RAPD-based analysis of differences between male and female genotypes of *Asparagus officinalis*

Y. Ii[1], A. Uragami[2], Y. Uno[1], M. Kanechi[1], N. Inagaki[1]

[1]*Plant Resource Science, Graduate School of Agricultural Science, Kobe University, Kobe, Japan*
[2]*National Institute of Vegetable and Tea Science, National Agriculture and Food Research Organization, Tsukuba, Japan*

Abstract

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Asparagus (*Asparagus officinalis* L.) plants are dioecious. All-male cultivars are desired because of their higher yields. To increase the proportion of male individuals planted in the field and expedite the breeding of all-male cultivars in asparagus, development of generally applicable molecular markers to distinguish male and female individuals is required. Bulked genomic DNA samples from ten male (XY) and ten female (XX) plants was screened with 10-bp random primers. Of the 188 primers tested, the primer T35R54 produced a 1600-bp fragment observed only in male individuals. The specificity of this T35R54-1600 marker was verified using DNA from one supermale (YY) and one female (XX) breeding line and their four F_1 progenies (XY). The T35R54-1600 marker fragment was observed in both supermale and all-male lines. The sequence of the T35R54 primer (5'-TTCACGGTGG-3') was absent among the sequences of primers or amplified fragments from previous studies. Therefore, this marker could be useful as a sex-related marker in future studies to increase the reliability of sex determination in asparagus.

Keywords: all-male; bulked segregant analysis; RAPD; sex marker; supermale

Asparagus (*Asparagus officinalis* L.) is an economically important perennial dioecious vegetable crop. Male plants show higher yields and vigor than those of female individuals and do not create a weed problem that results from seed production by female plants in the field (Ellison 1986). However, gender determination of seedlings must be waited for till the flowering, which takes 1–2 years after transplanting (Sneep 1953). The sex chromosomes of asparagus are designated X and Y. Female plants are homogametic (XX), whereas males are heterogametic (XY). Supermales (YY) are desirable to produce all-male asparagus progenies and ensure female plants are not transplanted into the field. Self-pollination of andromonoecious plants (Sneep 1953), or doubled haploid lines produced by anther and/or microspore culture (Falavigna 1979; Inagaki et al. 1980; Torrey, Peirce 1983; Falavigna et al. 1990, 1996; Shiga et al. 2009) made it possible to breed supermale asparagus plants. A population composed of male and female plants of the same cultivar exhibits high heterogeneity, and production of inbred lines takes many years (Ellison 1986).

The development of molecular markers linked to sex-determining chromosome segments would enable simplification and promotion of the breeding of supermale individuals. Many attempts to iden-

tify genetic markers linked to sex determination in asparagus were undertaken. Such studies utilized isoenzyme markers (Maestri et al. 1991), restriction fragment length polymorphism (RFLP) markers (Biffi et al. 1995), random amplified polymorphic DNA (RAPD) markers (Gebler et al. 2007), RAPD and sequence-characterized amplified region (SCAR) markers (Jiang, Sink 1997), and amplified fragment length polymorphism (AFLP) and/or SCAR markers (Reamon-Büttner et al. 1998; Reamon-Büttner, Jung 2000; Jamsari et al. 2004; Nakayama et al. 2006; Shiobara et al. 2011). Almost all previous reports described Y-linked markers except for one report of an X-linked marker by Gebler et al. (2007). However, application of all of these markers was limited to experimental populations. Nakayama et al. (2006) modified the Y-linked SCAR marker Asp1-T7 (Jamsari et al. 2004) and applied the modified marker to commercial cultivars. It is important to develop additional sex-related molecular markers in asparagus because the marker sequence might vary among cultivars. A polymerase chain reaction (PCR)-based RAPD analysis is one of the simplest and easiest methods to retrieve markers. In this study, we investigated the newly identified Y-linked RAPD marker in combination with bulked segregant analysis (BSA) of a commercial asparagus cultivar. A haploid individual raised from a polyembryonic seed, and supermale and all-male lines were used to verify the utility of the marker.

