Chromosomal Locations of Microsatellites in Onion

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Abstract. Bulb onion (*Allium cepa* L.) has a very large genome composed of a high proportion of repetitive DNAs. Genetic analyses of repetitive sequences may reveal microsatellites in order to increase the number of genetic markers in onion. Thirty microsatellites were previously isolated from an onion genomic library (Fischer and Bachmann, 2000). A complete set of Japanese bunching onion (*A. fistulosum*) – shallot (*A. cepa* Aggregatum group) monosomic addition lines were used to assign these microsatellites to the chromosomes of *A. cepa*. Simplified PCR conditions for each microsatellite were determined and 28 of the 30 primer pairs amplified DNA fragments, of which 21 microsatellite markers were assigned to chromosomes of *A. cepa*. Subsequent mapping of these microsatellites will enable us to establish the chromosomal distribution of these markers.

Microsatellites, or simple sequence repeats (SSRs), are tandemly repeated motifs of one to a few nucleotides (Field and Wills, 1996; Tautz and Renz, 1984) and are present at both protein-coding and noncoding regions of the genome (Toth et al., 2000). Microsatellites are highly polymorphic among individuals of a population (Morgante and Olivieri, 1993; Tautz, 1989). Polymorphism may be caused by slippage events during DNA replication (Schlötterer and Tautz, 1992). The information content among molecular markers is high for microsatellite markers due to intraspecific polymorphism, co-dominance, and high reliability and reproducibility (Bohn et al., 1999; Jones et al., 1997; Powell et al., 1996; Russell et al., 1997). Microsatellite markers have been used

for the construction of genetic maps (Barrett et al., 2004; Blair et al., 2003; Cregan et al., 1999; Röder et al., 1998; Somers et al., 2004) and are useful for plant breeding (Gupta and Varshney, 2000).

There have been few reports of microsatellites in the genus *Allium*. Fischer and Bachmann (1998) were the first to report microsatellites from onion (*A. cepa* L. Common onion group) and developed 30 primer pairs flanking microsatellite motifs to assess interspecific taxonomic analyses in *Allium* (Fischer and Bachmann, 2000). Because of the intricate PCR conditions required to reveal these microsatellites, including touch-down PCR and the use of fluorescein-labeled primers, it is difficult for a small laboratory to score these microsatellites.

The objectives of the present study were 1) to simplify the PCR conditions for 30 microsatellites developed by Fischer and Bachmann (2000) and 2) to assign microsatellite markers to the chromosomes of shallot (*A.*

cepa L. Aggregatum group) by using the *A. fistulosum*–shallot monosomic addition lines (Shigyo et al., 1996).

Materials and Methods

Plant materials. Two complete sets of *A. fistulosum*–shallot monosomic additions (Shigyo et al., 1996) were used to assign microsatellite markers to the chromosomes of shallot. The sets were analyzed together with their parents: shallot (*A. cepa* L. Aggregatum group) and Japanese bunching onion (*A. fistulosum* L. ‘Kujo Hoso’). Shallot was used to determine the PCR conditions for each of 30 primer pairs (AMS01 to AMS30; GenBank accession numbers, AJ391666 to AJ391725) described by Fischer and Bachmann (2000). Total genomic DNA was isolated from fresh leaf tissue using a mini-prep DNA-isolation method (van Heusden et al., 2000a).

