Megasporogenesis and Megagametogenesis in Autotetraploid Indica/Japonica Hybrid Rice

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Abstract: Autotetraploid indica/japonica hybrid combines both the advantages of polyploidy and the heterosis between indica and japonica. Embryo sac abortion is an important factor influencing spikelet fertility in autotetraploid rice hybrid. To clarify the cytological mechanism of embryo sac abortion, the megasporogenesis and megagametogenesis in an autotetraploid japonica/indica hybrid were examined by WE-CLSM technique. Abnormalities were observed from megasporocyte stage to mature embryo sac stage. The degeneration of the tetrad cells and the functional megaspore were characteristics of abnormalities during megasporogenesis. Abnormal small embryo sac and disordered number of nucleus were frequently observed during embryo sac development. Some interesting phenomena, such as two functional megaspores, the diplospory-like megasporocyte, and five-nucleate embryo sac were found. The abnormalities that occurred during female gametophyte development resulted in more than five types (i.e. embryo sac degeneration, embryo sac without female germ unit, embryo sac without egg apparatus, embryo sac with abnormal polar nuclei and abnormal small embryo sac) of abnormal embryo sacs in autotetraploid japonica/indica hybrid. Embryo sac fertility was lower in diploid japonica/indica hybrid than in autotetraploid japonica/indica hybrid although many abnormal phenomena were observed in autotetraploid hybrid.

Key words: Oryza sativa; japonica/indica hybrid; embryo sac; autotetraploid; abnormality; WE-CLSM

Autotetraploid rice (2n=4x=48) derived from diploid rice (2n=2x=24) through chromosome doubling with colchicine has larger kernels, higher biomass yield, higher protein and better amino acid content, compared with their original diploid counterparts (Hu et al, 2009; Luan et al, 2007; Song and Zhang, 1992; Tu et al, 2003; Tu et al, 2007). Autotetraploid indica/japonica hybrid, obtained by crossing 4x indica variety with 4x japonica variety, combines both the advantages of polyploidy and heterosis between indica and japonica (Cai et al, 2001). It has been reported that most of the 4x indica/japonica hybrids have higher seed set than their original 2x indica/japonica hybrids (Hu et al, 2009), which attracts many interests in China, since some researchers believed that the poor seed set of 2x indica/japonica hybrid can be improved at the 4x level (Huang et al, 2004). However, the seed set of most of the 4x indica/japonica hybrids were still much lower than the normal level. Embryo sac abortion was reported to be one of the most important factors influencing spikelet fertility of 4x inter-subspecific hybrids (Hu et al, 2009; Xiao et al, 2005). Little information was available concerning the embryo sac abnormality in 4x or 2x indica/japonica hybrid. Since the rice embryo sac is enclosed within the ovary, it is technically challenging to observe the embryo sac using conventional sectioning or whole stain-clearing technique. To facilitate observation of rice embryo sac, a WE-CLSM (whole-mount eosin B-staining confocal laser scanning microscopy) technique was developed in our laboratory (Guo et al, 2006; Xiao et al, 2005; Zeng et al, 2007; Zhang et al, 2003). WE-CLSM technique is one of the most convenient and effective techniques for embryo sac observation in rice. Diversity of abnormal embryo sacs was detected in both 4x indica/japonica hybrid and its corresponding 2x hybrid by using WE-CLSM technique (Hu et al, 2009; Zeng et al, 2007). The embryo sac formation and development in 2x indica/japonica hybrid was studied, and it was reported that the abnormalities that occurred during female gametophyte development
resulted in five major types of abnormal embryo sacs (Zeng et al., 2009). Although different types of abnormal embryo sacs were detected in the mature embryo sacs of 4x indica/japonica hybrid, the stage when the abnormalities occurred is much less understood, and it is still not clear about the differences between 4x and 2x indica/japonica hybrid during female gametophyte development.

