Occurrence of a Spontaneous Triploid Progeny from Crosses between Diploid Asparagus (Asparagus officinalis L.) Plants and Its Origin Determined by SSR Markers

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The origin of a spontaneous triploid asparagus plant from crosses of 2x × 2x was investigated by SSR and flow cytometric analyses. One hundred and twenty-four progeny were obtained from crosses between a diploid female ‘Gold Schatz’ and a diploid male ‘Hokkai 100’. SSR analysis proved that two and one genes were transmitted from the maternal and paternal parents, respectively, at each SSR locus of one progeny, 07M-61, whereas one gene each was from the female and male parents in the other diploid progeny. Triploidy of 07M-61 was confirmed by flow cytometric analysis. It was suggested that the triploid plant was derived from fertilization between an unreduced egg and reduced sperm nuclei, given its SSR genotypes. It was also suggested that the unreduced maternal gamete was derived from first division restitution (FDR) or second division restitution (SDR) with chiasma occurrence during meiosis. There were no noticeable morphological differences between the triploid and diploid progeny.

Key Words: flow cytometry, gamete, SSR analysis, unreduced egg.

Introduction

Triploid plants have been obtained by crosses between 4x × 2x or 2x × 4x, such as in apple (Laurens et al., 2000), watermelon (Cushman and Huan, 2008), and grape (Wakana et al., 2008). They have advantages in terms of their larger size and seedlessness of the fruit. Asparagus (Asparagus officinalis L.) is dioecious and most of its commercial cultivars are diploid (2n = 2x = 20). A triploid asparagus cultivar ‘Hiroshima Green’ was developed in Japan by interloid crosses between tetraploid female and diploid male plants of ‘Mary Washington 500’ (Hasegawa et al., 1987), and some other triploid asparaguses have also been obtained from reciprocal diploid-tetraploid crosses (Moreno et al., 2010; Ozaki et al., 2004). Triploid asparagus has advantages over the diploid type. A large size of spears was expected in triploid asparagus, and unnecessary seedlings, which are often treated as weeds in diploid asparagus production, are not produced from female triploid asparagus because of the low seed production ability.

Spontaneous triploids from diploid parents were reported in intra- and inter-specific crosses in Citrus (Esen and Soost, 1971), Malus (Sedysheva, 1995), and Rhododendron (Ureshino and Miyajima, 2002). The mechanism by which these spontaneous triploids arose was thought to be the production of 2n egg cells or 2n pollen grains, resulting from abnormal meiosis, and their union with a monoploid (n) gamete. Mixed populations including normal diploids, variable rates of triploids, and other rare polyploids can be simultaneously generated by sexual hybridization (Carputo et al., 2003; Wu, 1995). In the genus Populus, triploidy was observed in progeny from crosses among diploid parents (Bradshaw and Stettler, 1993). The type and frequency of 2n gametes are related to the species, genotype, and environmental conditions (Barcaccia et al., 1997). Uno et al. (2002) reported that one haploid, one triploid, and 32 diploids were obtained from polyembryonic seeds of the diploid asparagus ‘Mary Washington 500W’. This is the report on spontaneous
triploid asparagus appearance, but there is no information on the mechanism behind the occurrence of triploidy in this species. If the appearance of triploidy from diploid crosses can be controlled, genetic study as well as practical breeding should progress in this species.

Simple sequence repeat (SSR) is used for genetic and phylogenetic studies in many plants because of its high polymorphism and co-dominant expression (Fukuda et al., 2013; Goto-Yamamoto et al., 2013; Honsho et al., 2012). SSR markers have been established from expressed sequence tag (EST) in asparagus (Caruso et al., 2008). We performed 2x × 2x crosses in order to evaluate the applicability of SSR as genetic markers in asparagus, and showed that it is useful for hybrid confirmation and for evaluating the genetic purity of clonal hybrid cultivars, as well as for the genotyping of triploid and tetraploid cultivars (Takeuchi et al., 2012). We report here the occurrence of a triploid asparagus plant from a diploid-diploid cross and its origin determined by analyzing SSR markers.

Materials and Methods

Plant materials and DNA extraction

One hundred and twenty-four progeny of ‘Gold Schatz’ (an old diploid cultivar bred in former East Germany and conserved in Hokkaido University, Japan) × ‘Hokkai 100’ (a diploid cultivar bred by Hokkai Can Co. Ltd., Japan) and their parents were used for SSR analysis. DNA was extracted from young cladophylls of each plant cultivated in the University Farm, Kyushu University, using a modified version of the CTAB method (Stajner et al., 2002). The DNA concentration was adjusted to approx. 20 ng·μL⁻¹.

