Cytological evidence for chromosome elimination in wheat $\times$ *Imperata cylindrica* hybrids

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Haploid induction of wheat by crossing with *Imperata cylindrica* pollen is an efficient method for doubled haploid breeding. We investigated the process of wheat haploid formation after crossing with *I. cylindrica*. Our cytological observations of zygotes showed the successful fertilization of parental gametes. Wheat haploids were formed by complete elimination of *I. cylindrica* chromosomes. Missegregation of *I. cylindrica* chromosomes was observed in the first cell division of zygote. At metaphase *I. cylindrica* chromosomes did not congress onto the equatorial plate. The sister chromosomes did not move toward the poles during anaphase, though their cohesion was released normally. *I. cylindrica* chromosomes were still in the cytoplasm at telophase and eliminated from daughter nuclei. After two-celled stage, we could find no *I. cylindrica* chromosome in the nuclei but micronuclei containing *I. cylindrica* chromatin in the cytoplasm. These observations indicate that *I. cylindrica* chromosomes are completely eliminated from nuclei in the first cell division probably due to lack of functional kinetochores.

Key words: chromosome elimination, GISH, haploid, *Imperata cylindrica*, wheat

INTRODUCTION

Interspecific hybrids often show various abnormalities in their development due to the incompatibilities between their parental genomes (Jones and Pasakinskiene, 2005). Several hybrids are karyotypically unstable. Uniparental chromosomes, paternal in almost all cases examined, are eliminated partially or completely from the hybrid nuclei. This phenomenon is called as “chromosome elimination” and has been observed in several interspecific hybrids (Kasha and Kao, 1970; Barclay, 1975; Laurie and Bennett, 1988, 1989; Laurie, 1989a; Riera-Lizarazu et al., 1996; Fujiwara et al., 1997).

The combination of parental species mainly determines the degree of chromosome elimination. For example, in oat $\times$ maize, and wheat $\times$ barley (Thomas and Pickering, 1983; Riera-Lizarazu et al., 1996), paternal chromosomes are eliminated incompletely and several chromosomes can be found in mature plants. Complete elimination of paternal chromosomes occurs in wheat $\times$ *Hordeum*

bulbosum, maize, pearl millet, or sorghum, and barley $\times$ *H. bulbosum*, and so on (Kasha and Kao, 1970; Barclay, 1975; Laurie and Bennett, 1988, 1989; Laurie, 1989a). In these crosses, complete loss of paternal chromosomes results in the production of maternal haploids.

Cytological analyses performed to reveal the mechanism of chromosome elimination enunciated similar mitotic abnormalities in many hybrids (Laurie and Bennett, 1988, 1989; Laurie, 1989a; Mochida et al., 2004). Chromosomes being eliminated tend to be apart from the equatorial plate at metaphase. Sister chromosomes fail to move toward the poles at anaphase and are retained in the cytoplasm to be eliminated from the nuclei. These chromosomes form extra-micronuclei and are finally degraded. In addition to mitosis dependent elimination, another pathway of chromosome elimination was proposed in wheat $\times$ pearl millet crosses (Gernand et al. 2005). Micronuclei containing pearl millet chromatin were directly budded and eliminated from interphase nuclei. There might be an active mechanism to distinguish and to eliminate alien chromatin from nuclei. It remains to be seen whether such elimination pathway is common in other hybrids.

Haploid induction through chromosome elimination has

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been a practical technique in wheat breeding using doubled haploid, and therefore many researchers have investigated the pollinators which can induce haploid efficiently, and whose fresh pollens are easy to obtain. Recently, it was reported that wheat haploids are formed by crossing with *I. cylindrica*, a wild grass remotely related to wheat (Chaudhary et al., 2005; Pratap et al., 2005). This novel haploid induction method has two great advantages in practical application, (1) high frequency of haploid induction irrespective of wheat genotypes, and (2) natural coincidence of flowering period of wheat and *I. cylindrica* under subtemperate condition. Wheat genotypes affect the crossability with alien species (Inagaki and Mujeeb-Kazi, 1995; Pratap et al., 2005). For example, wheat varieties with dominant *Kr* genes, which inhibit alien pollen tube growth, show greatly reduced crossability with many alien species (Riley and Chapman, 1967; Snape et al., 1979; Falk and Kasha, 1981, 1983; Sitch et al., 1985). This restricts the application of haploid induction method by crossing with *H. bulbosum* or rye to only some wheat varieties. Although maize pollen is relatively insensitive to wheat genotypes, and therefore maize system has been a practical haploid induction method by chromosome elimination, it is necessary to adjust the flowering period using greenhouse to obtain fresh maize pollen for crossing in subtemperate condition, which increases the costs of this method. *I. cylindrica* also shows genotype nonspecific crossability with wheat, and the efficiency of haploid induction is comparable to that in using maize. In addition, the coincidence of flowering period between wheat and *I. cylindrica* enables us to obtain fresh pollen of *I. cylindrica* freely. From these points, *I. cylindrica* is expected to be employed in wheat haploid production alternative to maize (Chaudhary et al., 2005; Pratap et al., 2005).

