

# Wide hybridization between oat and pearl millet belonging to different subfamilies of Poaceae

Takayoshi Ishii · Hiroyuki Tanaka ·  
Amin Elsadig Eltayeb · Hisashi Tsujimoto

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**Abstract** Oat (*Avena sativa* L.) and pearl millet (*Pennisetum glaucum* L.) belong to different subfamilies of Poaceae. When emasculated oat was pollinated by millet, fertilization took place and all seven millet chromosomes were retained along the complete haploid oat complement during early stages of embryogenesis. Fourteen days after pollination, we cultured 170 embryos onto rescue medium, of which 99 were attached with endosperm tissue. Twenty-one embryos germinated and showed shoot growth. One of them also developed roots. The shoots of the rootless embryos elongated, but rolled to the scutellum side and eventually died in light conditions. Chromosome observations and marker analyses indicated that the seedling plants were true hybrids that retained all of the oat and millet chromosomes. One exceptional embryo with shoot and root grew under light conditions. This was a haploid of oat and developed to a fertile adult plant. One embryo generated a callus after 6 months cultivation, and it was found to harbor four out of the seven millet chromosomes corresponding to linkage groups 2, 4, 6, and 7. The callus grew vigorously but did not develop shoots or roots.

**Keywords** Chromosome elimination · Photosynthesis · Necrosis · Embryo rescue · Double fertilization

## Introduction

In the initial steps of interspecific hybridization, various abnormalities known as crossing barriers are often encountered. Barriers that occur before fertilization, such as failure of pollen germination or pollen tube elongation, are not very common in wide hybridization between wheat, oat, and related species, whereas those that occur after fertilization are common. Generally, the more distant the relationship between two species, the more severe the endosperm abnormality becomes in the hybrid. For example, a cross between wheat and rye, belonging to different genera in the same subtribe, produces shriveled but germinable seeds. However, a cross between more distantly related species such as wheat and *Leymus racemosus*, belonging to different subtribes of the same subfamily, produces an embryo but no endosperm. Thus, embryo rescue is needed to obtain hybrid plants (Kishii et al. 2004). In an even more distantly related cross such as between wheat and sorghum, belonging to different subfamilies of the same family (Poaceae), elimination of paternal chromosomes takes place and no true hybrid carrying the genomes of both parents appears.

In wheat and maize (*Zea mays*) or sorghum (*Sorghum bicolor*) crosses, chromosomes of maize and sorghum are eliminated during early zygotic cell division (Laurie and Bennett 1986, 1988a, 1989). In crosses of wheat and pearl millet (*Pennisetum glaucum*), the millet chromosomes are gradually eliminated over 3 weeks (Gernand et al. 2005; Ishii et al. 2010). Following chromosome elimination, haploid wheat plants can be recovered by embryo rescue

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T. Ishii  
The United Graduate School of Agricultural Sciences,  
Tottori University, Tottori 680-8550, Japan

H. Tanaka  
Faculty of Agriculture, Tottori University,  
Tottori 680-8550, Japan

A. E. Eltayeb · H. Tsujimoto (✉)  
Arid Land Research Center, Tottori University,  
Hamasaka, Tottori 680-0001, Japan  
e-mail: tsujim@alrc.tottori-u.ac.jp

(Inagaki and Mujeeb-Kazi 1995; Laurie 1989; Laurie and Bennett 1986, 1988a). In wheat  $\times$  maize or barley (*Hordeum vulgare*)  $\times$  *H. bulbosum* crosses, chromosome elimination is caused by the lack of attachment of spindle fibers to the centromeres of maize or *H. bulbosum* (Mochida et al. 2004; Sanei et al. 2011).

Oat and maize belong to different subfamilies of family Poaceae, namely Pooideae and Panicoideae, respectively. Crosses between oat and maize produce plants with one or more stable maize chromosome(s) in the oat genetic background (Kynast et al. 2001; Riera-Lizarazu et al. 1996; Rines et al. 2009). These oat–maize chromosome addition lines have since been used for physical mapping of the maize genome (Okagaki et al. 2001) and sorghum sequences in maize chromosomes by fluorescence in situ hybridization (FISH) (Koumbaris and Bass 2003). Moreover, these lines have also been used for the studies of centromere-specific histone (CENH3), gene expression (Jin et al. 2004), and meiotic chromosome behavior (Bass et al. 2000). These lines are also useful for introducing maize traits, such as the  $C_4$  photosynthetic system, to oat (Kowles et al. 2008).

