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Sex-conversion from Male to Female during Somatic Embryogenesis from Protoplasts in *Asparagus officinalis* L.)

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Sex-converting mutation from male to female was identified during somatic embryogenesis from protoplasts in asparagus. Although modification of allozyme genotypes from heterozygous to homozygous occurred in three loci, *Mdh-1*, *Mdh-2* and *Idh-1*, accompanied with sex-conversion, genotypes of sex-converted and control plants in *Pgm-1* and *Skdh-1* loci were identically heterozygous. Relative DNA contents of sex-converted and control plants estimated by flow cytometric analysis were similar, and gametophytic fertility of the sex-converting mutant as female parent was also confirmed by artificial pollination. From these results, the sex-conversion in the current study might be the result of the somatic mutation, such as somatic crossing-over, one of the chromosomal rearrangements.

INTRODUCTION

Asparagus officinalis L.) is a dioecious plant, and its sex expression is controlled by a single locus located on the L5 chromosome (Löptien, 1979). The genotypes of male and female are heterozygous (Mm) and homozygous recessive (mm), respectively (Sneep, 1953). There are generally three types of asparagus hybrid, namely clonal, double and population hybrids, depending on seed harvesting methods. Besides them, there are a few vitroclone cultivars. The vitroclone cultivars are asexually propagated through somatic embryogenesis from callus tissue originated from young spears or protoplasts.

Specific genetic modifications responsible for somaclonal variation have been discussed extensively (Larkin and Scowcroft, 1981; Evans *et al.*, 1984). Commercial micro-propagation of clones requires regenerated plantlets to be genetically uniform. It is, therefore, necessary to establish reliable techniques to maintain their genetic stability. If *in vitro* techniques promote genetic variation, it is important to identify the factors caus-

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ing the variation and its extent. Factors for somaclonal variation include single nucleotide changes (Dennis *et al.*, 1984), chromosome number changes (Karp *et al.*, 1982; McCoy *et al.*, 1982), chromosomal rearrangements (McCoy *et al.*, 1982; Orton, 1983) and so on. Polyploidy and aneuploidy have been often observed among plants regenerated from tissue cultures (Hammerschlag, 1992) and these changes have been shown to lengthen the duration of cell culture (Skirvin and Janick, 1976). Determination of the ploidy of cultured tissues is one of the most effective methods to monitor and minimize the somaclonal variants. Ploidy levels were determined by counting the somatic chromosomes, and also estimated by measuring the length of stomatas. The procedures are still difficult and/or time consuming in asparagus, so that the efficient analytical methods for determination of ploidy levels have long been desired. Recently, flow cytometry has become a rapid and useful tool for estimation of genome size and ploidy levels in several crops (Baird *et al.*, 1994; Martínez *et al.*, 1994; O'Brien *et al.*, 1996; Ollitrault-Sammarcelli *et al.*, 1994; Ozias-Akins and Jarret, 1994). Ozaki *et al.* (1998) reported that flow cytometry was also applicable to asparagus.

Sex-converted asparagus plants from male to female were found during somatic embryogenesis from protoplasts in this study. Allozyme analysis and flow cytometry were applied to clarify the mechanism of the sex-converting mutation during somatic embryogenesis.

MATERIALS AND METHODS

Protoplast culture

Callus induction and protoplast cultures were carried out as described by Kunitake and Mii (1990). Embryogenic calli were obtained from soft calli of vitroclone asparagus male cultivar 'Festo' by subculturing on MS medium supplemented with 1 mg/l 2,4-D, 1 g/l L-glutamine, 3% sucrose and 0.2% Gelrite. After embryogenic calli were pretreated for 4–7 days by suspending in 1/2 MS liquid medium with 1% sucrose, they were gently squashed and incubated with 10 ml of filter-sterilized enzyme solution containing 2% Cellulase Onozuka RS (Yakult Pharmaceutical Co. Ltd., Japan), 0.5% Macerozyme R-10 (Yakult Pharmaceutical Co. Ltd., Japan), 0.05% Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Japan) and 0.6 M sorbitol at pH 5.7. The mixture was incubated for 6 h at 25 °C to liberate protoplasts. The protoplasts were collected by filtration through a nylon sieve (60 µm) and washed twice with 0.6 M mannitol solution after centrifugation (100 × g for 5 min).

