Running Title: Development of polyspermic rice zygotes

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Research area: Cell Biology
Development of polyspermic rice zygotes produced in vitro

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Polyspermic rice zygote, which is produced by fusion of an egg cell with two sperm cells, has the potential to develop into triploid embryos and plants through typical nuclear and cell divisions.
Footnotes

Author contributions: E.T. and T.O. designed the experiments; E.T. performed most of the experiments; Y.O. provided technical assistance to E.T. and performed some parts of the experiments; E.T. and T.O. conceived the project and wrote the article.

Financial sources: This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grants-in-Aid no. 21112007 and 26113715 to T.O.) and the Japan Society for the Promotion of Science (Grant-in-Aid no. 25650083 to T.O.).

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Fertilization is a general feature of eukaryotic uni- and multicellular organisms to restore a diploid genome from female and male gamete haploid genomes. In most animals and fucoid algae, polyspermy block occurs at the plasmogamy step. Because the polyspermy barrier in animals and in fucoid algae is incomplete, polyspermic zygotes are generated by multiple fertilization events. However, these polyspermic zygotes with extra centrioles from multiple sperms show aberrant nuclear and cell division. In angiosperms, polyspermy block functions in the egg cell and the central cell to promote faithful double fertilization, although the mechanism of polyspermy block remains unclear. In contrast to the case in animals and fucoid algae, polyspermic zygotes formed in angiosperms are not expected to die because angiosperms lack centrosomes. However, there have been no reports on the developmental profiles of polyspermic zygotes at cellular level in angiosperms. In this study, we produced polyspermic rice zygotes by electric fusion of an egg cell with two sperm cells, and monitored their developmental profiles. Two sperm nuclei and an egg nucleus fused into a zygotic nucleus, and the triploid zygote divided into a two-celled embryo via mitotic division with a typical bipolar microtubule spindle, as observed during mitosis of a diploid zygote. The two-celled proembryos further developed and regenerated into triploid plants. These findings suggest that polyspermic plant zygotes have the potential to form triploid embryos. Polyspermy in angiosperms might be a pathway for the formation of triploid plants, which can contribute significantly to the formation of autopolyploids.
INTRODUCTION

Fertilization is a general feature of eukaryotic uni- and multicellular organisms to restore a diploid genome from female and male gamete haploid genomes. In most animals and fucoid algae, multiple microtubule-organizing centers (MTOCs) are occasionally generated by polyspermy-derived extra centrioles, and the multiple MTOCs in zygotes cause aberrant nuclear and cell division, resulting in zygotic or early embryonic lethality (Schuel, 1984; Navara et al., 1994; Nagasato et al., 1999; Santelices 2002). To ensure producing diploid zygotes, animals have developed two kinds of responses. One is the block of fusion of additional sperms, which operates in monospermic zygotes at the step of membrane fusion, and the other is selective use of one sperm nucleus from several sperm nuclei in polyspermic zygotes, which is known as events of physiological polyspermy (reviewed in Snook et al. 2001).

In angiosperms, the sporophytic generation is initiated by double fertilization to form seeds (reviewed in Raghavan, 2003). In double fertilization, one sperm cell from the pollen grain fuses with the egg cell, and the resultant zygote develops into an embryo that can transmit genetic material from the parents to the next generation. The central cell fuses with another sperm cell to form a triploid primary endosperm cell, which develops into the endosperm that nourishes the developing embryo/seedling (Nawaschin, 1898; Guignard, 1899; Russell, 1992). It has been indicated that polyspermy block functions in the egg cell (Faure et al., 1994; Kranz et al., 1995; Scott et al., 2008; Hamamura et al., 2011) and the central cell (Hamamura et al., 2011) to promote faithful double fertilization (Spielman et al., 2008), although the mechanism of polyspermy block remains unclear. Egg:sperm ratio in angiosperms is generally considered as 1:1, since one pollen tube containing two sperm cells reaches the embryo sac harboring two...
female gametes, an egg cell and a central cell (Russell, 1992). However, the egg:sperm ratio can be variable, because additional pollen tubes can invade the embryo sac (Sprague, 1929; Sprague, 1932; Kato, 1997) when sperm cell–egg cell fusion and/or sperm cell–central cell fusion does not progress successfully (Kasahara et al., 2012; Maruyama et al., 2013), providing the opportunity for multiple sperm cells to fuse with an egg cell. In addition, the possibility that polyspermic zygotes arise via multiple fusion has been reported (Rhoades, 1936; Suarez et al., 1992; Ramsey and Schemske, 1998). Interestingly, regarding developmental fate of polyspermic zygote, it has been considered that polyspermic plant zygotes formed in angiosperms do not die because plant cells lack centrosomes and do not use them as spindle pore for chromosome separation during nuclear division (Lloyd and Chan, 2006). However, the fate of polyspermic zygotes has not been studied in angiosperms, because it is extremely difficult to identify and prepare polyspermic zygotes for analysis. One exception is the study in which polyspermic maize zygotes were prepared by sequential electric fusion of an egg cell with two sperm cells (Kranz et al., 1995), but the development of the polyspermic maize zygotes was not investigated. In this study, we produced polyspermic rice zygotes by electric fusion of an egg cell with two sperm cells, and monitored their developmental profiles, including karyogamy, nuclear division, spindle formation, cell division and regeneration.
RESULTS

