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Development of Microsatellite Markers in Bunching Onion (*Allium fistulosum* L.)

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Since few genetic markers are available in bunching onion, many DNA markers are needed for the construction of a primary basis for the breeding of this crop. We report here the development of microsatellite markers in bunching onion. A size-fractionated genomic library was constructed from genomic DNA of bunching onion cv. 'Kujo Futo' and screened with a mixture of $(GA)_{15}$ and $(GT)_{15}$ oligonucleotide probes. From approximately 180,000 clones we isolated and sequenced 94 positive clones. A total of 52 clones were identified as having microsatellite repeats. Of them 49 had a GT motif, while only one had a GA motif, which is known to be more frequent than GT in most plant species. This is the first report of the abundance of GT over GA in *Allium* spp. Of 50 specific PCR primer pairs designed for the microsatellite-containing clones, 33 primer pairs amplified polymorphic loci in nine cultivars of bunching onion, 115 alleles detected in total. These results indicate that microsatellites with dinucleotide motifs, especially with GT, are promising sources of highly informative genetic markers in bunching onion.

Key Words: ***Allium fistulosum*, bunching onion, dinucleotide motif, microsatellite.**

Introduction

Bunching onion (*Allium fistulosum* L.) is one of the most popular vegetables in East Asia and surpasses bulb onion (*A. cepa* L.) in economic importance in Japan, ranking fourth among vegetable crops in annual output (MAFF, Japan 2000).

Major targets of bunching onion breeders are high yield, late bolting, disease resistance, suitability for mechanized farming and quality (low pungency, high sugar content, soft texture, etc.) although the mode of inheritance of these traits is unknown. Literature on genetics in bunching onion is very limited due to the disadvantages of this crop, such as cross-pollinating nature, biennial generation time and severe inbreeding depression. Moue and Uehara (1985) have described the mode of inheritance of cytoplasmic male sterility in bunching onion. To our knowledge, that is the only paper published on the genetics of agriculturally important characteristics in bunching onion. Although several polymorphic isozyme loci in bunching onion have been found (Haishima and Ikehashi 1992, Haishima *et al.* 1993, Mangum and Peffley 1994), many more are needed for the construction of a primary basis for bunching onion breeding.

Since many of the targeted traits are probably controlled by quantitative trait loci (QTLs), bunching onion breeders place their hopes on modern DNA techniques, which will provide them an unlimited number of genetic markers. Marker-assisted selection using DNA markers closely linked to QTLs should improve the efficiency of breeding.

In recent years, microsatellites, also termed simple sequence repeats, have been widely used as genetic markers in plants (Lagercrantz *et al.* 1993) as well as in man (Weber and May 1989), animals (MacHugh *et al.* 1997) and fungi (Lian *et al.* 2003). The increasing interest in microsatellite markers for a broad range of applications is based on their extreme variability, co-dominant inheritance, and greater reliability and reproducibility compared to RAPDs and AFLPs (Jones *et al.* 1997, Powell *et al.* 1996).

Our paper reports on the isolation and characterization of microsatellites with dinucleotide motifs and the development of microsatellite markers in bunching onion. To our knowledge, this is the first report concerning the isolation of microsatellites in bunching onion.

Materials and Methods

Bunching onion cv. 'Kujo Futo' was used for the isolation of microsatellites. Polymorphisms of microsatellite loci were analyzed among nine varieties including 'Kujo Futo' (Table 1). We chose these varieties from three major intraspecific groups; i.e., Kaga, Senju and Kujo. Genomic DNA was extracted from 0.1 g of a fresh leaf of a single plant of each variety with Nucleon PhytoPure (Amersham Biosciences, NJ, USA).