Pearl millet belongs to family Poaceae, subfamily Panicoideae, together with maize and sorghum, and possesses  $C_4$ -type photosynthesis that allows it to adapt to hot and dry conditions. We previously found that millet chromosomes are not eliminated during embryogenesis in crosses with oat (Ishii et al. 2010). Here, we report the chromosome dynamics in the embryo, endosperm, necrotic plant, and callus of hybrids between oat and millet.

## Materials and methods

### Plant materials

Oat (*Avena sativa*;  $2n = 6x = 42$ ) cultivar ‘Best Enbaku’ was used as female parent, and pearl millet (*Pennisetum glaucum*;  $2n = 2x = 14$ ) cultivar ‘Ugandi’ as male parent. Oat was grown in the field under natural conditions. Millet was grown in pots ( $\varnothing 27$  cm) under greenhouse conditions at a temperature of  $\geq 20$  °C.

### Inter-subfamily crosses and embryo rescue

Emasculation and pollination were carried out as previously described (Ishii et al. 2010). After pollination, the panicle was cut about 15 cm below a panicle including a node and covered with a plastic bag and then placed in a plastic bottle containing sucrose (4 %) and 100 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), the concentrations of which have been optimized by Machan et al. (1995) and Ishii et al. (2010). The panicles were kept at 23 °C in continuous light. To prevent spoiling of the solution, the bottle was put in

water kept at 4 °C (Ishii et al. 2010). The solution was renewed every 3 days, and the cutting edge of the culm was washed under running tap water before placing it in fresh solution. Fourteen days after pollination, immature seeds were sterilized in 70 % ethanol for 1 min and then rinsed three times in distilled water for 1 min each time. Embryos were excised and placed scutellum side down on rescue medium in Petri dishes ( $\varnothing 9$  cm, 20 ml medium for a dish). The medium consisted of a modified 1/2 Linsmaier and Skoog (LS) medium with amino acids (L-glutamine 0.4 g/l, L-alanine 0.05 g/l, L-cysteine 0.02 g/l, L-arginine 0.01 g/l, L-leucine 0.01 g/l, L-phenylalanine 0.01 g/l, L-tyrosine 0.01 g/l, see Norstog 1973), 30 g/l sucrose and 6 g/l agar TC (Phyto Technology Laboratories, Kansas City, KS). For callus growth, 2,4-D 2 mg/l and kinetin 1 mg/l were added to the medium, and the agar TC was replaced with 4 g/l gellan gum. The callus was dissected every month, transferred to new medium, and kept at 20 °C under dark conditions. Petri dishes containing embryos were also incubated in the dark at 20 °C. Once shoots developed, the dishes were transferred to a growth chamber under 12 h light (2800 Lux)/12 h dark at a constant temperature of 20 °C.

### Cytological analysis

Embryo, endosperm, necrotic plants, and callus samples were fixed in 3:1 (v/v) ethanol/glacial acetic acid at room temperature for 7 days for GISH/FISH observation. Methods for slide preparation, GISH probed with pearl millet genomic DNA, and FISH probed with pearl millet centromere satellite DNA sequences were as described by Ishii et al. (2010). To check the chromosomes of a normally grown plant, the root tips cells were pretreated with cold water for 24 h before fixation. To observe starch in the endosperm, an iodine test was performed using solution containing 1 % potassium iodide, 0.18 % iodine, and 50 % glycerol under a stereomicroscope (Zeiss Stemi 2000-C) or compound microscope (Olympus BX41). Photographs were taken using an Olympus DP12 camera.

### DNA extraction

Genomic DNA was extracted from leaves of oat and millet and from a hybrid callus using the cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980). Genomic DNA was extracted from two hybrid seedlings before they became severely necrotic using a DNeasy Plant Mini Kit (Qiagen, Maryland City, MD).