Production and development of polyspermic zygotes

Using isolated rice gametes, we first fused an egg cell with a sperm cell, and the resulting fused egg cell (zygote) was further fused with a second sperm cell within 10 min after the first fusion (Fig. 1A). In this study, sperm cells heterologously expressing histone H2B-GFP under the control of the ubiquitin promoter were used, since the chromatin in the sperm cells and subsequent zygotes/embryos was fluorescently labeled (Ohnishi et al., 2014), allowing us to precisely observe the developmental fate of the polyspermic zygote after electric fusion. As shown in Figure 1B and C, a zygote with two sperm nuclei was successfully prepared. The polyspermic zygote developed into a globular embryo-like structure consisting of 12–16 cells (Fig. 1D and E; 2 days after fusion). The embryo further divided into cell mass (Fig. 1F; 8 days after fusion) and white callus (Fig. 1G; 17 days after fusion). From the white callus, multiple shoots regenerated (Fig. 1H; 25 days after fusion) and plantlets were obtained (Fig. 1I; 105 days after fusion). The speed of growth from the polyspermic zygote to plantlet (Fig. 1B-I) was almost same as that of a diploid zygote produced by the fusion of female and male gametes (Uchiumi et al., 2007). The plantlets regenerated from polyspermic zygotes were capable of growing in soil into mature plants (Fig. 1J). Their flowers were larger than those of diploid plants (Fig. 1K) and formed well-developed awns, which were not obvious in diploid flowers (Fig. 1L; Supplemental Fig. S1). Although the plants flowered, mature seeds were hardly formed on these plants. These floral characteristics and the sterility of the plants are typical of triploid rice plantlets (Hu and Ho, 1963).
It has been reported that, in the case of diploid zygotes, approximately 80% of zygotes produced in vitro grew into globular embryo-like structures, and white calli and...
plantlets were obtained efficiently from the diploid globular embryo-like structures (Uchiumi et al., 2007). In the study, we monitored the developmental profiles of 14 polyspermic zygotes. Out of 14 zygotes, seven developed into globular-like embryos (Table 1). However, remaining seven polyspermic zygotes showed defects in karyogamy or first cell division (Supplemental Fig. S2). These defects detected at early stage of zygote development may be caused by physiological shock from two times electric fusion for polyspermic zygotes, or by delivery of excess cellular materials from two sperm cells. Among seven globular embryo-like structures, five grew into white calli, and three of the white calli regenerated to plants (Table 1). The regeneration efficiency from triploid embryo-like structures was relatively lower than that of diploid globular embryo-like structures (Table 1; Uchiumi et al., 2007). This difference in the efficiency of developmental growth between triploid and diploid zygotes may be caused by the unbalanced ratio of paternal to maternal genomic material in the zygotes, 2:1 in triploid zygotes and 1:1 in diploid zygotes.

Next, the ploidy level of the mature plants that regenerated from polyspermic zygotes was determined. When nuclei were extracted from leaves of wild-type rice plants (diploid) and DNA content per nucleus was measured by flow-cytometry, single peak of 2C was detected (Fig. 1M). In case of measurement of nuclei from leaves of wild-type rice plants and plants regenerated from polyspermic zygotes, the peak corresponding to 3C level was detected in addition to a 2C peak (Fig. 1N). These indicated that the plants derived from polyspermic zygotes were triploid, being consistent with their sterile phenotype and floral characteristics (Fig. 1K and L). In addition, the results suggested that the two sperm nuclei in the polyspermic zygote fused with the egg nucleus, resulting in a triploid zygotic nucleus. Therefore, we further
analyzed the karyogamic profiles and nuclear division of the polyspermic zygotes.

