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Original Research Article

Development of a SCAR Marker for Sex Identification in Asparagus

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Abstract - A sex-linked random amplified polymorphic DNA (RAPD) marker was identified from *Asparagus officinalis* L. and was converted into a sequence-characterized amplified regions (SCAR) marker for the large-scale screening of male and female plants. A total of 100 arbitrary decamer oligonucleotide primers were used for the RAPD analysis. Among them, the primer UBC347 amplified one female-specific 400 base pair DNA. Subsequently, the amplified RAPD fragment was cloned and sequenced. The fragment was abundant in AT and shared sequence homology with retrotransposon elements. On the basis of the sequence obtained, a pair of SCAR primer was designed. The amplification product, named F400, was the same size as the respective RAPD fragment from which it was derived. The F400 SCAR marker resulted to be female-specific in the three asparagus varieties tested in this study. This SCAR marker can be used for an early and rapid identification of female and male plants during breeding programs of asparagus.

Key words - *Asparagus*, Dioecious, RAPD marker, SCAR

Introduction

Asparagus (*Asparagus officinalis* L.) is an important perennial species with several functional substances and is native to Europe and Eastern Asia. Asparagus rarely produces hermaphroditic flowers and has homomorphic sex chromosomes (Telgmann-Rauber *et al.,* 2007; Jamilena *et al.,* 2008). The cultivated asparagus is dioecious with 1:1 sex ratio plant (male: M/m, female: m/m). Male plants are desired for yield, uniformity, and crop longevity; besides they do not produce seeds which give 'asparagus weed' in the production fields (Yeager and Scott, 1938; Ellison, 1986). On the other hand, it takes 2~3 years to distinguish male and female plants until asparagus plants flower. Therefore, early selection of a male asparagus among young seedlings is very important. Rapid identification of male and female asparagus varieties become also one of the main goals for breeding of this vegetable crop. If it is a purpose of super-male plants in breeding programs, the time generally takes about 5~6 years containing test cross processes (Reuther, 1984). Sexual dimorphism is known apparently controlled by gene(s) located on a pair of homomorphic L5 chromosomes in asparagus (Löptien, 1979). Telgmann-Rauber *et al.* (2007) determined and characterized genetic and physical maps around the sex-determining M-locus of the dioecious plant asparagus. Molecular markers have several advantages over traditional phenotypic markers. They can be detected in all phases of plant growth, whereas the latter can be identified only at a specific stage. The use of molecular markers can also improve the efficiency of cross-breeding because they are not modified by the environment. Their development has been greatly improved through the use of random amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Del Rio and Bamberg, 2000; Kim *et al.,* 2004), restriction fragment length polymorphism (RFLP) (Lízal and Relichová, 2004; Zhong *et al.,* 2003), amplified fragment length polymorphism (AFLP) (Lin and Kuo, 1995; Negi *et al.*, 2000), and simple sequence repeats (SSR) (Powell *et al.,* 1996). The RAPD technique, combined with BSA (bulked segregant analysis), can identify markers that are closely linked to economically important traits (Michelmore

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et al., 1991; Paran and Michelmore, 1993). To facilitate MAS (marker-assisted selection), RAPD markers have been converted into valuable SCAR (sequence-characterized amplified region) markers (Barzen *et al.,* 1997; Gill *et al.,* 1998; Kim *et al.,* 2000; Vidal *et al.,* 2000). In case of asparagus, sex identification through the induction of early flowering was previously performed (Abe and Kameya, 1986; Yanosaka *et al.,* 1989). Since molecular marker techniques were developed, male- or female-specific molecular markers also have been developed for asparagus breeding program (Jamsari *et al.,* 2004; Lu *et al.* 2006; Gao *et al.,* 2007; Gebler *et al.,* 2007).

In this study, we report a SCAR marker to identify female and male plants in the dioecious asparagus.