SSR analysis

PCR amplifications were carried out in a total volume of 12.5 μL of solution containing 6 ng of genomic DNA, 0.2 mM each dNTP, 0.5 μM forward and reverse primers, 1.25 μL of 10× PCR buffer (100 mM Tris-HCl, pH 9, 50 mM KCl, and 15 mM MgCl₂), and 0.25 U Taq DNA polymerase. We used five SSR primer pairs generating AG2, AG3, AG7, AG10, and TC1 regions developed by Caruso et al. (2008), and the forward primers were labeled at the 5’ end with fluorescent dye (6-FAM or HEX). PCR was performed on a thermocycler (PC-808-02; Astec Co. Ltd., Fukuoka, Japan) with one cycle of 5 min at 95°C as initial denaturation, 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C for amplification, and one cycle of 15 min at 72°C as final extension. Reaction solutions were diluted 15–20 times with sterilized distilled water, and 0.5 μL of the solution was mixed with 12 μL of deionized formamide (Wako Co. Ltd., Tokyo, Japan) and 0.25 μL of GeneScan 500 ROX (Applied Biosystems, Foster City, CA, USA). The mixtures were denatured (95°C) for 2 min and then cooled on ice immediately. Genotyping was conducted by using the capillary sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems) and fragment sizes were determined by using GeneScan version 3.7 (Applied Biosystems), as reported previously (Takeuchi et al., 2012). Each SSR was tested for single-locus Mendelian segregation of crossed progenies using chi-square goodness-of-fit test.

Flow cytometric analysis

Ploidy levels of twenty progeny from the above-mentioned crosses, including a progeny showing a triploid-like genotype (07M-61) in five SSR regions, were investigated by flow cytometry. A diploid control ‘Hokkai 100’ was also used for the analysis. Immature cladophylls were chopped with a sharp razor blade in nucleus extraction buffer (High Resolution DNA Kit; Partec, Görlitz, Germany), and the suspension including released nuclei was passed through a 50 μm filter. The nuclei in the filtrate were stained with four volumes of staining solution (High Resolution DNA Kit; Partec) containing 4’-6-diamidino-2-phenylindole (DAPI). Samples were analyzed with a flow cytometer (PA Ploidy Analyzer; Partec) after shaking the solution gently. Relative DNA content was estimated based on the most prominent peaks by counting from 4000 to 5000 nuclei.

Morphological characteristics

Lengths of the three longest cladophylls of 07M-61 and 15 other progeny were investigated 2.5 years after seed sowing.

Results and Discussion

Table 1 shows the SSR genotypes of the parents and the segregation of their progeny in AG2, AG3, AG7, AG10, and TC1 loci. The genotype of the plant was identified as 147/151 in AG2 locus when two peaks representing 147 and 151 showed a similar relative fluorescent intensity (RFI) with each other as previously reported (Takeuchi et al., 2012). The other genotypes were also identified in a similar manner. It was also confirmed that the segregation of the progeny genotypes in which data for 07M-61 were excluded fitted with monogenic inheritance by χ²-test (Takeuchi et al., 2012).

Although two peaks of 151 and 157 bp were also presented in one progeny 07M-61, the RFI of former peak was higher than the latter (Fig. 1). Thus, the genotype was identified as 151/151/157, with two 151s from the female parent and one 157 from the male parent.

Similarly, two peaks, one higher than the other, were observed at AG7, AG10, and TC1 loci in 07M-61 (Fig. 1), while the other progeny had one peak or two peaks with similar RFI and segregated into four (AG7), two (AG10), or three (TC1) genotypes (Table 1), all in accordance with Mendelian expectations (Takeuchi et al., 2012). The genotype 174/174/178 at the AG7 locus
in 07M-61 is composed of one 174 from the female, another 174 from the male, and 178 from the female, excluding a 172 gene from the male parent, since the two alleles (151/151) were from the seed parent in AG2. At the AG10 locus, it could be determined that, for the two 177s, one was from the female and one from the male, and 183 was from the female, similarly to AG2. Alleles of TC1 were not decidable from which parent they were derived. That is to say, there were two possibilities; either two alleles (219/219) were from the female and an allele (225) from the male or (219/225) were from the female and (219) from the male. Three peaks were observed at the AG3 locus in 07M-61 (Fig. 1), the allele 216 being from the female, 218 from the male, and 214 supposedly from the female, judging from the AG2 genotype, whereas the other progeny fitted with monogenic inheritance by \( \chi^2 \)-test (Table 1; Takeuchi et al., 2012).

