Molecular Tagging of the Ms Locus in Onion

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ABSTRACT. Cytoplasmic-genic male sterility (CMS) is used to produce hybrid onion (Allium cepa L.) seed. For the most widely used source of onion CMS, male sterility is conditioned by the interaction of the male-sterile (S) cytoplasm and the homozygous recessive genotype at a nuclear male-fertility restoration locus (Ms). Maintainer lines are used to seed propagate male-sterile lines, possess normal (N) male-fertile cytoplasm, and are homozygous recessive at the Ms locus. Due to the biennial nature of onion, it takes 4 to 8 years of crossing and scoring of progeny phenotypes to establish if maintainer lines can be extracted from an uncharacterized population or family. Identification of nuclear markers tightly linked to the Ms locus would allow for molecular-facilitated selection of maintainer lines. We evaluated testcross progenies from a segregating family for nuclear restoration of male fertility over at least three environments. Although segregations in the F2, family fit the expected 1:2:1 ratio ($P = 0.973$), the proportion of male-sterile testcross progenies showed significant ($P < 0.01$) year effects and it is therefore imperative to score male-fertility restoration over environments. Too many male-sterile testcross progenies were often observed, indicating that the dominant allele conditioning male-fertility restoration for S cytoplasm may not show complete penetrance. Segregations of amplified fragment length polymorphisms and restriction fragment length polymorphisms (RFLPs) revealed RFLPs flanking the Ms locus at 0.9 and 8.6 cM. An onion cDNA showing highly significant homology to the aldehyde dehydrogenase conditioned by the e2 locus of maize was identified and mapped to linkage group I, independent of the Ms locus. A sample of commercial onion germplasm was evaluated for putative allelic diversity at the RFLP loci linked to Ms. The genomic region corresponding to the cDNA (AOB272) revealing the closest RFLP to Ms was sequenced to reveal numerous single nucleotide polymorphisms. Single-stranded conformational polymorphisms and single nucleotide extensions were developed that revealed genomic variation at AOB272-EcoRI. The use of these molecular markers to select maintainer lines in onion is discussed.

The production of hybrid onion (Allium cepa L.) seed is economically feasible using systems of cytoplasmic-genic male sterility (CMS). The male-sterile (S) cytoplasm, first described by Jones and Emsweller (1936), is the primary source of onion CMS used worldwide. Male fertility in plants with S cytoplasm is restored by a dominant allele at the nuclear male-fertility restoration locus (Ms). Maintainer lines are used to seed propagate male-sterile lines, possess normal (N) male-fertile cytoplasm, and are homozygous recessive at the Ms locus ($S_{msms}$). Male-sterile inbred lines are seed propagated by crossing with a maintainer line possessing normal (N) male-fertile cytoplasm and the homozygous recessive genotype at the restorer locus ($N_{msms}$). Although examples of asexual propagation of individual male-sterile plants exist (Jones et al., 1949; Pike and Yoo, 1990), the large-scale production of hybrid onion seed is dependent on the availability of maintainer lines ($N_{msms}$) to seed-propagate male-sterile ($S_{msms}$) lines (Jones and Davis, 1944). Extraction of maintainer lines from some onion populations, e.g. the cultivars ‘Texas 1015Y’ (United States), ‘Sapporo-Ki’ (Japan), or ‘Pukekohe Longkeeper’ (New Zealand), has not been successful because of a high frequency of the dominant allele at Ms (Davis, 1957; Havey and Randle, 1996; Little et al., 1944) or the prevalence of S cytoplasm (Havey, 1993; Havey and Bark, 1994; Satoh et al., 1993). As a result, few hybrid cultivars have been developed from economically important onion populations.

Due to the biennial generation time of onion, 4 to 8 years are required to determine if maintainer lines ($N_{msms}$) can be extracted from an uncharacterized population or segregating family (Havey, 1995). The nuclear and cytoplasmic genotypes can be classified as $N_{msms}$, $N_{MsMs}$, or $S_{MsMs}$ by testcrossing to a male-sterile plant ($S_{msms}$), self pollinating the male-fertile plant, and separately scoring the male fertility of S, and testcross progenies. The same procedure will produce all male-fertile S, and testcross progenies for $N_{MsMs}$ and $S_{MsMs}$. For these genotypes, a maintainer plant ($N_{msms}$) must be crossed with $N_{MsMs}$ and $S_{MsMs}$ plants and progenies from these crosses self-pollinated and scored for male fertility to establish their cytoplasms.