Materials and Methods

Seeds of asparagus cultivars 'Welcome', 'Greentower' and 'Shower' were supplied by Sakata Seed Co. in Yokohama, Japan. Plants were cultivated from 2001 to 2004 at National Research Center for Climate Change, National Institute of Horticultural and Herbal Science, Rural Development Administration (NIHHS, RDA), Korea. Asparagus is a dioecious plant species containing separate staminate flowers and pistillate flowers on different individuals. Staminate flower looks superficially hermaphroditic, but its pistil size is much smaller than female flower due to a developmental arrest. In contrast, pistillate flowers develop only pistil without stamen (Fig. 1). We collected three male and female plants from three varieties after flowering and immediately frozen in liquid nitrogen and stored at -70℃. Total DNA was purified from approximately 0.5 g of fresh leaves using the protein precipitation method of Dellaporta *et al.* (1983).

RAPD analysis was performed with random decamer primers (Biotechnology Laboratory, University of British Columbia, Canada; UBC 301 to 400). Twenty nanograms of DNA was used as template in a total reaction volume of 25 ml that contained 2.5 ml of 10 × reaction buffer, 2.5 mM $MgCl_2$, 400 mM of each dNTP, 4 pM of each primer, and 2.0 units *Taq* DNA polymerase (MBI Fermentas, St. Leon-Roth, Germany). Amplification was performed in a Gene Amp PCR System 9600 Thermal Cycler (Perkin Elmer Cetus,

Table 1. A SCAR primer designed from sequences of RAPD-PCR fragmant produced by UBC347 primer

Primer name	Primer sequences (5'→3')	Amplicon size (bp)
F400	F, 5'-TTGCTTGGCGGATCATATTATG-3' R, 5'-TTGCTTGGCGATGTCCCTTTTG-3'	400

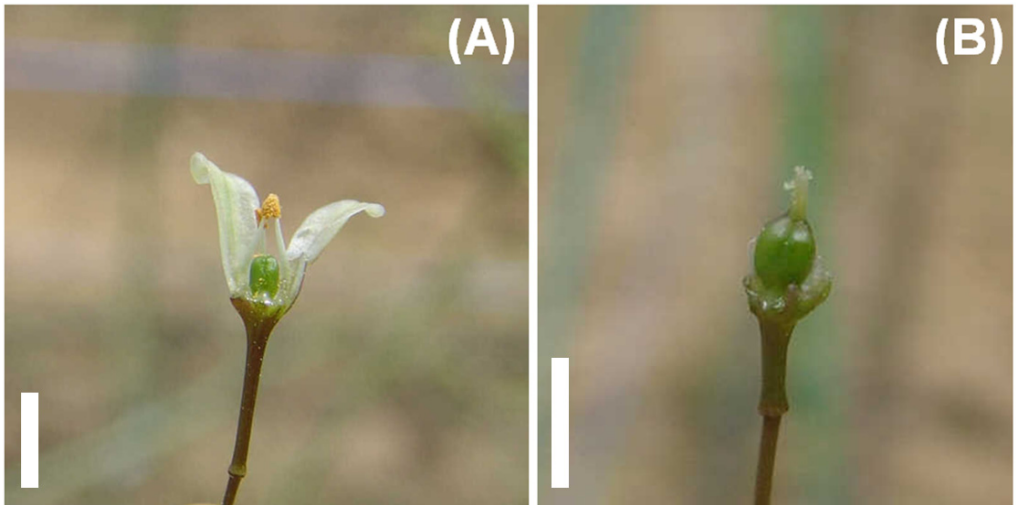

Fig. 1. Male (A) and female flowers (B) of asparagus. In case of female flower, petal was completely removed to show ovule and stigma. Scale bar = 5 mm.