A small-insert genomic library of 'Kujo Futo' was developed for the isolation of microsatellites. Genomic DNA was digested completely by *Sau*3AI and fractionated in a 1.0 % agarose gel. DNA fragments of 200 to 1,000 bp in length were recovered from the gel and ligated into the *Bam*HI site of a lambda phage vector, ZAP Express (Stratagene, CA, USA). The library was screened by two cycles of plaque hybridization with a mixture of digoxygenin-labeled synthetic $(GA)_{15}$ and $(GT)_{15}$ oligonucleotide probes. The subsequent

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Table 1. Bunching onion varieties used

	Variety	Variety group	Accession no.
1	Shimonita	Kaga	JP127028
2	Matsumoto Nebuka Futo	Kaga	JP133872
3	Iwatsuki	Kaga	JP133914
4	Yoshikura	Senju	JP133875
5	Tokyo Fuyuguro Ippon Futo	Senju	JP133906
6	Nishida	Senju	JP127027
7	Koshizu	Kujo	JP 25474
8	Kujo Futo	Kujo	JP133847
9	Asagikei Kujo	Kujo	JP133852

procedure for microsatellite isolation was the same as that described by Suwabe *et al.* (2002). We selected sequences containing at least five di-nucleotide or four tri-nucleotide repeats.

For the amplification of microsatellite loci, we designed specific PCR primers from the microsatellite flanking regions using Primer 3 (Rozen and Skaletsky 2000), web-based software available at http://www.genome.wi.mit.edu/genome_software/other/primer3.html. PCR was performed in a 10-μl reaction mixture containing 100 ng of genomic DNA, 250 nM of each primer, 0.25 mM of dNTPs, 1 × reaction buffer, and 1 unit of Takara *Taq* polymerase (Takara Shuzo, Osaka). The reaction mixture was subjected to initial denaturation at 94°C for 1 min, then 45 cycles of amplification at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec, and a final extension at 72°C for 4 min in a GeneAmp PCR system 9700 (Applied Biosystems, CA, USA). The products of PCR were then fractionated in a 4.0% denatured polyacrylamide gel containing 30% formamide. After electrophoresis, we stained the polyacrylamide gel with SYBR Green I Nucleic Acid Gel Stain (Molecular Probes, OR, USA), and then detected product bands using an image analysis system FMBIO II (Hitachi Software Engineering, Tokyo).

For reference to microsatellite markers, we calculated the polymorphic information content (PIC) for each locus according to Anderson *et al.* (1993).

Results and Discussion

We screened approximately 180,000 clones in the phage library for GA and GT repeats. Two cycles of screening isolated 94 positive clones. Fifty of them (53%) were confirmed as containing these motifs (Table 2). Forty-nine of the clones had a GT motif, while only one had a GA motif. The frequency of candidate regions for microsatellite markers containing GT repeats was therefore estimated to be 49 times higher than that for microsatellite markers containing GA repeats in bunching onion. This is a rather rare phenomenon in plants.

Based on database searches, Lagercrantz *et al.* (1993) found that the GA motif is more frequent than the GT motif in plants in general, while the latter is the most abundant di-nucleotide motif in mammals. Practically on a developed-marker basis, high $(GA)_n$: $(GT)_n$ ratios have been reported in *Brassica* spp., e.g., 6.5 : 3 (Szewc-McFadden *et al.* 1996), 8 : 4 (Uzunova and Ecke 1999), and 45 : 12 (Suwabe *et al.* 2002), as well as in maize, 13 : 8 (Taramino and Tingey 1996). Also in rice and barley high $(GA)_n$: $(GT)_n$ ratios have been reported on a positive-plaque basis: 1,136 : 524 in rice (Wu and Tanksley 1993) and 485 : 285 in barley (Struss and Plieske 1998). In plants the reverse situation has been reported only in tobacco, one $(GA)_n$ per 170 kb versus one $(GT)_n$ per 150 kb on a positive-plaque basis (Lagercrantz *et al.* 1993).

