that of the diploid counterparts.

Fluorescence in situ hybridization analysis

The FISH procedure was essentially as described previously (Kato et al. 2006). A probe mixture (e.g., fluorescein-labeled knob 5 ng/μl and Texas red-labeled TAG microsatellite 2 ng/μl, total volume 2 μl) was dropped onto the slide and a plastic cover slip was applied. The slide was maintained at 100 °C for 5 min in humid conditions to denature the chromosomal DNA and the probes. The slide was incubated in a sealed storage container with a moist paper towel for 3 h at 55 °C.

After a stringent washing (55 °C, 2× SSC, 1 mM EDTA, 20 min), the slides were allowed to drain. A drop of Vectashield mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) was applied and the cells were covered with a 24 × 32 mm cover slip. Fluorescent signals from each of the three channels (DAPI, fluorescein and Texas red) were captured using a 100× plan apochromatic oil objective and a BX60 epifluorescence microscope (Olympus, Tokyo, Japan) with an EOS Kiss X2 camera (Canon, Tokyo, Japan) and a camera adaptor provided by MeCan Imaging, Inc. (Saitama, Japan, http://www.mecan.co.jp/index.html).

Staining of pollen grains in the anther

Anthers of PP1-50 diploid, PP1-50 haploid, and Oh43 haploid lines were fixed in 70 % ethanol and stored at −20 °C. The anthers were submerged in 3 % iodide solution for 1 h to stain starch in the pollen grains. The anthers were washed in 70 % ethanol and embedded in one drop of glycerol on a slide to make the anther wall translucent, and a cover slip was applied. Images were captured using a light microscope and camera system.

Statistical analyses

Analyses of variance and Tukey's multiple range tests were performed to evaluate the effects of the treatment on plant height and root length. The numbers of mutated sectors were not normally distributed; therefore,

Kruskal–Wallis tests were applied to evaluate the significance. In the ratios, i.e., germination ratio, ratio of pollen sample with defective pollen grains, mutated seedling ratio, early failure of the shoot meristem ratio, a method of approximation to normal distribution (e.g., Chi-square test) was applied if the expected value was more than five. If the expected value was less than five, the probability was calculated from the binomial distribution of the expected value.

Results

Effect of sodium azide on haploid and diploid maize plants

The B55 haploid seedlings treated with sodium azide solution (0.2–1.0 mM) at seed stage exhibited numerous sectors with chlorophyll mutations in the leaves (Fig. 1a). The sizes of the sectors were smaller on lower leaves, and fewer and larger on the upper leaves (Fig. 1b–d). We counted the