USA), with initial denaturation for 5 min at 94℃, then 30 cycles of 1 min at 94℃, 1 min at 38℃, and 1.5 min at 72℃; followed by 5 min of final extension at 72℃. The PCR products were separated on 1.2% agarose gels at 100 V for 40 min using 1 × Tris-borate-EDTA (TBE) buffer, and finally stained with ethidium bromide. After amplification, each RAPD marker fragment was purified with 1 × TBE (Tris-borate/EDTA) buffer on 2% low-melting-point agarose (Promega, USA) gel. After staining with ethidium bromide, the agarose block containing the DNA was excised from the gel under low-wavelength UV light. DNA was recovered using a Wizard® PCR Preps DNA purification system (Promega, USA), according to the manufacturer's protocol (Soltis and Soltis, 1997).

DNA fragments were cloned into the pT-Adv vector, with the aid of an AdvanTAge™ PCR Cloning Kit (Clontech, CA), according to the manufacturer's instructions. Nucleotide sequences of the cloned fragment were determined using Cy5-labeled vector primers -- M13 -40 and M13 reverse -- and a Cy5™ AutoCycle™ Sequencing Kit (Pharmacia, USA) on an ALFexpress II DNA Sequencer. The samples were separated on 6% acrylamide-7 M urea gels in 0.5 × TBE buffer at 1500 V for 700 min. Sequences were aligned with CLUSTAL W (Thompson et al., 1994) and then adjusted manually to align several conserved regions. Sites with missing data or gaps were excluded from all analyses. Four specific primers were designed from the sequences of the marker fragments. Each primer comprised a 10 base pair (bp) of original RAPD primer sequence and an additional 12 to 14 bp of internal sequence. The PCR reaction mixture (25 ml) contained 2.5 ml of 10 × reaction buffer, 2.5 mM $MgCl_2$, 400 mM of each dNTP, 4 pM of each primer, and 2.0 units *Taq* DNA polymerase. Amplification was performed in a GeneAmp PCR System 9600 Thermal Cycler, with initial denaturation for 5 min at 94℃; followed by 1 min at 94℃, 1 min at 57℃, and 1.5 min at 72℃ (total of 30 cycles); with 7 min of final extension at 72℃. The PCR products were separated on 1.2% agarose gels at 100 V for 40 min using 1 × TBE buffer before being stained with ethidium bromide.

Results and Discussion

We utilized a RAPD approach to find sex-linked molecular markers from asparagus. Out of 100 primers screened (UBC300 to UBC400), only two primers UBC319 and UBC347 generated polymorphism PCR bands between male and female plants. The UBC347 primer produced a 400 bp fragment $UBC400_{347}$ linked to female plants (Fig. 2). However, UBC319 primer showed a problem in reproducibility. Therefore, we performed further study only for PCR product amplified with UBC347 primer.

Since RAPD markers show poor reproducibility, they have to be converted into SCAR markers. For this purpose, we cloned and determined its nucleotide sequence and compared sequences of $UBC400_{347}$ fragments from female plants (Fig. 3A). A 24-mer SCAR primer was designed on the basis of the UBC319 RAPD primer which amplified specific fragments only in male plants and a 22-mer SCAR primer was designed on the basis of the UBC347 RAPD primer which amplified specific bands only in female plants. The primer named F400 (Table 1).

The fragment amplified with UBC347 primer was abundant in AT with a 38% of low GC content. BlastX search against NCBI nr database search using the nucleotide sequences from $UBC400_{347}$ showed that it shared a 45% sequence identity (76% sequence similarity and 3e-12 of E-value) with a putative retrotransposon protein of rice, belonging to Ty1-copia subclass (Fig. 3B). Various types of retroelements have been found in heteromorphic or homomorphic sex chromosomes of dioecious plants (Fraser and Heitman 2005; Telgmann-Rauber *et al.,* 2007; Urasaki *et al.,* 2012). In this context, $UBC400_{347}$ fragment is likely a part of female-specific

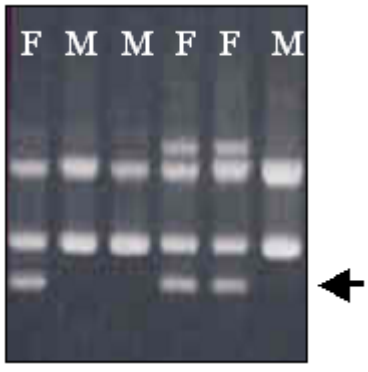

Fig. 2. RAPD marker UBC347 linked to sex. Arrow indicates a specific RAPD band for female plants.