MATERIAL AND METHODS

Plant material

Asparagus officinalis cv. Mary Washington 500W (Takii & Co., Kyoto, Japan) was used as a representative asparagus cultivar in this study. A haploid plant raised from a polyembryonic seed (Uno et al. 2002), ten male and ten female plants were selected from among progeny of cv. Mary Washington 500W for isolation of genomic DNA. The cultivar Zuiyu was selected as a representative of an all-male line. Cv. Zuiyu represents the F_1 progeny from the cross cvs Zuiyou-2 × ZM-19 and was bred for commercial production in a specific region of Japan (Uragami et al. 2011). The parental breeding lines were used as representatives of female and supermale genotypes, respectively. These stocks or seeds were kindly provided by the National Agricultural Research Center for the Hokkaido Region. All plant populations were grown in the experimental field of the Faculty of Agriculture, Kobe University.

Extraction of genomic DNA

Genomic DNA was extracted using the Nucleon Phyto Pure Plant DNA extraction kit (GE Healthcare, Little Chalfont, UK) or the CTAB method with minor modification (Doyle 1991). Female and male bulked-DNA samples were created by pooling an equal amount of DNA from each of the ten male and female individuals of cv. Mary Washington 500W.

PCR analysis for male-specific marker selection

A total of 188 random 10-bp primers were used to screen for DNA markers linked to the Y chromosome. The male and female bulked-DNA samples (cv. Mary Washington 500W) were used to screen each RAPD primer. The primers that produced distinctive bands only from male bulked DNA were selected for further analysis of additional genotypes. The RAPD reaction was carried out with MIR-D40 (Sanyo, Tokyo, Japan) or GeneAmp PCR System 9700 (Perkin Elmer, Waltham, USA) thermocyclers using the following conditions: denaturation for 4 min at 95°C, followed by 40 cycles of 94°C for 50 s, either 27, 31, 35, 40, 44 or 48°C for 50 s and 72°C for 2 min, and final elongation at 72°C for 10 min. The PCR reactions with each primer were carried out in a mixture containing 80 ng template DNA, 0.2mM dNTPs, 1× γTaq buffer, 1.0μM primer, and 0.5 U γTaq (Toyobo, Osaka, Japan).

Amplified products were electrophoresed alongside a molecular weight marker on a 1.5% agarose gel in 1× TBE buffer and stained with ethidium bromide. Gels were observed and photographed under ultraviolet light.

RESULTS AND DISCUSSION

To identify RAPD markers that differentiate male and female individuals, BSA was carried out. The male and female bulked-DNA samples were used to screen 188 random primers. Fig. 1 shows an example of RAPD banding patterns using eight primers. The banding patterns of the male and female bulked

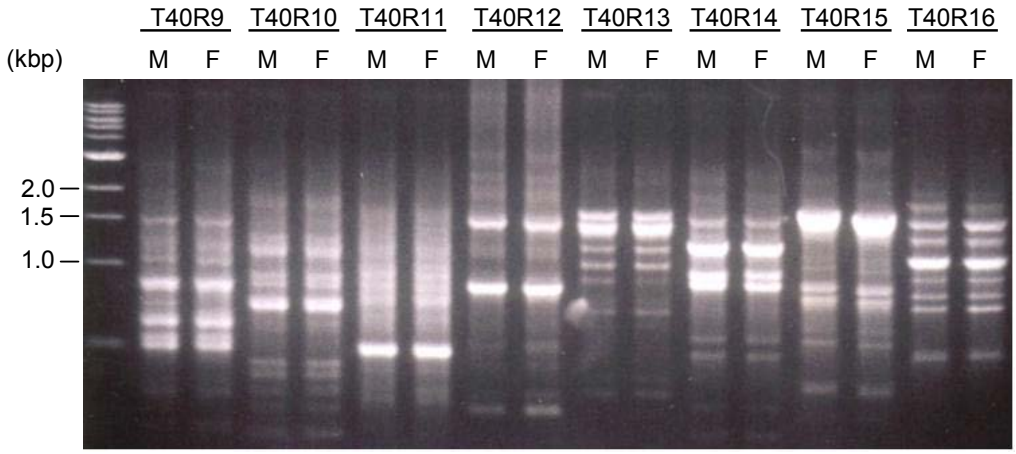

Fig. 1. RAPD banding patterns from bulked-DNA samples isolated from male and female progeny of *Asparagus officinalis* cv. Mary Washington 500W. M and F represent DNA bulked from ten male and ten female individuals, respectively. The leftmost lane represents a molecular marker. RAPD products were generated with eight primers from T40R9 to T40R16