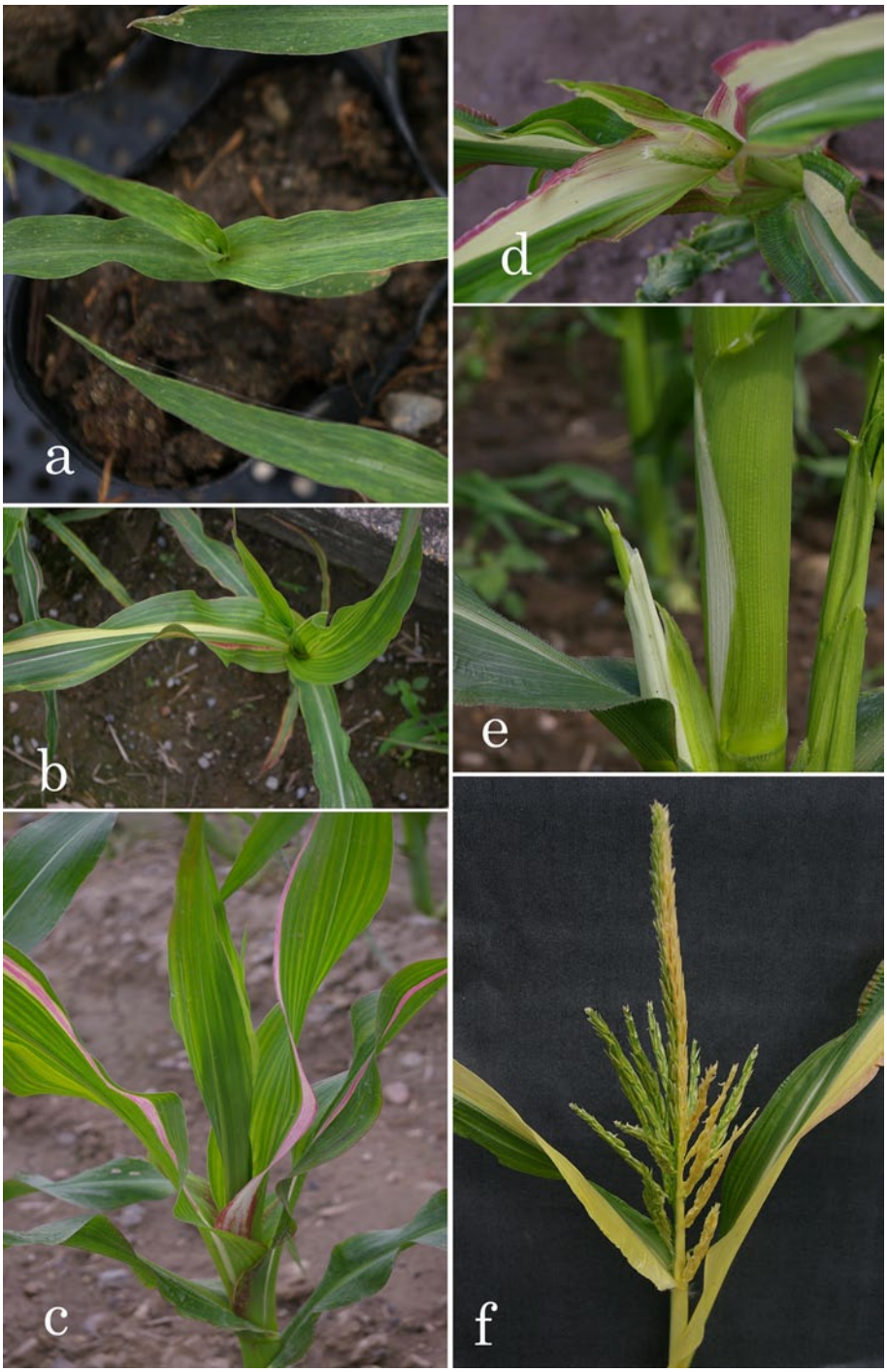

Fig. 1 Chlorophyll mutant sectors observed in B55 haploid plants after the sodium azide treatment (0.5 mM) of seeds. **a** Sectors observed in the seedling state; numerous small sectors are seen on the leaves. **b** The juvenile stage; the sectors are getting larger but the numbers have become smaller. **c**, **d** Albino sectors observed on multiple leaves. Note that the colors are different. **e** Albino sector observed on an ear shoot. **f** A quarter of the tassel exhibits albino coloration

Table 1 Growth and leaf sector mutation incidents after sodium azide (pH 3.0) treatment of maize B55 haploids

Sodium azide concentration (mM)	Germination rate (%) (no. germinated/ no. planted)	No. of chlorophyll mutation sectors (per leaf)		Root length at 72 h after germination (cm)[a]	Plant heights at flowering stage (cm)[a]	Early failure of shoot meristem (%) (no. failure/no. planted)
		4th leaf	5th leaf			
0	92 (25/27)	2.0	1.2	6.4 a	135 a	0 (0/25)
0.2	93 (24/26) ns	16.7**	3.0 ns	5.9 a	127 a	0 (0/22) ns
0.5	96 (23/24) ns	24.0**	6.9**	4.4 b	75 b	0 (0/23) ns
1.0	92 (23/25) ns	28.6**	8.0**	3.1 b	76 b	12 (2/17) ns
0.5 (pH 5.0)	89 (24/27) ns	27.9**	12.5**	–	–	–

Treatment was carried out on moistened kernels just before germination

ns not significant

** Significantly different at $P < 0.01$ from each control

[a] Numbers with the same letter are not significantly different

Table 2 Growths and mutation rates of sodium azide-treated hybrid maize kernels (Oh43 × B55 *R1-scm2*/*R1-scm2*)

Sodium azide concentration (mM)	Germination rate (%) (no. germinated/ no. plated)	Root length at 72 h after germination (cm)[a]	Plant height at flowering stage (cm)[a]	Rates of pollen samples (%) that segregate defective pollen grains (no. of samples with defective pollen/no. examined)	Chlorophyll mutation rate observed in haploid seedlings (%) (no. of mutants/no. examined)
0	95 (119/125)	9.6 a	283 a	0 (0/58)	0 (0/107)
1.5	90 (112/125)ns	6.5 b	201 b	7.0 (4/57) ns	2.9 (3/103) ns
2.0	81 (101/125)**	5.6 b	193 bc	18.3 (11/60)**	3.4 (4/116) ns
2.5	76 (95/125)**	5.1 b	193 bc	16.9 (10/59)**	15.3 (13/85)**
3.0	50 (63/125)**	4.5 b	180 c	25.0 (14/56) **	–