### Marker analysis

A total of 23 SSR markers (PSMP, 2006, 2008, 2043, 2056, 2059, 2078, 2084, 2090, 2227, 2231, 2233, 2237, 2246,

2248, 2251, 2255, 2263, 2266, 2267, 2270, 2271, 2273, and 2274; Allouis et al. 2001; Qi et al. 2004) and five STS markers (PSM, 305, 345, 716, 737, and 870; Millet Gene; <http://ukcrop.net/perl/ace/search/MilletGenes>) were used to detect millet chromosomes in two necrotic seedling plants and hybrid callus. These markers cover all seven millet linkage groups (LG1–7). PCR was performed in a total volume of 50  $\mu$ l containing 50 ng template DNA, 0.2  $\mu$ l (5 U/ $\mu$ l) TaKaRa *ExTaq* DNA polymerase, 5  $\mu$ l 10 $\times$  *ExTaq* buffer, 50 pmol each of forward and reverse primers, and 4  $\mu$ l dNTP mixture (2.5 mM each). Reactions were run with an initial denaturation step of 5 min at 94  $^{\circ}$ C; 35 cycles of 94  $^{\circ}$ C for 30 s, 57–60  $^{\circ}$ C for 30 s, and 72  $^{\circ}$ C for 30 s; and a final extension for 7 min at 72  $^{\circ}$ C. Electrophoresis was performed in 3 % agarose gel followed by staining with ethidium bromide to visualize the bands.

## Results

### Double fertilization in the oat–millet cross

Fourteen days after pollination of the oat pistil with millet pollen, we excised and observed the embryo and endosperm in the immature seeds (Table 1). In 2008 cross, an incomplete endosperm was observed in 63 of 115 embryos (54.8 %), and in 2009, in 36 of 55 embryos (65.5 %; Table 1; Fig. 1b, c). In addition, 10 seeds had an endosperm only. We excised an embryo with endosperm from an ovary each 7 and 14 days after pollination and, in addition, two embryos without endosperm 14 days after pollination and observed the chromosome constitution. In most of the embryo cells, seven millet chromosomes were seen as expected, but the number varied in many endosperm cells (Table 2). Thus, in this oat–millet cross, millet chromosomes are basically stable and are not eliminated as seen in case of a wheat–millet cross.

The endosperm cells included oat and millet chromosomes (Fig. 2a, b). In some cells, dicentric millet chromosomes (Fig. 2b, white arrowheads) and chromosome with oat and millet chromatins (Fig. 2b, open arrowhead and enlarged) were apparent. These indicate that translocations occurred between different millet chromosomes and between oat and millet chromosomes. Examination with iodine solution indicated inclusion of starch in the hybrid endosperms (Fig. 1d–f). These findings indicate that

double fertilization took place in more than half of the excised oat and millet embryos, though it is unclear whether the rest of the embryos without an endosperm were attributable to single fertilization or degeneration of the endosperm during development. In fact, all of the endosperms associated with the embryos were highly abnormal.

### Abnormal growth of hybrid embryos

Twenty-one embryos grew on the rescue medium; however, all except one showed diagnostic deformation whereby the shoot elongated but rolled to the scutellum side (Fig. 1g). When the embryos were exposed to light, the shoots first turned green but gradually became brown approximately 1 month after incubation (Fig. 1h), beginning from the top of the shoot to down (Fig. 1g–i). To determine whether light was the cause, some embryos were placed in the dark. The resulting shoots grew somewhat better than those grown under light conditions (Fig. 1j–l), but all plants finally became necrotic when transferred to light conditions. We further observed the chromosome constitution of three necrotic seedling plants. GISH/FISH analysis exhibited seven millet chromosomes in one of the plants, and PCR analysis indicated the presence of the seven millet chromosomes in the other two plants (Tables 2, 3; Fig. 2d–f).