Karyogamy and nuclear division in polyspermic zygotes

The karyogamic progression in monospermic diploid rice zygotes has been reported by Ohnishi et al. (2014). The sperm nucleus, which was fluorescently labeled with H2B-GFP, migrated adjacent to the egg nucleus after gamete fusion (Fig. 2Aa, b; 20 min after fusion). Then, the sperm nucleus fused with the egg nucleus, and the sperm chromatin began to decondense (Fig. 2Ac, d; 120 min after fusion). Sperm chromatin in the zygotic nucleus further decondensed (Fig. 2Ae, f; 180 min after fusion), and karyogamy was completed (Fig. 2Ag, h; 240 min after fusion, 2Ai, j; 360 min after fusion). When polyspermic zygotes were prepared, two sperm nuclei were clearly visible (Fig. 2Ba, b; 5 min after second fusion). We observed the karyogamic progression in six polyspermic zygotes in detail. In four of the six polyspermic zygotes, one of the two sperm nuclei first fused with egg nucleus and then sperm chromatin decondensed (Fig. 2Bc, d; 50 min after second fusion), but the other sperm nucleus remained adjacent to the diploid zygotic nucleus (Fig. 2Bc, d). Thereafter, the other sperm nucleus fused with the nucleus (Fig. 2Be, f; 160 min after second fusion) and karyogamy was mostly completed (Fig. 2Bg, h; 175 min after second fusion, 2Bi, j; 1 day after fusion). In the remaining two polyspermic zygotes, two sperm nuclei were visible in polyspermic zygote (Fig. 2Ca, b; 13 min after second fusion), came into contact (Fig. 2Cc, d; 47 min after second fusion) and fused together (Fig. 2Ce, f; 74 min after second fusion). United sperm nuclei further fused with the egg nucleus, and sperm chromatin began to decondense (Fig. 2Cg, h; 105 min after second fusion) for formation of zygotic nucleus (Fig. 2Ci, j; 1 day after fusion). The results shown in Figure 2 indicated that
two sperm nuclei can fuse with an egg nucleus via two different pathways. In both cases,

Figure 2. Two karyogamy pathways in polyspermic triploid zygotes. A, Karyogamy in a monospermic diploid zygote. An egg cell was fused with a sperm cell expressing H2B-GFP, and the resulting triploid zygote was serially observed. The sperm nucleus fluorescently labeled with H2B-GFP migrated adjacent to the egg nucleus after gamete fusion (panels a and b). Then, the sperm nucleus fused with the egg nucleus; the sperm chromatin began to decondense (panels c and d). Decondensation of sperm chromatin further progressed (panels e and f) and karyogamy was completed (panels g to j). B, One karyogamy pathway in polyspermic zygote. An egg cell was fused with a sperm cell expressing H2B-GFP, and then the fused gamete further fused with another sperm cell. The resulting triploid zygote was serially observed. Two sperm nuclei were clearly visible in the egg cell after its fusion with sperm cells (panels a and b). One of the two sperm nuclei first fused with the egg nucleus (panels c and d), and then the second sperm nucleus fused with the egg nucleus (panels e and f), resulting in a triploid zygote nucleus (panels g to i). C, Another karyogamy pathway in polyspermic zygote. Polyspermic zygote was prepared as in B and the triploid zygote was serially observed. Two sperm nuclei were observed in the egg cell after its fusion with sperm cells (panels a and b), and then these two sperm nuclei fused together (panels c to f). Then the united sperm nuclei further fused with the egg nucleus (panels g and h), resulting in a triploid zygotic nucleus (panels i and j). Upper panels are fluorescent images, and lower panels are merged bright-field and fluorescent images. Arrowhead in panel Bc indicates sperm chromatin which is decondensing in fused nucleus. Scale bars = 20 μm.

karyogamy was completed within 4 h, and the time-course for karyogamy in these polyspermic
zygotes was equivalent to that in diploid zygotes (Ohnishi et al., 2014). This suggests that two

![Diagram of sperm nuclei migration](image)

**Figure 3.** Organization of actin filaments and migration of sperm nuclei in polyspermic zygote during karyogamy. An egg cell expressing Lifeact-tagRFP was sequentially fused with two sperm cells expressing H2B-GFP, and resultant zygote was serially observed under a fluorescent microscope. Because two sperm nuclei were observed at different focal planes, images for two nuclei were separately captured and presented in panels A to C, and D to F. First sperm nucleus migrated toward egg nucleus (A and B) and fused with egg nucleus, resulting in decondensation of sperm chromatin in fused nucleus (C). Second sperm nucleus migrated toward egg nucleus (D to F) and fused with the diploid zygote nucleus, resulting in decondensation of sperm chromatin in the triploid nucleus (G). Insets in panels D and E show enlarged views of the sperm nuclei enclosed within the squares. Actin filaments in these Insets were represented by white color. The asterisk in panel A indicates the egg nucleus surrounded by actin filaments. Arrows in panels C and G indicate decondensing chromatin derived from first sperm nucleus. Arrowhead in panel G indicates decondensing chromatin derived from second sperm nucleus. Scale bars = 20 μm in A-G, 5 μm in the Insets.

sperm nuclei in polyspermic zygote migrate with same manner to sperm nucleus in diploid
zygote. It has been reported that migration of sperm nucleus in rice zygotes during karyogamy is dependent on actin filaments (Ohnishi et al., 2014). Therefore, two sperm cells expressing H2B-GFP were sequentially fused with an egg cell expressing Lifeact-tagRFP, which labeled actin filaments (Era et al., 2009), and distribution of actin filaments and migration of sperm nuclei in the polyspermic zygote were observed. Interestingly, actin filaments existed around or near sperm nuclei, and the sperm nuclei appeared to migrate along actin filaments (Fig. 3). These were consistent with the results of tracing the migration of the sperm nucleus and organization of actin filaments in a rice diploid zygote (Ohnishi et al., 2014), and suggested that actin filament-dependent migration of sperm nuclei normally progress in polyspermic zygotes.