The treatment was carried out on the moistened kernels just before germination. Haploid seedlings were obtained by pollinating the sodium azide-mutagenized hybrids with RWS pollen

ns not significant

** Significantly different at $P < 0.01$ from each control

[a] Numbers with the same letter are not significantly different

numbers of mutated sectors and found that 0.5–1.0 mM sodium azide was optimal for inducing mutations in maize B55 haploid seedlings (Table 1). At a concentration of 1.0 mM, plant growth was more retarded, and the occurrence of early failure of the shoot meristem was more frequent (12 %); however, the difference was not statistically significant. We conducted similar examinations of haploids induced from other inbred lines, i.e., Oh43 and B73, and found that in these inbred lines, 0.5 mM sodium azide was also optimal for the induction of sectors with chlorophyll mutations (data not shown). A sodium azide treatment (0.5 mM) at higher pH (pH 5.0) also induced numerous chlorophyll mutation sectors (Table 1). Mutated sectors were also observed on the ear shoot and tassels of treated B55 haploids (0.5 mM) (Fig. 1e, f), indicating that reproductive organs could carry the mutation, which is essential for successful mutagenesis. In the hope of producing a mutated line with a higher rate of spontaneous chromosome doubling, we treated 300 haploid B55 kernels with 0.5 mM sodium azide. There were 15 plants with doubled sectors on the tassels among the 300 haploids (M1), and the pollen grains obtained from the tassels were used to pollinate diploid B55 plants (M2). Next year we induced haploids from the M2 plants. The haploids plants (M3) were planted in the greenhouse but none of them exhibited a higher rate of spontaneous chromosome doubling on the tassel.

We tried sodium azide treatment (0.5–1.0 mM) on maize inbred lines B55 and B73, but the treated plants frequently became barren (up to 80 % with no seed set). We also tried the treatment on hybrid plants (Oh43 × B55, *R1-scm2*/*R1-scm2*); our preliminary experiments indicated that they are more tolerant to sodium azide and produced seeds even after severe damage caused by the chemical. The hybrid kernels were treated with 1.5–3.0 mM sodium azide and planted in the field (Table 2). The treated plants showed various abnormalities during growth, such as, early failure of shoot development, wrinkled leaves, bent stalks, and various dominant color sectors on the developing leaves. The pollen samples collected from a branch of the tassels showed segregation of defective pollen grains. In treatment

with 2.0–3.0 mM sodium azide, 17–25 % of samples contained defective pollen grains (Table 2). After pollination with RWS, we successfully harvested mature ears from the treated hybrid plants except for the plants treated with 3.0 mM sodium azide, where the plants were severely deformed. Haploid kernels were separated and after germination of the haploid kernels in a greenhouse, the numbers of seedlings with chlorophyll mutations were counted. The highest rate of mutation (15.3 % [13/85]) was recorded in the seedlings treated with 2.5 mM sodium azide.

In hybrid maize, sodium azide treatment at 2.5 mM was the optimum concentration to induce mutations. We tried treatments (2.0–2.5 mM sodium azide) on the Oh43 × B55 hybrid three times after the initial trial result and found that results varied from year to year considerably. The main problem was early failure of the shoot meristem, although even when this occurred, another shoot developed into tassel seeds and harvesting of kernels was possible. However, the amount of seeds was decreased significantly. Sometimes 50–90 % of plants ceased shoot growth. In the initial trial, only 4 % of plants exhibited early shoot growth failure.

Fertility of PP1-50 haploids and their meiotic behavior

During preliminary studies of sodium azide treatment (1.5 mM for 2 h) of the maize hybrid Oh43 × B55 (*R1-scm2*/*R1-scm2*) and the induction of haploids, we found a haploid plant that extruded anthers from the whole tassel and shed pollen grains among 100 haploids planted in summer 2009. We designated this plant as PP1-50 (purple aleurone and purple scutellum, a derivative of Oh43 × B55 hybrid, the 50th haploid plant from the edge). Fortunately, we obtained four selfed kernels of the PP1-50 haploid and planted two of them in a greenhouse. The two PP1-50 doubled haploid plants produced normal