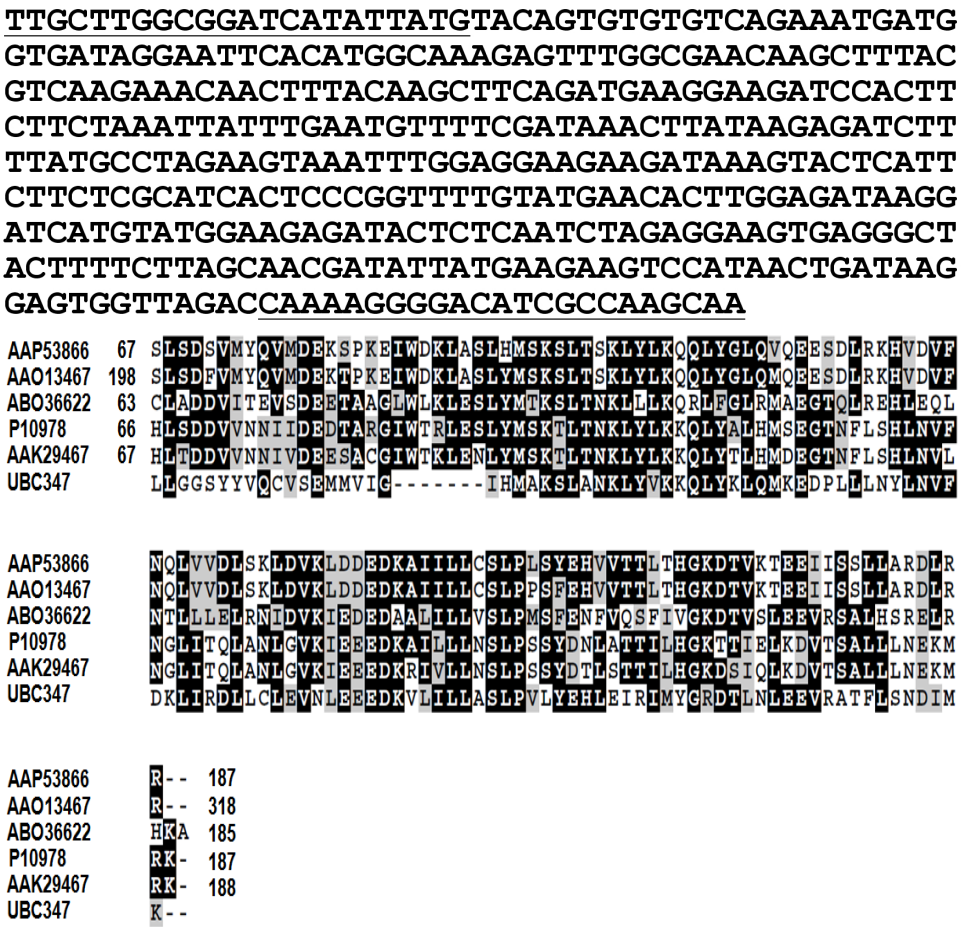

Fig. 3. Nucleotide sequences of a female specific fragment amplified with UBC347 primer and multiple sequence alignment. (A) Nucleotide sequences of a female specific fragment amplified with UBC347 primer. Underlines indicate primer sequences for SCAR primers. (B) Multiple sequence alignment of $UBC400_{347}$ fragment and plant retrotransposon proteins belonging to UBN2 superfamily. The sequence alignment and conserved residues were displayed using the CLUSTALW and the BOXSHADE programs. Identical amino acid sequences are highlighted in black, and similar sequences in grey. The retrotransposon protein sequences used in this alignment are as follows: UBC347, *Asparagus officinalis* L. (in this study, GenBank Acc. No. JX508289); *Oryza sativa* Japonica Group (GenBank Acc. Nos. AAP53866 and AAO13467, respectively); *Solanum lycopersicum* (GenBank Acc. No. ABO36622); *Nicotiana tabacum* (GenBank Acc. No. P10978); *Solanum chilense* (GenBank Acc. No. AAK29467).

retroelements.

We tested the SCAR primer in eighteen male/female plants of the three cultivars ('Welcome', 'Greentower' and 'Shower'). PCR amplification with the SCAR primer F400 revealed a 400 bp fragment with the same size as the cloned RAPD marker, which was female-specific in all the plants belonging to the three cultivars (Fig. 4).

There is no way to identify morphological differences between male and female at seedling stages (Kubituki and Rudall, 1998). And then, sex-linked molecular markers in asparagus are not only important for breeding but also for understanding the genetic mechanism of sex determination. SCAR markers are also advantageous over RAPD markers because they detect only a single locus and their amplification is less sensitive to reaction conditions (Weeden, 1994). Several sex-linked molecular markers have been identified from asparagus (Jiang and Sink, 1997; Lu *et al.,* 2006; Gao *et al.,* 2007; Gebler *et al.,* 2007).