samples were identical for each primer, but banding patterns varied among the primer sets. Twenty-five bands specific to male or female plants amplified by 16 primers were observed (data not shown). These 16 primers were selected for further analysis of additional asparagus genotypes to evaluate their efficacy to distinguish the male and female sexes.

Of the 16 random primers, 15 primers showed different results from the BSA, i.e. no correlation with male and female sex was observed. This finding might be a result of unstable annealing of the primers to bulked heterologous DNAs and bias during amplification. Only the T35R54 primer (5'-TTCACGGTGG-3') distinguished the sexes. The amplified PCR product was about 1600-bp in length at 35°C annealing temperature. Each individual showed different banding patterns because of heterogeneity (Fig. 2). The 1600-bp fragment was observed in all ten males, but not in the ten females and the haploid. The sex of the haploid was unknown because the plant did not survive to flowering. It was expected to be a female as the haplotype was derived from a homogametophytic (XX) female plant. Indeed, it was reported that all haploids raised from polyembryonic seeds of asparagus produce only pistillate flowers (Randall, Rick 1945). These results indicated that the T35R54 fragment (T35R54-1600) was located on the Y chromosome in cv. Mary Washington 500W.

The utility of the T35R54-1600 fragment as a sex marker was tested by analyzing other asparagus cultivars and breeding lines possessing sex-specific genotypes. Cv. Zuiyu is an all-male hybrid raised by crossing cv. Zuiyou-2 and the supermale cv. ZM-19. Among these genotypes, T35R54-1600 was amplified from genomic DNA of cvs ZM-19 and Zuiyu but not from cv. Zuiyou-2 (Fig. 3). This result confirmed that T35R54-1600 might be located on the Y chromosome in asparagus, and has potential utility as a sex-related marker among asparagus cultivars. Although RAPD markers are sensitive to reaction

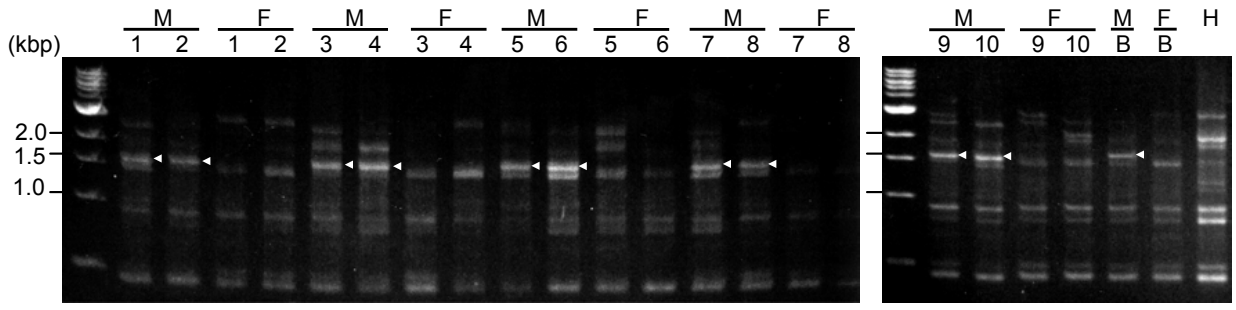

Fig. 2. RAPD banding patterns from DNA of *Asparagus officinalis* cv. Mary Washington 500W generated with the primer T35R54. M, F and H represent male, female, and haploid, respectively. Each number indicates an individual plant. Bulked DNA samples (B) were pooled from each of ten male and ten female individuals. The leftmost lane represents a molecular marker. The male-specific 1600-bp fragment (T35R54-1600) is indicated by white arrowheads