The protoplasts were embedded in 0.1% Gelrite-solidified 1/2 MS media containing 1 mg/l NAA, 0.5 mg/l zeatin, 1 g/l L-glutamine, 0.6 M glucose and cultured at a density of 1×10^5 cells/ml in 60 × 15 mm plastic petri-dishes containing 3 ml of culture medium. All dishes were sealed with Parafilm® and maintained at 25 °C in the dark for 30 days. Formed colonies were subcultured on MS medium with 3% sucrose for 30 days, and embryogenic calli developed from the colonies were also subcultured on the medium of the same constitution until plant regeneration through somatic embryogenesis.

Three hundred and twenty five plants were established from 650 germinated embryos and transplanted in an open field. Sex expression of the regenerated plants was investigated in the field. There were two female plants considered to be sex-converted

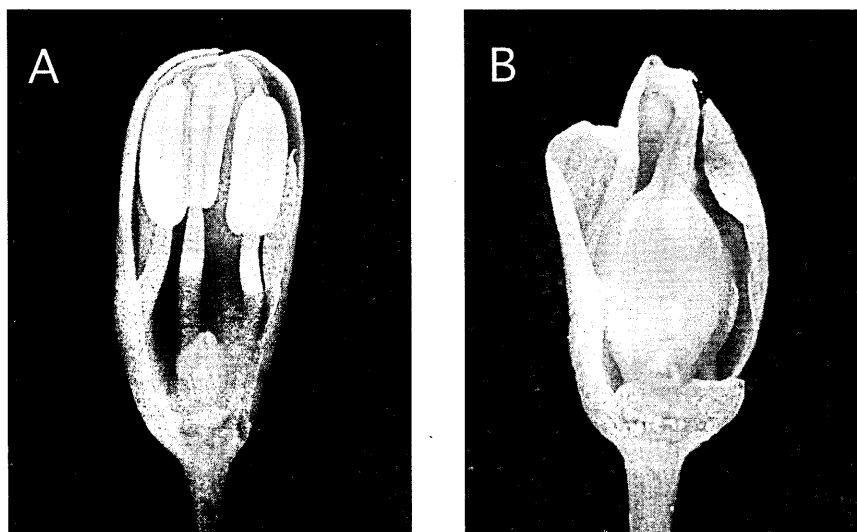


Fig. 1. Flowers of control (A) and sex-converted (B) plants in 'Festo'.

plants, although almost all the flowered plants were male (Fig. 1).

Ploidy, allozymes and gametophytic fertility of one sex-converted female plant (coded as FP-1) and five male plants, (coded as FP-2, FP-3, FP-4, FP-5 and FP-6) were analyzed.

Allozyme analysis

Spears of the sex-converted (FP-1) and control (from FP-2 to FP-6) plants were used for enzyme extraction. Each sample was homogenized in pre-cooled extraction buffer prepared as described by Wendel and Parks (1982), and crude enzyme extracts were prepared for horizontal starch gel electrophoresis. Genotypes of eight enzyme loci in five enzyme systems, AAT, IDH, MDH, PGM and SKDH, were scored. Genetic basis and allozyme determination of the eight loci followed the previous reports (Ozaki *et al.*, 2000a, 2000b).

Ploidy analysis

Flow cytometric analyses were conducted with a Partec PA Ploidy Analyzer (Germany) equipped with DAPI filter and Multicycle for MS-DOS software to measure nuclear DNA content. Pieces of phylloclade in each plant were chopped using a sharp razor blade in nuclei extraction buffer (High Resolution DNA Kit, Partec). The suspension containing the released nuclei was passed through a 50 μ m filter. The nuclei filtrates were stained with four times volumes of staining solution (High Resolution DNA Kit, Partec) containing 4'-6-diamidino-2-phenylindole (DAPI) for more than one min. Fluorescent intensities of sex-converted and control plants were compared in this experiment. Haploid (97SA-003, a gynogenetic haploid plant obtained from the crosses

between diploid and tetraploid), diploid ('Cito'), triploid ('Hiroshima Green') and tetraploid ('Seto Green') plants were also examined as standards. At least 7,000 nuclei were counted for each sample, and relative DNA content was determined according to the prominent peak in each sample.

Gametophytic fertility of sex-converted plant

The FP-1 and another control female plant, Welcome-9201-6 (selected from 'Welcome') were used for investigating gametophytic fertility as seed parents. Artificial pollination with three male plants, $GLS_F1-16-2-6-3$, $GLS_F1-16-2-6-6$ and $GLS_F1-16-2-6-17$, randomly selected from the progenies of 'Gijnlim', was made in the middle of March 1999 in a greenhouse. Fruits were harvested at maturity on 24 June 1999, and the number of seeds and the average seed weight were investigated in each cross.

