Identification of Homozygous Male Plants by Quantitative Analysis of a Nucleotide Sequence Linked to the Sex-determination Locus in Asparagus officinalis L.

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Discrimination of homologous and heterozygous plants at the male-determination locus in asparagus (Asparagus officinalis L.) was examined by quantitative analyses of a diagnostic marker for male asparagus. The quantitative difference of a sequence, AspTaq1, derived from the Asp1-T7 diagnostic marker (Jamsari et al., 2004), was measured using the real-time monitoring PCR and normalized based on the quantitative difference of a sequence, AODEF-Taq1, derived from a single copy gene, AODEF. The normalized difference was higher in established supermales than in regular males grown from commercially available seeds. The averaged values in respective groups are in the approximate ratio of 2:1. Homozygous male (MM), heterozygous male (Mm), and female (mm) genotypes were obtained by selfing of an andromonoecious line. The progeny were divided into three groups by the normalized difference; plants showing high values and intermediate values were thought to be homozygous and heterozygous males, respectively. Significant amplification of AspTaq1 was not detected in several plants; therefore, they were judged to be females. Then, to identify supermales, pollen from male plants was crossed separately with female plants, and the sex of the next generation was determined. Pollen from two male plants showing high values of the normalized difference generated only males in the next generation, while pollen from 12 male plants showing intermediate values generated both female and male plants. Pollen from one male plant that showed intermediate values generated only male plants. The normalized difference in homozygous males was comparable to the value obtained using a recombinant sequence that contains an equal number of AspTaq1 and AODEF-Taq1. These results indicate that homozygous and heterozygous males can be identified by quantitative analysis of a nucleotide sequence linked to the sex-determination locus and that the use of an authentic sequence for calibration facilitates the discrimination process of those genotypes in asparagus plants.

Key Words: garden asparagus (Asparagus officinalis), homozygous male, quantitative PCR, sex-determination locus, supermale.

Introduction

Garden asparagus (Asparagus officinalis L.) is a dioecious plant, whose young shoots are utilized worldwide as a vegetable (Drost, 1997). In economic production, male plants are mainly cultivated because they have higher marketable yields (Ellison and Schermerhorn, 1958; Moon, 1976), small but regular shoot size (Ellison and Sheer, 1959), and earlier sprouting in spring (Ellison and Schermerhorn, 1958; Ellison et al., 1960). Male plants are also advantageous for maintaining in fields; they do not produce seeds that will germinate and grow into weedy plants. Thus, producing hybrid seed populations consisting of only males (all-male hybrid seeds) has been attempted (Ellison and Kinelski, 1985).

Conventionally, female and male asparagus plants have been distinguished only by flower morphology. The sex-determining locus has been known to lie on homomorphic chromosome pair LV (Löptien, 1979; for review see Jamilena et al., 2008), and a dominant gene (M) at the locus determines the male phenotype (Marziani et al., 1999; Uno et al., 2002). Females are recessive homozygous (mm) and regular males are...
heterozygous (Mm) at the sex-determining locus, while homozygous males (MM) are viable and fertile, like heterozygous males. For producing hybrid seed populations containing only males, the pollen grains from homozygous male lines at the sex-determination locus (MM) are crossed with female plants. These homozygous male lines are therefore called supermale by breeders. Supermale lines can be established from andromonoecious asparagus plants through self-fertilization of hermaphrodite flowers and the selection of homozygous males (Sneep, 1953); however, homozygous males are morphologically indistinguishable from heterozygous males, even at the flowering stage. Thus, to identify homozygous males, the occurrence of only males in the next generation must be tested after crossing with females. The whole procedure to establish a useful supermale line therefore requires many years and much effort (Sonoda et al., 2003; Nakayama et al., 2006).

Recently, a few sets of PCR primers that amplify nucleotide sequences tightly linked to the sex-determination locus have been reported (Gebler et al., 2007; Jamsari et al., 2004, Nakayama et al., 2006; Reamon-Büttner and Jung, 2000). Molecular markers associated with the female gender have also been reported (Gebler et al., 2007), although trials are very limited and their applicability to detect the m-locus remains to be examined. Thus, we considered that only markers linked to the male determination locus are presently reliable for establishing a molecular marker-based method to select homozygous males in asparagus.