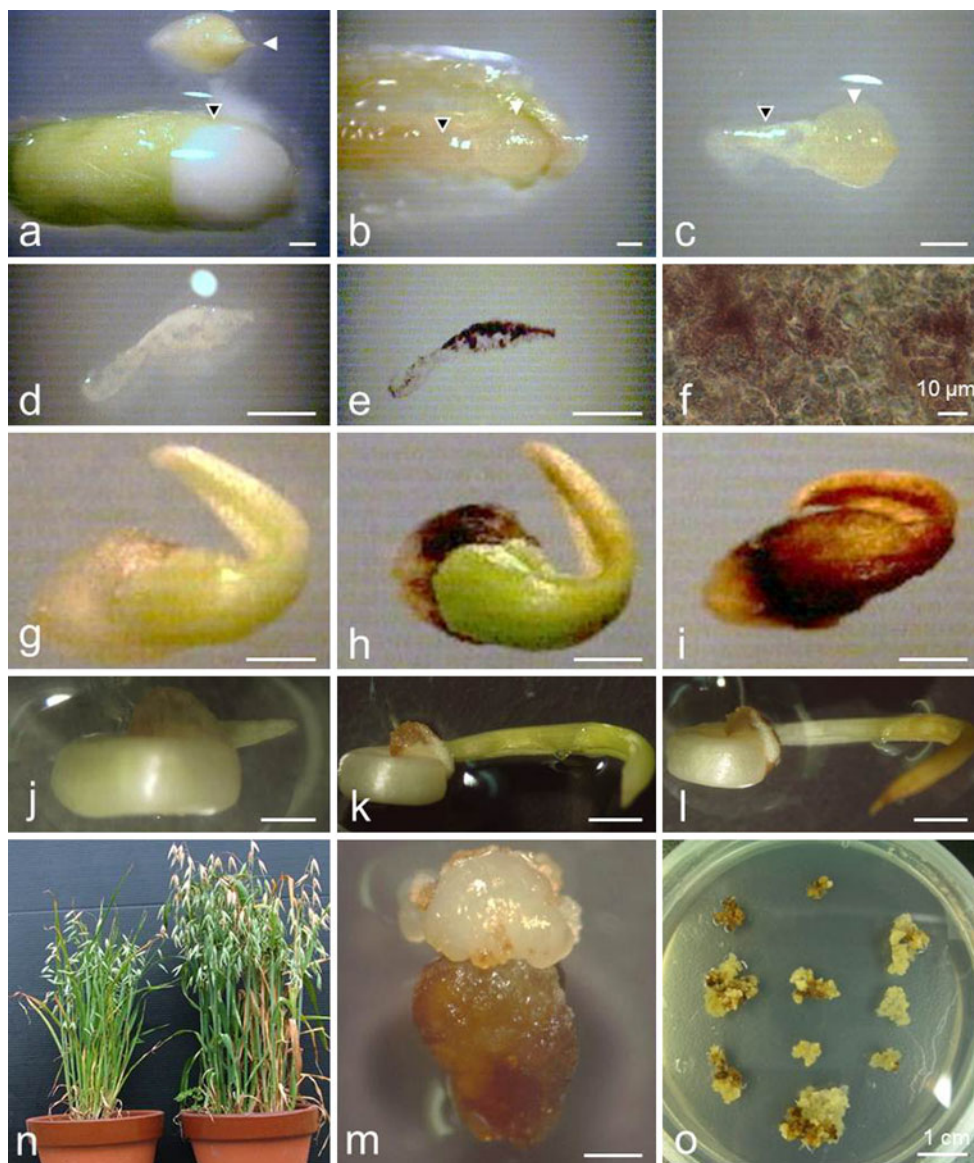
There was one exceptional plant that grew to adult size; however, it was smaller than a normal plant. A chromosome count revealed it to be a haploid containing only 21 oat chromosomes (Figs. 1n, 2c), but it bore nine seeds perhaps by fertilization of unreduced gametes. GISH/FISH detected no millet chromosomes.