In the present study, we investigated the female gametophyte formation and development in a 4x japonica/indica hybrid. The objective was to clarify the cytological mechanism of embryo sac abortion in 4x indica/japonica hybrid.

MATERIALS AND METHODS

Plant materials

Two diploid varieties, Taichung 65 (japonica) and Guanglu’ai 4 (indica) were used. The autotetraploid rice (2n=4x=48) was derived from the diploid variety (2n=2x=24) through chromosomes doubling with colchicine. The 4x japonica variety was crossed to 4x indica variety to produce 4x japonica/indica hybrid.

The F₁ of 4x japonica/indica hybrid (Taichung 65-4x × Guanglu’ai 4-4x) was used to investigate the female gametophyte formation and development. The diploid variety Guanglu’ai 4 was planted at the same time to study the megasporogenesis and megagametogenesis in rice. All the plant materials were cultivated in July 2005 at the experimental farm of South China Agricultural University, Guangzhou, China (23°16′N, 113°8′E).

Fixation

Florets were collected at different developmental stages. When the hybrid was flowering, florets with open glumes, i.e., in which embryo sacs were mature and ready for fertilization, were collected at noon each day. All the samples were fixed in FAA (formaldehyde:acetic acid:50% ethanol=5:6:89) for at least 24 h, then washed with 50% ethanol and stored in 70% ethanol at 4°C.

Eosin B staining and embryo sac scanning

The eosin B staining procedure for embryo sac scanning using laser scanning confocal microscope (WE-CLSM technique) was described by Zeng et al. (2007). The measurement of embryo sac size was according to Zeng et al. (2007).

RESULTS

Megasporogenesis and megagametogenesis in autotetraploid japonica/indica hybrid

The female gametophyte developmental stages in 4x japonica/indica hybrid (Taichung 65-4x × Guanglu’ai 4-4x) were similar to its diploid parent ‘Guanglu’ai 4’. The megasporogenesis and megagametogenesis in 4x japonica/indica hybrid were described as the following stages: megasporocyte stage (Fig. 1-A, B), meiotic division stage (Fig. 1-C, D), functional megaspore stage (Fig. 1-E), mono-nucleate stage (Fig. 1-F), two-nucleate stage (Fig. 1-G, I), four-nucleate stage (Fig. 1-J), eight-nucleate stage (Figs. 1-K, N) and mature embryo sac stage (Fig. 3-B).

Abnormalities occurred during megasporogenesis in autotetraploid japonica/indica hybrid

Abnormalities were observed at megasporocyte stage, meiotic division stage and the functional megaspore stage.

In some ovaries, the megasporocyte degenerated, and a new megasporocyte was developed instead (Fig. 1-O). During the meiotic division process, two degenerating dyad cells were observed (Picture not shown). In some ovaries, four cells of the tetrad were degenerated (Fig. 1-P). The degeneration of the functional megaspores was frequently observed in ovaries at this stage (Fig. 2-A, B). In some cases, two functional megaspores were detected synchronously in one ovary (Fig. 2-C), and one functional megaspore and a diplospory-like megasporacyte were observed together in one ovule (Fig. 2-D).

Abnormalities occurred during megagametogenesis in autotetraploid japonica/indica hybrid

Abnormalities were also detected from the mono-nucleate stage to the mature embryo sac stage in 4x japonica/indica hybrid.

Some of the degenerated mono-nucleate embryo
sacs (Fig. 2-E) or two-nucleate embryo sacs were found. Some abnormal two-nucleate embryo sacs were detected with both nuclei located at the micropylar-most region (Fig. 2-F, G). Moreover, a mono-nucleate embryo sac and a two-nucleate embryo sac were detected simultaneously within an ovary.

Different kinds of five-nucleate embryo sacs were found. Some of the five-nucleate embryo sacs contained three nuclei at the micropylar pole and two nuclei at the chalazal pole (Fig. 2-H, I), or three nuclei at the chalazal pole and two nuclei at the micropylar pole (Fig. 2-J). Some of the embryo sacs had five nuclei, which were located at the micropylar pole, and the cells at the chalazal pole were degenerated (Fig. 2-K).