In bulb onion, Fisher and Bachman (2000) developed 30 microsatellite markers, the majority of which have GT repeats. However, they did not study the $(GA)_n$: $(GT)_n$ ratio, since the probes they used for plaque screening did not include $(GA/CT)_n$. We are the first to report on the surprisingly low $(GA)_n$: $(GT)_n$ ratio in *Allium* spp. This information should be very useful for efficient development of microsatellite markers from genomic libraries, and even from microsatellite-enriched libraries. Our ongoing trial has shown that a GT-enriched library is less redundant than a GA-enriched library in bunching onion: 170 sequenced clones from the GT-enriched library included 63 unique $(GT)_n$-containing clones, while 138 sequenced clones from the GA-enriched

Table 2. Microsatellite motif and other characteristics of clones isolated with $(GA)_{15}$ and $(GT)_{15}$ probes from a genomic library of bunching onion 'Kujo Futo'

Motif(s) in core sequence	No. of clones isolated	Mean (range) of repeat number in the longest repeat sequence		No. of clones amplified on 'Kujo Futo' template	No. of clones polymorphic among nine varieties
		GA or GT	Others		
GA	1	15 (15)		1	1
GA; other(s)	0				
GT	29	8.3 (5–16)		27	19
GT; other(s)	20	9.0 (5–13)	8.0 (5–22)	19	13
GA; GT	0				
GA; GT; other(s)	0				
Subtotal (legitimate clones)	50	8.5 (5–16)	8.0 (5–22)	47	33
Other(s)	2		6.0 (5–7)	1	0
Total	52			48	33

library included only 37 unique $(GA)_n$-containing clones (H. Tsukazaki, National Institute of Vegetable and Tea Science, personal communication). In the forage grass timothy (*Phleum pratense* L.), in which the GA motif is thought to be more common than the GT motif, a GA-enriched library was far less redundant than a GT-enriched library (Cai *et al.* 2003).

Levinson and Gutman (1987) suggested that the prevalence of GT repeats in animals could be explained by a high rate of transitions of methylated C residues in 5′-CG-3′ nucleotides to T resulting in an increase of GT motifs. Since in plants cytosine is methylated not only at 5′-CG-3′ but also at 5′-CNG-3′, Lagercrantz *et al.* (1993) mentioned a possibility that the absence of a specific 5′-CG-3′ hotspot in plants could prevent the numerous occurrence of GT repeats. These

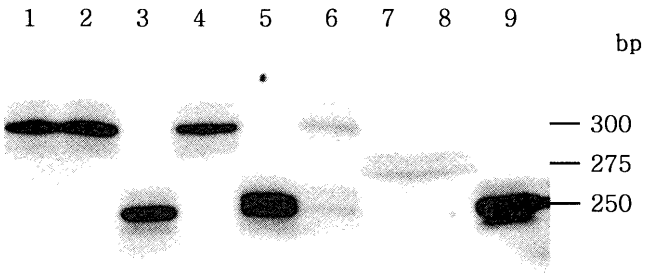

Fig. 1. Polymorphisms detected among nine bunching onion varieties at AFS105, a microsatellite locus. PCR products were separated on 4% denatured polyacrylamide gel plates. Lanes: 1, 'Shimonita'; 2, 'Matsumoto Nebuka Futo'; 3, 'Iwatsuki'; 4, 'Yoshikura'; 5, 'Tokyo Fuyuguro Ippon Futo'; 6, 'Nishida'; 7, 'Koshizu'; 8, 'Kujo Futo'; and 9, 'Asagikei Kujo'.

Table 3. Core and primer sequences of 33 microsatellite markers developed from bunching onion (*Allium fistulosum*) 'Kujo Futo', and polymorphisms detected among nine bunching onion varieties