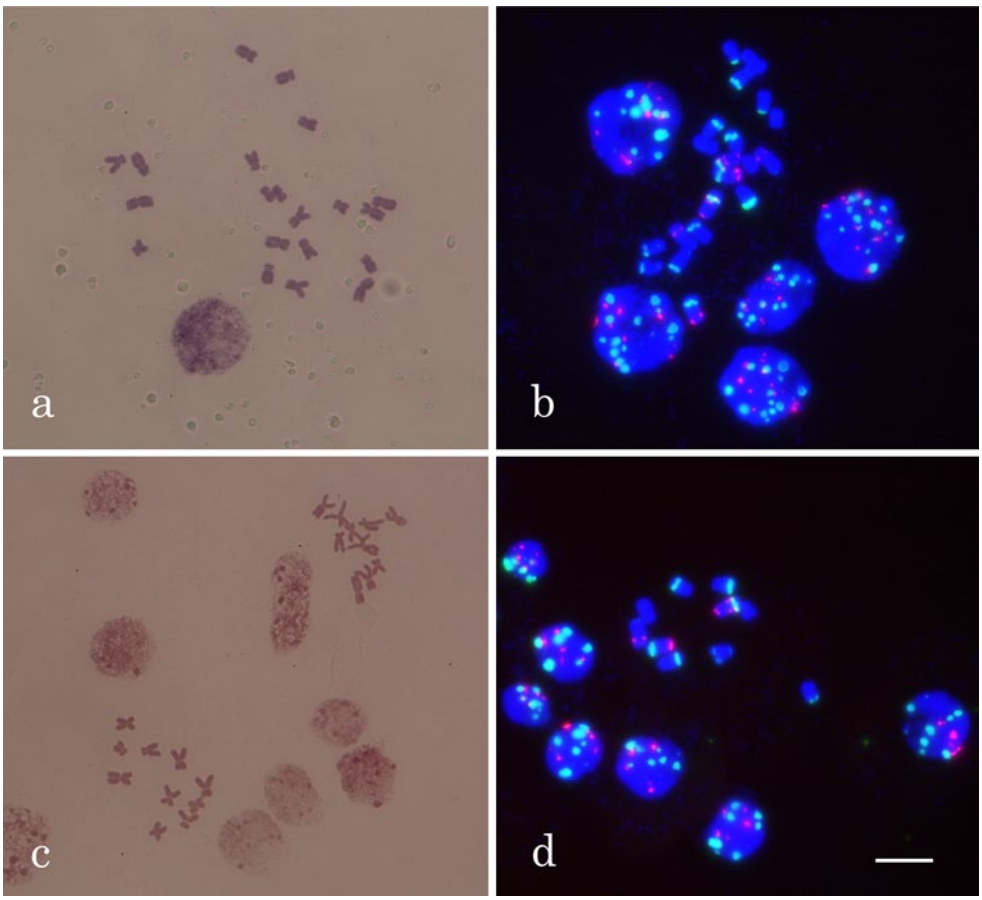

Fig. 2 Chromosome number and ploidy determined by acetic orcein staining or FISH. **a** Metaphase chromosomes of a PP1-50 diploid root tip after acetic orcein staining. **b** Metaphase chromosomes of a PP1-50 diploid root tip observed by FISH; five interphase cells are also observed. The *red signals* are TAG microsatellites and the *green signals* are knobs. **c** Metaphase chromosomes of a PP1-50 haploid root tip after acetic orcein staining. **d** Metaphase chromosomes of a PP1-50 haploid root tip observed by FISH; seven interphase cells are observed. The number of FISH signals is exactly half of the diploid counterparts. The ploidy is clearly determined from interphase cells. *Bar* 10 μm