From a hereditary system proposed for sex determination in asparagus (Löptien, 1979), the molecular ratio of the male-determination gene in homozygous and heterozygous males is inferred to be 2 to 1, and the dose level of the male-determination locus in diploid asparagus genomes is thought to depend on this ratio. We expected that heterozygous and homozygous males might be discriminated by quantitative analysis of the male-specific nucleotide sequence. In this study, we examined the applicability of QR-PCR (quantitative real-time PCR or real-time monitoring PCR; Higuchi et al., 1993), which has been employed for determination of gene copy numbers (De Preter et al., 2002), to estimate the relative number of male-determination loci in diploid asparagus genomes. We constructed recombinant DNA that contains an authentic sequence combined with a reference sequence to normalize measurements and tried to use it to estimate the dose level of the male-determination locus. The reliability and reproducibility of diagnosing supermales by the dose level of a nucleotide sequence linked to the male-determination gene will be discussed.

Materials and Methods

Plant materials

Asparagus (Asparagus officinalis L.) plants grown from seeds and maintained through vegetative propagation were used throughout experiments. Female and male plants of ‘Mary Washington 500W’ and ‘New Jersey 264’ were grown from commercially available seeds (Nozaki Seeds, Sapporo, Japan). A supermale line, ‘MM’, and female and male lines of ‘Gold Schatz’ were maintained at an experimental farm of Hokkaido University through vegetative propagation. An andromonoecious line of ‘Gijnlim’ was a gift from Mr. Y. Minagawa (Training Center for Agricultural Technology, Biei, Hokkaido, Japan) and grown at an experimental farm.

DNA extraction from asparagus cladophylls

Young cladophylls (60–100 mg) were harvested from plants, frozen in liquid nitrogen, and pulverized with a mortar and pestle. The powder was homogenized in ten times volume of medium consisting of 20 mM MES-KOH, pH 5.8, 0.5 mM spermidine, 0.15 mM spermine, 1.0% (w/v) Triton X-100, 150 mM KCl, and 4 mM EDTA. Removing divalent cations caused erosion of DNA from nuclei, which could be avoided by the addition of polyamines (Willmitzer and Wagner, 1981). The homogenate was centrifuged at 12,000 g for 10 min at 4°C. The supernatant was discarded, and DNA was extracted from the pellet with Plant DNAzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. DNA was dissolved in TE solution (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored at −40°C until use.

Quantitative real-time PCR

Primers were designed to amplify a nucleotide sequence (AspTaql) of 146 bp within Asp1-T7 (Jamsari et al., 2004), a diagnostic marker for male asparagus plants. The sequence of primers was as follows: AspTaqlF, 5'-GGCGAGGCTTTGAAAGCAC-3'; AspTaqlR, 5'-AA GGCTATCCACTCTCTCAACAG-3'. For normalization, a partial sequence (AODEF-Taql) of 97 bp within AODEF (accession no. AB180962; Park et al., 2003) was amplified using the following primers: AODEF-TaqlF, 5'-CCATGATCTCCGTCTTGCATG-3'; AODEF-TaqlR, 5'-AATTAAGGTGTAAGCATAGTGCTAG-3'. TaqMan probes (URL: http://www.roche-diagnostics.jp/products/md/taq/pcr/index.html), Asp-probe F (5' FAM- AAATCAAGGACTACTCTGCACTGAGGAC-3'), Asp-probe H (5'HEX-AACAGTACT TCACCCCTACCTGACAAGTG-3') for AODEF-Taql were used for the detection of amplification during PCR. Fluorescent dyes liberated from quenchers were monitored using a quantitative PCR analyzer, Chromo 4 (MJ Research, Waltham, Ma, USA), and the data were analyzed using Opticon Monitor v.3 (MJ Research). The levels of AspTaql were normalized based on levels of AODEF-Taql. The normalized difference was calibrated with the value obtained for ‘New Jersey 264’ (NJ 264).
Construction of a plasmid containing authentic sequences

A partial sequence of Asp1-T7 was ligated with the 5’ terminus of a nucleotide sequence derived from AODEF after adding the cleavage site for Hind III. Additional sequences for cleavage were introduced by PCR using primers 5’-GAGCTACTCCACGGTGCAAC-3’ (AoS3A-F) and 5’-GTAAGCTTTGCTATGGGTAATA GTTGTC-3’ (AoS3A-RH) to amplify the internal sequence of Asp1-T7, and 5’-TGAAAGCTTACACACAG GATTTGGCTGGTAG-3’ (Aodef-2FH) and 5’-GTT GGTGTAATTCCCTAGT-3’ (Aodef-2R) for the internal sequence of AODEF. The whole sequence, which includes Asp1-Taq1 and AODEF-Taq1, was then amplified by PCR using primers AoS3A-F and Aodef-2R. The PCR product was inserted into pGEMT-Easy vector (Promega Corp., Madison, USA) using the TA-cloning method, and transformed into Escherichia coli strain DH5-alpha. The plasmid (designated pT7DEF) was extracted from E. coli and used to determine the dose of AspTaq1.