After karyogamy, chromosomes originated from triploid zygote nucleus were arranged at the equator (Fig. 4A–H) and then separated to form two daughter nuclei through bi-polar mitotic division (Fig. 4I–N), as occurs in diploid zygotes (Ohnishi et al., 2014). In the case of animal and fucoid algae polyspermic zygotes, abnormal and lethal nuclear division/cytokinesis is induced by aberrant and extra spindle structures derived from the centrioles of extra sperms (Schuel, 1984; Navara et al., 1994; Nagasato et al., 1999). To observe spindle formation in rice polyspermic zygotes, we next conducted immunocytochemical analyses of zygotes at appropriate mitotic stages.

**Microtubule organization in polyspermic zygotes during mitosis**

Polyspermic rice zygotes were fixed at each mitotic phase, and microtubule structure and chromosome organization were visualized by immuno-fluorescent staining with anti-α-tubulin antibody and 4',6-diamidino-2-phenylindole (DAPI) staining, respectively. In zygotes at
prophase, short microtubules were present around the periphery of the nucleus (Fig. 5A–C).

**Figure 4.** Mitotic division of polyspermic zygote. After *in vitro* fusion of an egg cell with a sperm cell expressing H2B-GFP, the fused gamete was further fused with another sperm cell. The polyspermic zygote was then cultured and mitotic division was serially monitored. Chromatin and chromosomes were labeled with H2B-GFP in polyspermic zygote (A–D). Chromosomes originated from triploid zygote nucleus were arranged at the equator (E–H), and then separated to form two daughter nuclei (I–N). Upper and lower panels are fluorescent and bright-field images, respectively. Scale bar = 20 μm.

Chromosomes were arranged at the equator and a microtubule spindle was formed in the zygote
at metaphase (Fig. 5D–F). In anaphase zygotes, the chromosomes were evenly separated towards each pole, possibly via the action of the microtubule spindle (Figure 5G–I).

**Figure 5.** Microtubule organization during mitotic division of polyspermic zygotes. Polyspermic zygotes were fixed at each mitotic phase, and microtubule structure and chromosome organization were visualized by immuno-fluorescent staining with anti-α-tubulin antibody and DAPI staining, respectively. Representative images are presented. Images in upper and middle panels show immuno-fluorescent staining and DAPI staining, respectively. Lower panels show merged images. Scale bars =10 μm in A–L, 20 μm in M–O.
Phragmoplasts were present at mid-telophase (Fig. 5J–L) and at late-telophase (Fig. 5M–O) in dividing polyspermic zygotes. The profiles of microtubule organization during mitosis were almost same as those observed in diploid rice zygotes (Supplemental Fig. S3), and were equivalent to those observed in *Arabidopsis* diploid zygotes (Webb and Gunning, 1991). The results suggested that the chromosomes in triploid zygotes were evenly divided into two daughter nuclei via the bi-polar microtubule spindle, and that polyspermy did not affect the mitotic profile in angiosperm zygotes. This is consistent with the expectation that polyspermic plant zygotes lacking centrosomes would divide normally, in contrast to those in animals and fucoid algae (Lloyd and Chan, 2006; Spielman and Scott, 2008). In addition to observation of microtubules in dividing polyspermic zygotes, the organization of actin filaments during cell plate formation was also monitored. When the polyspermic zygotes produced by the fusion of an egg cell expressing Lifeact-tagRFP with two sperm cells expressing H2B-GFP were cultured, intense signals derived from actin filaments were detected at the possible site of cell plate formation both in dividing polyspermic triploid zygotes and in monospermic diploid zygotes (Supplemental Fig. S4).
DISCUSSION