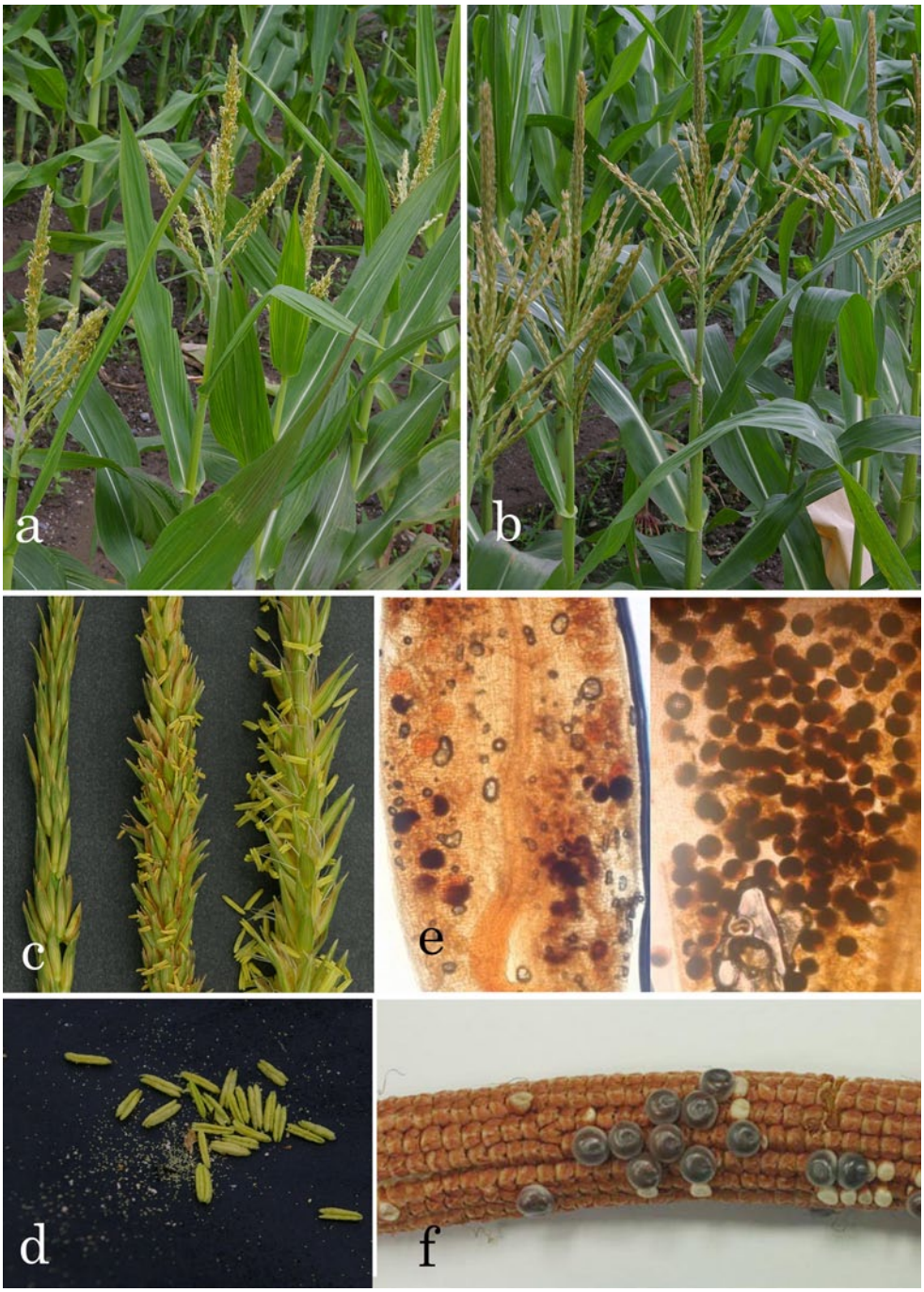

Fig. 3 The fertility of PP1-50 haploids. **a** PP1-50 haploids at flowering stage; many fertile anthers have been extruded. **b** B55 haploids; no anthers are extruded. **c** Tassel branches of B55 haploid (*left*), PP1-50 haploid (*center*) and PP1-50 diploid (*right*). The anther size of the haploid PP1-50 is smaller than the diploid counterpart. **e** Pollen grains in the anthers; Oh43 haploid (*left*) and PP1-50 haploid (*right*). **d** Pollen grains obtained from a PP1-50 haploid plant; many pollen grains remain in the anther. **f** An ear pollinated by the pollen shown in **d**. Plump kernels were obtained at maturity

plump kernels after self-pollination. Next year, we transferred RWS pollen to PP1-50 doubled haploid plants and obtained more than 100 kernels with haploid embryos. Nineteen kernels of putative PP1-50 haploids (purple embryos with a colorless aleurone) were germinated, and after chromosome counts all of them were proved to be haploids (chromosome number = 10, Fig. 2). Field observations showed that haploid PP1-50 protruded anthers on hot sunny days, and a small amount of fertile pollen was obtained (Fig. 3a, c, d). The pollen grains were transferred