Simplification of PCR conditions for 30 primer pairs. Polymerase chain reactions (PCRs) were performed with the 30 primer pairs in a program thermal cycler (iCycler; Bio-Rad, Hercules, Calif.) to simplify the complicated touch-down PCRs reported by Fischer and Bachmann (2000). Two conditions for simplification were as follows; PCR mixture I was made with 0.4 μ M of each of the primers, 0.1 mM dNTPs, 1 $\times$ *rTaq* buffer, 1 U *rTaq* polymerase (Takara, Shiga, Japan), and 100 ng template DNA in a volume of 25 μ L; mixture II differed in that 1 $\times$ PCR Gold buffer and 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, Calif.) were used instead of 1 $\times$ *rTaq* buffer and 1 U *rTaq* polymerase, and that additional 1 mM magnesium were used. PCR temperatures were as follows: 94 °C (3 min) followed by 35 cycles at 94 °C (30 s), 50 to 60 °C (1 min), and 72 °C (1 min), with a final extension at 72 °C for 7 min for the mixture I. For the mixture II, the sequence was 95 °C (11 min) followed by 40 cycles at 94 °C (30 s), 50 to 60 °C (30 s), and 72 °C (30 s), with a final extension at 72 °C for 10 min. The ramp times were carried out in the default conditions that adjusted temperatures at the maximum ramp rate with the minimum ramp time. The PCR products were separated on 2.0% agarose gel. The optimum annealing temperature for each primer pair was chosen after production of fewer nonspecific amplicons.

Assignment of microsatellite markers to chromosomes. The primer pairs with simplified PCR conditions were analyzed for shallot-specific amplicons between *A. fistulosum* and shallot. If products were monomorphic, they were subjected to denaturing polyacrylamide gel electrophoresis (PAGE) with silver staining. If they were also monomorphic after denaturing PAGE, they were subjected to capillary electrophoresis with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Simple polymorphic amplicons were analyzed with denaturing PAGE or SSCP analysis using electrophoresis and gel with silver staining. Denaturing PAGE and SSCP were performed as described by Martin et al. (2005). PCR for capillary electrophoresis was performed with one 5' fluorescent-labeled primers (NED for AMS01,

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Fig. 1. Amplification profiles for the primer pairs, AMS02 (a), AMS14 (b) and AMS17 (c), in *A. fistulosum* (F), shallot (A), and the two sets of *A. fistulosum*-shallot monosomic additions (1A to 8A) on agarose gel. M, molecular size marker. Arrows point to shallot chromosome specific markers. (a) AMS02-470 and AMS02-530 located on chromosome 7A; (b) AMS14-175 located on 2A; (c) AMS17-360 located on 1A and AMS17-360 located on 5A.

Fig. 2. Part of amplification profiles for the primer pair AMS29 in *A. fistulosum* (F), shallot (A), and the set of *A. fistulosum*-shallot monosomic additions (1A to 8A) on denaturing polyacrylamide gel. M, molecular size marker. Arrows indicate chromosome 8A specific marker.

FAM for AMS05 and HEX for AMS24) using 15 µl sample containing 1 pM fluorescent-labeled primer, 1 µM reverse primer, 0.2 mM dNTPs, 0.125 U AmpliTaq Gold, and 100 ng

template DNA in 1 × Gold buffer with 4 mM magnesium. PCR amplification was carried out for 35 cycles, 30-s denaturation at 94 °C; 30-s annealing at 56 to 60 °C; and 30-s primer extension at 72 °C. PCR was preceded by 11 min of pre-denaturation at 94 °C and followed by 10 min of postsynthesis at 72 °C. The analysis with the ABI PRISM 310 and subsequent analyses were conducted using a modification of the procedure of Araki et al. (2003). The amplified fragments from 100 to 700 bp were scored in each *A. fistulosum* and shallot. The primer pairs producing shallot-specific bands were used to assign chromosomal locations using the monosomic additions. The markers were designated according to the primer name and the molecular size of each band in base pairs.

Results

Simplification of PCR conditions. The PCR conditions for shallot were simplified for 28 primer pairs (Table 1). Amplicons of approximately the same sizes as reported by Fischer and Bachmann (2000), were observed for 19 primer pairs. Nine primer pairs amplified amplicons of different sizes from onion and shallot, and two primer pairs of AMS11 and AMS15 amplified no PCR products in shallot under all the PCR conditions. We simplified the PCR conditions for 21 of 22 primer pairs with the exception of AMS11. The optimum

Table 1. Simplified PCR conditions, electrophoresis for polymorphism detection, and fragment sizes amplified from *A. fistulosum* and shallot.