### Callus from a hybrid embryo

In the 2009 cross, one embryo generated a callus after about 6 months of culture (Fig. 1m). When this callus was excised and cultured on medium containing 2,4-D and kinetin, it grew vigorously for at least 2 years (Fig. 1o). GISH/FISH analysis revealed that most of the cells carried four millet chromosomes (Table 2; Fig. 2g–i). Moreover, millet markers analysis revealed that these chromosomes corresponded to millet linkage groups (LGs) 2, 4, 6, and 7 (Table 3). LG 4 primers PSM716, PSMP2008, and PSMP2084 showed different bands in the callus compared to those seen in millet. DNA was subsequently extracted from 20 millet plants, and PCR

**Table 1** Embryos, endosperms, and differentiated seedlings resulting from the cross between oat and pearl millet

	No. of crosses	No. of embryos	%	No. of endosperms	%	No. of differentiated seedlings	%
2008	4,748	115	2.4	63	54.8	12	10.4
2009	1,677	55	3.3	36	65.5	9	16.4
Total	6,425	170	2.6	99	58.2	21	12.4



**Fig. 1** Culture of oat-pearl millet hybrid embryos. An immature seed of (a) normal oat 14 days after self-pollination and (b) resulting from an oat-millet cross 14 days after pollination. c An embryo and endosperm excised from the hybrid seed resulting from the oat-millet cross. In plates (a–c), white and black arrowheads indicate the embryo and endosperm, respectively. d An endosperm excised from an immature hybrid seed. e Starch-iodine analysis showing the presence of starch in the hybrid endosperm. f Magnification of the cells shown in (e), but a lack of starch formation in several cells. The

images in (g–i) and (j–l) are from two embryos as examples, respectively. The embryo in (g–i) was kept in 12 h light after shoot emergence and that in (j–l) in continuous dark after shoot emergence. n A plant (left) showing normal growth after the oat-millet cross; its overall size was smaller than normal (right). It was found to be haploid. m A callus generated from a seed from the oat-millet cross. o The callus grew vigorously on medium containing hormones. Scale bars: 1 mm, except in (f)

performed using PSM716, PSMP2008, and PSMP2084 primers. The results indicated that the bands were attributable to the polymorphic nature of the millet population used for crossing (data not shown). When the callus reached 5–10 mm, it was transferred to hormone-free medium for the regeneration of the hybrid plants; however, it continued to show vigorous growth without differentiation even under this hormone-free condition.

## Discussion

### Generation of an endosperm from a wide cross

During double fertilization of angiosperms, each of the two sperm cells in a pollen grain fertilize with an egg cell or polar nuclei placed in the embryo sac. Since both the sperm cell and egg cell are haploid ( $n$ ) and the polar nuclei diploid

**Table 2** Frequency of pearl millet chromosomes in embryo and endosperm in ovaries 7 and 14 days after pollination, two embryos without endosperm 14 days after pollination, necrotic seedling plant, and callus cells

Tissue	No. of cells			No. of cells with indicated number of pearl millet chromosomes										
	Observed	With pearl millet chromosome(s)	%	1	2	3	4	5	6	7	8	9	≥10	
Embryo 1 (7 days after pollination) <sup>a</sup>	83	83	100	0	0	0	0	0	14	61	8	0	0	
Embryo 2 (14 days after pollination)	500	500	100	0	0	0	0	8	61	422	9	0	0	
Embryo 3 (14 days after pollination)	500	500	100	0	0	0	0	25	67	397	10	1	0	
Embryo 4 (14 days after pollination)	250	250	100	0	0	1	0	3	33	209	4	0	0	
Total	1,333	1,333	100	0	0	1	0	36	175	1,089	31	1	0	
Endosperm 1 (7 days after pollination)	256	256	100	0	1	2	7	9	26	53	146	12	0	
Endosperm 4 (14 days after pollination)	250	250	100	0	0	3	30	97	77	22	4	4	13	
Total	506	506	100	0	1	5	37	106	103	75	150	16	13	
Necrotic seedling plant (shoot)	200	200	100	0	0	0	0	5	11	149	31	4	0	
Necrotic seedling plant (shoot meristem)	200	200	100	0	0	0	0	1	13	166	15	5	0	
Necrotic seedling plant (scutellum)	200	200	100	0	0	0	0	1	6	185	8	0	0	
Total	600	600	100	0	0	0	0	7	30	500	54	9	0	
Two-month-old callus	500	500	100	0	13	67	402	15	1	1	1	0	0	
Four-month-old callus	250	250	100	0	5	31	210	3	1	0	0	0	0	
Total	750	750	100	0	18	98	612	18	2	1	1	0	0	