RESULTS

Allozyme analysis

No variation of allozyme genotypes was observed among six plants from FP-1 to FP-6

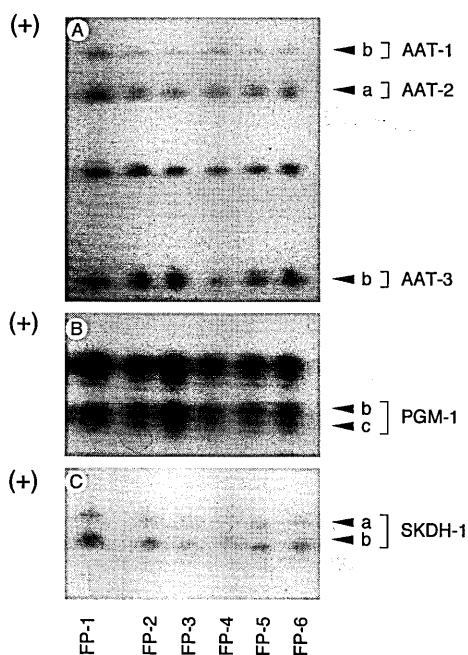


Fig. 2a. Allozyme banding patterns in sex-converted (FP-1) and control (FP-2, 3, 4, 5 and 6) plants in asparagus. A: AAT, B: PGM, C: SKDH.

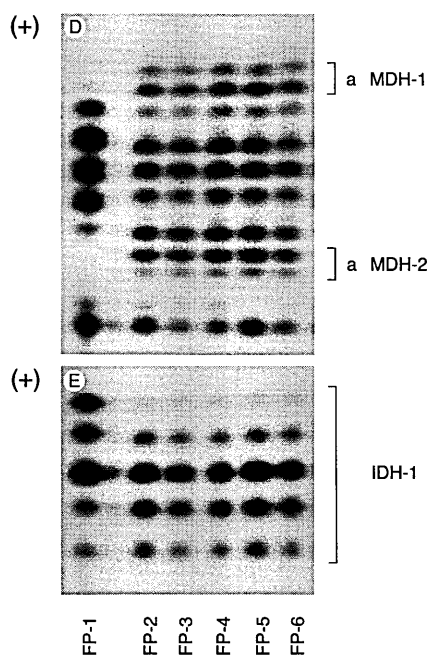


Fig. 2b. Allozyme banding patterns in sex-converted (FP-1) and control (FP-2, 3, 4, 5 and 6) plants in asparagus. D: MDH, E: IDH.

at five loci of "bb" in *Aat-1*, "aa" in *Aat-2*, "bb" in *Aat-3*, "bc" in *Pgm-1* and "ab" in *Skdh-1* (Fig. 2a). There was also no difference in the genotypes among the five male plants at three loci of "an" in *Mdh-1*, "an" *Mdh-2* and "an" in *Idh-1* (Fig. 2b). There were, however, differences of the genotypes between the males and the female at three loci, "an" vs. "nn" in *Mdh-1*, "an" vs. "nn" in *Mdh-2* and "an" vs. "aa" in *Idh-1*, respectively.

Ploidy analysis

Fluorescent intensities at prominent peaks in four standard plants, haploid, diploid, triploid and tetraploid, were 50.0, 100.4, 157.2, 215.8, respectively. Those in the sex-converted and control plants were 107.45 and 104.41, respectively, with no statistical significant difference to diploid value at $P=0.05$ by t-test (data not shown).

Gametophytic fertility of sex-converted plant

Table 1 indicates the results of crosses. Crosses with the three male parents to the FP-1 resulted high values (80.1–94.8%) of fruit set (Fig. 3) as well as the crosses to the control plant (88.6–100%). There were little differences in the average number of seeds

Table 1. Fruit and seed sets with artificial crosses in sex-converted and control plants.

Seed parent	Pollen parent	No. of flowers pollinated	No. of fruits harvested (%)	No. of seeds harvested	Average no. of seeds per fruit	Average weight of a seed (mg)
FP-1 (Sex-converted)	GIS,F ₁ -16-2-6-3	151	121 (80.1)	360	2.98	34.2
	GIS,F ₁ -16-2-6-6	149	121 (81.2)	360	2.98	31.9
	GIS,F ₁ -16-2-6-17	155	147 (94.8)	351	2.39	33.6
Welcome-9201-6 (Control)	GIS,F ₁ -16-2-6-3	175	155 (88.6)	384	2.48	31.3
	GIS,F ₁ -16-2-6-6	143	143 (100)	386	2.70	32.6
	GIS,F ₁ -16-2-6-17	170	157 (92.4)	695	4.43	35.7



Fig. 3. Fruit set in a sex-converted (FP-1) plant.

per fruit in the crosses except one control cross between Welcome-9201-6 and GIS_F₁-16-2-6-17. Average weight of a seed showed the similar values (31.3–35.7 mg) in all the six crosses.