Identification of female and male asparagus plants

Male plants included in the selfed progeny were crossed with a female line derived from ‘Franklin’ to determine whether they are homozygous or heterozygous genotypes at the sex-determination locus. Pollen grains were sampled from the plants in their second and third year after germination. The gender of plants in the next generation was judged by amplification of an Asp1-T7-derived diagnostic marker, Asp1-T7_sp, using a set of PCR primers 5’-ATATGCGAGGCATTTGGAAG-3’ and 5’-CTGTAGCAGGGGGTAGGTGA-3’. DNA was extracted from cladophylls as described above and used as a template for PCR. DNA extracted from male plants amplified a 216-bp fragment, which was detected with agarose gel electrophoresis.

Morphology of flowers

Flowers were sampled from the plants and examined under a dissecting microscope. Images were captured with a CCD camera (DP-12; Olympus, Tokyo). Dimensions of flower organs were measured on captured images using the computer program Canvas v.8.

Determining the dose of AspTaq1

The dose level of AspTaq1 in the selfed progeny plants numbered 1–21 (see the ‘Results’ section), an andromonoecious line of ‘Gijnlim’ that was used for obtaining those progeny plants, and a male plant of ‘New Jersey 264’ was determined. The dose level, which means the copy number of a nucleotide sequence in a genome, was calculated from the normalized differences and their ratios, assuming that the dose of pT7DEF is 1. Male plants with a dose value ≤0.693 were grouped into heterozygous males and those with values >0.693 were grouped into homozygous males. This threshold was obtained as the x-coordinate in the intersection of two normal distribution lines that represent distribution at μ = 0.5 with a variance of 0.01 and μ = 1.0 with a variance of 0.04. Plants showing values <0.307 were grouped into females.

Results

Quantitative analysis of a male determination locus-linked DNA sequence

We compared quantitative differences of a male-specific sequence, AspTaq1, in a supermale line, MM and female and male plants grown from commercially available seeds. The values are presented as ratios to male plants in cultivar ‘NJ 264’ (Table 1). Normalized differences in a supermale line ranged from 1.88 to 2.00, while those of regular males ranged from 1.00 to 1.19, showing an approximate ratio of 2 to 1 in the AspTaq1 amount. All amplified DNA appeared as single bands at the expected positions upon agarose gel electrophoresis (data not shown). DNA from females did not show significant amplification of AspTaq1 in PCR (Table 1).

Identification of homozygous and heterozygous males

Selfed progeny were obtained from a ‘Gijnlim’ andromonoecious line. To ensure self-fertilization, the rhizome was transferred to a greenhouse in fall, and plants were grown from sprouts until fruit set in the greenhouse. Quantitative differences in AspTaq1 of progeny were measured and normalized (Table 2). Significant amplification of AspTaq1 was detected in plants numbered 1, 3, 5, 6, 8–13, and 15–20. Normalized differences in plants 1 and 15 were higher than those in other plant lines. The difference in the value between plants 1 and 15 was not significant.

To know the dose levels of AspTaq1 in each plant, normalized differences were calibrated based on the value in pT7DEF, which includes equal numbers of AspTaq1 and AODEF-Taq1. Doses of AspTaq1 in plants 3, 5, 6, 8–13, and 16–20 ranged from 0.40 to 0.63 (Table 2), and those in 1 and 15 were very close to 1. Accordingly, these two groups that showed different dose

Table 1. Quantitative analysis of a male determination locus-linked DNA sequence in female, male and supermale asparagus plants.