The increase in the Ca\(^{2+}\)-level in a maize egg cell after \textit{in vitro} fusion with a sperm cell first suggested that mechanisms of Ca\(^{2+}\)-dependent egg activation function in plants as well as animals (Antoine et al., 2001). Recently, it was reported that the Ca\(^{2+}\) level in the egg cell embedded in the embryo sac increased immediately after fusion with a sperm cell (Denninger et al., 2014; Hamamura et al., 2014). The increased Ca\(^{2+}\) level is considered to activate the zygote to develop into an embryo. Moreover, it has been postulated that the increased Ca\(^{2+}\) level triggers the polyspermy block of fertilized egg cells via Ca\(^{2+}\)-dependent exocytosis of cell wall materials (Kranz et al., 1995; Bleckmann et al., 2014; Denninger et al., 2014). In the study, second sperm cell fusion was conducted within 10 min after first sperm cell fusion with an egg cell, since it has been found that zygotes start formation of cell walls after fusion with sperm cells (Kranz et al., 1995; Uchiumi et al., 2007), and that deposition of cell wall materials on whole surface of the electrofused maize zygote is generally observed 20 min after egg-sperm fusion (Kranz et al., 1995). We tested second sperm cell fusion with fused gametes (zygotes) at 10, 20 or 30 min after first sperm cell fusion. Polyspermic zygotes were efficiently obtained when zygotes were fused with second sperm cell at 10 min after first sperm fusion (n=12; fused 11, unfused 1). However, fusion was hardly observed when zygote were fused with second sperm cell at 20 min (n=4; fused 0, unfused 4) or 30 min (n=11; fused 1, unfused 10) after first sperm fusion. These suggest that cell wall formation would function as polyspermy block in rice zygotes around 20 min after gamete fusion as in case of maize zygotes (Kranz et al., 1995). The possible Ca\(^{2+}\)-dependent polyspermy block in plant zygotes, such as cell wall deposition, might be equivalent to the Ca\(^{2+}\)-dependent formation of the fertilization envelope in fertilized animal
eggs, a process mediated by exocytosis of cortical granules. This type of block is thought to be a slow or permanent polyspermy block. In addition, prior to this slow block, it has been known that a positive shift in the egg membrane induced by sperm fusion functions as a fast polyspermy block in animals and fucoid algae (Brawley, 1991; Wong and Wessel, 2006). In angiosperms, further studies will be required to clarify the details of the fast polyspermic block.

In addition to these fast and slow blocks, which operate at the plasmogamy step, the polyspermy barrier at karyogamy step has been known in polyspermic zygotes of some animal taxa, in which only one sperm nucleus fuses with the egg nucleus and the remaining extra sperm nuclei degenerate or disappear (Tarin, 2000; Wong and Wessel, 2006; Iwao, 2012). Interestingly, in some gymnosperms such as Pinus and Picea, two sperms enter the egg, but only one sperm nucleus migrates towards the egg nucleus and fuses with it (Blackman, 1898; McWilliam and Mergen, 1958; Runions and Owens, 1999). These observations indicate that selective karyogamy to produce a diploid zygote operates as a polyspermy barrier in some animals and plants. In angiosperm zygotes, however, such selective karyogamy to promote diploid progeny would not occur, because two sperm nuclei fused with an egg nucleus to form triploid zygote without degradation or rejection of excess sperm nuclei (Figs. 1 and 2), and no diploid plants was regenerated from the polyspermic zygotes in the present study (Fig. 1; Supplemental Fig. S1). Moreover, the present study indicated the existence of two karyogamy pathways; two sperm nuclei separately fuse with egg nucleus, or two sperm nuclei first fuse each other and the resulting fused nucleus fuses with egg nucleus (Fig. 2). This suggests that order of fusion among these three gamete nuclei is not strictly determined. Interestingly, two types karyogamy has been reported in maize primary endospermic cell, which is produced by fusion of central cell
with a sperm cell (Möl et al., 1994; Kranz et al., 1998). Upon the karyogamy of maize primary endospermic cell, two polar nuclei fuse each other and then the resulting secondary nucleus fuses with a sperm nucleus, or one of the two polar nuclei fuses with a sperm nucleus and the fused nucleus further fused with the remaining polar nucleus. These suggest that order of karyogamy of gamete nuclei in multi-nuclear gametic cell may be random in angiosperms.

In angiosperms, one pollen tube containing two sperm cells reaches the embryo sac in which two female gametes exist, thus the egg:sperm ratio in the embryo sac is considered as 1:1 (Russell, 1992). However, it has been reported that additional pollen tubes can invade the embryo sac (Sprague, 1929; Sprague, 1932; Kato, 1997; Kasahara et al., 2012; Maruyama et al., 2013). This suggests that the egg:sperm ratio can be variable, and that penetration of extra pollen tubes into the embryo sac provides the opportunity for multiple sperm cells to fuse with an egg cell. However, there was no experimental evidence addressing whether plant polyspermic zygotes show aberrant mitosis/cell division as is the case for animal polyspermic zygotes or develop without arrest. The results of this study clearly showed that a zygote prepared from one egg cell and two sperm cells has the potential to divide and regenerate into a mature triploid plant. This suggested that polyspermic zygotes in the embryo sac can develop into mature triploid embryos if the balance of male to female genomes in the endosperm is within a permissible range (Köhler et al., 2010).