Polymorphisms in the chloroplast and mitochondrial DNAs distinguishing N and S cytoplasms of onion have been identified (de Courcel et al., 1989; Havey, 1993; Holford et al., 1991; Satoh et al., 1993) and allow breeders to establish cytoplasms without crosses. Havey (1995) and Sato (1998) developed molecular
markers revealed by the polymerase chain reaction (PCR) and it now takes only hours, as opposed to years, to establish the cytoplasm of a single onion plant. Cytoplasmic determinations are especially important given that open-pollinated populations may exclusively or predominantly possess S cytoplasm (Havey, 1993; Havey and Bark, 1994) and will commonly occur after hybrid onion seed has been retained and used to develop open-pollinated populations.

The development of maintainer lines would be greatly facilitated by identifying molecular markers flanking the nuclear Ms locus. Such markers would allow breeders to retain only those plants that are maintainers (\( N_{msms} \)) or can be used to develop maintainer lines (\( N_{Ms} \)). Reducing the numbers of plants to be testcrossed to a male-stereile line. In this study, we evaluated amplified fragment length polymorphisms (AFLPs) and restriction fragment length polymorphisms (RFLPs) for linkage to the Ms locus, assessed putative allelic diversity for molecular markers tightly linked to Ms, and converted the closest linked RFLP to PCR-based markers.

**Materials and Methods**

A segregating population of 58 F\(_2\) onion families from BYG15-23 \( \times \) AC43 has been described (King et al., 1998a). Individual F\(_2\) plants or F\(_3\) families were testcrossed to one of five male-stereile S-cytoplasmic lines (MSU611-1A \( \times \) MSU611B, MSU5718A \( \times \) MSU8155B, B1731A \( \times \) MSU5785B, B3350A \( \times \) B2352B, or B1828A) using standard techniques (Pike, 1986). At least 20 testcross bulbs were grown, vernalized, randomly selected for flowering, randomly assigned to field plots, and scored for male-fertility restoration by visual inspection of umbels over at least 3 years in Madison, Wis. (1995 to 2000). The significance of years and family by year interactions were tested using SAS (SAS Institute, Cary, N.C.) and adjusted mean proportion of male-stereile testcross progenies calculated. Genotypes of F\(_2\) families at the Ms locus were based on segregations for male-fertility restoration among testcross progenies. A second segregating family (1064B \( \times \) 1616C) was a gift (Dr. Richard Jones, Seminis Seed Co.) and used to establish segregation of additional polymorphic fragments revealed at AOB272-EcoRI.

Genomic DNAs of parental inbreds (BYG15-23 and AC43) and the F\(_3\) families were extracted from leaf tissue bulked from at least 50 seedlings and purified through CsCl gradients (Havey, 1991). Segregations of some RFLPs in the BYG15-23 \( \times \) AC43 family were previously described (King et al., 1998a). We used an onion RFLP map developed by the former Native Plants, Inc. (Gilroy Foods, Inc.) and made available by Gilroy Foods, Inc., to identify additional onion cDNAs [Allium Gilroy Food (AGF) 109, AGF120, AGF122, AGF125, AGF136, AGF147, AGF148, AGF152, AGF158, AGF161, AGF166, AGF167, AGF173, and AGF176 or Allium Gilroy (AGI) 063, AGI101, AGI106, AGI128, AGI131, AGI151, AGI156, and AGI178] revealing RFLPs putatively linked to the Ms locus. These cDNAs were hybridized first to EcoRI, EcoRV, and HindIII digests of BYG15-23 and AC43 DNAs; second to BamHI, BglII, DraI, KpnI, PstI, PvuII, SacI, XbaI, and XhoI digests; and third to AluI, Apal, Ascl, AvaI, AvaiI, BanI, BglII, BstEII, DdeI, FspI, HaeIII, HinII, MboI, MspI, Nael, Ncol, NdeI, NotI, SalI, Sall, Scal, SpeI, SwaI, and XmaI digests to reveal RFLPs segregating in the BYG15-23 \( \times \) AC43 mapping family and evaluated for linkage to Ms. All cDNAs were hybridized to DNAs of elite commercial inbreds or populations (King et al., 1998b) to determine the numbers of polymorphic fragments revealed by each probe–enzyme combination. RFLP loci were named using the probe (e.g., AOB272), restriction enzyme used to reveal the polymorphism (E1 = EcoRI, E5 = EcoRV, D1 = DraI, or H3 = HindIII), and the sizes in kilobases of the segregating DNA fragments for AC43 and BYG15-23.