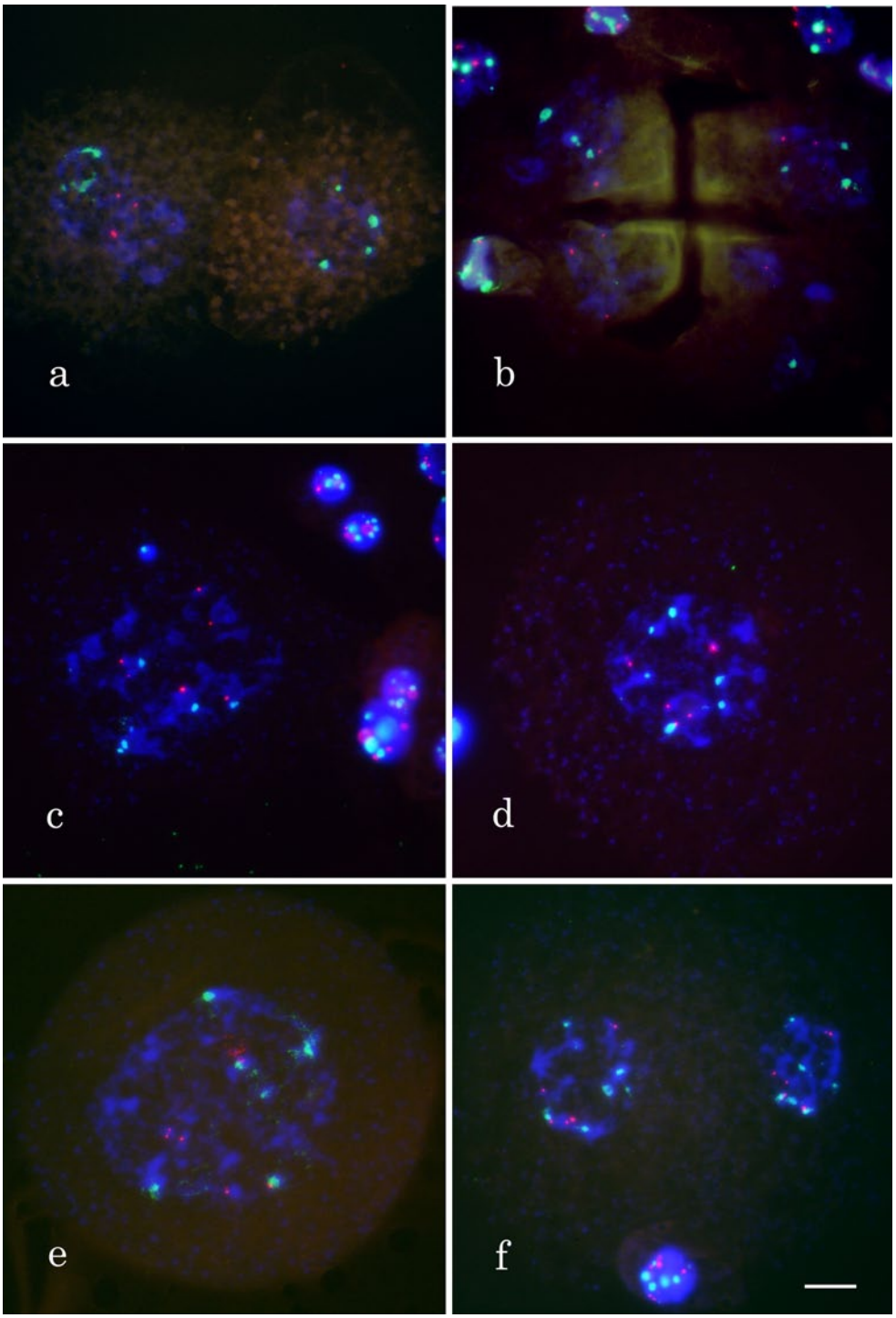

Fig. 4 Fluorescence in situ hybridization pictures of meiocytes observed in B55 haploids and PP1-50 haploids. The *red signals* are TAG microsatellites and the *green signals* are knobs. **a** Dyad of a B55 haploid plant; all *red signals* are present in the *left* daughter cell. **b** Tetrad of a B55 haploid; signals are unequally distributed. Five haploid anther cells surround the tetrad. **c** Meiocyte of a PP1-50 haploid plant. The chromosomes failed to separate in late metaphase of meiosis I. **d–e** Restituted meiocytes after FDR observed in PP1-50 haploid plants. **f** A dyad after the division of a restituted nucleus. The signals are equally distributed. A haploid anther cell is observed at the *bottom*. *Bar* 10 μm

Table 3 Examination of meiotic division behavior from the end of meiosis I to the tetrad by in situ hybridization

Genotype	No. of restituted nuclei	No. of dyads		No. of tetrads	
		Balanced	Unbalanced	Balanced	Unbalanced
PP1-50 haploid	77	123	49	0	164
B55 haploid	0	0	56	0	91
PP1-50 diploid	0	88	0	81	0
KYS diploid	0	36	0	39	0

Two sequences, TAG microsatellite (*red*) and knob repeat (*green*) were used to detect FISH signals. If the daughter cells of tetrad or dyad had the same number of signals, they were considered to be balanced; if they were different, unbalanced