Polyploidization is a common phenomenon in angiosperms. It has been suggested that 60–70% of flowering plants have polyploid ancestry (Masterson, 1994; Blanc and Wolfe, 2004; Cui et al., 2006), and that polyploidy has played a major role in the long-term diversification and evolutionary success of plants (Leitch and Bennett, 1997; Comai, 2005).
There are two distinct types of polyploids: autoployploids, which arise within a population of an individual species; and allopolyploids, which are the product of interspecific hybridization (Kihara and Ono, 1926; Ramsey and Schemske, 1998). In the formation of autoployploid plants, triploid plants are considered as the intermediate stage in the formation of stable tetraploid plants, and this pathway of tetraploid formation is known as the triploid-bridge (Ramsey and Schemske, 1998; Comai, 2005). Triploid plants spontaneously emerge in natural populations and in cultivated plants, and are produced frequently (Sandfaer, 1979). In tomato, the frequency of triploids was approximately 0.1%; 45 triploids in approximately 55,000 field-grown plants (Rick, 1945). Similarly, in 22 varieties of barley, the frequency of triploid plants was 0.01–0.29% (Sandfaer, 1975). As for the mechanism of triploid formation, it is generally accepted that an unreduced gamete with the somatic number of chromosomes fuses with a reduced gamete with one set of chromosomes, producing a triploid zygote (Bretagnolle and Thompson, 1995). In addition to fusion of unreduced and reduced gametes, the possibility of polyspermy has been proposed for maize and wheat (Rhoades, 1936; Suarez et al., 1992) and some orchids (Hagerup, 1947). However, it has been regarded as an uncommon mechanism of polyploid formation (Grant, 1981; Ramsey and Schemske, 1998). One of the reasons why polyspermy is regarded as uncommon is because it is difficult to provide evidence for this phenomenon at the cellular level. That is, it is difficult to reproduce the polyspermy situation in zygotes and to analyze the developmental profiles of polyspermic zygotes. In the present study, these difficulties overcome by using an in vitro fertilization system, and it was indicated that a polyspermic zygote can develop into a mature embryo after the fusion of multiple sperm cells with one egg cell. This suggests that polyspermy may be an a pathway for formation of triploid...
plants.
MATERIALS AND METHODS

Plant materials, isolation of gametes and zygotes

*Oryza sativa* L. cv. Nipponbare plants were grown in an environmental chamber (K30-7248; Koito Industries, Yokohama, Japan) at 26°C under a 13-h light/11-h dark photoperiod. Transformed rice plants expressing the histone H2B-GFP fusion protein and Lifeact-TagRFP were prepared as previously described (Abiko et al., 2013; Ohnishi et al., 2014). The isolation of egg cells and sperm cells from rice flowers were conducted as described Uchiumi et al. (2006).

Electro-fusion of gametes, and subsequent culture of zygotes

Electro-fusion of isolated gametes for zygote production was conducted as previously reported (Uchiumi et al., 2007) with some modifications. To prepare a polyspermic zygote, an isolated egg cell and a sperm cell were transferred into a 0.5–1.0 µL fusion droplet of mannitol solution (370 mosmol kg \(^{-1}\) H\(_2\)O) overlaid with mineral oil on a coverslip, and electrofusion was conducted (Uchiumi et al., 2007). The fused egg cell was transferred into a fresh mannitol droplet (370 mosmol kg \(^{-1}\) H\(_2\)O) in which a sperm cell was already transferred. The second fusion was conducted as described above, and the resulting polyspermic zygote was further cultured. Two methods were used to culture zygotes. For fluorescent observation of the zygotes, cells were cultured by the ‘in-drop zygote culture’ method described previously (Nakajima et al., 2010). Alternatively, zygotes were cultured using a Millicell-based method to observe their development into globular-like embryos, cell masses, and white calli (Uchiumi et al., 2007).
Plantlets were regenerated from white calli as described (Toki et al., 2006).

**Microscopic observations**

Zygotes expressing H2B-GFP were observed under a BX-71 inverted fluorescence microscope (Olympus, Tokyo, Japan) with 460–490-nm excitation and 510–550-nm emission wavelengths (U-MWIBA2 mirror unit; Olympus). The fluorescence of Lifeact-tagRFP proteins was observed with 520–550-nm excitation and >580-nm emission wavelengths (U-MWIG mirror unit; Olympus). Digital images of gametes, zygotes, and their resulting embryos were obtained using a cooled charge-coupled device camera (Penguin 600CL; Pixcera, Los Gatos, CA, USA) and InStudio software (Pixcera). Zygotes were also observed under a BZ9000 microscope (Keyence, Osaka, Japan) with 440–470-nm excitation and 535–550-nm emission wavelengths (OP-66836 GFP-BP filter set, Keyence) for H2B-GFP proteins and 525–540-nm excitation and 605-655-nm emission wavelengths (OP-66837 TRITC filter set, Keyence) for Lifeact-tagRFP proteins.