The abnormalities that occurred at the eight-nucleate stage were diverse. Some embryo sacs contained one more nucleus at the micropylar pole at late eight-nucleate stage (Fig. 2-L). In a few cases, many nuclei were observed at both the micropylar and the chalazal pole. In some cases, all cells at both the micropylar and the chalazal pole were degenerated (Fig. 2-M, N). At late eight-nucleate stage, some embryo sacs were detected with the polar nuclei located at abnormal position (Fig. 2-O), some embryo sacs with abnormal numbers of polar nuclei were also observed (Fig. 2-P). The degenerated egg apparatus was observed in some ovaries (Fig. 3-A) at late eight-nucleate stage. At eight-nucleate stage, some abnormal embryo sacs with smaller sizes (Fig. 3-I, J)
Abnormalities of mature embryo sacs in autotetraploid japonica/indica hybrid

A normal mature embryo sac in 4x japonica/indica hybrid had one egg apparatus that consisted of one egg cell and two synergids at the micropylar pole, two polar nuclei located above the egg apparatus and a group of antipodal cells at the chalazal pole (Fig. 3-B). The egg apparatus and two polar nuclei formed the female germ unit (Huang and Russell, 1992). We examined 307 mature embryo sacs in 4x japonica/indica hybrid, and five major categories of abnormal embryo sacs were frequently observed: (1) Embryo sac degeneration (ESD, Fig. 3-C); (2) Embryo sac without female germ unit (ESWF, Fig. 3-D), but with antipodals; (3) Embryo sac without egg apparatus (ESWE, Fig. 3-E, F), but with polar nuclei and antipodals; (4) Embryo sac with abnormal polar nuclei (ESWA). This abnormal type had normal egg apparatus and antipodals, but the polar nuclei either located at an abnormal position (Fig. 3-G), or with abnormal numbers of polar nuclei (Fig. 3-H); (5) Abnormal small embryo sac (ASES). This type had less than 1/2 of the normal embryo sac size, and often accompanied with abnormal polar nuclei (Fig. 3-K, L).

Besides these five types mentioned above, other abnormal types were also found. For example, twin...
ovules with two abnormal embryo sacs were observed at the mature stage in 4x hybrid.

The frequencies of various abnormal embryo sac types in 4x japonica/indica hybrid were listed in Table 1. ESD was the most frequent type of abnormality, followed by ESWA (Table 1).

### DISCUSSION

**Abnormalities that occurred during female gametophyte development resulted in five major types of abnormal embryo sacs in autotetraploid japonica/indica hybrid**

Five major types of abnormal embryos sacs were detected in mature embryo sacs of 4x hybrid, i.e. ESD, ESWF, ESWA, ESWE and ASES. After the investigation of female gametophyte development, it was obvious to find that the ESD was mainly resulted from the degeneration of the functional megaspores, because many degenerated functional megaspores were found at the functional megaspore stage. The degeneration of mono-nucleate embryo sac or two-nucleate embryo sac also led to ESD. But abnormalities that occurred at mono-nucleate embryo sac stage or two-nucleate embryo sac stage were not so often as that at the functional megaspore stage. ESWF, ESWA, ESWE and ASES mainly occurred at the eight-nucleate stage, and we had cytological proof to support this view. For example, we found that some embryo sacs with egg apparatus degenerated at late eight-nucleate stage. Embryo sacs with abnormal polar nuclei were also observed at late eight-nucleate stage. At the eight-nucleate stage, some of the abnormal embryo sacs were found with smaller sizes compared to normal ones, suggesting that ASES occurred at this stage.

