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 *rf2* 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* (Jones and Clarke, 1943). Male-sterile plants possess S 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 popula-

tions, 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 S₁ and testcross progenies. The same procedure will produce all male-fertile S₁ 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 cytoplasm.

Polymorphisms in the chloroplast and mitochondrial DNAs distinguishing N and S cytoplasm 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 cytoplasm without crosses. Havey (1995) and Sato (1998) developed molecular

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable. The technical help of Mark Petrashek is gratefully acknowledged. This research was supported in part by the generous support of Gilroy Foods and the Novartis, Rio Colorado, Sakata, Seminis, and Shippo Seed Companies. A.F. Gökçe was supported by a fellowship from the Turkish Ministry of Education.

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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 Msms*), reducing the number of plants to be testcrossed to a male-sterile 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_3 onion families from BYG15-23 x AC43 has been described (King et al., 1998a). Individual F_2 plants or F_3 families were testcrossed to one of five male-sterile S-cytoplasmic lines (MSU611-1A x MSU611B, MSU5718A x MSU8155B, B1731A x MSU5785B, B3350A x 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-sterile testcross progenies calculated. Genotypes of F_2 plants at the *Ms* locus were assigned based on segregations for male-fertility restoration among testcross progenies. A second segregating family (1064B x 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 x AC43 family were previously described (King et al., 1998a). We used an onion RFLP map developed by the former Native Plants, Inc. (Salt Lake City, Utah) 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*, *ApaI*, *AscI*, *AvaI*, *AvaII*, *BanI*, *BglI*, *BstEII*, *DdeI*, *FspI*, *HaeIII*, *HincII*, *MboI*, *MspI*, *NaeI*, *NcoI*, *NdeI*, *NotI*, *SalI*, *ScaI*, *SpeI*, *SwaI*, and *XmnI* digests to reveal RFLPs segregating in the BYG15-23 x 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 *rf2* 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 x 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 among 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'-CTCGTAGACTGCGTACC-3' and 5'-AATTGGTACGCAGTCTAC-3') and *MseI* (5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3') primers having a single selective nucleotide and eight selective *EcoRI* primers (AAC, AAG, ACA, ACC, ACG, ACT, AGC, AGG) and eight selective *MseI* primers (CAA, CAC, CAG, CAT, CTA, CTC, CTG, CTT) carrying three selective nucleotides were purchased from Gibco-BRL. A *PstI* adapter (5'-TGTACGCAGTCTAC-3') and a preselective *PstI* primer having a single selective nucleotide (5'-CTCGTAGACTGCGTACATGCA-3') and five selective *PstI* primers carrying three selective nucleotides (GGA, GGT, GGG, GAG, GAT) were synthesized and evaluated with the eight selective *MseI* 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 0.10 (Manly et al., 2001). A second analysis treated male-fertility restoration as a quantitative trait and significant effects across the onion linkage map were estimated using MapManager QTX version b13 (Manly and Olson, 1999). A likelihood ratio statistic over 20 was considered as significant for the F_2 family (Manly et al., 2001).

The cDNA clones revealing RFLPs linked to *Ms* were sequenced as described by Lilly and Havey (2001). Oligonucleotide primers AOB272-F (5'-GCCAATTGCTAACTTACTCTTCAAG-3') and AOB272-5R (5'-CATATTTTCTCTCATACCAA-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

using Sequencer 3.1.2 (Genecodes). Putative homologies were assessed using TBLASTX searches of the EST databases.

Single nucleotide polymorphisms (SNPs) among the nine onion populations (Table 1) were identified after sequence alignments. Oligonucleotide primers were designed to flank eight of these SNPs [primers listed by Gökçe (2001) and in Fig. 1]. Allele specific primer pairs were used to amplify SNP alleles at AOB272 using PCR conditions of 2 mM of $MgCl_2$, 0.2 mM each dNTPs, 0.6 mM each primer, 0.6 unit Gold Taq DNA polymerase, and 20 ng sample DNA in 30 mL final volume. Annealing temperatures were varied taking into consideration the melting temperatures of oligonucleotides at 1.2 mM $MgCl_2$ as calculated by the Oligo 6.1 program. Cycling conditions were 30 s at 94 °C, 45 s at varied annealing temperatures (45 to 72 °C), and 90 s at 72 °C with cycles of 25, 30, 35, or 40 cycles.