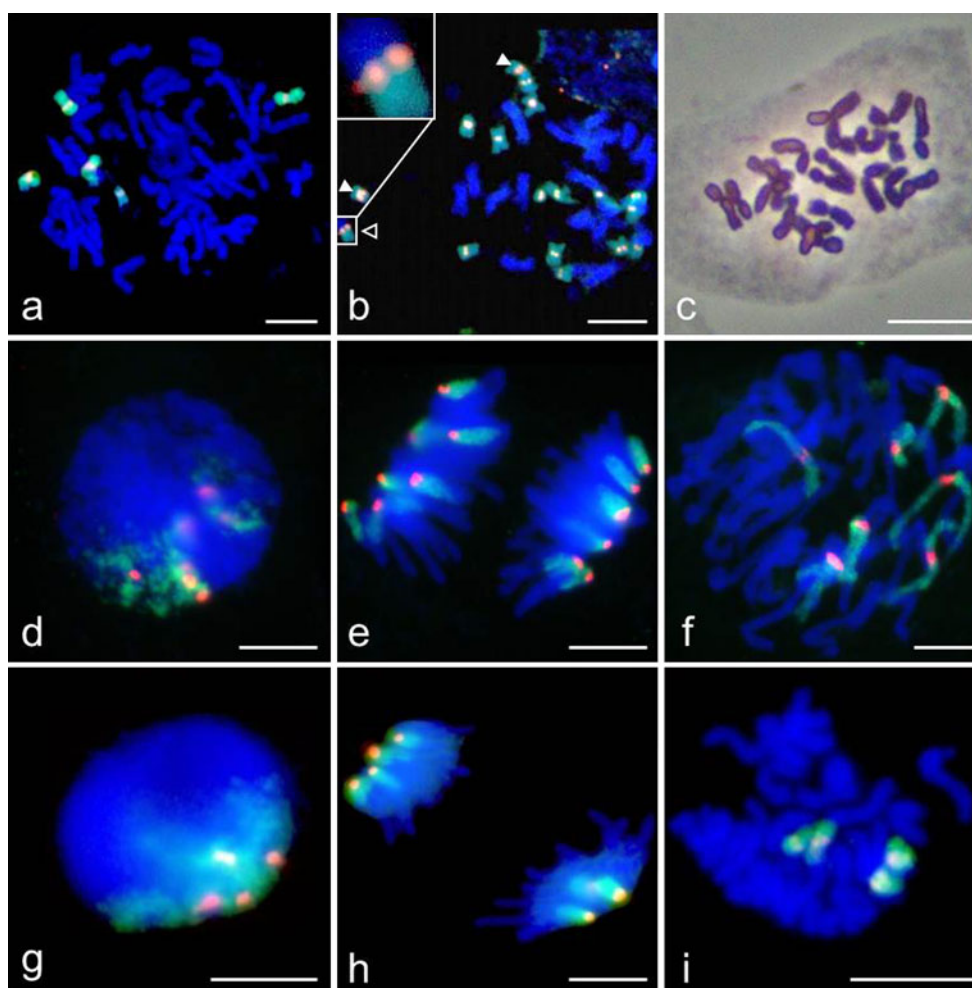
<sup>a</sup> The embryo and endosperm with a same number are derived from the same ovary

( $2n$ ), ploidy of the embryo and endosperm after fertilization with a haploid sperm cell ( $n$ ) is  $2n$  and  $3n$ , respectively.