Two peaks reflecting \( G_0/G_1 \) (prominent peak) and \( G_2/M \) (minor peak) stages of somatic cells appeared in the diploid cultivar ‘Hokkai 100’ (Fig. 2a) and 20 progeny. Relative fluorescent intensities of the prominent peaks in ‘Hokkai 100’ and 19 progeny except for 07M-61 were approximately 195. In contrast, the value in 07M-61 was approximately 295, indicating the triploidy of 07M-61 (Fig. 2b). There were no noticeable

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**Table 1. Genotypes and goodness-of-fit tests of the progeny from the cross of ‘Gold Schatz’ and ‘Hokkai 100’ at AG2, AG3, AG7, AG10, and TC1 loci.**

<table>
<thead>
<tr>
<th>SSR</th>
<th>Genotypes of parents</th>
<th>Progeny genotype (Number of plants)</th>
<th>Goodness-of-fit testz</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Expected ratio</td>
</tr>
<tr>
<td>AG2</td>
<td>151/155 × 147/157</td>
<td>147/151 (34) 147/155 (30) 151/157 (32) 155/157 (27) 151/157 (1)y</td>
<td>1:1:1:1:1</td>
</tr>
<tr>
<td>AG10</td>
<td>177/183 × 177/177</td>
<td>177/177 (58) 177/183 (65) 177/177/183 (28) 177/173 (1)y</td>
<td>1:1</td>
</tr>
<tr>
<td>TC1</td>
<td>219/225 × 219/225</td>
<td>219/219 (40) 219/225 (59) 225/225 (24) 219/219/225 (1)y</td>
<td>1:2:1</td>
</tr>
</tbody>
</table>

* Goodness-of-fit tests in which data for 07M-61 were excluded (Takeuchi et al., 2012).

1 07M-61.

* Highlighted data are cited from a previous report (Takeuchi et al., 2012).
morphological differences between the triploid and diploid progeny (data not shown).

The occurrence of spontaneous triploids from crosses between diploids was reported in some plant species. Triploids of *Citrus* (Chen et al., 2008) and carnation (*Dianthus caryophyllus* L.) (Nimura et al., 2008) were derived from the fertilization of 2n (female) and n (male) gametes, whereas those of tulip (Okazaki et al., 2005) and poplar (*Populus tomentosa* Carr.) (Zhang et al., 2007) were from the fertilization of n (female) and 2n (male) gametes. Unreduced gametes, irrespective of where they are derived from, are generally considered to contribute to the occurrence of spontaneous polyploid individuals from diploid parents, although the frequency of triploid progeny from diploid parents is usually very low (Esen and Soost, 1971).

Unreduced gametes in angiosperms have been considered mainly to originate from abnormal meiosis, first division restitution (FDR) or second division restitution (SDR) (Ramanna, 1983). In the first case, failure of spindle formation occurs because of the nuclear membrane reforming around the chromosomes without movement to two opposite poles during meiosis I. In the case of SDR, normal first meiotic division occurs, that is, the chromosomes divide and cytokinesis occurs, producing a dyad. No cell plate is, however, formed at the separation of sister chromatids during meiosis II. Upon the absence of a chiasma, diploid gametes from FDR are identical to the complete heterozygous genotypes of gametes at a ratio of 1:1 at each locus. Genotypes of diploid gametes with a chiasma are segregated into heterogeneous (50%) and homogeneous (50%) types, including two different homozygous genotypes of gametes at a ratio of 1:1 in each locus from FDR, completely heterozygous genotypes from SDR.

Spontaneous triploidy has been detected among plantlets established from polyembryonic seeds in asparagus (Uno et al., 2002), but the mechanism behind its appearance was not investigated. Since the population hybrid ‘Mary Washington 500W’ was used, whose seeds were obtained from crosses among females and males with several genotypes, it was quite difficult to determine the involved mechanism using molecular markers. The present investigation is the first on a mechanism generating spontaneous triploidy derived from crosses between diploids in asparagus, and it was found that the spontaneous triploid progeny originated from the fusion of a reduced microspore and an unreduced diploid egg, since one gene from the paternal parent and two genes from the maternal one were transmitted at the AG2 locus. The suggestion derived from the result for the AG2 locus is not contradicted by the genotypes of the remaining loci in 07M-61. The genotype of the diploid egg that contributed to the production of triploid 07M-61 was heterozygous at AG3, AG7, and AG10 and homozygous at AG2. The genotype of TC1 in the diploid egg could not be determined from the genotype of 07M-61. If the rate of chiasma occurrence is low in asparagus, it is expected that the triploid 07M-61 was derived from an FDR-derived unreduced egg.

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**Literature Cited**