Microsatellite	Core sequence	Primer sequence (5′-3′)	Expected size in 'Kujo Futo' (bp)	Polymorphism in 9 varieties	
				No. of alleles	PIC
AFS060	$(GC)_5(AC)_8(AT)_8$, $(GT)_{13}$	CCTATCCCCATGCATCACAACTTAT ATTCCTTCACCACCGTTGATTTATT	133	5	0.66
AFS062	$(AG)_{15}$	TTTCTCTCATTCCACAATTACCACTG CGTTGAGACAAACAAACAAACAAGA	157	4	0.69
AFS063	$(AT)_8(GT)_{10}$	CTGCACACCAATACTTCAACCATTT TTTCAAAACAAGCAAAAATTCAAGC	219	2	0.76
AFS065	$(GT)_{12}$	GCAATGGTTTTAGACGTGTCGATAG ATGTTGGATTTTGACAAATTTTGGA	246	5	0.37
AFS067	$(GT)_{10}$	CAAAATTTCTCCTTCTACCCCATTG ATGACGACCGCATATAGTGACATTT	270	3	0.65
AFS068	$(AT)_5(GT)_{10}$	CAGTGGTTAGTGTGGTTTTGCTTTT TCAGAATTTATAGAGTTTGGCGTACAA	190	2	0.50
AFS069	$(GT)_{10}$	AATCGGGTTTTCTGACATTCATTTT TAACCAGAATCCATAAGCCACTCAA	286	4	0.72
AFS070	$(TG)_7TA(TG)_5$, $(TG)_7$	TTTGAAGTCATAGACCGAATCAAAA GGATTTGGGTGTTAGTGACCAATTT	230	5	0.72
AFS075	$(GT)_5$, $(TA)_3TG(TA)_5$	ACAGCGCATATACTGAGACTGCTCT TGTTTGTCAATTATCCCGAATAACG	256	3	0.54
AFS080	$(TG)_6$	GGGTGTGTGGCTCTGTGTGT TCCACGTTCTAATGCTAGGTAACCA	138	3	0.60
AFS084	$(TG)_5CG(TG)_1CG(TG)_4$	ACTTGTTTTGCTTTCTGTTTGTTGC TCTTAATGTCCTATGAAGCGTTACCC	180	4	0.72
AFS085	$(TG)_4TA(TG)_9(TA)_{11}$	TATCTAGGTCCATAAGTGGACGATG TATCAGAACCTGCCCTTCTAAATCC	230	2	0.38
AFS086	$(GT)_4GGGG(GT)_5$	TTTTTGCACACATTTACAAACACTG ACATGGATACTCACTCTCTCCA	201	2	0.22
AFS087	$(TG)_8CG(TG)_2$	GATCAACTGATAGAAGGACGGGTTC ATACACACATGGTATGACGAAGCAA	249	2	0.30
AFS088	$(GT)_{11}$	TATCTTCGAGCACGGTTCTTCTTGT ATGGCTTCGATGATGGATAGTTGTA	167	4	0.66
AFS089	$(GT)_3AT(GT)_8$	TCGACCTCTAGATCGACCTCCT TATCAAAATTGAGCCACTGGATTTG	179	3	0.60
AFS092	$(TA)_8(TG)_9$	GTCTGTTTTAGGGCATGAGTGTGAG AAATTTGCAGAGGGAGTCAGATAGG	296	2	0.56
AFS095	$(TG)_8(TA)_6$	GTCCAACGACATCAAGTAAGCAGTT ATACGTCCTGCACAAAATCAGAAAA	222	3	0.62
AFS097	$(GT)_8$	AAAGGAAGATGGGAATGGTTGAATA AACATATAAGACGCCAGGCAGTACA	197	6	0.75

Table 3. (continued)