<table>
<thead>
<tr>
<th>Cultivars/Lines</th>
<th>Phenotype</th>
<th>Normalized difference (average ± SD, n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NJ 264&lt;sup&gt;4&lt;/sup&gt;</td>
<td>female</td>
<td>0.00 ± 0.01</td>
</tr>
<tr>
<td>MJ 264&lt;sup&gt;4&lt;/sup&gt;</td>
<td>male</td>
<td>1.00 ± 0.03</td>
</tr>
<tr>
<td>MW500W&lt;sup&gt;5&lt;/sup&gt;</td>
<td>female</td>
<td>0.07 ± 0.09</td>
</tr>
<tr>
<td>MW500W&lt;sup&gt;5&lt;/sup&gt;</td>
<td>male</td>
<td>1.05 ± 0.06</td>
</tr>
<tr>
<td>GS&lt;sup&gt;6&lt;/sup&gt;</td>
<td>female</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>GS&lt;sup&gt;6&lt;/sup&gt;</td>
<td>male</td>
<td>1.19 ± 0.35</td>
</tr>
<tr>
<td>MM&lt;sup&gt;6&lt;/sup&gt;</td>
<td>supmale</td>
<td>1.94 ± 0.10</td>
</tr>
</tbody>
</table>

<sup>4</sup> ‘New Jersey 264’.
<sup>5</sup> ‘Mary Washington 500W’.
<sup>6</sup> ‘Gold Schatz’.
<sup>6</sup> Established supermale line ‘MM’. 
levels of AspTaq1 were estimated to correspond to heterozygous (Mm) and homozygous (MM) genotypes with respect to the male-determination locus, respectively. PCR using DNA from plants 2, 4, 7, 14, and 21 as a template did not amplify AspTaq1 significantly, and hence these progeny plants were identified as female.

**Identification of supermale plants**

All progeny were grown to flowering. Of these, plants 2, 4, 7, 14, and 21 bore female flowers, as expected. All other progeny had male flowers and therefore heritability of gender in these plants to the next generation was examined to determine whether they were homozygous or heterozygous genotypes on the sex-determination locus. Pollen grains from male plants were applied separately to female flowers in a greenhouse. Mature fruits that developed from pollinated flowers were harvested, and plants were grown from the seeds in order to identify the gender. Finally, 2 to 13 plants were obtained for each pollinator. Their gender was identified upon electrophoresis of PCR products amplified using a diagnostic marker, Asp1-T7_sp (Fig. 1).

The progeny generated using pollen from plants 3, 5, 6, 8–12, and 16–20 included female plants, while progeny generated using pollen from 1, 13, and 15 were all male plants. This indicated that 1, 13, and 15 are supermales. Electrophoretograms of the amplified PCR products showed that PCR amplified a DNA fragment with the expected length of 216 bp (data not shown).

**Morphology of flowers born on an andromonoecious plant and its progeny plants**

Homogynous and heterozygous males at the male-determination locus bore flowers with similar morphology (Fig. 2A). Plants 2, 4, 7, 14, and 21 bore typical female flowers with a developed ovary and degenerated stamina (Fig. 2A). Additionally, we found no significant variation in flowers borne on the andromonoecious plant that had been used to obtain a series of female, homogynous male, and heterozygous male progeny. Some dimensions representing the pistil shape varied very close to the normal distribution (quantitative data not shown, but see Fig. 2B-1 for flower appearance), which indicated that hermaphrodite and male flowers cannot be distinguished by morphology until ovarian development begins.

Expression of the andromonoecious phenotype in the

Table 2. Identification of homozygous males based on quantitative analysis of a nucleotide sequence linked to male-determination locus in Asparagus officinalis.

<table>
<thead>
<tr>
<th>Individual number</th>
<th>Phenotype (Male/Female)</th>
<th>Normalized difference (average ± SD, n = 9)</th>
<th>Dose/genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.94 ± 0.17</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.02 ± 0.04</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.05 ± 0.08</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.01 ± 0.00</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.97 ± 0.07</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.75 ± 0.07</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.01 ± 0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.02 ± 0.08</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.12 ± 0.09</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1.00 ± 0.08</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1.11 ± 0.05</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.80 ± 0.05</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1.02 ± 0.04</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.05 ± 0.00</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1.91 ± 0.16</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1.15 ± 0.20</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>0.95 ± 0.04</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>1.17 ± 0.19</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>1.06 ± 0.04</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.94 ± 0.00</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>0.00 ± 0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Gijnlim-Ax</td>
<td>1.10 ± 0.07</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>NJ264x</td>
<td>1.00 ± 0.03</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>pT7DEF</td>
<td>—</td>
<td>1.87 ± 0.03</td>
<td>1</td>
</tr>
</tbody>
</table>

* Progeny generated by selfing of an andromonoecious plant of ‘Gijnlim’.

† An andromonoecious line of ‘Gijnlim’ used for obtaining a series of progeny.

‘New Jersey 264’.