Microsatellite ^z	PCR mixture	Annealing temp (°C)	Electrophoresis ^y	Expected size (bp) ^x	Observed size (bp) in shallot ^w	Observed size (bp) in <i>A. fistulosum</i> ^w
AMS01	II	60.0	C	126	101, 103, 105, 113, 115, 121, 127	101, 126
AMS02	I	54.0	A	530	470, 530	---
AMS03	II	56.0	P	121	203, 242	127, 197, 228, 271, 342
AMS04	I	56.9	P	204	181, 205, 213	181, 192, 205, 213
AMS05	II	56.0	C	229	117, 127, 135, 140, 147, 152, 155, 162, 207, 214, 354, 365	101, 102, 118, 135, 159, 355
AMS06	I	55.0	P	147	105	149
AMS07	I	50.6	P	114, 174	108, 163, 270	108, 163, 179, 212, 270
AMS08	I	56.0	P	205	190, 350, 685	210, 350, 685
AMS09	I	54.2	P	278	236, 278	236
AMS10	II	52.9	P	157	150, 255, 285	255
AMS11 ^v	---	---	---	92	---	---
AMS12	II	56.0	P	274	237, 274	---
AMS13	I	58.0	P	168	138	139
AMS14	II	60.0	A	169	175	157
AMS15	II	58.0	P	229	---	122, 187, 211, 261, 529
AMS16	I	58.0	P	261	253, 261	253, 279, 445
AMS17	I	58.0	A	264	270, 360	---
AMS18	I	54.2	P	195	165	---
AMS19	I	58.0	P	131	125, 130	128, 289, 311, 372, 389, 408, 513
AMS20	I	55.0	P	372	350, 372	350, 410
AMS21	I	56.0	P	264	141, 194, 220, 235, 250	235, 250
AMS22	II	57.4	P	310	270, 282, 310, 330, 360	282, 360
AMS23	II	58.0	A	157	150, 218	135
AMS24	II	57.4	C	161	111, 125, 127, 133, 142, 147, 151, 153, 155, 173, 197, 210, 220	138, 144, 145, 238, 265
AMS25	I	52.5	P	235	200, 220	200, 210
AMS26	I	52.5	P	213	206, 225	---
AMS27	II	54.0	S	318	177, 235, 338, 390, 430, 550	235, 288, 430, 550
AMS28	II	58.0	P	250	150, 242	150, 230, 362, 571
AMS29	I	54.0	P	310	315	332
AMS30	II	57.4	P	342	342	---

^zThe STMS numbers were described by Fischer and Bachmann (2000).

^yA, P, C, and S indicate agarose gels, denaturing polyacrylamide gels, capillary and SSCP, respectively.

^xExpected size in each microsatellite is the fragment size in onion that Fischer and Bachmann (2000) mentioned.

^wThe observed size in *A. fistulosum* and shallot in AMS27 shows the fragments detected on denaturing PAGE.

^vThe primer pair of AMS11 amplified no DNA fragment in *A. fistulosum* and shallot under all the conditions used in this study.

Table 2. Chromosomal locations of microsatellite markers in shallot.

Chromosome	Microsatellite marker
1A	AMS01-113 ^z
	AMS09-278 ^z
	AMS16-261
2A	AMS17-270
	AMS08-190
	AMS10-150 ^z
	AMS14-175
3A	AMS27-390
	AMS12-274
4A	AMS23-218
	AMS01-127 ^z
5A	AMS20-372
	AMS23-150
	AMS17-360
6A	AMS21-141 ^z
	AMS09-278 ^z
7A	AMS21-141 ^z
	AMS01-113 ^z
	AMS02-470
	AMS02-530
8A	AMS12-237
	AMS24-210
	AMS01-127 ^z
	AMS10-150 ^z
	AMS26-225
	AMS29-315

^zThe footnotes show the markers located on the two chromosomes.

annealing temperature differences between onion (Fischer and Bachmann, 2000) and shallot (these results) varied from -17.4 to 1.3 °C.