Wheat haploid induction using *I. cylindrica* pollen has been reported only in India so far (Chaudhary et al., 2005; Pratap et al., 2005). *I. cylindrica* is merely a wild grass and therefore has uneven genotypes (Matumura et al., 1983; Matumura and Nakajima, 1988; Cheng and Chou, 1997), which does not ensure that *I. cylindrica* distributed in other regions can induce wheat haploid. It is thought that wheat haploids are formed by elimination of *I. cylindrica* chromosomes in accordance with precedents. If this is so, revealing the timing of chromosome elimination is important in terms of plant breeding, especially, gene transfer of *I. cylindrica* into wheat genome by inducing intergenomic translocation. The cytological analysis is yet to be done, and the actual mechanism of haploid induction is completely unknown. In this study, the availability of pollen of Japanese *I. cylindrica* in wheat haploid induction and the mechanism of haploid formation in wheat $\times$ *I. cylindrica* crosses were investigated.

MATERIALS AND METHODS

Plant materials and crossing procedures Hexaploid wheat, *Triticum aestivum* L. cv. 'Chinese Spring' ($2n = 6x = 42$) and *Imperata cylindrica* ($2n = 2x = 20$) were used as female and pollen parents, respectively. Crossing was performed essentially as described by Chaudhary et al. (2005). Wheat plants were grown in a field in the city of Kashiwara, Osaka, Japan. The wheat spikes were emasculated before anthesis. The upper small spikelets of the wheat spikes and the central florets of each spikelet were removed, and then anthers were detached from remaining florets. A few days after emasculation, the top of the florets was cut off to expose stigmas. Fresh pollen of *I. cylindrica* was collected from wildy growing plants around the field and applied on the stigmas with a soft brush. The uppermost internodes of wheat stems were injected with 100 mg/l 2,4-D solution, and 20 mg/l 2,4-D solution was dropped onto the ovaries 24 h after pollination. The 2,4-D treatments were repeated for two more consecutive days. Embryos were excised from immature seeds 13 to 16 days after pollination and grown at 20°C for several weeks on the MS medium supplemented with 30 g/l sucrose, 100 mg/l myo-inositol, 1 mg/l thiamine HCl, 0.5 mg/l pyridoxine HCl, 0.5 mg/l nicotinic acid, 2 mg/l glycine and 8 g/l agar, and adjusted to pH 5.8 with NaOH. When the regenerated plants grew enough to transplant, they were transferred to soil in a field.

Chromosome preparation of root tip cells Vigorously growing root tips were collected from regenerated plants, wheat and *I. cylindrica*. To accumulate metaphase cells, the root tips were pretreated in ice water for 16 to 20 h for regenerated plants and wheat, and in 2 mM 8-hydroxyquinoline for 3 to 4 h for *I. cylindrica*. The root tips were then fixed in 3:1 ethanol/acetic acid. Chromosome preparations were made as described by Mukai et al. (1990).

Cytological analyses of embryos Ovaries of pollinated wheat were fixed in 3:1 ethanol/acetic acid and stored at -20°C. The embryos at the stages later than 2 to 4 days after pollination were mainly exploited for cytological analyses. The embryo was dissected out from the ovary in 45% acetic acid under a stereomicroscope using fine needles and transferred on a glass slide with a micropipette. Before the embryo dried out, a drop of acetocarmine was added and covered with coverslip supported at the one side by a razor blade. Then we removed the razor blade and squashed the embryo. Optical images of the squashed or unsquashed embryo were captured using a BX51 microscope (Olympus) with digital camera. For *in situ* hybridization, after squashing the embryo, the slide was placed on dry ice to freeze. The coverslip was removed, and the slide was immediately

dipped into 45% acetic acid for 15 min. After air-drying, the slide was stored at -20°C .