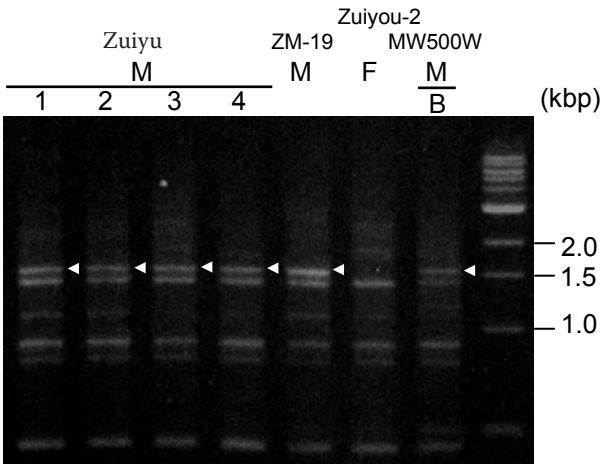

Fig. 3. Verification of the male-specific RAPD marker in additional asparagus cultivars and a breeding line showing sex-specific genotypes in *Asparagus officinalis* L. M and F represent male and female, respectively. Each number represents an individual plant of the cultivar Zuiyu (all-male). Individuals of ZM-19 (supermale) and Zuiyou-2 (female), the parent lines of Zuiyu, were analyzed. Bulked DNA (B) from ten male individuals of cv. Mary Washington 500W (MW500W) was used as a positive control. The rightmost lane represents a molecular marker. The male-specific 1600-bp fragment (T35R54-1600) is indicated by white arrowheads

conditions (Jiang, Sink 1997), the T35R54 primer showed stable amplification in the present study. If newly identified markers are confirmed with normal genotypes, it takes some years to test their utility because samples must be prepared after determination of the sex by flowering. However, shoots of seedlings could be used in the case of sex-specific genotypes. To our knowledge, this is the first report that utilization of haploid and supermale lines enabled rapid verification of the specificity of RAPD markers in asparagus.

A number of previous studies investigated sex-related markers in asparagus (Maestri et al. 1991; Biffi et al. 1995; Jiang, Sink 1997; Reamon-Büttner et al. 1998; Reamon-Büttner, Jung 2000; Jamsari et al. 2004). However, the markers identified were able to distinguish male and female individuals only in a single population. For example, the Y-linked SCAR marker identified in a segregating population was not applicable to other clones (Jiang, Sink 1997). The Asp1-T7 marker (Jamsari et al. 2004) failed to detect male specificity in some male individuals of the commercial cv. Mary Washington 500W (Nakayama et al. 2006). A modified sequence-specific marker amplified with the Asp1-T7spf and Asp1-T7spr primers successfully distinguished male and female individuals among six commercial cultivars and two breeding lines (Nakayama et al. 2006). The T35R54 marker was identified from a commercial cultivar and our results demonstrate it is applicable to other asparagus cultivars or breeding lines.

Although the amplified 1600-bp fragment was not cloned or sequenced, the sequence of the T35R54 primer (5'-TTCACGGTGG-3') was not present among the sequences of primers or amplified fragments from previous studies (OC15 primer, Jiang, Sink 1997; Asp1-T7 primers, Jamsari et al. 2004; 396-bp fragments amplified with Asp1-T7spf and Asp1-T7spr primers, Nakayama et al. 2006; E31M56, E41M50, and E33M53 from AFLP adaptor primers, Reamon-Büttner et al. 1998). Our results indicate the T35R54-1600 fragment might be useful as a sex-related marker for other asparagus cultivars. Its use in conjunction with other molecular markers will improve the reliability of distinction between male and female individuals. Moreover, markers are often utilized in multiplex PCR with additional primer sets to distinguish PCR error, diagnose infectious diseases, and/or discriminate sex. The use of a combination of primer pairs that amplify overlapping fragments offers the possibility to improve the sensitivity and specificity of multiplex PCR analysis.

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Corresponding author:

Yuichi Uno, Ph.D., Kobe University, Graduate School of Agricultural Science, Plant Resource Science, Rokko, Kobe 657-8501, Japan
phone: + 81 78 803 5832, e-mail: yuno@kobe-u.ac.jp