**Immunostaining of microtubules in zygotes during mitosis**

Polyspermic zygotes were prepared by sequential *in vitro* fusion of an egg cell with two sperm cells expressing H2B-GFP, and then cultured using ‘in-drop zygote culture’ method as described above. Because the chromosomes in the zygotes were fluorescently labeled with H2B-GFP, the movement of chromosomes during mitosis could be observed under a fluorescence microscope as described above. After 18 h of fusion, observations of the zygote were conducted every 30 min. When zygotes were judged to be in prophase, observations were conducted at 5–10 min intervals to obtain zygotes with appropriate mitotic stages for immunostaining. Immunostaining
of microtubules was conducted as previously described (Smertenko et al., 2004) with modifications. Zygotes at various stages during mitosis were fixed in 4% paraformaldehyde, 5 mM EGTA, 2 mM MgCl₂, and 0.4% Triton X-100 in 0.1 M PIPES (pH 6.8) for 30 min at room temperature. The fixed cells were washed with PBS buffer, and then treated with 0.8% macerozyme R-10, 0.2% pectolyase Y-23, 0.4 M mannitol, 5 mM EGTA and Protease inhibitor cocktail (1:100 dilution, Nacalai tesque, Kyoto, Japan) in 15 mM MES-KOH (pH 5.0) for 5 min at room temperature. The cells were washed with PBS buffer, incubated with 1% w/v BSA in PBS for 30 min, and then incubated with monoclonal anti-α-tubulin mouse antibody (clone DM1A) (1:200 dilution, Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature or overnight at 4°C. The zygotes were then washed three times for 10 min in PBS and incubated with donkey anti-mouse IgG antibody labeled with Alexa 488 (1:200 dilution, Life technologies, Carlsbad, CA, USA) for 1 h. After washing three times for 5 min in PBS buffer, the zygotes were incubated for 10 min with 5 µg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Dojindo Laboratories, Kumamoto, Japan). The zygotes were washed once or twice in PBS buffer and then observed under an LSM 710 CLS microscope (Carl Zeiss, Jena, Germany) with 488-nm excitation and 505-530-nm emission wavelengths for Alexa-488, and with 405-nm excitation and >420-nm emission wavelengths for DAPI.

Flow-cytometry analyses

To examine the ploidy level of plants regenerated from polyspermic zygotes, the DNA content per nucleus was measured by flow-cytometry using a CyFlow Ploidy Analyzer PA-II (Partec, Münster, Germany) and a CyStain UV Precise P Kit (Partec). For this analysis, fresh leaf
materials (5 mm²) were chopped with a sharp razor in 200 µl extraction buffer from the kit. Then, 1 ml staining solution from the kit was added to the chopped tissues, which were stained for 1 min. The crushed tissue and buffers were filtered through a 30-µm nylon mesh (Partec), and the filtered samples were loaded into the Ploidy Analyzer. Approximately 1,000–2,000 nuclei were measured for each sample. Measurements were conducted twice for most samples. Diploid plants (2n = 24, Oryza sativa L. cv. Nipponbare) were used as the control.

SUPPLEMENTAL DATA

Supplemental Figure S1. Flowers of possible triploid rice plants which were derived three independent triploid zygotes.

Supplemental Figure S2. Developmental defects of polyspermic zygotes at the step of karyogamy and first cell division.

Supplemental Figure S3. Microtubule organization in diploid zygotes during mitotic division.

Supplemental Figure S4. Actin organization in diploid and polyspermic triploid zygotes during cell plate formation.

ACKNOWLEDGMENTS

We thank Ms. T. Mochizuki (Tokyo Metropolitan University) for isolating rice egg cells, and the RIKEN Bio Resource Center (Tsukuba, Japan) for providing cultured rice cells (Oc line). We also thank Dr. K. Yamamoto (Tsukuba Botanical Garden, National Museum of Nature and Science, Japan), Mr. K. Hori and Dr. T. Murakami (Tokyo Metropolitan University) for flow-cytometry.
Table 1. Developmental profiles of polyspermic triploid zygotes and monospermic diploid zygotes