To obtain sex-linked diagnostic markers and to better understand molecular and evolutionary mechanism of sex determination in asparagus, a BAC library was constructed and several BAC clones containing sex-linked markers were

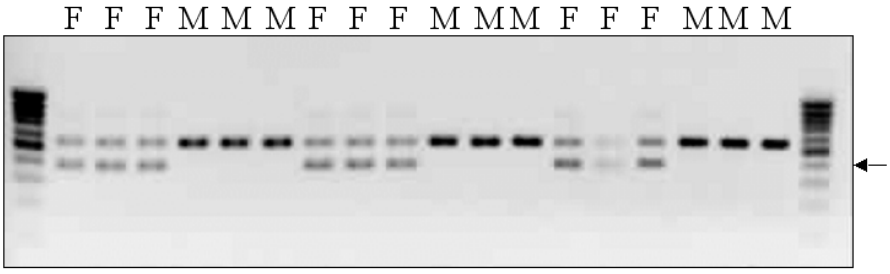

Fig. 4. Identification of sex using SCAR primer F400 for male and female plants of asparagus. Lane 1 and Lane 20, GeneRuler™ 100-bp DNA Ladder Plus; Lane 2 to 4, female of cv. 'Welcome'; Lane 5 to 7, male of cv. 'Welcome'; Lane 8 to 10, female of cv. 'Greentower'; Lane 11 to 13, male of cv. 'Greentower'; Lane 14 to 16, female of cv. 'Shower'; Lane 17 to 19, male of cv. 'Shower'. Arrow indicates a specific female fragment amplified with a SCAR primer F400.

identified (Jamsari *et al.,* 2004). Telgmann-Rauber *et al.,* (2007) determined genetic and physical maps around the sex-determining M-locus using 4 closely linked BAC clones and also found an accumulation of repetitive sequences and a low gene density in the sex-determining region $UBC400_{347}$ fragment has no sequence homology with any other nucleotide sequences derived from asparagus on NCBI nr database and previously published literatures, indicating that the fragment is a new female-linked molecular marker located on a novel locus. Jiang and Sink (1997) reported that SCAR markers linked to the sex expression locus M from asparagus could be used for only one parents and their progeny. But F400 SCAR marker was found to be applicable to three asparagus germplasm. We suggest that the SCAR marker will be a useful marker for cultivation and breeding of a new variety in three asparagus varieties ('Welcome', 'Greentower' and 'Shower').

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