Polymorphisms between *A. fistulosum* and shallot. The 28 primer pairs revealed polymorphic amplicons between *A. fistulosum* and shallot, and of which two for AMS02, one for AMS14, two for AMS17, and two for AMS23 were obtained (Table 1; Fig. 1). For 16 primer pairs out of the remaining 24, a total of 27 shallot-specific bands were detected on denaturing PAGE with silver staining (Table 1; Fig. 2). Three primer pairs (AMS01, AMS05, and AMS24) showed complex amplification products after denaturing PAGE were subjected to capillary electrophoresis yielding 6, 11, and 13 shallot-specific bands, respectively (Table 1). Nine primer pairs that were monomorphic between *A. fistulosum* and shallot using denaturing PAGE were analyzed using SSCP with silver staining, and one shallot-specific band was detected only for AMS27 (data not shown). In total, 65 amplicons specific to shallot were observed for 24 out of the 28 primer pairs (Table 1).

Chromosomal locations of microsatellite markers. The 65 amplicons were assigned to shallot chromosomes. Twenty-one out of the 65 shallot-specific amplicons were detected in a single monosomic addition or two additions (Figs. 1 and 2). The microsatellite marker AMS14-175 was detected in monosomic additions with chromosome 2A but not in any other monosomic additions (Fig. 1b). In the same way, the chromosomal locations of the remaining 20 markers were revealed (Table 2; Figs. 1 and 2). Of the 21 chromosome-specific markers, 16 were located on single chromosomes, and the other five primers produced amplicons located on two chromosomes each. Seven markers, i.e., AMS01-113, AMS02-

470, AMS12-237, AMS17-360, AMS21-141, AMS23-218 and AMS24-210, did not correspond with the amplicon size in onion reported by Fischer and Bachmann (2000). These seven markers produced additional amplicons of different sizes to those reported by Fischer and Bachmann (2000). Of the 44 amplicons not assigned to shallot chromosomes, 23 were absent from all monosomic additions, and 21 were detected in three or more additions (data not shown).

Discussion

We produced 65 amplicons from shallot carrying microsatellites (Table 1) and assigned 21 amplicons to shallot chromosomes (Table 2). Linkage maps of onions have been produced based on AFLPs (van Heusden et al., 2000a, 2000b), and SSRs, SNPs, RAPDs and RFLPs (King et al., 1998; Martin et al., 2005). These two maps may be expanded by the 65 microsatellite amplicons obtained in this study. Of 14 linkage groups of the RFLP map, 12 have been assigned to chromosomes using the *A. fistulosum*-shallot monosomic addition set (Martin et al., 2005). The remaining two unassigned linkage groups might be assigned to chromosomes using 21 chromosome-specific microsatellite markers. Assigning markers to the physical chromosomes of *A. cepa* by linkage mapping is still difficult because of lack of mapping stocks and high levels of heterozygosity. This means that assignment of polymorphic markers to specific chromosomes via our monosomic additions provide a very useful set of genetically characterized markers for application in *A. cepa*. The appearance of more than two amplicons in diploid shallot means that the locus must be duplicated. Of 28 primer pairs, 18 produced one or two amplicons and the results represent high frequency of single locus PCR markers by comparison with 23% duplication rate in onion described by King et al. (1998).

Song et al. (2004) reported the isolation of microsatellites with dinucleotide motifs from Japanese bunching onion (*A. fistulosum* L. 'Kujo Futo') and the suitability of microsatellite markers for genetic analysis in bunching onion. This is the only report of microsatellites in *A. fistulosum*. In this study, 23 out of 30 primer pairs amplified DNA fragments both in shallot and *A. fistulosum* indicating that the 23 primer pairs may be useful for genetic analysis of *A. fistulosum* (Table 1). In addition, we obtained 45 microsatellite amplicons specific to *A. fistulosum* for 19 out of 23 primer pairs. Eight shallot-*A. fistulosum* monosomic additions ($2n = 2x + 1 = 17$) and 20 shallot-*A. fistulosum* single alien deletions ($2n = 3x - 1 = 23$) have been produced (Hang et al., 2004) and it will be possible to assign these 45 microsatellite amplicons to the chromosomes of *A. fistulosum*.

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