<table>
<thead>
<tr>
<th>Gametes used for fusion</th>
<th>No. of produced zygote</th>
<th>No. of zygote developed into each growth stage</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Karyogamy</td>
</tr>
<tr>
<td>Egg + Sperm + Sperm</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Egg + Sperm</td>
<td>4</td>
<td>4</td>
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</table>
Figure 1. Production and development of polyspermic rice zygotes. A, Schematic illustration of procedure to produce polyspermic rice zygotes. An egg cell was fused with a sperm cell to produce monospermic zygote (first fusion). Within 10 min after first fusion, the monospermic zygote was fused with a second sperm cell to produce polyspermic zygote (second fusion). B to L, Development of polyspermic rice zygotes produced by in vitro fusion. The polyspermic zygote (B, bright-field image; C, fluorescent image) developed into a globular embryo-like structure (D, bright-field image; E, fluorescent image), cell mass (F), and white callus (G) during culture in liquid N6D medium. When the white callus was subsequently cultured on solid regeneration medium, it formed multiple shoots (H) and then plantlets were obtained (I). Plantlets were grown in soil and formed flowers (J). The flowers (3X in K) were larger than those of diploid plants (2X in K), and awns were well-developed in possible triploid flowers (3X in L). M and N, Ploidy level of rice plant regenerated from polyspermic zygote. After nuclei were extracted from leaves of wild-type rice plants (M) or from leaves of wild-type rice plants and plants regenerated from polyspermic zygotes (N), DNA content per nucleus was measured by flow-cytometry. Light-green and pink colored circles in A indicate sperm and egg nuclei, respectively. Arrowheads in panel L indicate awns. Scale bars = 20 μm in B–E, 100 μm in F, 1 mm in G and H, and 1 cm in I, K, and L.

Figure 2. Two karyogamy pathways in polyspermic triploid zygotes. A, Karyogamy in a monospermic diploid zygote. An egg cell was fused with a sperm cell expressing H2B-GFP, and
the resulting triploid zygote was serially observed. The sperm nucleus fluorescently labeled
with H2B-GFP migrated adjacent to the egg nucleus after gamete fusion (panels a and b). Then,
the sperm nucleus fused with the egg nucleus, the sperm chromatin began to decondense (panels
c and d). Decondensation of sperm chromatin further progressed (panels e and f) and
karyogamy was completed (panels g to j). B, One karyogamy pathway in polyspermic zygote.
An egg cell was fused with a sperm cell expressing H2B-GFP, and then the fused gamete further
fused with another sperm cell. The resulting triploid zygote was serially observed. Two sperm
nuclei were clearly visible in the egg cell after its fusion with sperm cells (panels a and b). One
of the two sperm nuclei first fused with the egg nucleus (panels c and d), and then the second
sperm nucleus fused with the egg nucleus (panels e and f), resulting in a triploid zygotic nucleus
(panels g–j). C, Another karyogamy pathway in polyspermic zygote. Polyspermic zygote was
prepared as in B and the triploid zygote was serially observed. Two sperm nuclei were observed
in the egg cell after its fusion with sperm cells (panels a and b), and then these two sperm nuclei
fused together (panels c–f). Then the united sperm nuclei further fused with the egg nucleus
(panels g and h), resulting in a triploid zygotic nucleus (panels i and j). Upper panels are
fluorescent images, and lower panels are merged bright-field and fluorescent images.
Arrowhead in panel Bc indicates sperm chromatin which is decondensing in fused nucleus.
Scale bars = 20 μm.

**Figure 3.** Organization of actin filaments and migration of sperm nuclei in polyspermic zygote
during karyogamy. An egg cell expressing Lifeact-tagRFP was sequentially fused with two
sperm cells expressing H2B-GFP, and resultant zygote was serially observed under a fluorescent
microscope. Because two sperm nuclei were observed at different focal planes, images for two nuclei were separately captured and presented in panels A to C, and D to F. First sperm nucleus migrated toward egg nucleus (A and B) and fused with egg nucleus, resulting in decondensation of sperm chromatin in fused nucleus (C). Second sperm nucleus migrated toward egg nucleus (D to F) and fused with the diploid zygote nucleus, resulting in decondensation of sperm chromatin in the triploid nucleus (G). Insets in panels D and E show enlarged views of the sperm nuclei enclosed within the squares. Actin filaments in these Insets were represented by white color. The asterisk in panel A indicates the egg nucleus surrounded by actin filaments. Arrows in panels C and G indicate decondensing chromatin derived from first sperm nucleus. Arrowhead in panel G indicates decondensing chromatin derived from second sperm nucleus. Scale bars = 20 µm in A-G, 5 µm in the Insets.

Figure 4. Mitotic division of polyspermic zygote. After in vitro fusion of an egg cell with a sperm cell expressing H2B-GFP, the fused gamete was further fused with another sperm cell. The polyspermic zygote was then cultured and mitotic division was serially monitored. Chromatin and chromosomes were labeled with H2B-GFP in polyspermic zygote (A–D). Chromosomes originated from triploid zygotic nucleus were arranged at the equator (E–H), and then separated to form two daughter nuclei (I–N). Upper and lower panels are fluorescent and bright-field images, respectively. Scale bar = 20 µm.

Figure 5. Microtubule organization during mitotic division of polyspermic zygotes. Polyspermic zygotes were fixed at each mitotic phase, and microtubule structure and
chromosome organization were visualized by immuno-fluorescent staining with anti-α-tubulin antibody and DAPI staining, respectively. Representative images are presented. Images in upper and middle panels show immuno-fluorescent staining and DAPI staining, respectively. Lower panels show merged images. Scale bars =10 μm in A–L, 20 μm in M–O.


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