A 1900-bp full-length onion cDNA (ACB75-jm8.e05, Genbank accession AF465823) showing highly significant homology to the aldehyde dehydrogenase encoded by the r2 locus of maize (Cui et al., 1996) was identified during random sequencing of cDNAs from an onion bulb cDNA library (McCallum and Pither-Joyce, unpublished) and was hybridized to digests of the 32 restriction enzymes listed above. Segregations of an RFLP revealed by SwaI were established using the BYG15-23 \( \times \) AC43 mapping family.

For AFLPs, we initially evaluated DNA from the parental inbreds and a subset of 11 DNAs, four families known to be homozygous dominant, three heterozygous, and four homozygous recessive at Ms. AFLPs segregating in these 11 F\(_3\) family DNAs were evaluated using all F\(_3\) family DNAs. The AFLP protocol was as reported by Vos et al. (1995). Preselective EcoRI (5'-CTCGTAGACTGCTGTAACC-3' and 5'-AATTGGTACGCAGTCTAC-3') and Msel (5'-GAGCAGTAGCTCCTGAG-3' and 5'-TCTCAGAGGCTCAT-3') primers having a single selective nucleotide and eight selective EcoRI primers (AAC, AAG, ACA, ACC, ACG, ACT, AGC, AGG) and eight selective Msel primers (CAA, CAC, CAG, CAT, CTA, CTC, CTG, GTT) carrying three selective nucleotides were purchased from Gibco-BRL. A PsiI adapter (5'-TGTCACGCAGTCTAC-3') and a preselective PsiI primer having a single selective nucleotide (5'-CTCGTAGACTGCTGTAACC-3') and five selective PsiI primers carrying three selective nucleotides (GGA, GGT, GGG, GAG, GAT) were synthesized and evaluated with the eight selective Msel primers.

Segregations of AFLPs, RFLPs, and male-fertility restoration were tested for goodness-of-fit ratios by chi-square analyses. Linkage arrangements were estimated using MapManager XP version b13 (Manly and Olson, 1999). A likelihood ratio statistic over 20 was considered as significant for the F\(_3\) family (Manly et al., 2001).

The cDNA clones revealing RFLPs linked to Ms were sequenced as described by Lilly and Havey (2001). Oligonucleotides AOB272-F (5'-GCCAATTTGCAACTTCTTTCAAG-3') and AOB272-5R (5'-CATATTCTTCTTCACTACCA-3') specific to the ends of cDNA AOB272 were designed using Oligo 6.1 (Molecular Technology) and used to amplify genomic regions from AC43, BYG15-23, and a diverse set of seven elite onion populations (Table 1). PCR reaction conditions were 1.5 mM of MgCl\(_2\), 0.2 mM each dNTPs, 1 mM of each primer, 1 unit Gold Taq DNA polymerase (Applied Biosystems), and 100 ng of DNA in 50 mL final volume. PCR cycles were 10 min at 94 °C, then 40 cycles at 30 s at 94 °C, 45 s at 45 °C, and 3 min at 68 °C, and a final extension for 15 min at 72 °C. PCR products were loaded on a 1% agarose gel and visualized by EtBr staining (Sambrook et al., 1989). Amplified genomic DNAs were cloned by TA tailing using the pCR 2.1 vector (Invitrogen), transformed into bacteria, and plated on LB medium with ampicillin (Sambrook et al., 1989). Five transformed bacterial colonies from each of the nine onion populations were selected, plasmid DNAs were isolated, and inserts were sequenced. Raw sequences in both directions were edited and contigs formed
Results and Discussion

Segregation at the Ms locus. King et al. (1998a) previously reported segregation at the Ms locus in the cross BYG15-23 x AC43 scored for male-fertility restoration from at least 20 testcross bulbs at one location (Madison, Wis.) in 1 of 3 years (1994 to 1996). No RFLPs were linked closer than 13.8 cM to Ms. Because male-fertility restoration may be affected by the environment (van der Meer and van Bennekom, 1969), we evaluated male-fertility restoration using these same testcross families over at least three environments (1995 to 2000 at Madison, Wis.). The putative scores of 12 F₂ plants were changed, four from homozygous recessive to heterozygous, six from heterozygous to homozygous dominant, one from homozygous dominant to heterozygous and one from unclassified to homozygous dominant [segregations reported by Gökçe (2001)]. Segregations at the Ms locus fit the expected 1:2:1 ratio (14:28:13; P = 0.973). Individual plants in some testcross families appeared to be male-sterile early during dehiscence, but shed pollen after over half the flowers in the umbel had dehisced. Across environments, 17 out of 28 testcross families generated using paternal plants scored as heterozygous at Ms (Fig. 2) segregated for significantly more male-sterile than male-fertile progenies. Three out of nine families reported by Jones and Clarke (1943) showed segregations significantly different from the expected and, as in our study, they also reported tendencies towards too many male-sterile progenies. For parental plants heterozygous at the Ms locus, we observed significant (P < 0.01) year effects for the proportion of male-sterile testcross progenies, indicating that male-fertility restoration was affected by the environment. The family × year interaction was not significant. Our experiences indicate that the tendency to observe too few male-fertile plants may be due to scoring too early the restoration of male fertility, scoring within only one environment, and/or reduced penetrance of the dominant allele at Ms. We highly recommend scoring male-fertility restoration in S cytoplasm over environments and over the entire dehiscence period of each umbel.