Specific alleles at AOB272 were revealed by single nucleotide extensions using the SNaPshot (ABI) system. Genomic DNAs from the nine onion cultivars (Table 1) were amplified using the AOB272 forward and reverse primers and purified by column chromatography (Qiagen). Primers flanking eight SNPs were synthesized (Fig. 1). Single nucleotide extensions used 1× SNaPshot Ready Reaction Premix, 4 mM of the primer, 20 ng preamplified template DNA in a 5 mL final volume. Cycling conditions were 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s for a total of 25 cycles. After the single nucleotide extension, one unit of calf intestinal phosphatase was added to the PCR reaction mix, incubated for one hour at 37 °C, and heat inactivated at 72 °C for 15 minutes. One milliliter of the final product was mixed with 1.2 mL of formamide buffer and 0.3 mL of size standard for analysis on the ABI377 automatic sequencer. Peaks representing single nucleotide extensions were identified using the GeneScan 3.1 software.

Single stranded conformational polymorphisms (SSCP) were revealed as previously described by (McCallum et al., 2001) except that polymorphisms were resolved in 0.5× MDE gels (FMC BioProducts). Primers used to amplify the AOB272 region for SSCPs were 5'-TGTTGCTGTTCTTGCTTGG-3' and 5'-CACATTCCGTTAAGTAATTTGTTTCAG-3'.

Results and Discussion

SEGREGATION AT THE *Ms* LOCUS. King et al. (1998a) previously reported segregation at the *Ms* locus in the cross BYG15-23 ×

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 F_2 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 F_3 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

Table 1. Origins, cytoplasm, and genotypes at the *Ms* locus of onion populations evaluated for sequence variation at the AOB272 genomic region.

Population ^z	Origin ^y	Source ^x	Cytoplasm ^w	Genotype at <i>Ms</i> ^v
AC43	USDA	417-2	N	<i>Msms</i>
BYG15-23	USDA	125-1	N	<i>msms</i>
SK1a	USDA	16588	N	<i>MSMS</i>
SK1b	USDA	16664	N	<i>msms</i>
CN1	Asgrow	1604B	N	<i>msms</i>
CN2	Asgrow	1607B	N	<i>msms</i>
CN6	Asgrow	1673	S	<i>MsMs</i>
CN7	Asgrow	1674	S	<i>MsMs</i>
CN8	Asgrow	1616	S	<i>MsMs</i>
PLK	May-Ryan	NA	S	<i>MsMs</i>

^zAC43 and BYG15-23 are parents of the onion mapping family (King et al., 1998); SK1a and b are S_1 families from 'Sapporo-Ki' (Gökçe, 2001); CN1, 2, 6, 7, and 8 are short-day onion populations from provided by Asgrow Seed Company; and PLK is the open-pollinated population 'Pukekohe Longkeeper' from New Zealand.

^yAsgrow and May-Ryan are seed companies in the United States and New Zealand, respectively.

^xNA = source not available.

^wNormal (N) male-fertile or male-sterile (S) cytoplasm (Jones and Clarke, 1943).

^vGenotypes at *Ms* are homozygous dominant (*MsMs*), heterozygous (*Msms*), or homozygous recessive (*msms*).

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.

cDNA clone ^z	Restriction enzyme	Genbank accession no.	Putative homology	Sizes of allelic fragments ^y	Anonymous fragments ^y
AJK295	<i>Hind</i> III	AA451599	ACC oxidase	3.0, 20.0	4.3, 5.0, 18.0
AOB186	<i>Eco</i> RV	AA451597	Ubiquitin	2.5, 6.7	6.0
AOB210L	<i>Hind</i> III	AA451567	Rubisco	8.0, 9.0	Numerous
AOB232	<i>Eco</i> RV	AA451596	Sterol methyl-transferase	24.0, 30.0	4.0, 35.0
AOB272	<i>Eco</i> RI	AA451592	Sexual differentiation protein	6.0, 10.0, 12.0	14.0, 5.0
API27	<i>Eco</i> RI	AA451547	No hits	3.0, 7.0	None
API63	<i>Eco</i> RV	AF366456	Phosphoglucosyltransferase 2	5.0, 7.5	Numerous
API65L	<i>Dra</i> I	AA451551	Rubisco	3.0, 3.5	Numerous

^zL = 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.