Most interspecific crosses produce seeds with an abnormal embryo and/or endosperm. This is caused by an imbalance in the species-specific polar nuclei activation value or maternal and paternal genome ratio. Generally, the ‘genome amount’ derived from the female and male must be at a ratio of 2:1 in the endosperm and 1:1 in the embryo. However, in interspecific crosses the ratio shifts (Johnston and Hanneman 1982; Lin 1984; Nishiyama and Yabuno 1978). Furthermore, proper expression of imprinting genes is important for successful endosperm development (Haig and Westoby 1991). This imprinting phenomenon is controlled by DNA methylation and/or histone modification (Kinoshita et al. 2004, Lawrence et al. 2004). Ishikawa et al. (2011) reported that endosperm malformation in interspecific rice crosses is caused by abnormality of imprinting genes.

In inter-subfamily crosses in Poaceae, double fertilization does not always take place. At 48 h after pollination in a wheat–sorghum combination, only 10 % of crosses show double fertilization, while 57 and 2 % show single fertilization with the egg cell or polar nuclei, respectively (Laurie and Bennett 1988a). However, even when double fertilization does take place, the endosperm stops growing. This may be caused by chromosome elimination from the endosperm, as observed by Laurie and Bennett (1988a). Chromosome elimination causes an imbalance in the genome ratio in the endosperm and embryo, resulting in

incomplete endosperm development. In wheat–maize, wheat–sorghum and wheat–millet crosses, the paternal chromosomes are completely eliminated during embryogenesis, and thus, the resulting plants become maternal haploids that can be rescued 16–21 days after pollination. Endosperms are rarely detectable in these crosses (Inagaki and Mujeeb-Kazi 1995, Laurie and Bennett 1988b, Ishii unpublished data). On the other hand, in oat–maize crosses, endosperms were observed 16 days after pollination together with an embryo or alone (Rines et al. 1997). In the present study, more than 50 % of the crosses between oat and millet resulted in generation of an endosperm (Table 1). This frequency is much higher than observed in other inter-subfamily crosses. For example, in oat–maize crosses, maize chromosomes are not stable in the endosperm and are eliminated within 48 h of pollination (Rines and Dahleen 1990). However, in the present oat–millet combination, the chromosomes were not eliminated from the endosperm (Table 2; Fig. 2a, b), allowing normal early endosperm development. However, an imbalance in imprinting genes may occur during development, resulting in ceased development. In this study, we often observed chromosome aberration and various number of millet centromeres in the endosperm cells (Fig. 2a, b; Table 2). This may have been caused by dysfunction of the cell cycle check point during endosperm development or, alternatively, incorrect modification of DNA or histone as a result of inhibition of DNA methylation, which induces chromosome breakage during wheat mitosis (Cho et al. 2011).



**Fig. 2** Cytological analysis of endosperm, haploid plant, necrotic seedling plant, and callus cells from the oat–pearl millet hybrid (**a**) and (**b**). Chromosomes in the endosperm from the oat–millet cross 14 days after pollination. In (**b**), two translocated chromosomes are shown: one translocation involved two different millet chromosomes (*white arrowhead*) and the other, oat and millet chromosomes (*open arrowhead*; an enlarged image is included). **c** A mitotic cell from a plant growing normally. This plant had 21 oat chromosomes,

indicating that it was a haploid. **d–f** Mitoses in a hybrid plant showing necrosis at interphase (**d**), anaphase (**e**), and prometaphase (**f**). **g–i** Mitoses in the callus at interphase (**g**), anaphase (**h**), and metaphase (**i**). In all except (**c**), *green* represents the GISH signal from the probe for millet genomic DNA, and *red* the FISH signal from the probe for centromere-specific repetitive sequences of millet. *Blue* shows counterstained DNA from DAPI. Scale bar: 10  $\mu$ m (color figure online)

Ishii et al. (2010) previously observed chromosome aberration in early embryo cells resulting from a cross between wheat and millet and suggested the involvement of a malfunction of cohesin degradation during anaphase. It is unclear whether chromosome aberration in the endosperm in the present hybrid between oat and millet caused by a malfunction of cohesin degradation. In early endosperm development, retention of paternal chromosomes is indispensable for maintaining normal genome balance, and subsequently, correct functioning of imprinting genes can be requested.