Microsatellite	Core sequence	Primer sequence (5′-3′)	Expected size in 'Kujo Futo' (bp)	Polymorphism in 9 varieties	
				No. of alleles	PIC
AFS099	$(TA)_8(GT)_{13}$	TTAATCGCATTGACAAAGTTTATTT TGCCCCTCATTAATAACAACATGAC	237	5	0.81
AFS102	$(CG)_5$, $(TGCG)_5(TG)_6$	ATGCAGTTGCACTTCACG CGTACTTAACATTCATACACACAAACACA	113	3	0.76
AFS103	$(AT)_{10}(GT)_9$	TTTTACCTAGATATTTTCGAATTTCA CATCTTTCTTTTTCACTAGCTTCCTG	209	3	0.63
AFS104	$(TGTA)_8(TG)_7$	CTCGCCCTTTTAGATTCATTTCCTA GCCACTAAGTTGGTCGTGTAAAGAA	201	5	0.69
AFS105	$(GT)_{10}TT(GT)_3$, $(GT)_5$	GTGGAGTTTACTTCCCCAAATGAAG TTTCGTTGCAAACATTTACACACAC	275	4	0.76
AFS109	$(GT)_4TT(GT)_7$	CCTATGTCTTTACCTATCCAACCAACA CCGAATTTCAAGTGTGTCAAGTTTT	193	4	0.71
AFS110	$(GT)_9$	TTTTGAAGTTTTGCCCTTTTTATCC TCATGACACCATGTGTCTCTATGTCT	232	3	0.66
AFS111	$(GT)_8$	TGTTTAATGGACTTTCAATGCCTGT GCATTAAAATGAAGAAATCCCGAAG	223	3	0.70
AFS112	$(GT)_9$	CCTCTCTGCATGCTCTCTTTCATTA ATAAAAGTTCCAATCCCACTTCGAG	265	3	0.62
AFS118	$(TG)_5CG(TG)_4$	TTGTACGTGACCATAGTGTTCCATC ATTCCAAATGAGATTCCTTCTTTCG	267	2	0.49
AFS119	$(TG)_8$	TGAAATCTAAATTCATTTCGAACTTTTCT AATATACTGGGCCTTCTAGGGGAAA	252	2	0.35
AFS122	$(TG)_9$	TTTTTAAGTAATGCAACATTATGTATGTG AAGGAAATATCGAAGAGGAGCTTCA	204	2	0.49
AFS123	$(GT)_9$, $(TA)_9$	TTTATCTGTATATCAATGTTTTTGTTCA TGGGATTTACATCGCAACATACATA	180	4	0.68
AFS128	$(TA)_8(TG)_{13}$	TTGTTTGAATCATTGGTGTTGAATG CCCCTATCATTTTTGGTGTCTTTAC	157	2	0.47
Total				115	
Average				3.3	0.59

assumptions, however, cannot explain the GT abundance observed in bunching onion. If the transitions of methylated C residues to T could play a significant role in the generation of GT repeats, a higher GC content might enhance the occurrence of GT repeats. However, the GC content of bulb onion, a close relative of bunching onion, was estimated at 32%, the lowest known for any angiosperm (Kirk *et al.* 1970, Stack and Comings 1979). The cause of the prevalence of GT repeats in bunching onion may be a challenging subject of molecular biology.

We found 5 to 16 motif repeats in the longest repeat sequence for each clone, with an average of 8.5 for legitimate motifs, GA and GT (Table 2). For other motifs, we found 5 to 22 repeats, with an average of 8.0. Besides these legitimate clones, we obtained two illegitimate clones that had GC or AT repeats, but no GA or GT repeats.

We designed primer pairs for all the microsatellite clones except two, which had motif repeats near one end of their sequence. The amplification product size designed on the clone sequences ranged from 113 to 296 bp. In the PCR that used genomic DNA of 'Kujo Futo' as a template, 48 primer pairs amplified one fragment of the expected size (Table 2) and occasionally an additional fragment. This is thought to reflect the heterozygosity retained in 'Kujo Futo', an open-pollinated variety. No fragment was amplified for the two remaining primer pairs.

Of the 48 loci, 33 were polymorphic (Table 2 and Fig. 1). We detected a total of 115 reproducible bands in the nine varieties (Table 3). The number of alleles per polymorphic locus ranged from 2 to 6, and the PIC value ranged from 0.22 to 0.81. The average number of alleles and the average PIC values at these polymorphic, dinucleotide-motif-containing loci were 3.3 and 0.59, respectively, which were as high as those reported in 32 rape seed varieties and breeding lines (Plieske and Struss 2001), 3.2 and 0.50, respectively, and fairly comparable to those reported in 58 maize inbred lines (Smith *et al.* 1997), at 5.6 and 0.70, respectively. These results indicate that dinucleotide microsatellite markers are as highly informative in bunching onion as in *Brassica* spp.

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