^yKnown 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.

at *Ms* for the 11 families, a 158-bp fragment from primer pair E-ACT 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 ($P = 0.637, 0.084, \text{ and } 0.432$, 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 primers with additional selective nucleotides to reduce the numbers of fragments appearing on autoradiograms.

SEGREGATIONS AND SEQUENCE ANALYSES OF RFLPS NEAR THE *Ms* LOCUS. We evaluated for linkage to *Ms* using the segregation of previously described (King et al. 1998a) and newly identified RFLPs (Table 2). We initially scored restoration of male fertility as a qualitative trait (Fig. 3) and the *Ms* locus was placed at the highest confidence (LOD = 3.0) between RFLPs AOB272-E1-10.0/12.0 (0.9 cM) and two cosegregating RFLPs AOB186-E5-6.7/2.5 and AOB232-E5-24.0/30.0 (8.6 cM). Other closely linked RFLPs were revealed by cDNA clones AOB210L, API63, and API65L (Fig. 3). Accession numbers and sequence homologies of cDNA clones revealing linkage to *Ms* are listed in Table 2. Hybridizations of AOB272, API63, API65L, and AOB210L to DNA-gel blots from a collection of elite onion populations revealed numerous polymorphic fragments (Table 2). For AOB272, fragments of 6.0, 10.0 and 12.0 kb are genetically characterized alleles; fragments of 10.0 and 12.0 kb segregated in BYG15-23 x AC43 (King et al., 1998a) and fragments of 6.0 and 12.0 kb segregated in the Seminis family (autoradiogram not shown). An additional fragment of 14.0 kb was observed among inbred onion populations and may be one more allele at this locus (Table 2). API63-E5-5.0/7.5 was linked at 1.7 cM from AOB272-E1 (Fig. 3); putative alleles of 14 and 6.7 kb were identified among the elite onion germplasms (Table 2). RFLPs revealed by AOB210L-H3-8.0/9.0 and API65L-D1-3.0/3.5 were mapped 2.7 and 3.6 cM, respectively, from API63-E5-5.0/7.5 (Fig. 2). However one must be careful when scoring polymorphisms revealed by AOB210 and API65; these clones showed significant homology to RuBISCO and revealed numerous fragments segregating independently of *Ms* (King et al., 1998a, Table 2).

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-*Eco*RI showed highly significant effects (likelihood ratio statistic = 112), in agreement with the qualitative trait mapping (Fig. 3).

An RFLP (ACB75-jm8.e05-*Swa*I-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. Møller (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 (PID g729859) associated with sexual differentiation (Sato et al., 1994) and an *Arabidopsis* homolog (AY054590). The AOB272 genomic region was amplified using prim-

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.

Population ^z	Nucleotide position in AOB272 genomic sequence ^y														
	498	503	512	550	658	933	985	1158	1191	1209	1256	1266	1279	1296	1303
AC43	T	A	C	T	A	T	C	A	A	C	C	T	A	G	A
CN1	T	A	T	T	A	T	C	A	A	T	A	T	A	G	A
CN2a	T	A	G	T	A	T	C	A	A	T	A	T	A	G	A
CN6	T	A	T	T	A	T	C	A	A	T	A	T	A	G	A
BYG15-23	A	G	T	A	C	T	T	G	G	T	A	C	A	T	G
CN2b	A	G	G	A	C	T	T	G	G	T	A	T	A	T	G
CN8	A	G	C	A	C	C	T	G	G	T	A	T	A	T	G
PLK	A	G	G	A	C	T	T	G	G	T	A	T	A	T	G
SK1a	A	G	G	A	C	T	T	G	G	T	A	T	A	T	G
SK1b	A	G	G	A	C	T	T	G	G	T	A	T	C	T	G

^zPopulations are listed in Table 1. CN2a and CN2b are two alleles in population CN2.