 AFLP segregations. Sixty-four EcoRI and MseI primer pairs and 40 PstI and MseI primer pairs were evaluated using DNAs of 11 F₂ families, four each of MsMs and msms and three Msms. For EcoRI and MseI primer pairs, approximately 20 to 40 of 200 to 250 fragments were polymorphic among this subset of four progenies. For PstI and MseI primer pairs, 30 to 50 of 100 to 150 fragments were polymorphic among the 11 progeny families. Although numerous AFLPs segregated among the families, we identified only three AFLPs consistent with known segregations
Fig. 1. Genomic DNA sequences for the two major alleles at AOB272. Positions and sequences of oligonucleotides for detection of single nucleotide polymorphisms (SNP) are shown in bold. Specific SNPs are underlined. Upper line is AC43 sequence; lower is BYG15-23 sequence.

Fig. 2. Mean proportion of male-sterile progenies adjusted for years (y-axis) for all testcross families from BYG15-23 x AC43 mapping population (King et al., 1998a). Family numbers are shown on the x-axis. Assigned genotypes for parental plants at the Ms locus were homozygous dominant (gray), heterozygous (black), or homozygous recessive (white). Segregations of 1:1 were expected for parental plants scored as heterozygous at Ms and significantly greater numbers of male-sterile progenies were observed for means from 17 out of 28 testcross families from heterozygous parents. Bars show experiment-wide standard errors.
at Ms for the 11 families, a 158-bp fragment from primer pair E-ACCT and M-CAG, a 120-bp fragment from primer pair E-AGG and M-CTC, and a 335-bp fragment from primer pair P-GAG and M-CAT. Segregations of these AFLPs fit the expected 3:1 ratio (P = 0.637, 0.084, and 0.432, respectively); however, none were closely linked (<10 cM) to Ms. Although we were optimistic that the AFLP technique would enable us to establish closer linkages to Ms because of the significantly greater numbers of fragments, as compared to RFLPs and randomly amplified polymorphic DNA (RAPD) markers, we experienced difficulties scoring segregations due to the plethora of closely migrating fragments. Van Heusden et al. (2000) successfully mapped AFLPs in onion using DNA (RAPD) markers, we experienced difficulties scoring segregations due to the plethora of closely migrating fragments. Van Heusden et al. (2000) successfully mapped AFLPs in onion using

### Table 2. Sequence homologies, Genbank accession numbers, and sizes of polymorphic DNA fragments revealed by onion cDNAs revealing restriction fragment length polymorphisms linked to the Ms locus in onion.

<table>
<thead>
<tr>
<th>cDNA clone</th>
<th>Restriction enzyme</th>
<th>Genbank accession no.</th>
<th>Putative homology</th>
<th>Sizes of allelic fragments</th>
<th>Anonymous fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJK295</td>
<td>HindIII</td>
<td>AA451599</td>
<td>ACC oxidase</td>
<td>3.0, 20.0</td>
<td>4.3, 5.0, 18.0</td>
</tr>
<tr>
<td>AOB186</td>
<td>EcoRV</td>
<td>AA451597</td>
<td>Ubiquitin</td>
<td>2.5, 6.7</td>
<td>6.0</td>
</tr>
<tr>
<td>AOB210L</td>
<td>HindIII</td>
<td>AA451567</td>
<td>Rubisco</td>
<td>8.0, 9.0</td>
<td>Numerous</td>
</tr>
<tr>
<td>AOB232</td>
<td>EcoRV</td>
<td>AA451596</td>
<td>Sterol methyl-transferase</td>
<td>24.0, 30.0</td>
<td>4.0, 35.0</td>
</tr>
<tr>
<td>AOB272</td>
<td>EcoRI</td>
<td>AA451592</td>
<td>Sexual differentiation protein</td>
<td>6.0, 10.0, 12.0</td>
<td>14.0, 5.0</td>
</tr>
<tr>
<td>API27</td>
<td>EcoRI</td>
<td>AA451547</td>
<td>No hits</td>
<td>3.0, 7.0</td>
<td>None</td>
</tr>
<tr>
<td>API63</td>
<td>EcoRV</td>
<td>AF366456</td>
<td>Phosphoglucosomutase 2</td>
<td>5.0, 7.5</td>
<td>Numerous</td>
</tr>
<tr>
<td>API65L</td>
<td>DraI</td>
<td>AA451551</td>
<td>Rubisco</td>
<td>3.0, 3.5</td>
<td>Numerous</td>
</tr>
</tbody>
</table>

*L* = long fragment from putative chimeric cDNA clone. AOB210L corresponds to fragment from 178 to 407 bp; API65L corresponds to fragment from 427 to 746 bp.