DISCUSSION

Numerous reviews have summarized the variability observed in plant regenerants from tissue cultures (Evans *et al.*, 1984; Hammerschlag and Bauchen, 1984; Hammerschlag, 1992; Daub, 1986). Genetic stability is required for technical and commercial micropropagation system, whereas research on somaclonal variation has culminated in the release of improved cultivars of blackberry (McPheeters and Skirvin, 1989), celery (Heath-Pagliuso *et al.*, 1989), geranium (Skirvin and Janick, 1976) and sweet potato (Moyer and Collins, 1983).

Sex-converting mutation was recognized in asparagus during somatic embryogenesis from protoplasts in the current study. Although the genotypes of sex-converted (female) plant and the control male plants were identically heterozygous in *Pgm-1* and *Skdh-1* loci, the genotypes of sex-converted plant were homozygous in contrast to the heterozygous genotypes of control male plants in the three loci, *Mdh-1*, *Mdh-2* and *Idh-1*. The allozyme mutation in a part of loci might have been resulted from nuclear genomic mutations, such as single nucleotide mutation, decrease of chromosome number and chromosomal rearrangements.

Single nucleotide change was reported in maize (Dennis *et al.*, 1984). The change brings only one (or a few) modification of genes, so that this hypothesis would be neglected in the current investigation because the modification occurred in allozyme genes on three different loci and one sex determination gene.

Chromosome analysis in the somatic tissues during tissue cultures has been reported in asparagus. Kong and Chen (1988), Elmer *et al.* (1989) and Kunitake and Mii (1990) reported the stability of somatic chromosome number in the regenerants from protoplasts of asparagus. In contrast, Elmer *et al.* (1989) reported that the regenerants possessed aneuploid chromosome numbers ($2n=22-38$), and Kunitake *et al.* (1998) reported that polyploidization in embryogenic calli-derived plants increased with increasing duration of subculture, particularly when plants with low ploidy levels were used as explants. There have been, however, no reports on decrease in chromosome number during somatic embryogenesis in asparagus.

It was reported that deletion of a part of a chromosome was resulted by chromosome arm damage and often induced by radiation (Marcotrigiano and Gradziel, 1997). In case of the loss of the dominant alleles on the damaged chromosome, the hemizygous cells express the recessive traits.

Somatic crossing-over occurs when corresponding chromosome parts are exchanged between homologous chromosomes by breakage and reunion (Poethig, 1987; Suzuki *et al.*, 1989). Somatic crossing-over was reported in soybean and tobacco (Evans and Paddock, 1976). Because of their low frequency, the likelihood of a crossing-over event occurring at the terminus of the shoot apical meristem is minimal, and most somatic crossing-over events occur somewhere else in the plant body. Visual changes of the phenotypes, such as codominant chlorophyll mutations, often provide a helpful evidence

of somatic crossing-over since somatic crossing-over would generally go unnoticed (Evans and Paddock, 1976).

The difference of the estimated relative DNA contents between male (control) and female (sex-converted) plants was not statistically significant. Gametophytic fertility of the sex-converted plant as seed parents was also similar to that of the control female plant. If the sex-converting mutation was resulted from decrease of chromosome number or deletion of a part of a chromosome, relative DNA contents in the sex-converted and the control plants might have been different and the gametophytic fertility might have become lower in the sex-converted plant than in the control plants. Somatic crossing-over could be one of the explanations of the sex-converting mutation in this study. In this case, the sex-conversion might be the result of the change of the genotypes from "Mm" to "mm" in sex determining locus.

The three linkage pairs of *Aat-1/Mdh-1*, *Aat-1/Idh-1* and *Pgm-1/Skdh-1* were recognized previously (Ozaki *et al.*, 2000b), and the linkage of *Mdh-1* and sex determination loci was also reported (Maestri *et al.*, 1991). In this investigation, both of the two loci, *Pgm-1* and *Skdh-1*, were identically heterozygous in the sex-converted and control plants, whereas genotypes of the two loci, *Mdh-1* and *Idh-1*, changed to be homozygous from heterozygous in accordance with the mutation. The result of genotypic mutation in the current investigation conformed to the estimated linkage pairs of allozyme and sex determination loci.

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