onto the silks of a Oh43 × B55 plant, and plump kernels were produced on the ear (Fig. 3f). On rainy or cloudy days, they protruded smaller numbers of anthers and pollen grains were retained within the anther with no pollen being shed. The anther size was smaller than PP1-50 diploid counterparts (Fig. 3c). Iodide staining showed that anthers of PP1-50 haploids contained abundant pollen grains. In the anthers of haploid PP1-50, an average 288 pollen grains were observed using a microscope (an average of 5 anthers, range 212–381, Fig. 3e). On the other hand, the anthers of haploid Oh43 plants contained 15 pollen grains per anther (an average of 5 anthers, range 6–32), anthers of diploid PP1-50 contained 1170 pollen grains per anther (an average of 3 anthers). The open-pollinated ears of haploid PP1-50 produced 0–3 kernels on the ears and no indication of higher fertility at the female site. We attempted self-pollination of 14 PP1-50 haploids, but only two plants produced 1–2 kernels. We planted 50 diploid PP1-50 plants and they were morphologically identical because of their doubled haploid nature. All of these plants produced abundant pollen grains and plump kernels after pollination, and there was no sign of elevated chromosome numbers (triploidy, etc.). We crossed pollen produced by the diploid PP1-50 plant with B55 *R1-scm2* and Oh43 *R1-scm2* and induced haploids; 55 haploid plants were grown in a greenhouse and the fertile/sterile haploid ratio was recorded. A total of 27 haploids were male fertile and 28 were male sterile, this ratio fits the 1:1 segregation ratio after Chi-square test ($p > 0.89$). From FISH analyses of meiotic-stage anthers, it was found that B55 haploid meiocytes routinely underwent irregular meiosis compared with diploids. There are no homologous chromosomes in the meiocytes of haploids, with ten univalent chromosomes observed in the metaphase of meiosis I. FISH analyses revealed that these univalent chromosomes separated randomly and produced an unbalanced dyad after unequal division (Fig. 4a; Table 3). In meiosis II, the dyad divided and produced a tetrad composed of four unbalanced daughter cells (Fig. 4b). In PP1-50 haploids, half of the meiocytes followed unequal division at meiosis I, resulting in unbalanced dyads or tetrads (49 + 164 = 213, Table 3). However, the other half (48 ± 2.5 %) of the meiocytes failed to separate during meiosis I and produced restituted nuclei (Fig. 4c–e). These restituted nuclei underwent meiosis II and produced a dyad with two balanced daughter cells (Fig. 4f) (77 + 123 = 200, Table 3) after equal division. The results were consistent between two anther samples from different individual haploid PP1-50 plants (restituted + balanced:unbalanced, 102:108 and 98:105, respectively, statistically not significant between the two samples after Chi-square test, $p > 0.9$). In PP1-50 diploid meiocytes, meiotic division was normal as was that of the control line (KYS, Table 3).

The parental lines of PP1-50 are B55 and Oh43, and B55 and Oh43 haploids are highly sterile. Sodium azide mutagenesis generated a mutation that makes haploid anthers fertile and can be maintained in a homozygous state in diploid PP1-50 plants. The 1:1 fertile/sterile plant segregation ratio indicated that there is a single factor that is responsible for high anther fertility in the haploid state. We designated the mutated genetic factor as *fdr1*. The mutant phenotype is expressed only in the haploid state, and in diploid plants homozygous for *fdr1*, the meiotic behavior was normal.

Discussion

In this study, a valuable mutant, PP1-50 carrying *fdr1*, was obtained. This line undergoes first division restitution in males during meiosis I at a rate of 48 % and produces high numbers of fertile pollen grains. The fertility rate is extremely high for a haploid; however, its pollen-shedding ability was not as high. The rate of pollen grain formation was lower than that in normal plants because PP1-50 haploids undergo FDR meiosis at a rate of 48 % (52 % reduction compared with 100 % FDR) and produce dyads as the final product meiotic division instead of tetrads. In normal meiotic divisions, if there are 100 meiocytes 400 pollen grain will be produced. In PP1 haploids, if there are 100 meiocytes, 48 meiocytes undergo FDR and 48 dyads will be produced. These

dyads develop into 96 pollen grains. Hence, the number of pollen grains contained in an anther is about 24 % ($0.48 \times 0.5 = 0.24$) of a diploid counterpart. In maize, a 24 % pollen fertility rate is semi-sterile and generally such plants do not shed pollen easily. Therefore, it is desirable to obtain a new mutant with a 100 % FDR rate in the haploid state.

Meiotic mutations that skip the meiotic process have been discovered in maize (Singh et al. 2011) and *Arabidopsis* (Ravi et al. 2008); those mutations exhibit effects in the diploid state and show elevated chromosome numbers after fertilization. Unlike those mutations, the *fdr1* mutation does not show any abnormality in the diploid state. In this sense, the phenomenon is similar to FDR shown by wheat haploids, where doubled plants show no mutated phenotype (Jauhar 2007). This factor is important for the utilization of the *fdr1* mutation for breeding purposes to generate doubled haploids. In order to generate superior maize hybrid cultivars, diploid inbred lines and hybrid plants must be absolutely normal; any further elevation of chromosome number in the diploid state will make cultivar unusable. The *fdr1* mutation carried by PP1-50 has a desirable character that can be utilized as part of breeding programs, i.e., doubled haploid production and fixing mutations in one generation. The poor pollen-shedding ability and low seed set on the female side are limitations for the use of this mutation. The female side fertility can be improved by crossing B73 to PP1-50, because B73 routinely produces 5–25 kernels after pollination in the haploid state (Kato 2002). The male side fertility may be improved by inducing another new mutation, or combining different mutations into one line by crossing.