Genomic *in situ* hybridization (GISH) was performed according to Mukai and Gill (1991) and Mukai et al. (1993) with some modifications. For probes, genomic DNA was extracted from the leaves of hexaploid wheat and *I. cylindrica* and labeled with fluorescein-12-dUTP, tetramethyl-rhodamine-5-dUTP or cyanine 5-dUTP using Nick Translation Mix kit (Roche Diagnostics). Chromosomal DNA was denatured in 70% formamide for 2 min at 69°C , dehydrated in an ethanol series at -20°C and air-dried. The hybridization mixture (100 μl) consists of 50% formamide, 10% dextran sulfate, 50 μg blocking DNA from salmon testis, $2 \times \text{SSC}$, 1 μg each of the labeled hexaploid and *I. cylindrica* genomic DNA. The mixture was denatured for 10 min at 100°C and immediately quenched in ice for at least 10 min. A 10- μl aliquot of the mixture was applied to each slide. The slides were incubated overnight in a moist chamber at 37°C . After hybridization, the slides were washed with $2 \times \text{SSC}$ at room temperature for 5 min, 50% formamide- $2 \times \text{SSC}$ at 40°C for 15 min, $1 \times \text{SSC}$ at room temperature for 15 min, and $2 \times \text{SSC}$ at room temperature for 5 min. The slides were mounted in a fluorescence antifade solution (1.25% DABCO, 90% glycerol). Each fluorescent signal was captured with a Zeiss Axioskop fluorescence microscope coupled to a cooled CCD camera (Hamamatsu Photonics, model 4880). The images were pseudo-colored and merged by Photoshop 5.0 software (Adobe).

RESULTS

Wheat haploid induction using Japanese *I. cylindrica* pollen

Utilization of pollen of Japanese *I.*

cylindrica in wheat haploid induction was confirmed by using wheat variety 'Chinese Spring' (Table 1), and most of the frequencies of various haploid induction parameters in the present study were similar to the previous report using other wheat varieties (Chaudhary et al., 2005). The embryo formation rate was 32.7% ranging from 0.0 to 90.5% among spikes. Embryos were rescued from the endosperm-lacked seeds and 37.2% of the rescued embryos recovered as plants. The chromosome constitution was checked for 9 regenerated plants. All were wheat haploids and had 21 wheat chromosomes but no *I. cylindrica* chromosome (Fig. 1a).

Fertilization between wheat and *I. cylindrica*

Penetration of the *I. cylindrica* pollen tube into the wheat stigma was observed after pollination in the haploid induction process (data not shown). To investigate whether the fertilization occurred between wheat and *I. cylindrica*, we examined the karyotypes of zygotes. Wheat has 42 chromosomes and *I. cylindrica* has 20 chromosomes in a somatic cell (Fig. 1b and 1c). Thus the expected combination of chromosomes of their hybrid zygote is 21 wheat and 10 *I. cylindrica* chromosomes. Even the largest *I. cylindrica* chromosome is much smaller than the smallest wheat one (compare Fig. 1b and 1c). Although the chromosomes of both the parents can be distinguished easily according to their size, the parental origins of chromosomes were also confirmed by GISH. The absence of cross-hybridization between wheat and *I. cylindrica* was confirmed in preliminary examinations (data not shown). Zygotes had the chromosomes of both the parents. The expected hybrid combination of 21 wheat and 10 *I. cylindrica* chromosomes was observed in zygotes at prometaphase and metaphase (Fig. 2a and 2c),

Table 1. Results of crossing between 'Chinese Spring' and *I. cylindrica*

Florets pollinated	Seed formation (%)	Embryo formation (%)	Regenerated plants (%)
336	336 (100)	110 (32.7)	41 (12.2)

Fig. 1. Metaphase chromosomes of root tip cells. (a) A regenerated wheat haploid plant, (b) wheat and (c) *I. cylindrica*. Bars represent 10 μm .

Fig. 2. Elimination of *I. cylindrica* chromosomes in the wheat $\times$ *I. cylindrica* hybrids. (a), (c), (f) and a right panel of (g) are cells after GISH, and wheat and *I. cylindrica* chromatins are represented in green and red, respectively. Note that (b) and (d) show a focal plane of unsquashed zygotes and not all chromosomes are visible. (a) Zygote at prometaphase. (b, c) Metaphase cells at the first cell division. Some *I. cylindrica* chromosomes (arrows) are clearly apart from the equatorial plate. Arrowheads indicate the other *I. cylindrica* chromosomes. (d) Unsquashed zygote at telophase. Four *I. cylindrica* sister chromosomes are visible in this focal plane. Probable pairs of sister chromosomes are indicated by arrows or arrowheads. (e) Squashed zygote at telophase. Arrows show *I. cylindrica* chromosomes. (f) One of two cells of a two-celled embryo at the second cell division. Signals from *I. cylindrica* chromatins were detected (arrows). (g) Interphase cells at 14-celled stage before (left panel) and after GISH (right panel). One cell has a micronucleus (arrows) containing *I. cylindrica* chromatin and the other does not. Bars represent 10 μ m.