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**Table 1. Frequencies of embryo sac (ES) abnormalities in 4x japonica/indica hybrid (All the ovaries were collected at mature stage).**

<table>
<thead>
<tr>
<th>Cross</th>
<th>ES fertility (%)</th>
<th>ESD (%)</th>
<th>ASES (%)</th>
<th>ESWF (%)</th>
<th>ESWE (%)</th>
<th>ESWA (%)</th>
<th>OAT (%)</th>
<th>Number of ovaries observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taichung 65-4x×Guanglu'ai 4-4x</td>
<td>61.89</td>
<td>12.70</td>
<td>6.19</td>
<td>6.19</td>
<td>3.26</td>
<td>6.84</td>
<td>2.93</td>
<td>307</td>
</tr>
</tbody>
</table>

ESD, embryo sac degeneration; ASES, abnormal small embryo sac; ESWF, embryo sac without female germ unit; ESWE, embryo sac without egg apparatus; ESWA, embryo sac with abnormal polar nuclei; OAT, other abnormal type.
Differences between autotetraploid japonica/indica hybrid and its corresponding diploid hybrid during female gametophyte development

In the present study, we studied the female gametophyte development in 4x japonica/indica hybrid (Taichung 65-4x×Guanglu’ai 4-4x). The developmental process in the 2x japonica/indica hybrid (Taichung 65×Guanglu’ai 4) was reported by Zeng et al (2009). So it provided an opportunity to discuss the differences between the 4x hybrid and the 2x hybrid.

The embryo sac developmental phases were similar in both the 4x and the 2x hybrids. The abnormalities that occurred during megasporogenesis and megagametogenesis in 2x hybrid can also be found in 4x hybrid, but the abnormalities occurred in the 4x hybrid were more complex compared to the 2x hybrid, and some distinct phenomena were detected only in the 4x hybrid. Abnormalities were not observed during the meiosis of the megasporeocyte in 2x hybrid (Zeng et al, 2009), but we found abnormalities during the meiotic process in 4x hybrid in the present study.

The doubling of chromosomes in the 4x hybrid can lead to doubling of functional megaspores. For example, we found double functional megaspores within an ovary (Fig. 2-C). Since the doubling of megaspores was not detected in ovaries in 2x hybrid, it suggested that the dosage effect of tetraploid resulted in new abnormalities in 4x hybrid.

Various numbers of nuclei were detected in some abnormal embryo sacs during megagametogenesis in 4x hybrid. At four-nucleate stage, we found five-nucleate embryo sac (Fig. 2-H, J). Some five-nucleate embryo sac had one more nucleus at the micropylar pole or chalazal pole compared to the normal four-nucleate embryo sac. At eight-nucleate stage, we found nine-nucleate embryo sac that contained one more nucleus at the micropylar pole compared to the normal eight-nucleate embryo sac. The abnormal five-nucleate embryo sacs were not observed in 2x hybrid.

Therefore, the doubling of the functional megaspores and disordered number of nuclei were characteristics of 4x japonica/indica hybrid during female gametophyte development. Furthermore, we found diplosory-like megasporocyte (Fig. 2-D) in 4x hybrid. It seemed that there were connections between 4x hybrid and the apomixis, and it need to be confirmed in future studies.

Comparing the mature embryo sacs between 4x and 2x hybrids, we found that the embryo sac fertility in 2x hybrid (24.3%) (Zeng et al, 2007) was lower than in 4x hybrid (61.89%). Therefore, although the embryo sac abnormalities were more complex in 4x hybrid, the embryo sac fertility was higher in 4x hybrid than 2x hybrid.

Hu et al (2009) reported that the embryo sac fertility and the seed set were higher in most of the 4x indica/japonica hybrids than in 2x hybrids, and they believed that 4x indica/japonica hybrids have potential in rice breeding. In the present study, we studied the female gametophyte development in 4x japonica/indica hybrid, and the results broadened our understanding of embryo sac abortion in tetraploid hybrid and provide a theoretical basis for use of 4x indica/japonica hybrid in production.

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