Known alleles at RFLP locus were established by King et al. (1998a) or this research. Anonymous fragments were polymorphic among a sample of elite commercial inbred lines (King et al., 1998b) and may be alleles at these loci or at other loci. Fragment sizes are in kilobases.

Because testcross families tended to possess too many male-sterile progenies (Fig. 2), misclassification of genotypes would affect the placement of Ms on the onion linkage map. We treated male-fertility restoration as a quantitative trait and scanned for linkage groups showing significant effects. Only the region adjacent to AOB272-EcoRI showed highly significant effects (likelihood ratio statistic = 112), in agreement with the qualitative trait mapping (Fig. 3).

An RFLP (ACB75-jm8.e05-Swal-10.0/12.0) revealed by the onion cDNA homologous to the aldehyde dehydrogenase encoded by the rf2 locus of maize (Cui et al., 1996) segregated independently of Ms, mapping to the end of linkage group I (LOD > 4) terminal to API10 (King et al., 1998a). This clone gave simple banding patterns across numerous restriction enzymes and is therefore not likely duplicated in the onion genome. Moller (2001) proposed that aldehyde dehydrogenase may play a general role in restoring male fertility when CMS is caused by mutations compromising mitochondrial electron transport; however in onion, variation at this structural locus does not explain any significant component of the phenotypic variation for male-fertility restoration.

**Sequence variation at AOB272.** The sequence of cDNA AOB272 (Genbank accession AA451592) showed significant homology to the yeast ISP4 protein (P1D g792859) associated with sexual differentiation (Sato et al., 1994) and an Arabidopsis homolog (AY054590). The AOB272 genomic region was amplified using primers

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Fig. 3. Linkages among restriction fragment length polymorphisms (RFLPs) and Ms locus of onion. Genetic distances in centiMorgans shown on left. Nomenclature for RFLPs described in Materials and Methods. Only the center portion of linkage group B (King et al., 1998a) is shown.
Table 3. Single nucleotide polymorphisms in the AOB272 genomic region for nine onion populations.

<table>
<thead>
<tr>
<th>Population</th>
<th>Nucleotide position in AOB272 genomic sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC43</td>
<td>T  A  C  T  A  T  C  A  A  C  C  T  A  G  A</td>
</tr>
<tr>
<td>CN1</td>
<td>T  A  T  T  A  T  C  A  A  T  A  T  A  G  A</td>
</tr>
<tr>
<td>CN2a</td>
<td>T  A  G  T  A  T  C  A  A  T  A  T  A  G  A</td>
</tr>
<tr>
<td>CN6</td>
<td>T  A  T  T  A  T  C  A  A  T  A  T  A  G  A</td>
</tr>
<tr>
<td>BYG15-23</td>
<td>A  G  T  A  C  T  T  G  G  T  A  C  A  T  G</td>
</tr>
<tr>
<td>CN2b</td>
<td>A  G  G  A  C  T  T  G  G  T  A  T  A  T  G</td>
</tr>
<tr>
<td>CN8</td>
<td>A  G  C  A  C  C  T  G  G  T  A  T  A  T  G</td>
</tr>
<tr>
<td>PLK</td>
<td>A  G  G  A  C  T  T  G  G  T  A  T  A  T  G</td>
</tr>
<tr>
<td>SK1a</td>
<td>A  G  G  A  C  T  T  G  G  T  A  T  A  T  G</td>
</tr>
<tr>
<td>SK1b</td>
<td>A  G  G  A  C  T  T  G  G  T  A  T  A  T  G</td>
</tr>
</tbody>
</table>

Notes: Positions refer to Genbank accession number AF366455.

Fig. 4. Single-stranded conformational polymorphisms amplified from the AOB272 genomic region. The first lane contains size marker. The following lanes contain denatured PCR products from AC43, BYG15-23, CN1, CN2, CN6, CN7, and CN8 (left to right). Origins of populations are listed in Table 1.
Literature Cited