#### Necrosis in hybrid plants

The oat–millet hybrids showed severe necrosis promoted by light irradiation. This necrosis may have been caused by

a hypersensitive response-like reaction similar to that seen in complementary hybrid necrosis genes (Mizuno et al. 2010). Alternatively, it may have been due to the different photosynthetic systems in oat and millet, namely  $C_3$ - and  $C_4$ -type photosynthesis, respectively. However, the detailed mechanism responsible for this necrosis is unknown. Production of plants with a  $C_4$ -type photosystem has been attempted by introducing genes from  $C_4$  to  $C_3$  plants. For example, Tsuchida et al. (2001) produced rice ( $C_3$ ) transformants with genes of maize ( $C_4$ ) and observed expression of the transgenes. The plants showed photoinhibition and stunting even in weak light. Moreover, Kynast et al. (2001) produced a series of oat lines containing maize chromosomes using the wide-hybridization method. They observed varying degrees of necrosis in the resulting lines.

**Table 3** Presence or absence of pearl millet genome markers in pearl millet, oat, necrotic seedling plants, and callus

Marker	Linkage group	Pearl millet	Oat	Necrotic plant 1	Necrotic plant 2	Callus
PSMP2006	1	+	–	–	+	–
PSMP2090	1	+	–	+	+	–
PSMP2246	1	+	–	+	+	–
PSMP2273	1	+	–	–	–	–
PSMP2059	2	+	–	+	+	+
PSMP2231	2	+	–	+	+	+
PSMP2237	2	+	–	–	–	+
PSMP2255	2	+	–	+	+	+
PSMP2056	3	+	–	+	+	–
PSMP2227	3	+	–	+	+	–
PSMP2251	3	+	–	+	+	–
PSMP2267	3	+	–	+	+	+
PSM305	4	+	–	–	+	+
PSM716	4	+	–	–	–	+a
PSMP2008	4	+	–	+a	+a	+a
PSMP2084	4	+	–	+b	+b	+a
PSM345	5	+	–	+	+	–
PSMP2078	5	+	–	–	–	–
PSMP2233	5	+	–	+	+	–
PSMP2274	5	+	–	+	+	–
PSM737	6	+	–	–	+	+
PSM870	6	+	–	–	+	+
PSMP2248	6	+	–	+	+	+
PSMP2270	6	+	–	+	+	+
PSMP2043	7	+	–	–	+	+
PSMP2263	7	+	–	+	+	+
PSMP2266	7	+	–	–	+	+
PSMP2271	7	+	–	+	+	+

+: presence, –: absence. The longer and shorter bands compared to the original pearl millet bands are indicated by a and b, respectively

Kowles et al. (2008) also identified oat–maize chromosome addition lines with photosynthetic-related PPDK ( $C_4$ -specific pyruvate orthophosphate dikinase) and PEPC ( $C_4$ -specific phosphoenolpyruvate carboxylase) genes of maize and confirmed expression of these enzymes in the mesophyll cells. However, these plants showed  $C_3$ -type photosynthesis. These findings indicate that  $C_3$  plants with  $C_4$ -type photosynthesis genes become necrotic or alternatively survive through suppression of  $C_4$ -type photosynthesis.

The hybrid callus obtained in this study grew vigorously even under light conditions. This was possible because it was white and, therefore, unable to perform photosynthesis (Fig. 1o). It also grew vigorously in medium without hormone, but no plants were regenerated, indicating that the callus produced excessive hormones.

Riera-Lizarazu et al. (1996) and Rines et al. (2009) reported that oat–maize crosses produced plants containing one or very few maize chromosomes because maize chromosomes are unstable during hybrid embryogenesis. On the other hand, with oat–millet crosses, millet chromosomes are stable, and therefore, all millet chromosomes

are retained together with the oat chromosomes. Coexistence of genomes from oat and millet within the same cell did not allow the plants to grow to adult size. However, eliminating three millet chromosomes from the callus reduced the load but was still insufficient in generating plants. Haploid plant that lost all their millet chromosomes had no load and, therefore, was able to grow to adult plant. Thus, partial chromosome elimination during embryogenesis is required for the introduction of millet genes into oat.

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