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**Fig. 1.** Identification of supermales in progeny obtained by selfing of andromonoecious asparagus. Progeny generated by self-fertilization of an andromonoecious line of asparagus were grown to flowering, and pollen was collected separately from each plant to pollinate female plants. The plants in the following generation were subjected to gender identification by amplification of a DNA-based diagnostic marker, Asp1-T7_sp, using PCR. Numbers in boxes correspond to those of individual plants indicated in Table 2. Electrophoretograms enclosed by a box indicate PCR products derived from different plants originating from the same parents.
progeny generated through self-pollination was also followed. Of these progeny, heterozygous male plants numbered 3 and 17 (Fig. 2B-3) bore many fruits, which was comparable to the mother plant (Fig. 2B-2), despite less fruit setting than female plants (Fig. 2B-4). Plants 13 and 15 had only a few fruits (data not shown). These supermales were thus thought to be andromonoecious.

**Discussion**

Quantitative determination of a DNA marker linked to the male-determination locus in the sex chromosome allele combined with analysis of gender in progeny plants revealed that QR-PCR is applicable for identifying homozygous and heterozygous males in garden asparagus. When applying QR-PCR to discriminate heterozygous and homozygous males, the use of the pT7DEF plasmid, which carries sequences for measuring and normalizing AspTaq1 amounts, improved the reliability. Incorporating an authentic sequence was thought to facilitate comparison of data from separate trials using different markers and/or dyes and allow for efficient interpretation of correct results. The AspTaq1 dose levels in regular males grown from commercially distributed seeds were approximately half the values recorded for pT7DEF. This indicates that the diagnostic marker resides as a single copy DNA sequence in the Y chromosome-containing genome and confirms that the heterozygous genotype on sex chromosomes (XY) represents the male flower phenotype.

Conventional procedures for identifying supermales require observations of sex expression through two generations (Sonoda et al., 2003). The observation that some homozygous and heterozygous males from an andromonoecious line produced fruits implies that all-male hybrid seed populations contain seeds expressing an andromonoecious phenotype. We are far from understanding the technology to inactivate pistils in male asparagus flowers; thus, we must employ an empirical approach and consider a large number of progeny to establish superior pollinators that produce all-male hybrids without expressing the andromonoecious phenotype. The diagnostic method presented here used only progeny vegetative tissues in the generations after selfing and will greatly reduce the time and effort required for selecting supermale plants. Therefore, this method will be most useful when applied to an initial selection step, as most males can then be excluded through screening. Early selection of homozygous males is expected to reduce the number of plants that must be examined in the field and facilitate the establishment of useful supermale lines.

A male plant numbered 13 showed a dosage level corresponding to heterozygous plants with respect to the AspTaq1 amount but represented a supermale genotype in tests to check the sex of progeny plants. This result obviously contradicts our assumption, and we speculate that some chromosomal aberration in the Y chromosome occurred during meiosis or fertilization. Deletion of a relatively long region including AspTaq1 or, more markedly, loss of the entire Y-chromosome from the homozygous male genome may have caused the type of variation found in plant 13. It is known that recombination occurs between the sex-determining locus and close markers in asparagus (Falavigna et al., 1983;
Reamon-Büttner and Jung, 2000; Telgmann-Rauber et al., 2007); however, amplification of AspTaq1 dosage levels between other heterozygous and homozygous males indicates that the variance in plant 13 was not due to experimental variations or the method per se. Other approaches will be required to elucidate the event in plant 13. Determining the nucleotide sequence around AspTaq1 and results of fluorescence in situ hybridization (FISH) (de Melo and Guerra, 2001; Reamon-Büttner et al., 1999) may provide a clue to revealing the case example that occurred in plant 13. Apart from our interest, this type of variant may be practically eliminated from the population after screening homozygous males or supermales.

Along with establishing an efficient method for identifying supermales, a control to bias pistil function toward suppression or complete loss in all male hybrid seeds is desired. Morphologically, male flower differentiation in asparagus occurs during the late stages of maturation. Degeneration of the pistil in developing flowers causes male flowers, and hence a fully developed ovary and style with stigma remain (Bracale et al., 1990; Caporal et al., 1994; Marziani et al., 1999). The morphological similarity of hermaphrodite, homozygous male, and heterozygous male flowers implies that the hermaphrodite flower results from a limited modification of pistil functions only during development. Identifying genes involved in the degeneration of pistil function in male flowers may advance our knowledge about pistils that remain active in hermaphrodite flowers of andromonoecious asparagus plants and provide insight into how we can bias pistil function towards complete inactivation in flowers of all-male hybrid seed-derived plants.

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Literature Cited