^yPositions refer to Genbank accession number AF366455.

ers AOB272-F and AOB272-5R and yielded a 1019-bp fragment. Together with the flanking cDNA sequences, the AOB272 genomic region was 1375 bp in size and carried two introns. No insertions, deletions, or simple sequence repeats were present among AC43, BYG15-23, and the seven onion populations (Table 1). *TaqI* cut sites were present at positions 1098 and 1146 in the AC43 allele, but were absent in the BYG15-23 allele. Within the AOB272 genomic region, there were two major alleles among these germplasms differing for several single nucleotide polymorphisms (SNPs) (Fig. 1, Table 3). Nine of these SNPs formed two alleles between the 324th and 1342nd nucleotides of the AOB272 genomic region (Fig. 1, Table 3).

DEVELOPMENT OF SSCP FOR AOB272. Because there are numerous SNPs within the AOB272 genomic region, these may be readily detected by nonradioactive SSCPs. Fig. 4 shows SSCP banding patterns typical of all onion germplasm surveyed to date using the AOB272 primers. Four single stranded DNA fragments occasionally were revealed on high-resolution gels, suggesting that this primer set may amplify products from two loci. King et al. (1998a) observed that tandem duplications of cDNAs may commonly occur in the onion genome. SSCPs are especially useful because the specific polymorphisms in our germplasm sample may not be polymorphic in other elite commercial populations. We observed double-stranded duplex polymorphisms on long gels, suggesting that heteroduplex analysis (Nataraj et al., 1999) may also be useful for nonradioactive genotyping at this locus.

ATTEMPTS TO DEVELOP DUAL-PRIMER PCR MARKER FOR AOB272. Many primer pairs were synthesized flanking specific SNPs in the AOB272 genomic regions [Fig. 1, other primers were listed by Gökçe (2001)]; however all primer pairs occasionally amplified

both the target and nontarget alleles across a plethora of annealing temperatures (gels not shown). AC43 allele specific primers often produced weak products from BYG15-23 DNA samples, even though they did not produce products from other samples homozygous for the BYG15-23 allele. The most likely explanation is that BYG15-23 possesses the AC43 allele at low frequency. The AC43 and BYG15-23 allele specific primer pairs produced the target products at 74 to 70 °C and 72 to 68 °C with lower temperatures during earlier cycles. However, amplification was not consistent and we concluded that SNP detection using flanking primers was not reliable. Flanking primers may successfully distinguish alleles at AOB272 if PCR cycling conditions were adjusted for each primer pair and reactions replicated to reduce the error rate. However, this may not be realistic because of variations in DNA template purity from minipreparations of large numbers of onion plants in a breeding program.

DEVELOPMENT OF SINGLE NUCLEOTIDE EXTENSIONS FOR SNPs AT AOB272. Five SNPs were used to distinguish the AC43 and BYG15-23 alleles by single nucleotide extensions. Oligonucleotide primers flanking SNPs were synthesized (Fig. 1). Dideoxynucleotides (ddNTP) carrying different fluorescent tags (ABI SNaPshot kit) were mixed with primers flanking the SNPs and allowed to anneal to the amplified genomic fragments. A specific fluorescent ddNTP was added to the primers, corresponding to the nucleotide at the specific SNP in the target DNA fragment. Specific extension products were well resolved on an automatic sequencing machine and revealed codominant polymorphisms. However it is imperative that the primers used for single nucleotide extensions be purified to remove contaminating shorter oligonucleotides.

USE OF CYTOPLASMIC MARKERS AND RFLPS LINKED TO THE *Ms* LOCUS. The molecular markers flanking the *Ms* locus can be used for marker-facilitated selection of maintainer lines in onion. Because alleles at AOB272 (Table 3) were not associated with any specific genotype at *Ms* across a diverse set of onion germplasms (Table 1), one must establish which polymorphisms are present in the parents used in crosses to identify fragments in coupling-phase linkage with the recessive *ms* allele. The segregation of the dominant and recessive alleles at *Ms* can then be predicted using these linked, phase-known markers. Although molecular identification of nuclear genotypes and the cytoplasm does not shorten the onion-breeding cycle (2 years per generation are still required), the technique represents a more judicious use of resources by reducing the number of testcrosses required to identify maintainer plants.

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.

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