Fig. 3. Failure of double fertilization. (a) Dissected ovule with hybrid embryo and unfertilized central cell. The polar nucleus is not fertilized (white arrow), though the zygote has developed to several cells (arrowhead, the cells are indistinguishable in this figure). Black arrow indicates pollen tube penetrating the micropyle. (b) Elongating pollen tube carrying two sperm cells. GISH image is shown in the inset, and *I. cylindrica* signals are colored with green. Bars represent 60 μ m in (a) and 20 μ m in (b).

demonstrating the successful fertilization of parental gametes.

However, fertilization between the central cell and the sperm cell seemed not to occur. We could not find any developing endosperm cells in the ovules, though we examined more than four hundred ovules including about one hundred ovules possessing hybrid embryo. Unfertilized polar nuclei were sometimes observed instead (Fig. 3a). The lack of endosperms in the seeds was likely due to the failure of double fertilization rather than early abortion of endosperm. The possibility that only one sperm cell was transported into the ovules might be excluded, as two sperm cells were observed in the elongating pollen tube (Fig. 3b).

Wheat haploid formation through chromosome elimination Abnormal behavior of *I. cylindrica* chromosomes was observed in the first zygotic division. Fig. 2b shows metaphase cell of an unsquashed zygote stained with acetocarmine. Although all wheat chromosomes were assembled on the equatorial plate, several *I. cylindrica* chromosomes were clearly apart from the plate (Fig. 2b and 2c). At telophase, wheat sister chromosomes reached to the both poles and formed daughter nuclei. By contrast, the separated sister chromatids of *I. cylindrica* failed to move toward the poles (Fig. 2d and 2e). Two *I. cylindrica* chromosomes were often observed in proximity (Fig. 2d). Each adjacent chromosome pair was likely to be the pair of sister chromosomes. At least 18 missegregated *I. cylindrica* chromosomes were countable in squashed zygote (Fig. 2e), though decondensation of the *I. cylindrica* chromosomes and excess amount of the cytoplasm made it difficult to discern individual *I. cylindrica* chromosomes in telophase zygotes. It was supposed that all chromosomes of *I. cylindrica* might be in elimination process in the first cell division.

We examined whether *I. cylindrica* chromosomes rema-

ined in the daughter nuclei of the zygotes in the second cell division. Two-celled stage cells at metaphase and anaphase were obtained. GISH signals of *I. cylindrica* chromosomes were detected in the anaphase cell (Fig. 2f). However, their morphology was quite different from that in the first cell division. These chromosomes might correspond to micronuclei, which were formed from the *I. cylindrica* chromosomes left in the cytoplasm in the first cell division. We were not able to get any evidence that *I. cylindrica* chromosomes were in the nuclei after two-celled stage. Thus, it is probable that loss of *I. cylindrica* chromosomes is completed in the first zygotic division.

Micronuclei were observed in the cells after two-celled stage. GISH clearly revealed that these micronuclei contained *I. cylindrica* chromatin (Fig. 2g). In our observations, micronuclei were found in 66.7% of two to four-celled stage cells ($n = 21$ cells) 2 days after pollination, and only 4.2% of the cells ($n = 120$ cells) in the embryos 3 to 4 days after pollination (14 to 20-celled stage), indicating that micronuclei were lost from most of the cells during embryo development.

Rates of fertilization and embryo development In all, we dissected 403 ovaries fixed 2 to 4 days after pollination (Table 2). The average fertilization rate was 33.5% (range 0.0 to 82.6% among spikes) and not significantly different from the embryo formation rate (32.7%) calculated in the embryo rescue (see Table 1). This indicates that most of the fertilized egg cells could grow to form embryos.

Table 2. Frequency of fertilization observed in ovaries fixed 2 to 4 days after pollination

Ovaries analyzed	Embryo formation	Fertilization frequency (%)
403	135	33.5

DISCUSSION

Utility of *I. cylindrica* in wheat breeding We succeeded to produce wheat haploids using Japanese *I. cylindrica* pollen following Chaudhary et al. (2005). The embryo formation rate (32.7%) was higher than that in works of other researchers using 'Chinese Spring' female and maize male (28.6% in Matzk and Mahn, 1994, 26.0% and 18.4% in Inagaki and Mujeeb-Kazi, 1995). This supports the data of Chaudhary et al. (2005) and Pratap et al. (2005) that the *I. cylindrica* can be employed to produce haploid alternative to maize. The availability of *I. cylindrica* of two geographically separated countries, India and Japan, in haploid induction might be indicative of availability of *I. cylindrica* in other countries.