The chemical agent EMS has been used extensively to generate mutations in maize (Bird and Neuffer 1987). Compared with the mutation rate of EMS (24 %), the rate of chlorophyll mutations resulting from sodium azide treatment (2.5 mM) was 15 % (Table 2), which was considered adequate for these studies. Sodium azide-treated haploids exhibited numerous sectors with chlorophyll mutations and they provided numerically similar concrete data within 4 weeks. Haploid maize kernels can be utilized to evaluate the mutagenic effects of potentially hazardous chemicals in a short period of time. The possible causes of the variable results in sodium azide treatment could be (1) slight differences in room temperature during treatment and (2) the intensity of washing after the treatments.

Author contribution N. S. performed characterization of PP1-50 haploids, T. H. and D. A. conducted sodium azide mutagenesis and collected data, and A. K. designed the experiments and prepared the manuscript.

Acknowledgments The authors thank H. H. Geiger for providing the RWS line.

Conflict of interest None of the authors have any conflicts of interest associated with this study.

References

Barret P, Brinkmann M, Beckert M (2008) A major locus expressed in the male gametophyte with incomplete penetrance is responsible for in situ gynogenesis in maize. Theor Appl Genet 117:581–594

Bird RM, Neuffer MG (1987) Induced mutations in maize. Plant Breed Rev 5:139–180

Chase SS (1969) Monoploids and monoploid-derivatives of maize (*Zea mays* L.). Bot Rev 5:117–167

Coe EH (1959) A line of maize with high haploid frequency. Am Nat 93:381–382

Conger BV, Carabia JV (1977) Mutagenic effectiveness and efficiency of sodium azide versus ethyl methanesulfonate in maize) induction of somatic mutations at the yg2 locus by treatment of seeds differing in metabolic state and cell population. Mutat Res 46:285–296

Eder J, Chalyk S (2002) In vivo haploid induction in maize. Theor Appl Genet 104:703–708

Geiger HH, Gordillo GA (2009) Doubled haploids in hybrid maize breeding. Maydica 54:485–499

Jauhar PP (2007) Meiotic restitution in wheat polyhaploids (Amphihaploids) a potent evolutionary force. J Hered 98:188–193

Kato A (1999) Air drying method using nitrous oxide for chromosome counting in maize. Biotech Histochem 74:160–166

Kato A (2000) A safe procedure for EMS treatment of pollen in maize. Maize Genet Coop Newsl 74:19–22

Kato A (2002) Chromosome doubling of haploid maize seedlings using nitrous oxide gas at the flower primordial stage. Plant Breed 121:370–377

Kato A, Albert PS, Vega JM, Birchler JA (2006) Sensitive fluorescence in situ hybridization signal detection in maize using directly labeled probes produced by high concentration DNA polymerase nick translation. Biotech Histochem 81: 71–78

Kleiberab D, Priggeac V, Melchinger AE, Burkarda F, Vicentec FS, Palominod G, Gordillob GA (2012) Haploid fertility in temperate and tropicalmaize germplasm. Crop Sci 52:623–630

Lashermes P, Beckert M (1988) Genetic control of maternal haploidy in maize (*Zea mays* L.) and selection of haploid inducing lines. Theor Appl Genet 76:405–410

Neuffer MG, Coe EH (1978) Paraffin oil technique for treating mature corn pollen with chemical mutagens. Maydica 23:21–28

Olsen O, Wang X, Wettstein DV (1993) Sodium azide mutagenesis, preferential generation of A.T $\geq$G.C transitions in the barley Ant18 gene. Proc Natl Acad Sci USA 90:8043–8047

Prigge V, Xu X, Li L, Babu R, Chen S, Atlin GN, Melchinger AE (2012) New insights into the genetics of in vivo induction of maternal haploids, the backbone of doubled haploid technology in maize. Genetics 190:781–793

Ravi M, Marimuthu MP, Siddiqi I (2008) Gamete formation without meiosis in *Arabidopsis*. Nature 451:1121–1124

Rober FK, Gordillo GA, Geiger HH (2005) In vivo haploid induction in maize-performance of new inducers and significance of doubled haploid lines in hybrid breeding. Maydica 50:275–283

Sarkar KR, Coe EH (1966) A genetic analysis of the origin of maternal haploids in maize. Genetics 54:453–464

Singh M, Goel S, Meeley RB, Dantec C, Parrinello H, Michaud C, Leblanc O, Grimanelli D (2011) Production of viable gametes

without meiosis in maize deficient for an ARGONAUTE protein. Plant Cell 23:443–458

Szarejko I, Forster BP (2007) Doubled haploidy and induced mutation. Euphytica 158:359–370

Xu X, Li L, Dong X, Jin W, Melchinger AE, Chen S (2013) Gametophytic and zygotic selection leads to segregation distortion through in vivo induction of a maternal haploid in maize. J Exp Bot 64:1083–1096