Interestingly, the rates of embryo formation/seeds and plant regeneration/embryos were highly variable among spikes (0.0 to 90.5% and 7.7 to 100.0%, respectively). Inagaki and Bohorova (1995) and Laurie (1989b) showed that several factors, such as the developmental age of pistils and culture medium for embryo rescue, greatly affect on the efficiency of haploid production. This implies that under optimal conditions it might be possible to obtain haploid plant at much higher efficiency in wheat $\times$ *I. cylindrica* than that in this study.

These, together with two advantages (genotype nonspecific crossability, haploid production at low cost) mentioned earlier (Chaudhary et al., 2005, Pratap et al., 2005), suggest that haploid production using *I. cylindrica* has potential to be the most useful method in exercising doubled haploid breeding in wheat for accelerating the genetic upgradation programmes with enhanced precision and efficiency.

Mechanism of chromosome elimination Elimination of *I. cylindrica* chromosomes is clearly due to the aberrant movement during mitosis. *I. cylindrica* chromosomes don't assemble on the equatorial plate at metaphase. Sister chromatids are separated normally but fail to move toward the poles at anaphase. Some sister chromosomes were still in proximity at telophase, indicating the complete lack of the movement during anaphase. We could find *I. cylindrica* chromosomes in the nuclei only in zygotes. It is likely that all are eliminated from nuclei very rapidly, within one division cycle. These observations suggest that the kinetochores of *I. cylindrica* do not function completely.

Chromosome elimination in hybrids is likely due to the lack of kinetochore activity, which leads to missegregation of chromosomes in mitosis. This hypothesis is supported by the observations that the centromeres of chromosomes being eliminated had small or invisible centromeric constriction (Finch, 1983; Laurie and Bennett, 1988, 1989; Laurie, 1989a), and spindle microtubule did not attach to the maize chromosomes that should be

eliminated in wheat $\times$ maize hybrids (Mochida et al., 2004).

As in our observation, the origin of eliminated chromosome is paternal in almost all cases investigated. A possible explanation for paternal kinetochore inactivation is the incompatibility of paternal centromere and maternal kinetochore proteins. Although the contribution of paternal genes in early development remains unclear in plant, most of the cytoplasm of zygote is derived from egg cell, and delayed and/or attenuated transcription of paternal genes in early embryogenesis has been reported in *Arabidopsis* (Viell-Calzada et al., 2000, 2001; Weijers et al., 2001). In this case, it might be critical for paternal kinetochore activity whether maternal kinetochore components, such as CENP-A, can compensate for the lack of paternal ones. There is no problem in intraspecific hybrids because both the parents use essentially the same kinetochore proteins, but in the case of interspecific hybrids it might depend on the similarity of those proteins between their parents. If paternal centromeres can recruit maternal kinetochore proteins to form functional kinetochores, paternal chromosomes would be able to be transmitted stably.

Intriguingly, Jin et al. (2004) showed that oat CENP-A was successfully incorporated into the centromeres of maize chromosomes in place of maize CENP-A in oat-maize chromosome addition lines derived from oat $\times$ maize hybrids. In oat $\times$ maize crosses, maize chromosomes are sometimes eliminated incompletely and one or more maize chromosome(s) are retained in the recovered plants (Riera-Lizarazu et al., 1996). Maize chromosomes become stable at unknown timing, and once they become stable, most of them can be transmitted for generations (Riera-Lizarazu et al., 1996; Kynast et al., 2001). Taking together these findings and the fact that every maize chromosome can be added to oat without other maize chromosomes (Kynast et al., 2001), it is highly conceivable that all oat protein complexes responsible for chromosome segregation can substitute for maize ones. Nevertheless, maize chromosomes are still eliminated in early developmental stage after hybridization. Although it is unknown whether there is a coincidence between the timing of oat CENP-A recruitment and that of maize chromosome stabilization, the stabilization might depend on the compensation of maternal kinetochore proteins for paternal ones.

The process of chromosome elimination is similar across different interspecific hybrids. In all cases examined so far, chromosome elimination is accompanied by the missegregation of uniparental chromosomes. The stability of chromosomes, however, varies from hybrid to hybrid. This might depend on the difference in the way of kinetochore inactivation. Completion of chromosome elimination within the first cell division cycle might be indicative of complete lack of kinetochore function. Only

wheat $\times$ sorghum hybrid has been known to show such elimination pattern so far (Laurie and Bennett, 1988). Wheat $\times$ *I. cylindrica* hybrid might be another example of complete loss of kinetochore function. Comparative studies on the kinetochore proteins among hybrids will be necessary to understand the molecular background of chromosome elimination.

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